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Selective chromogenic and fluorogenic signalling of Hg^{2+} ions using a fluorescein-coumarin conjugate

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ABSTRACT

A novel, Hg^{2+}-selective chemosensor was prepared via Mannich reaction of dichlorofluorescein with piperazinyl-coumarin moiety. The dichlorofluorescein–coumarin derivative exhibited well-defined Hg^{2+}-selective chromogenic behavior, indicated by a green to pink colour change in solution, as well as fluorogenic signalling. Significant changes in fluorescence of the dichlorofluorescein subunit were analyzed in reference to the rather constant coumarin emission as an internal standard yielding Hg^{2+} selectivity. The Hg^{2+} selectivity of the chemosensor was not appreciably affected by the presence of common coexisting alkali, alkaline earth, and transition metal ions. The detection limit of the dichlorofluorescein–coumarin conjugate for the determination of Hg^{2+} ions was 4.3×10^{-6} mol L^{-1} and the conjugate dye could be used as a chemosensor for the analysis of Hg^{2+} ions in aqueous environments.

1. Introduction

Selective and efficient Hg^{2+} sensing is a critical topic in view of the extensive impact of Hg^{2+} on human health and natural ecosystems [1]. A number of interesting systems capable of Hg^{2+}-selective chromogenic or fluorogenic chemosensing have been reported [2]; many of these systems are based on well-established and unique molecular frameworks, such as crown ethers [3-6], calix[4]arenes [7], cyclams [8], squaraines [9], 8-hydroxyquinolines [10–12], 1,4-disubstituted azines [13], thioureas [14,15], 1,3-dithiole-2-thione [16], and fluoresceins [17,18]. More recently, a number of efficient Hg^{2+}-selective chemosensing systems have been devised based on rhodamine lactam or thiolactone derivatives [19–22], and have utilized the selective ring opening or chemical transformation processes of the rhodamine hydrazide or lactam subunit [23]. However, the chemosensor systems for Hg^{2+} ions have typically suffered from unfavorable fluorescence quenching due to spin orbit interactions [3]. In view of the selective signalling of Hg^{2+} ions in common turn-off type chemosensing systems, the use of an internal standard with a well-defined and useful emission maximum for ratiometric analysis might be helpful.

Fluorescein and its related compounds have attracted much research interest for the construction of many interesting supramolecular systems for chemosensing and molecular visualization of biological systems [24,25]. Utilization of the Mannich reaction, employing the dichlorofluorescein (DCF) molecular scaffold and amines having suitable binding subunits, has afforded many useful supramolecular systems [26]. In particular, a series of DCF-based chemosensors having dipicolylamine or thioether binding moieties have been successfully devised for the sensing and/or visualization of Zn^{2+} ions in living cells [27]. Dipicolylamine-appended fluorescein has also been reported as a highly effective fluorescent and colorimetric sensor for pyrophosphate in 100% aqueous solution [28]. A fluorescein derivative bearing a boronic acid group also was developed as a fluorescent chemosensor for fluoride ions [29]. In contrast, Sparano et al. reported on the effect of conformation on fluorescence quenching in a series of DCF derivatives in which nitrogen atoms are possible quenching agents [30] and subsequent utilization of the DCF derivatives as fluorescent sensors for specific RNA [31].

This paper concerns a simple, Hg^{2+}-selective chemosensor **1** based upon the DCF molecular framework with appended coumarin moieties, the latter acting as an internal standard for the selective ratiometric signalling of Hg^{2+} ions using dissimilar fluorescence changes in DCF and the coumarin fluorophores of **1**.

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2. Experimental section

2.1. General

2′,7′-Dichlorofluorescein, piperazine, and 4-bromomethyl-6,7-dimethoxycoumarin were purchased from Aldrich Chemical Co. and used without further purification. All solvents used for the measurements of UV–vis and fluorescence spectra were purchased from Aldrich Chemical Co. as ‘spectroscopic grade’. NMR measurements were performed with Varian Gemini-2000 (300 MHz) or Varian VNS (600 MHz) spectrometer. The solvents for NMR measurements were $CDCl_3$, CD_3OD, or DMSO-d_6. All peaks were given as δ in ppm and were related to the signals of residual non-deuterated peaks. UV–vis spectra were recorded with a Jasco V-550 spectrophotometer. Fluorescence spectra were measured on an Aminco-Bowman Series 2 Spectrophotometer. Mass spectra were recorded on a JEOL JMS-600W in FAB mode or Micromass Autospec mass spectrometer in EI mode. TLC analyses were performed on silica gel plates and flash chromatography was conducted by using silica gel column packages purchased from Biotage.

2.2. Preparation of **2**

To a solution of piperazine (870 mg, 10 mmol) in 100 mL of acetonitrile was added 4-bromomethyl-6,7-dimethoxycoumarin (298 mg, 1 mmol) and stirred overnight at room temperature. Volatiles were removed under reduced pressure and the residue was partitioned between dichloromethane and water. The organic phase was washed with water and evaporated and the crude product was purified by column chromatography (silica gel, CH_2Cl_2:MeOH = 5:1). The yield was 81%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.42 (s, 1H), 7.03 (s, 1H), 6.28 (s, 1H), 3.84 (s, 3H), 3.80 (s, 3H), 3.62 (s, 2H), 2.69 (s, 4H), 2.48 (s, 1H) 2.38 (s, 4H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 160.6, 152.9, 152.3, 149.1, 145.4, 111.0, 110.9, 106.4, 100.0, 58.9, 56.1, 55.9, 54.1, 45.7; HRMS (EI) *m/z* calcd for $[M]^+$, $C_{16}H_{20}N_2O_4$, 304.1423, found 304.1440.

2.3. Preparation of **1**

Compound **2** (88 mg, 0.28 mmol) was treated with para-formaldehyde (70 mg, 2.4 mmol) in CH_3CN (10 mL) and refluxed for 30 min. 2′,7′-Dichlorofluorescein (46 mg, 0.12 mmol) in 10 mL of H_2O/CH_3CN (1:1) was added to the solution. The reaction mixture was heated under reflux for 1 day. The solvents were removed completely under reduced pressure and the residue was purified by the column chromatography (silica gel, CH_2Cl_2:MeOH = 9:1) to afford compound **1** as an orange-red solid (64 mg, 54%). ^{1}H NMR (300 MHz, CD_3OD) δ 8.07 (d, $J = 6$ Hz, 1H), 7.64–7.60 (m, 2H), 7.20–7.13 (m, 5H), 6.79 (s, 2H), 6.29 (s, 2H), 4.17 (s, 4H), 3.86 (s, 6H), 3.81 (s, 6H), 3.68 (s, 4H), 3.02 (s, 8H), 2.72 (s, 8H); ^{13}C NMR (150 MHz, CD_3OD) δ 175.2, 173.8, 173.7, 163.8, 159.7, 157.0, 154.5, 154.7, 150.9, 147.7, 141.3, 134.3, 131.1, 130.8, 130.6, 130.5, 129.8, 129.1, 112.4, 112.1, 111.7, 106.8, 101.0, 59.2, 56.9, 56.8, 54.2, 53.2, 51.9; HRMS (FAB-negative, *m*-NBA) *m/z* calcd for $[M{-}H]^-$, $C_{54}H_{51}Cl_2N_4O_{13}$, 1033.2835, found 1033.2792.

2.4. General UV–vis and fluorescence spectra measurements

Since the chemosensor was not fully soluble in 100% aqueous media, minimum amount of DMSO was used as a solubilizing medium. Stock solution of **1** was prepared in DMSO and the UV–vis and fluorescence spectra were obtained in mixed DMSO/aqueous buffer (1:99) solution. For UV–vis measurements, sample solutions were obtained by mixing appropriate amount of stock solution of **1** (1.0×10^{-2} mol L^{-1} in DMSO) with stock solution containing metal perchlorate and acetate buffer (20×10^{-3} mol L^{-1}) in water and finally diluted with buffer to make the solution having desired concentrations of chemosensor **1**, metal ions, and buffer in aqueous 1% DMSO solution. Fluorescence measurements were carried out similarly with a slit width of 5 nm.

2.5. Association constant and detection limit

The binding stoichiometry of **1** with Hg^{2+} ions was determined by using Job's plot [32]. For the Job plot analyses, a series of solutions with varying mole fraction of metal ions were prepared by maintaining the total **1** and Hg^{2+} ion concentration constant (2.0×10^{-5} mol L^{-1}). The fluorescence emission was measured for each sample by exciting at 340 nm and spectra were measured from 350 to 650 nm. Fluorescence intensity at 527 nm was measured for each solution and the values of $I_o(1 - X_{Hg(II)}) - I$ (where $X_{Hg(II)}$ is the mole fraction of Hg^{2+}) were calculated and

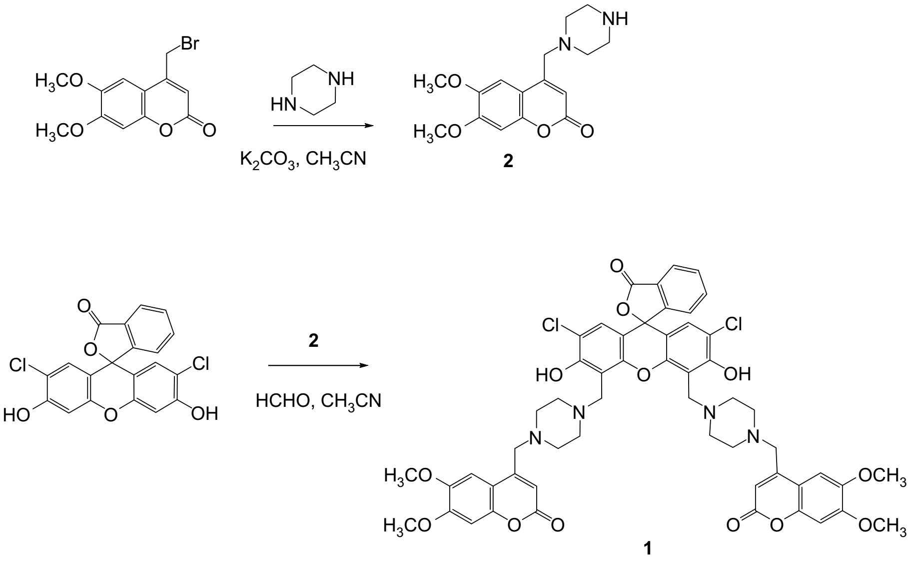

Fig. 1. Synthesis of DCF-coumarin chemosensor **1**.

plotted against the $X_{Hg(II)}$ of final solution. The association constant was calculated by a nonlinear least squares fit of the titration data of the chemosensor **1** with Hg^{2+} ions by using a DynaFit program obtained from Biokin [33]. Detection limit was calculated from a plot of the fluorescence changes as a function of log[Hg^{2+}] following the literature [34]. A linear regression curve was fitted to the intermediate values of the sigmoidal plot. The point at which this line crossed the ordinate axis was taken as the detection limit.

3. Results and discussion

DCF-coumarin **1** was prepared in moderate yield by Mannich reaction of DCF with piperazinyl-coumarin **2**, which was prepared by reaction of piperazine with 4-bromomethyl-6,7-dimethoxycoumarin (Fig. 1). In compound **1**, two fluorophore species – one DCF subunit and two coumarin subunits – were connected by piperazine spacers which can act as binding sites for selective complexation of the targeted metal ions. The chemosensing behavior of **1** was investigated by UV–vis and fluorescence measurements.

The UV–vis absorption spectrum of **1** in 1% DMSO solution (DMSO:H_2O = 1:99, v/v, buffered at pH 4.8 with acetate buffer) consisted of a moderate absorption band around 340 nm and a strong band around 505 nm, which are characteristics of the coumarin and DCF moieties, respectively (Fig. 2). The absorption spectrum did not change significantly in the presence of representative alkali (Na^+, K^+), alkaline earth (Mg^{2+}, Ca^{2+}), or transition metal ions (Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}). However, upon interaction with Hg^{2+} ions, the absorption of the DCF moiety was considerably broadened and red-shifted from 505 to 515 nm with a shoulder at 530 nm, while that of the coumarin moiety was not significantly affected. The color of the solution concomitantly changed from green to pink, allowing for facile naked-eye detection of Hg^{2+} ions (inset of Fig. 2).

To have an insight into the fluorescence signalling behavior of **1**, changes in fluorescence spectra in the presence of various metal ions were compared at three representative solutions having different pH (acetate, hepes, and tris buffered at pH 4.8, 7.0, and 8.0, respectively). The selectivity toward Hg^{2+} ions was most pronounced in acetate buffered solution and, therefore, the following investigation for the chemosensing behaviors of **1** were carried out in acetate buffered aqueous 1% DMSO solution at pH 4.8. The fluorescence spectrum of **1** in aqueous 1% DMSO solution consisted of a strong emission characteristic of the DCF moiety around 523 nm and a relatively moderate emission for the coumarin moiety around 439 nm (Fig. 3). The fluorescence of DCF unit in compound **1** should be quenched due to photoinduced electron transfer (PET) from piperazinyl unit [30]. However, in acetate buffer (pH = 4.8), the amino atoms of piperazinyl unit might be protonated and induced the blockage of PET process, that resulted in strong emission of DCF unit. Upon interaction with various metal ions, Hg^{2+} ions solely induced a markedly decreased emission of the DCF moiety at 523 nm and a slightly increased fluorescence for the coumarin moiety. The solution color under illumination with a UV lamp changed from bright green to blue (inset of Fig. 3). Other metal ions revealed relatively insignificant effects on the emissions of both fluorophores, with the exception of Cu^{2+} and Pb^{2+}.

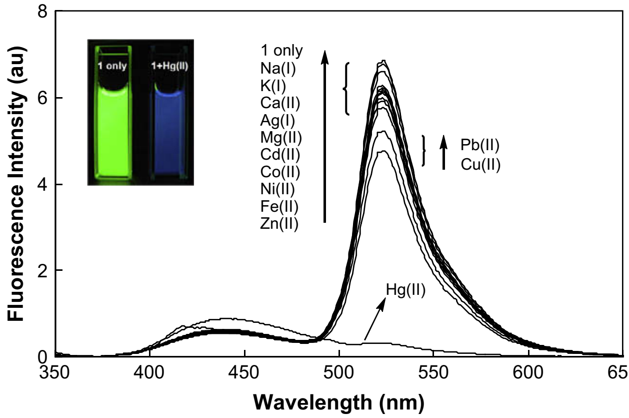

Fig. 3. Fluorescence spectra of **1** in the presence of various metal ions in aqueous 1% DMSO. Inset shows the picture of **1** in the absence and presence of Hg^{2+} ions under illumination with a UV lamp. [**1**] = 5.0×10^{-6} mol L^{-1}, [M^{n+}] = 5.0×10^{-4} mol L^{-1}, pH 4.8 acetate buffer (10×10^{-3} mol L^{-1}). λ_{ex} = 340 nm.

The responses in DCF region were somewhat scattered in the presence of various tested metal ions, however, the changes in the fluorescence were more conveniently analyzed using the relatively invariant coumarin emission as an internal standard. As shown in Fig. 4, the fluorescence intensity ratio of the DCF and coumarin subunits (I_{525}/I_{445}), which was calculated using the fluorescence intensities at 525 and 445 nm, clearly demonstrated the pronounced Hg^{2+} selectivity of **1**. Upon interaction of **1** with Hg^{2+} ions, the ratio (I_{525}/ I_{445}) for the **1**-Hg^{2+} system (0.36) decreased

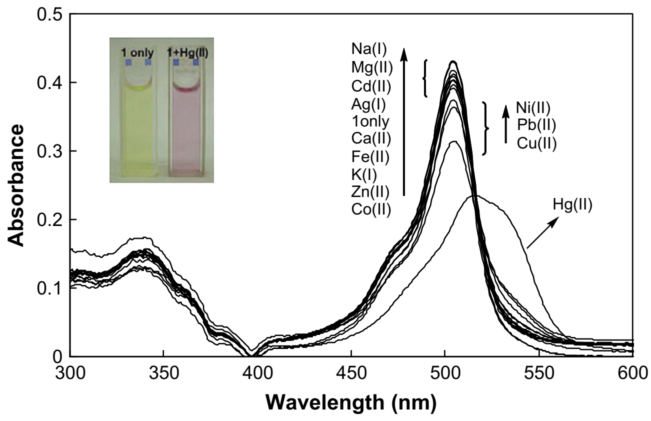

Fig. 2. UV–vis spectra of **1** in the presence of various metal ions in aqueous 1% DMSO. Inset shows the picture of **1** in the absence and presence of Hg^{2+} ions. [**1**] = 1.0×10^{-5} mol L^{-1}, [M^{n+}] = 1.0×10^{-3} mol L^{-1} in DMSO:H_2O = 1:99, pH 4.8 (acetate buffer, 10×10^{-3} mol L^{-1}).

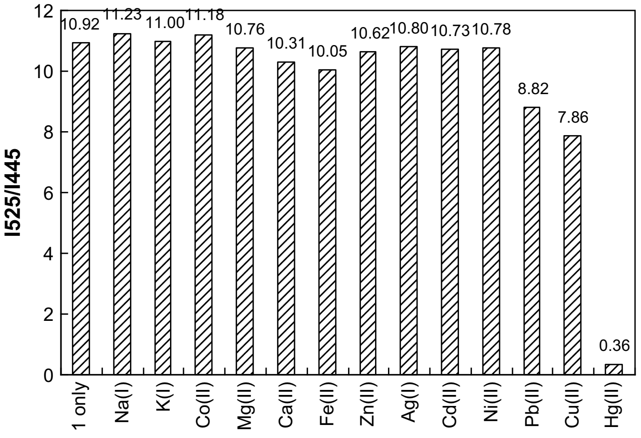

Fig. 4. Ratiometric behavior (I_{525}/I_{445}) of **1** in the presence of various metal ions in aqueous 1% DMSO solution. [**1**] = 5.0×10^{-6} mol L^{-1}, [M^{n+}] = 5.0×10^{-4} mol L^{-1}, pH 4.8 (acetate buffer, 10×10^{-3} mol L^{-1}). λ_{ex} = 340 nm.

more than 30-fold compared with free **1** (10.92). Although Cu^{2+} (7.86) and Pb^{2+} (8.82) also induced slight changes in I_{525}/I_{445}, the other metal ions exhibited relatively small variations within the range between 10.05 (Fe^{2+}) and 11.23 (Na^{+}). Generally the on–off type signalling system due to the quenching nature of the complex formation suffers from uncertainty in absolute fluorescence measurements. The use of internal reference which has relatively invariant response, such as coumarin moiety in the present system, might enhance the reliability of the signalling in quantitative analytical viewpoint.

To gain more insight into the practical applicability of compound **1** in Hg^{2+} signalling, competition experiments on the signalling of Hg^{2+} ions by **1** in the presence of common coexisting metal ions were carried out. Fluorescence changes I_{525}/I_{445} of the **1**-Hg^{2+} system, which was obtained by the treatment of **1** with 10 equiv of Hg^{2+} ions, were not significantly affected by the background presence of 100 equiv of other metal ions (Fig. 5). The intensity ratios for the surveyed metal ions varied only in a relatively limited range of 0.42 (Ni^{2+}) to 0.96 (Fe^{2+}), except for a ratio of 4.97 for somewhat interfering Ag^{+} ion.

The quantitative analytical behavior of **1** for Hg^{2+} signalling was investigated by fluorescence titration with $Hg(ClO_4)_2$ in the same solvent (Fig. 6). As the concentration of Hg^{2+} ions increased, the fluorescence of the DCF unit (I_{525}) decreased significantly. However, the fluorescence of the coumarin moieties (I_{445}) increased moderately. The ratio (I_{525}/I_{445}) decreased as a function of Hg^{2+} ions from 9.7 to 0.26 for **1** in the absence and presence of 20 equiv of Hg^{2+} ions, respectively; after 20 equiv, the ratio did not significantly change (inset of Fig. 6). This observation implies that compound **1** could quantitatively signal Hg^{2+} ions in concentrations up to 2.0×10^{-4} mol L^{-1}. A Job's plot [32] indicated that a 1:1 complexation of the **1**-Hg^{2+} system (Fig. 7). The association constant, K_{assoc}, was calculated by nonlinear curve fitting procedure of the titration results [33] and found to be 4.5×10^{4} mol^{-1} L. The detection limit [34] for sensing Hg^{2+} ions by **1** in aqueous 1% DMSO was also estimated from the titration results and found to be 4.3×10^{-6} mol L^{-1}.

The selective Hg^{2+} signalling by **1** seems to be due to the selective complexation of Hg^{2+} ions with two adjacent piperazine moieties of **1**, similar to that observed for the complexation of Hg^{2+} and Cu^{2+} with 2,6-bis(piperazinylmethyl)pyridyl-based systems having two convergent piperazine moieties as the binding site [35,36]. Piperazine moiety has been utilized as an efficient ligand for the complexation of transition metal ions such as in open-chain polyamine ligands with rigid double connecting bridges [37] and in catalysts based on Cu^{2+} complexes of 1,4-bis(pyridin-2-ylmethyl)piperazine for the olefin aziridination [38]. Complexation of Hg^{2+} ions resulted in quenching of the fluorescence of the DCF moiety and slightly enhanced fluorescence of the coumarin moiety. That might be due to the complexation induced modulation of the PET and fluorescence resonance energy transfer (FRET) processes as well as quenching nature of the complexed mercury ions [3]. Quenching of the DCF moiety by the complexed Hg^{2+} ions was evidenced by the observation that the fluorescence response of **1** in the presence of Hg^{2+} ions, when directly exciting DCF unit ($\lambda_{ex} = 523$ nm) under the identical conditions, was not significantly different from Fig. 3 ($\lambda_{ex} = 340$ nm). On the other hand, a slight increase in coumarin fluorescence might be due to the Hg^{2+}-induced suppression of FRET between coumarin and DCF moiety [39]. For a combination of coumarin and DCF units, there exists a distinct FRET from coumarin unit to DCF unit due to the somewhat overlap between the emission of coumarin unit and absorption of DCF unit (400–480 nm). Upon interaction with Hg^{2+} ions, the absorption of DCF unit was considerably broaden with a red shift and concomitantly the overlap between the emission of coumarin unit and absorption of DCF unit became lower. Therefore, the emission of DCF unit became lower, while the emission of

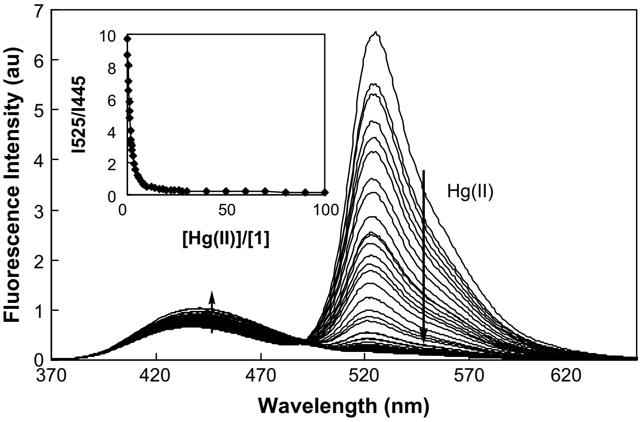

Fig. 6. Titration of **1** with Hg^{2+} ions. Inset shows the changes in the fluorescence intensity ratio (I_{525}/I_{445}). $[\mathbf{1}] = 2.0 \times 10^{-5}$ M in aqueous 2% DMSO solution, pH 4.8 (acetate buffer, 10×10^{-3} mol L^{-1}). $\lambda_{ex} = 340$ nm.

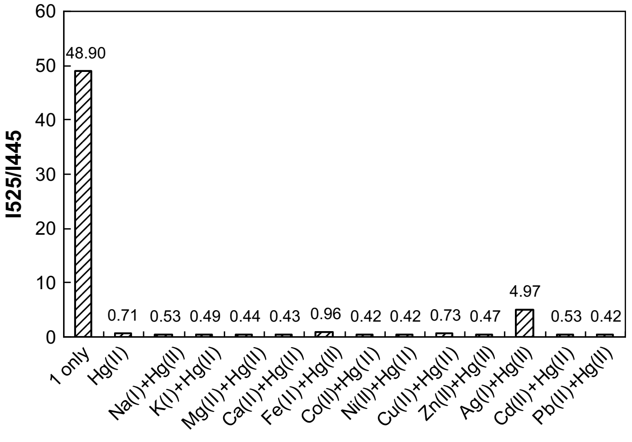

Fig. 5. Ratiometric behavior (I_{525}/I_{445}) of **1** in the presence of Hg^{2+} and various coexisting metal ions in aqueous 1% DMSO solution. $[\mathbf{1}] = 1.0 \times 10^{-5}$ mol L^{-1}, $[Hg^{2+}] = 1.0 \times 10^{-4}$ mol L^{-1}, $[M^{n+}] = 1.0 \times 10^{-3}$ mol L^{-1}, pH 4.8 (acetate buffer, 10×10^{-3} mol L^{-1}). $\lambda_{ex} = 340$ nm.

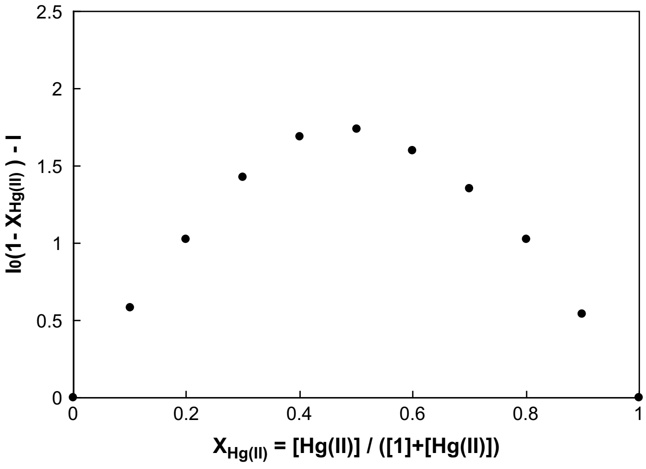

Fig. 7. Job's plot of 1-Hg^{2+} system. $[\mathbf{1}] + [Hg^{2+}] = 2.0 \times 10^{-5}$ mol L^{-1}. In aqueous 1% DMSO solution at pH 4.8 (acetate buffer, 10×10^{-3} mol L^{-1}).

coumarin unit exhibited a little higher due to less efficient energy transfer.

4. Conclusions

A novel chemosensor was prepared by combining two well-known fluorophores, coumarin and dichlorofluorescein. The compound effectively signals Hg^{2+} ions in both chromogenic and fluorogenic chemosensing modes. The conjugate dye exhibited well-defined, visible, Hg^{2+}-selective chromogenic behavior in turning from green to pink, which allowed naked-eye detection of Hg^{2+} ions in aqueous 1% DMSO solution. The turn-off type changes in fluorescence of the dichlorofluorescein moiety can be conveniently analyzed by a ratiometric approach using the coumarin emission as an internal reference. Hg^{2+} selectivity of the chemosensor was not significantly affected by the presence of common physiologically and environmentally important alkali, alkaline earth, and transition metal ions. The dichlorofluorescein–coumarin conjugate dye could signal Hg^{2+} ions in submillimolar concentration range in the presence of common coexisting metal ions in aqueous environment.

Acknowledgments

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