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Selective Ratiometric Signaling of Hg^{2+} Ions by a Fluorescein-Coumarin Chemodosimeter

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The design of selective and sensitive signaling systems for biologically important metal ions has attracted much research interest.[1] Hg^{2+} signaling is particularly important, because of its toxic impact on biological systems and the environment.[2] Many of the Hg^{2+} signaling systems which have been designed suffered from on-off type signaling, due to the intrinsic quenching nature of Hg^{2+} ions.[3] In view of this, a signaling system which exhibits turn-on type signaling or ratiometric sensing is highly desirable for the development of new Hg^{2+}-selective signaling systems.

Among the many well-designed signaling approaches, chemodosimeters are particularly attractive, due to their advantages of high selectivity and characteristic accumulative effect for analyte determination.[4] Particularly, the chemodosimetric determination of Hg^{2+} ions has attracted much research interest.[5] This technique is based on well-known molecular transformations, such as Hg^{2+}-induced ring opening or the cyclization of rhodamine-hydrazide,[6] regenerative chemodosimetric behavior based on Hg^{2+}-induced squaraine dye formation,[7] and the desulfurization of thiocarbonyl derivatives.[8]

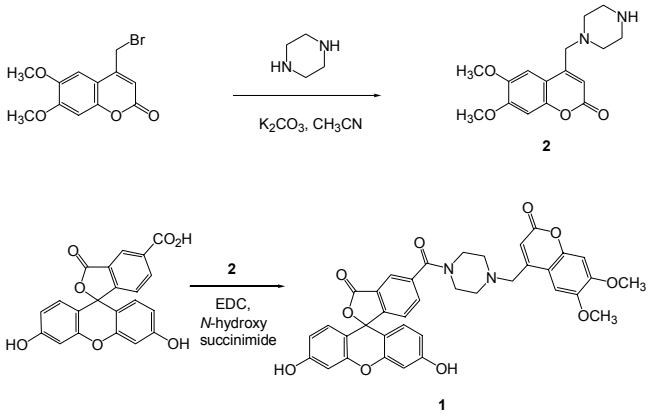
Ratiometric measurements have important features in that they permit a signal ratio approach and, thus, increase the dynamic range and provide a built-in correction for environmental effects.[9,10] If two-band wavelength ratiometric sensing with a single reporter dye is used as a fluorescence reporter, the ratio of the intensities of the two bands can provide a sensitive and convenient indicator of the analyte binding. Fluorescence resonance energy transfer (FRET) has been extensively used as a design tool for the construction of fluorescent signaling and imaging systems.[11] The FRET generally arises from the interaction between a pair of fluorophores and thus, it is an effective mechanism for regulating the states of a fluorophore. In fact, the fluorescence intensity of an independent fluorophore is able to be tuned by controlling the FRET process in response to external stimulants, so that the corresponding molecular-level signaling devices can be constructed.[12] Several sophisticated systems featuring the FRET between coumarin and fluorescein derivatives for the signaling of Zn^{2+} ions,[13] hydrogen peroxide,[14] and phosphodiesterase activity[15] have been developed.

We recently reported a simple Hg^{2+}-selective chemodosimeter consisting of 2',7'-dichlorofluorescein by utilizing the selective mercuration of the xanthene moiety.[16] The mercuration resulted in the chromogenic and fluorogenic signaling of the Hg^{2+} ions for simple fluorescein derivatives. In this paper, we report a new fluorescein-coumarin conjugate which showed ratiometric and chemodosimetric signaling behavior by FRET. Compound **1** was designed by combining the Hg^{2+}-selective chemodosimetric feature of the fluorescein moiety and the FRET donor capability of coumarin.

The fluorescein-coumarin derivative **1** was prepared in moderate yield by the condensation of 5-carboxyfluorescein (EDC, *N*-hydroxysuccinimide) with piperazinomethyl-coumarin **2**,[17] which was prepared by the reaction of 4-bromomethyl-6,7-dimethoxycoumarin with piperazine (Scheme 1). In compound **1**, the two fluorophoric species, coumarin and fluorescein, which have significantly overlapped emission and absorption profiles, were connected by a piperazine spacer.

Compound **1** showed two strong absorption bands at 457 and 480 nm corresponding to the fluorescein unit and a moderate absorption band at 349 nm for the coumarin moiety. Treatment of compound **1** with 100 equiv of various metal ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Ag^+, Cd^{2+}, Hg^{2+}, and Pb^{2+}) induced no significant changes in its UV-vis absorption spectra except for Hg^{2+} and Cu^{2+} ions (Figure 1). In the presence of Hg^{2+} ions, the absorption intensity of the fluorescein moiety was significantly diminished and concomitantly red shifted to 483 and 511 nm, respectively. The resulting solution color changed from green to reddish orange and was discernible by the naked-eye. On the other hand, the absorption band of the coumarin moiety was not appreciably affected.

The fluorescence spectra of **1** in aqueous 10% DMSO revealed a strong emission at 525 nm which is characteristic of the fluorescein subunit (Figure 2). Although the excitation was carried out at 340 nm for the coumarin fluorophore, a weak



Scheme 1. Preparation of chemodosimeter **1**

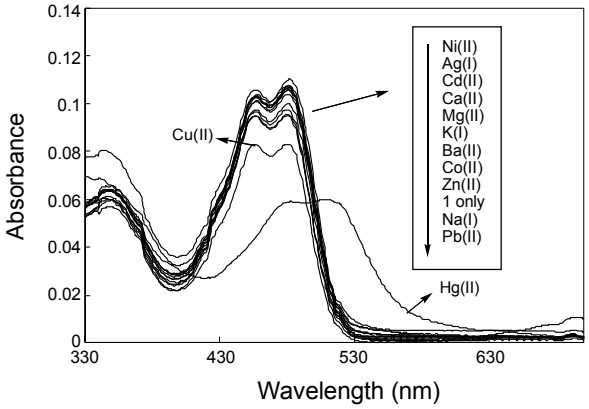


Figure 1. UV-vis spectra of **1** in the presence of various metal ions in aqueous 10% DMSO. [**1**] = 1.0×10^{-5} M, [M^{n+}] = 1.0×10^{-3} M in DMSO : H_2O = 10 : 90, v/v, pH 4.8 (acetate buffer, 10 mM).

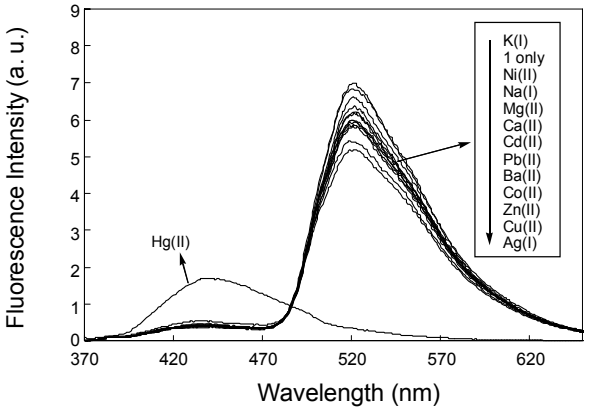


Figure 2. Fluorescence spectra of **1** in the presence of various metal ions in aqueous 10% DMSO. [**1**] = 5.0×10^{-6} M, [M^{n+}] = 5.0×10^{-4} M, pH 4.8 acetate buffer (10 mM). λ_{ex} = 340 nm.

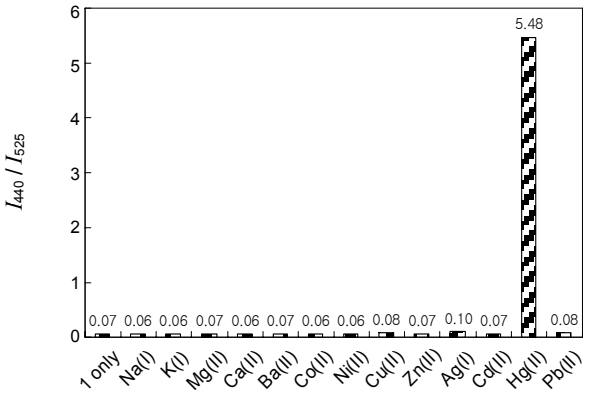
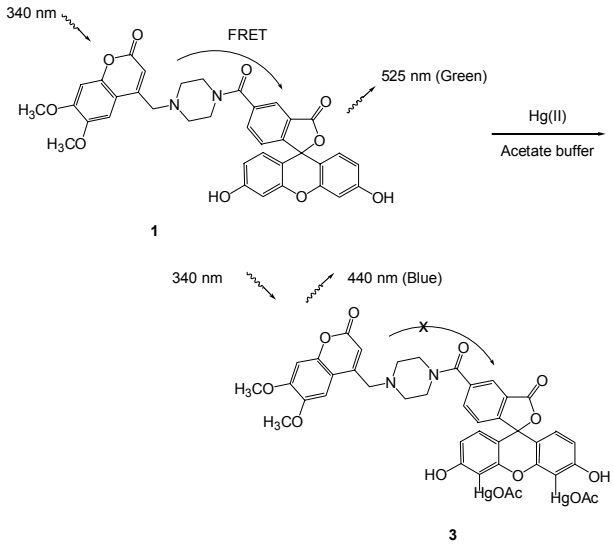


Figure 3. Ratiometric behavior of **1** (I_{440}/I_{525}) in the presence of various metal ions in aqueous 10% DMSO. [**1**] = 5.0×10^{-6} M, [M^{n+}] = 5.0×10^{-4} M, pH 4.8 (acetate buffer, 10 mM). λ_{ex} = 340 nm.

emission at around 440 nm was observed for the coumarin subunit. This implies that an efficient FRET was operative between the coumarin and fluorescein subunits of **1**. Upon its interaction with the Hg^{2+} ions, the fluorescence of the fluorescein unit was significantly reduced, while that of the coumarin unit moderately increased. The solution color under illumination with a UV lamp changed from green to blue. The other metal ions induced relatively insignificant changes in the emission spectra of **1** in the coumarin region, but with some responses in fluorescein region.



Scheme 2. Hg^{2+}-selective signaling mechanism of **1**. For the sake of clarity, only the dimercurated product **3** is shown.

The Hg^{2+}-selective signaling is due to the selective mercuration at the 2',4',5',7'-positions of the xanthene ring of **1** (Scheme 2). Upon mercuration, the fluorescence of the fluorescein unit disappeared almost completely. Subsequently, the FRET from the coumarin donor to the fluorescein acceptor is no longer possible and the fluorescence of the coumarin donor was enhanced. The Hg^{2+}-induced transformation was previously confirmed by the ^1H and ^{13}C NMR spectra for dichlorofluorescein.[16] In the present case, the mercuration is, in principle, possible up to tetramercuration successively at the 2'-, 4'-, 5'-, and 7'-positions of the xanthene ring of **1**. In scheme 2, only the dimercurated product **3** was shown for the sake of clarity.

The Hg^{2+} selectivity of **1** was well-illustrated again by the ratiometric analysis using the ratio of the fluorescence intensities of the coumarin and fluorescein moieties (Figure 3). With Hg^{2+} ions, the ratio of the fluorescence intensities observed at 440 and 525 nm (I_{440}/I_{525}) increased about 78-fold compared with **1** alone, while the other metal ions had no significant effects and the ratio varied within a relatively narrow region between 0.89-fold for the **1**-K^+ and 1.6-fold for the **1**-Ag^+ system.

The Hg^{2+}-selective signaling of **1** was not significantly affected by the presence of the other coexisting metal ions. The competition experiments for the signaling of the **1**-Hg^{2+} system were carried out by the treatment of **1** with 10 equiv of Hg^{2+} ions in the presence of 100 equiv of the coexisting metal ions. As can be seen from Figure 4, the fluorescence intensity ratio (I_{440}/I_{525}) of the **1**-Hg^{2+} system varied in relatively narrow region between 4.11 (Zn^{2+}) and 4.60 (Ba^{2+}), except for somewhat interfering Ag^+ ions (3.61).

Finally, the quantitative analytical behavior of **1** was investigated by fluorescence titration with Hg^{2+} ions. Upon the addition

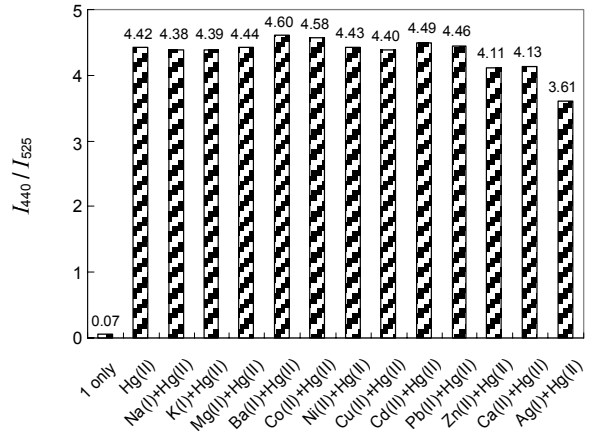


Figure 4. Ratiometric behavior (I_{440}/I_{525}) of **1** in the presence of Hg^{2+} and various coexisting metal ions in aqueous 10% DMSO. [**1**] = 5.0 × 10^{-6} M, [Hg^{2+}] = 1.0 × 10^{-4} M, [M^{n+}] = 5.0 × 10^{-4} M, pH 4.8 (acetate buffer, 10 mM).

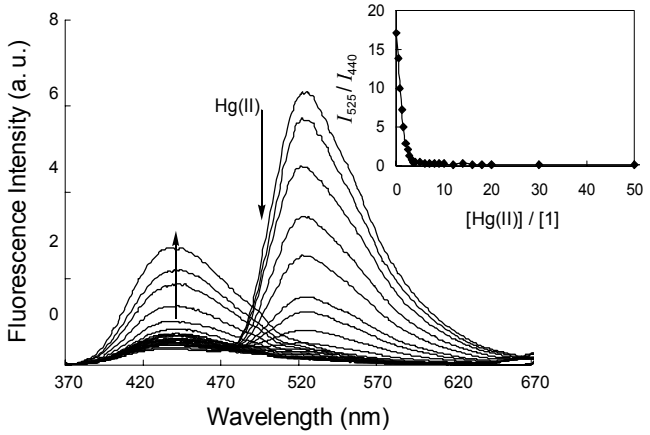


Figure 5. Titration of **1** with Hg^{2+} ions. The inset shows the changes in the fluorescence intensity ratio (I_{525}/I_{440}). [**1**] = 1.0 × 10^{-5} M in aqueous 10% DMSO, pH 4.8 (acetate buffer, 10 mM). λ_{ex} = 340 nm.

of incremental amounts of Hg^{2+} ions, the fluorescence of **1** steadily decreased, while that of the coumarin moiety increased concomitantly (Figure 5). From this concentration dependent fluorescence change, the detection limit of **1** for the determination of Hg^{2+} ions was estimated to be 9.25 μM.

In summary, we prepared a new chemodosimeter by combining the two well-known fluorophores, coumarin and fluorescein. The compound described herein effectively signals Hg^{2+} ions by the selective mercuration of the xanthene moiety in both chromogenic and fluorogenic signaling modes. The turn-off type changes in the fluorescence of the fluorescein moiety can be conveniently followed by the ratiometric approach in reference to the coumarin emission. The chemodosimeter can signal Hg^{2+} ions at submillimolar concentrations in the presence of common coexisting metal ions in aqueous environments.

Experimental Section

General. 5-Carboxyfluorescein, 4-bromomethyl-6,7-dimethoxycoumarin, *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC), *N*-hydroxysuccinimide, and piperazine were purchased from Aldrich and used without further purification. All solvents used for the measurements of UV-vis and fluorescence spectra were purchased from Aldrich as 'spectroscopic grade'. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini-2000 spectrometer (300 and 75 MHz, respectively). UV-vis spectra were recorded with a Jasco V-550 spectrophotometer. Fluorescence spectra were measured on an Aminco-Bowman Series 2 Spectrophotometer. Mass spectral data were obtained with a Micromass Autospec mass spectrometer. Analytical TLC was performed on silica gel (60F-254) plates (0.25 mm) precoated with a fluorescent indicator. Flash chromatography was carried out by using commercial silica gel (32 - 63 μm) cartridge (Biotage).

Preparation of 2.[17] A mixture of 4-bromomethyl-6,7-dimethoxycoumarin (100 mg, 0.33 mmol) and piperazine (280 mg, 3.34 mmol) was stirred at room temperature for 2 h. The reaction mixture was partitioned between CH_2Cl_2 and water. The organic layer was separated and evaporated. The residue was purified by column chromatography (silica gel, CH_2Cl_2 : CH_3OH = 5 : 1) to yield white colored **2**. Yield: 82%. ^{1}H NMR (300 MHz, CD_3OD) δ 7.49 (s, 1H), 6.98 (s, 1H), 6.37 (s, 1H), 3.92 (s, 3H), 3.87 (s, 3H), 3.69 (s, 2H), 2.87 (m, 4H), 2.55 (m, 4H); ^{13}C NMR (75 MHz, CD_3OD) δ 163.9, 154.9, 154.6, 151.0, 147.7, 112.8, 112.4, 107.7, 101.0, 60.7, 56.9, 56.8, 55.0, 46.6; HRMS (EI) *m/z* calcd for $[M]^+$, $C_{16}H_{20}N_2O_4$, 304.1423, found 304.1413.

Preparation of 1. To a solution of EDC (62 mg, 0.40 mmol) and *N*-hydroxysuccinimide (37 mg, 0.32 mmol) in dry THF, 5-carboxyfluorescein (100 mg, 0.27 mmol) was added and the solution was stirred for 24 h at room temperature. To this mixture, piperazinomethyl-coumarin **2** (91 mg, 0.32 mmol) and *N*,*N*-diisopropylethylamine (0.23 mL, 1.33 mmol) were successively added and stirred for 12 h under nitrogen atmosphere. The reaction mixture was evaporated and the residue was partitioned between water and dichloromethane. The organic phase was separated and evaporated. Resulting product was purified by column chromatography (silica gel, CH_2Cl_2-CH_3OH) to yield **1** as orange colored powder. Yield: 37%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.06 (br s, 1H), 7.72 (br d, *J* = 7.2 Hz, 1H), 7.45 (s, 1H), 7.31 (d, *J* = 7.5 Hz, 1H), 6.95 (s, 1H), 6.86 (d, *J* = 8.7 Hz, 2H), 6.66 (d, *J* = 2.1 Hz, 2H), 6.57 (dd, *J* = 2.4 and 8.8 Hz, 2H), 6.40 (s, 1H), 3.91 (s, 3H), 3.90 (s, 3H), 3.86 (br m, 2H), 3.78 (s, 2H), 3.61 (br m, 2H), 2.73 (br m, 2H), 2.62 (br m, 2H); ^{13}C NMR (75 MHz, CD_3OD) δ 170.5, 169.8, 162.4, 157.0, 155.4, 153.3, 153.2, 149.6, 146.3, 136.4, 130.7, 129.1, 128.9, 127.1, 119.6, 113.0, 111.3, 111.1, 106.2, 105.2, 102.6, 99.5, 58.3, 56.6, 56.4, 53.4, 52.9, 47.8, 42.3; MS (FAB, *m*-NBA) *m/z* calcd for $[M+H]^+$, $C_{37}H_{31}N_2O_{10}$, 663.2, found 663.3.

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