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A highly selective turn-on fluorescence chemosensor for Hg(II) and its application in living cell imaging

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ABSTRACT

A new coumarin derivative (**MS1**) containing an imine moiety and a hydroxyl moiety exhibits an enhanced fluorescence in the presence of Hg^{2+} ions. Other metal ions Al^{3+}, Ca^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} produced only minor changes in the fluorescence values of **MS1**. The binding ratio of **MS1**–Hg^{2+} complexes was determined from the Job plot to be 1:1. The binding constant (K_a) of Hg^{2+} binding to **MS1** was found to be $6.85 \times 10^3\ M^{-1}$. The maximum fluorescence enhancement caused by Hg^{2+} binding to **MS1** was observed in the pH range of 6.5–9.0. Confocal fluorescence microscopy imaging using RAW 264.7 cells showed that **MS1** could be used as an effective fluorescent probe for detecting Hg^{2+} in living cells.

1. Introduction

The development of chemosensors that detect heavy metal ions, such as Cd^{2+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, Pb^{2+}, and Zn^{2+}, has been an important research issue on biological imaging, environmental monitoring, and medical diagnostics [1–3]. Among them, mercury is one of the most toxic heavy metal elements and exists in three forms: elemental, inorganic, and organic mercury. Mercury ions have shown high affinity for thiol groups in proteins, leading to the malfunction of cells and consequently causing many health problems in the brain, kidney, and central nervous system. Its accumulation in the body results in a wide variety of diseases, such as prenatal brain damage; serious cognitive and motion disorders; and Minamata disease [4]. Therefore, in order to specifically detect mercury ions in biological and environmental samples, the design of highly selective and sensitive mercury chemosensors is in high demand.

Several methods for the detection of mercury ions in various samples have been developed, including atomic absorption – emission spectroscopy [5], inductively coupled plasma mass spectroscopy (ICPMS) [6], and inductively coupled plasma–atomic emission spectrometry (ICP-AES) [7]. Although these methods are quantitative, most of these methods require expensive instruments and are not good for on-site analysis. Recently, more attention has been focused on the development of fluorescent chemosensors for the detection of Hg^{2+} ions in biological and environmental samples [8–23].

Numerous molecular probes using different receptors and fluorescent units have been developed for Hg^{2+} detection. Because Hg^{2+} is known as a fluorescence quencher due to spin–orbit coupling [24], most fluorescent chemosensors detect Hg^{2+} through a fluorescence quenching. Due to sensitivity concerns, fluorescent chemosensors detecting metal ions using fluorescence enhancement are more easily monitored than those using fluorescence quenching. This paper reports on a newly designed a coumarin-based fluorescent enhancement Hg^{2+} chemosensor.

In this study, a fluorescent chemosensor (**MS1**) containing an imine moiety and a hydroxyl moiety was designed for metal ion detection. **MS1** exhibits weak fluorescence due to the imine isomerization which has been known to have a non-radiative decay process in the excited state [25]. The binding of metal ions to the chemosensor blocks the imine isomerization and results in considerable fluorescence enhancement of coumarin. The metal ions Al^{3+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} were tested for metal ion binding selectivity with **MS1**, but Hg^{2+} was the only ion that caused a blue emission upon binding with **MS1**. The fluorescence microscopy experiments also demonstrated that **MS1** can be used as a fluorescent probe for detecting Hg^{2+} in living cells.

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2. Experiment

2.1. Materials and instrumentations

All solvents and reagents were obtained from commercial sources and used as received without further purification. UV/vis spectra were recorded on an Agilent 8453 UV/vis spectrometer. NMR spectra were obtained on a Bruker DRX-300 NMR and Varian Unity INOVA-500 NMR spectrometer. Fluorescence spectra measurements were performed on a Hitachi F-7000 fluorescence spectrophotometer. Fluorescent images were taken on a Leica TCS-SP5-X AOBS Confocal Fluorescence Microscope.

2.2. Synthesis of chemosensor MS1

Hydrazine (50%, 333 μL, 10.3 mmol) was added to a solution of 7-hydroxy-2-oxo-2H-chromene-8-carbaldehyde [26] (80 mg, 0.42 mmol) in MeOH (10 mL) and the reaction mixture was reflux for overnight. The solvent was removed under reduced pressure and the crude solid was taken up into a mixture of water and EtOAc. The combined aqueous layer was extracted four times with EtOAc. The combined organic layer was washed with brine, and then dried with Na_2SO_4. The solvent was removed under the reduced pressure and the residue was purified by column chromatography using hexane/ethyl acetate (v/v = 1:1) as an eluent to afford **MS1** as a yellow solid. Yield: 40 mg (47%); mp: 257–258 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ12.77 (b, 1H), 8.38 (s, 1H), 7.96 (d, J = 9.5 Hz, 1H), 7.47 (d, J = 8.5 Hz, 1H), 7.36 (s, 2H), 6.83 (d, J = 8.5 Hz, 1H), 6.26 (d, J = 9.5 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 160.3, 159.7, 151.3, 144.9, 134.6, 128.3, 113.2, 111.6, 110.9, 106.8. MS (EI): m/z (%) = 204.0 (100) $[M+H]^+$, 188 (52), 175 (48), 159 (39). HRMS (EI): calcd. for $C_{10}H_7NO_4$ $[M+H]^+$ 204.0535; found 204.0529.

2.3. Metal ion binding study by UV–vis and fluorescence spectroscopy

Chemosensor **MS1** (10.0 μM) was added with different metal ions (60.0 μM). All spectra were measured in 1.0 mL metanol–water solution (v/v = 4:1, 10 mM HEPES, pH 7.0). The light path length of cuvette was 1.0 cm.

2.4. The pH dependence on Hg^{2+} binding in Chemosensor MS1 studied by fluorescence spectroscopy

Chemosensor **MS1** (10.0 μM) was added with Hg^{2+} (10.0 μM) in 1.0 mL metanol–water solution (v/v = 4:1, 10 mM buffer). The buffers were: pH 3–4, AcONa/AcOH; pH 5–10, HEPES; pH 11–12, Tris.

2.5. Determination of the binding stochiometry and the apparent association constants K_a of Hg(II) binding in chemosensor MS1

The binding stochiometry of **MS1**–Hg^{2+} complexes was determined by Job plot experiments. The fluorescence intensity at 455 nm was plotted against molar fraction of **MS1** under a constant total concentration (20.0 μM). The fluorescence approached a maximum intensity when the molar fraction was 0.5. These results indicate that chemosensor **MS1** forms a 1:1 complex with Hg^{2+}. The association constant (K_a) of **MS1**–Hg^{2+} complexes was determined by the consequent Eq. (1) [27]:

$$\frac{1}{(I - I_0)} = \frac{1}{\{k_a \times (I_{max} - I_0) \times [Hg^{2+}]\}} + \frac{1}{(I_{max} - I_0)} \quad (1)$$

where I is the fluorescence intensity at 455 nm at any given Hg^{2+} concentration, I_0 is the fluorescence intensity at 455 in the absence of Hg^{2+}. The association constant K_a was evaluated graphically by plotting $1/(I - I_0)$ against $1/[Hg^{2+}]$. Typical plots $\{1/(I - I_0)$ *vs.* $1/[Hg^{2+}]\}$ are shown in Fig. 5. Data were linearly fitted according to Eq. (1) and the K_a value was obtained from the slope of the line.

2.6. Cell culture.

RAW 264.7 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 at 37 °C.

2.7. Cytotoxicity assay

The methyl thiazolyl tetrazolium (MTT) assay was used to measure the cytotoxicity of **MS1** in RAW 264.7 cells. RAW 264.7 cells were seeded into a 96-well cell-culture plate. Various concentrations (0, 5, 10, 15, 20, 30 μM) of **MS1** were added to the wells. The

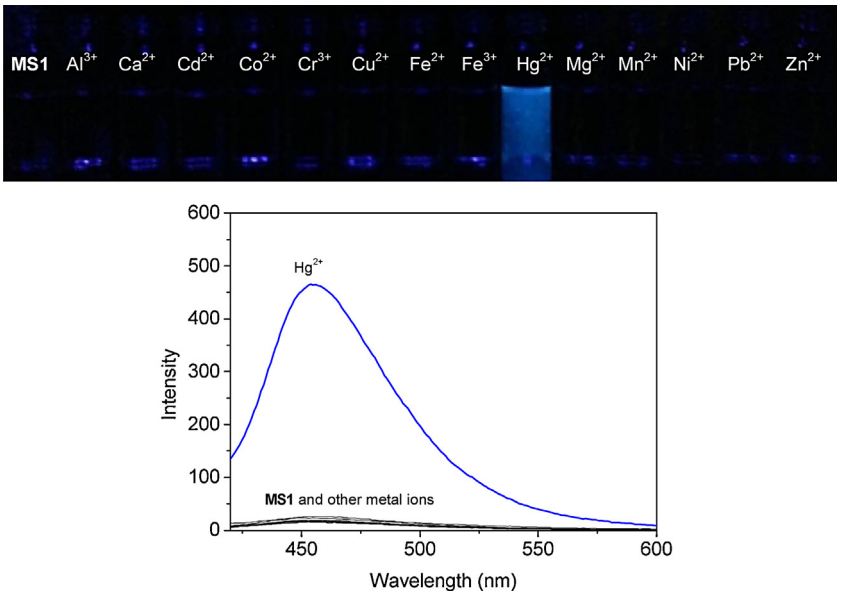
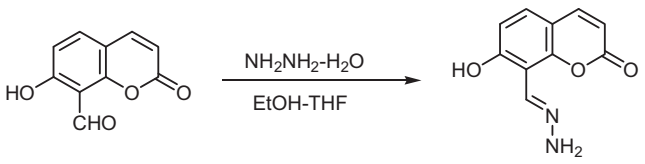


Fig. 1. Fluorescene change of chemosensor MS1 (10 μM) upon addition of various metal ions (60 μM) in methanol–H_2O (v/v = 4/1, 10 mM HEPES, pH 7.0) solutions. The excitation wavelength was 330 nm.



Scheme 1. Synthesis of chemosensor MS1.

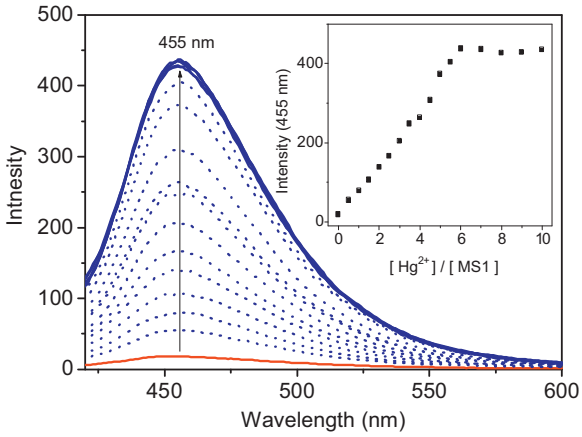


Fig. 2. Fluorescence response of MS1 (10.0 μM) to various equivalents of Hg^{2+} in methanol–water (v/v = 4:1, 10 mM HEPES, pH 7.0) solutions. The excitation wavelength was 330 nm.

cells were incubated at 37 °C under 5% CO_2 for 24 h. 10 μL MTT (5 mg/mL) was added to each well and incubated at 37 °C under 5% CO_2 for 4 h. Remove the MTT solution and yellow precipitates (formazan) observed in plates were dissolved in 200 μL DMSO and 25 μL Sorenson's glycine buffer (0.1 M glycine and 0.1 M NaCl). Multiskan GO microplate reader was used to measure the absorbance at 570 nm for each well. The viability of cells was calculated according to the following equation:

$$\text{Cell viability}\,(\%) = \frac{(\text{mean of absorbance value of treatment group})}{(\text{mean of absorbance value of control group})}.$$

2.8. Cell imaging

The cells cultured in DMEM were treated with 10 mM solutions of Hg^{2+} (2 μL; final concentration: 20 μM) dissolved in sterilized PBS (pH 7.4) and incubated at 37 ° for 30 min. The treated cells were washed with PBS (2 mL × 3) to remove remaining metal ions. DMEM (2 mL) was added to the cell culture, which was then treated with a 10 mM solution of chemosensor **MS1** (2 μL; final concentration: 20 μM) dissolved in DMSO. The samples were incubated at 37 ° for 30 min. The culture medium was removed, and the treated cells were washed with PBS (2 mL × 3) before observation. Fluorescence imaging was performed with a Leica TCS-SP5-X AOBS Confocal microscope. The cells were excited with a white light laser at 350 nm, and emission was collected at 460 ± 25 nm.

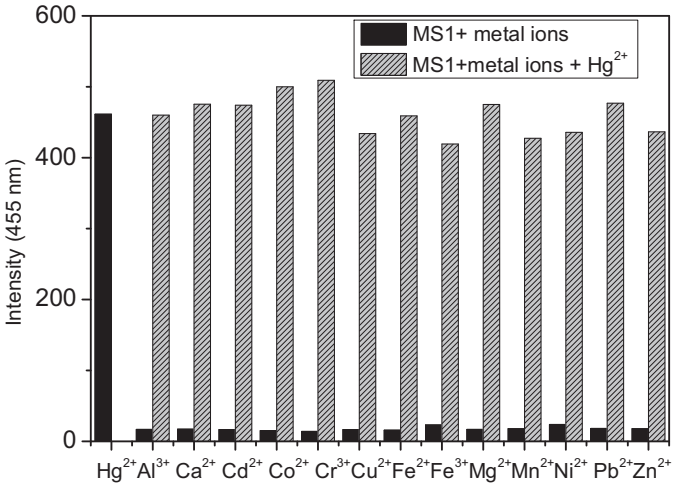


Fig. 3. Fluorescence response of chemosensor MS1 (10.0 μM) to Hg^{2+} (60.0 μM) or 60.0 μM of other metal ions (black bars) and to the mixture of other metal ions (60.0 μM) with 60.0 μM of Hg^{2+} (gray bars) in methanol–water (v/v = 4:1, 10 mM HEPES, pH 7.0) solutions.

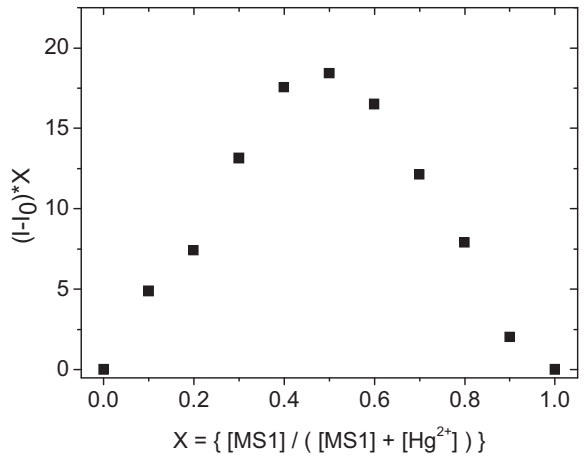


Fig. 4. Job plot of the **MS1**–Hg^{2+} complexes in a methanol–water (v/v = 4:1, 10 mM HEPES, pH 7.0) solutions. The total concentration of MS1 and Hg^{2+} was 20.0 μM. The monitored wavelength was 455 nm.

2.9. Computational methods

Quantum chemical calculations based on density functional theory (DFT) were carried out using a Gaussian 09 program. Ground state geometry optimization of **MS1** was performed using the B3LYP functional and the 6-31G basis set. Ground state geometry optimization of **MS1**–Hg^{2+} complexes was performed using the B3LYP functional and the LANL2DZ basis set.

3. Results and discussion

3.1. Characterization of chemosensor MS1

Chemosensor **MS1** was synthesized by the reaction of hydrazine and 7-hydroxy-2-oxo-2H-chromene-8-carbaldehyde to form an

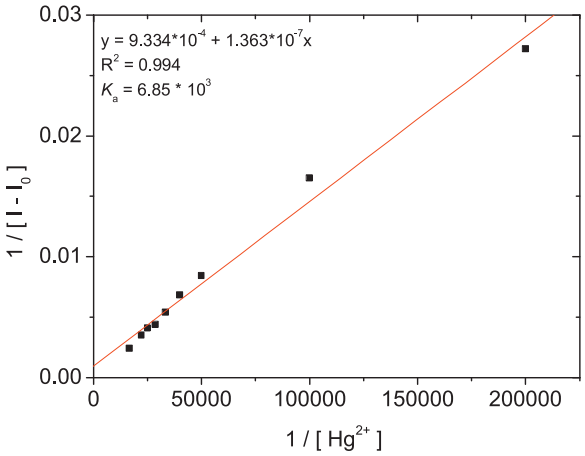


Fig. 5. Benesi–Hildebrand plot of MS1 with Hg^{2+} in methanol/water (v/v = 4:1, 10 mM HEPES, pH 7.0) solutions. The excitation wavelength was 300 nm and observed wavelength was 455 nm. The binding constant was 6.85 × 10^3 M^{-1} for Hg^{2+} binding in MS1.

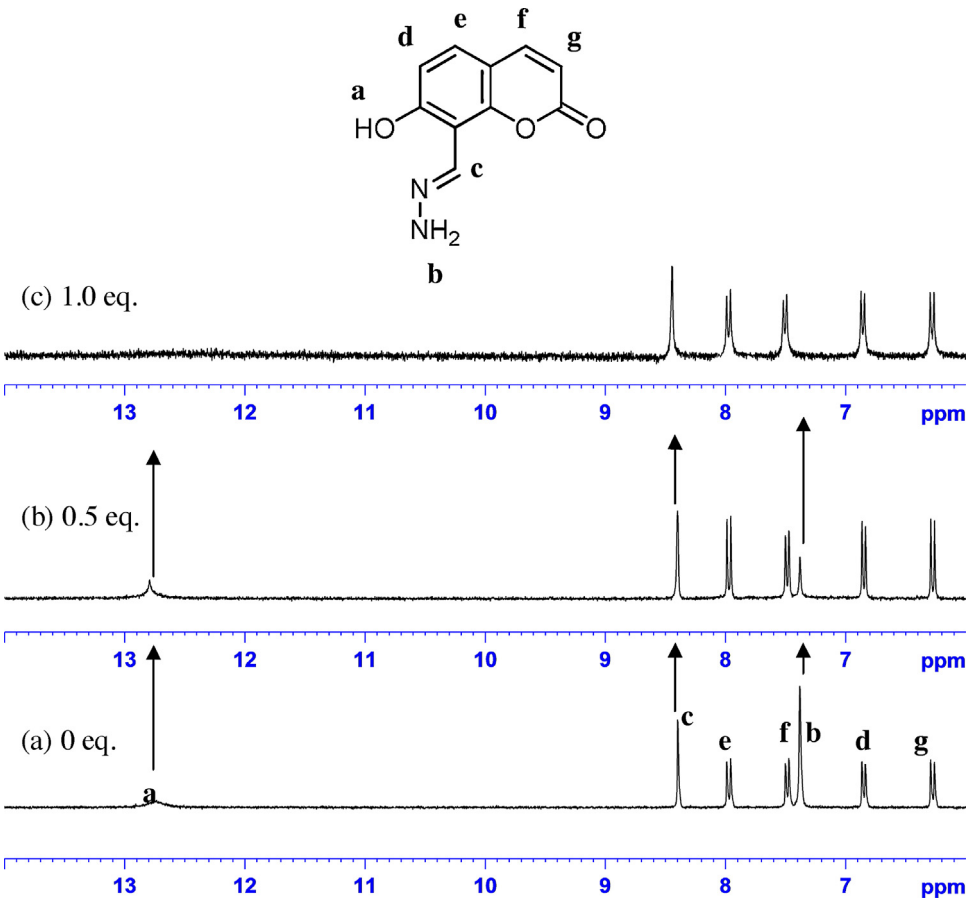


Fig. 6. 1H NMR 300 MHz spectra of MS1 (10.0 mM) upon titration with various equivalents of Hg^{2+} in DMSO-*d6*.

imine bond (Scheme 1). Chemosensor **MS1** is yellow and has an absorption band centered at 330 nm. Chemosensor **MS1** exhibits weak fluorescence ($\Phi = 0.002$) compared to coumarin ($\Phi > 0.5$). This is due to the imine isomerization which has been known to have a non-radiative decay process in the excited state [25]

3.2. Cation-sensing properties

The sensing ability of **MS1** was tested by mixing it with the metal ions Al^{3+}, Ca^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+}. Hg^{2+} was the only ion that caused a blue emission from **MS1** (Fig. 1). During Hg^{2+} titration with **MS1**, a new emission band centered at 455 nm was formed (Fig. 2). After adding 6 equivalents of Hg^{2+}, the emission intensity reached a maximum. The quantum yield of the emission band was 0.039, which is 19-fold that of **MS1** at 0.002. These observations indicate that Hg^{2+} is the only metal ion that readily binds with **MS1**, causing significant fluorescence enhancement and permitting highly selective detection of Hg^{2+}.

To study the influence of other metal ions on Hg^{2+} binding with **MS1**, competitive experiments were performed with other metal ions (60.0 μM) in the presence of Hg^{2+} (60.0 μM) (Fig. 3). It was found that fluorescence enhancement caused by the mixture of Hg^{2+} with most metal ions was similar to that caused by Hg^{2+} alone. None of the other metal ions were found to interfere with the binding of **MS1** with Hg^{2+}.

In order to understand the binding stoichiometry of **MS1**–Hg^{2+} complexes, Job plot experiments were carried out. In Fig. 4, the emission intensity at 455 nm is plotted against molar fraction of **MS1** under a constant total concentration (20.0 μM). Maximum emission intensity was reached when the molar fraction was 0.50. Results indicated a 1:1 ratio for **MS1**–Hg^{2+} complexes, in which one

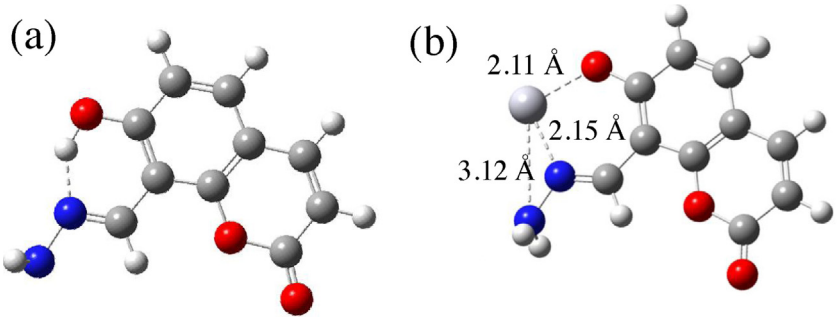


Fig. 7. DFT-optimized structures of (a) MS1 and (b) **MS1**–Hg^{2+} complexes. Blue atom, N; red atom, O; gray-white atom, Hg. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

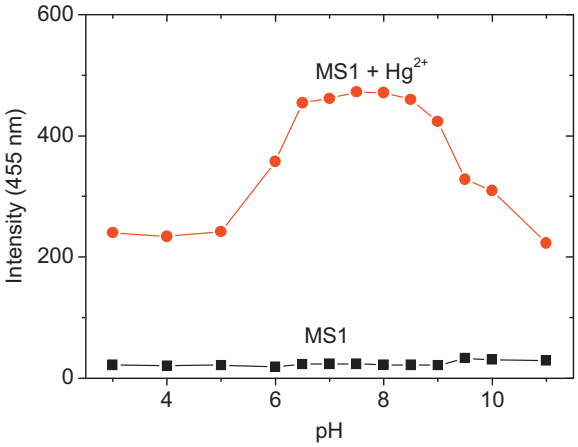


Fig. 8. Fluorescene response (455 nm) of free chemosensor MS1 (10 μM) and after addition Hg^{2+} (60 μM) in MeOH–H_2O solutions (v/v = 4/1, 10 mM buffer). The excitation wavelength was 330 nm.

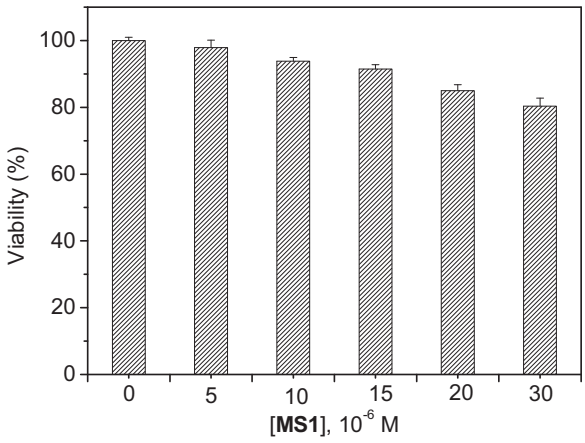


Fig. 9. Cell viability values (%) estimated by an MTT assay versus incubation concentrations of **MS1**. RAW 264.7 cells were cultured in the presence of **MS1** (0–30 μM) at 37 °C for 24 h.

Hg^{2+} ion was bound to one **MS1**. The association constant K_a was evaluated graphically by plotting $1/(I - I_0)$ against $1/[Hg^{2+}]$ (Fig. 5). The data was linearly fit and the K_a value was obtained from the slope and intercept of the line. The association constant (K_a) of Hg^{2+} binding in **MS1** was found to be $6.85 \times 10^3\ M^{-1}$. The detection limit of **MS1** as a fluorescent sensor for the analysis of Hg^{2+} was determined from the plot of fluorescence intensity as a function of the concentration of Hg^{2+} (see Fig. S3 in the supporting information). **MS1** was found to have a detection limit of 0.193 μM, which means it is able to detect Hg^{2+} concentrations in the micro-molar range.

To gain a clearer understanding of the structure of **MS1**–Hg^{2+} complexes, ^1H NMR spectroscopy was employed (Fig. 6). Hg^{2+} is a heavy metal ion and can affect the proton signals that are close to the Hg^{2+} binding site. The ^1H NMR spectra of **MS1** recorded with increasing amounts of Hg^{2+} show that the proton (H_a and H_b) signals at 12.75 ppm and 7.38 ppm disappear as Hg^{2+} was added. Addition of Hg^{2+} also caused the proton (H_c) signal at 8.38 ppm shifted downfield. These observations indicate that Hg^{2+} binds to **MS1** mainly through one oxygen atom and one nitrogen atom.

To elucidate the structures of **MS1** and **MS1**–Hg^{2+} complexes, density functional theory (DFT) calculations were undertaken using the Gaussian 09 software package. Chemosensor **MS1** and **MS1**–Hg^{2+} complexes were subjected to energy optimization by using B3LYP/6-31G and B3LYP/LANL2DZ, respectively. The global minima structures for **MS1** and **MS1**–Hg^{2+} complexes are shown in Fig. 7. The distances of Hg^{2+} from the two nitrogen atoms were 3.12 Å and 2.15 Å, and from the oxygen atom was 2.11 Å.

We performed pH titration of **MS1** to investigate a suitable pH range for Hg^{2+} sensing. As depicted in Fig. 8, the emission intensities of metal-free **MS1** were very low. After mixing chemosensor **MS1** with Hg^{2+}, the emission intensity at 455 nm suddenly increased at pH 6.5 and reached a maximum in the pH range of 6.5–9.0. The emission intensity decreases at pH > 9.0. This indicates poor stability of the **MS1**–Hg^{2+} complexes at high pH. For pH < 6.5, the emission intensity is very low due to the protonation on the amine group, which prevents the formation of **MS1**–Hg^{2+} complexes.

3.3. Living cell imaging

The potential of **MS1** for imaging Hg^{2+} in living cells was investigated next. First, an MTT assay with a RAW 264.7 cell line was used to determine the cytotoxicity of **MS1**. In Fig. 9, the cellular viability was estimated to be greater than 80% after 24 h, which indicates that **MS1** (<30 μM) has low cytotoxicity. Furthermore, the images of cells were obtained using a confocal fluorescence microscope. When RAW 264.7 cells were incubated with **MS1** (10 μM), no fluorescence was observed (Fig. 10a). After the treatment of Hg^{2+}, bright

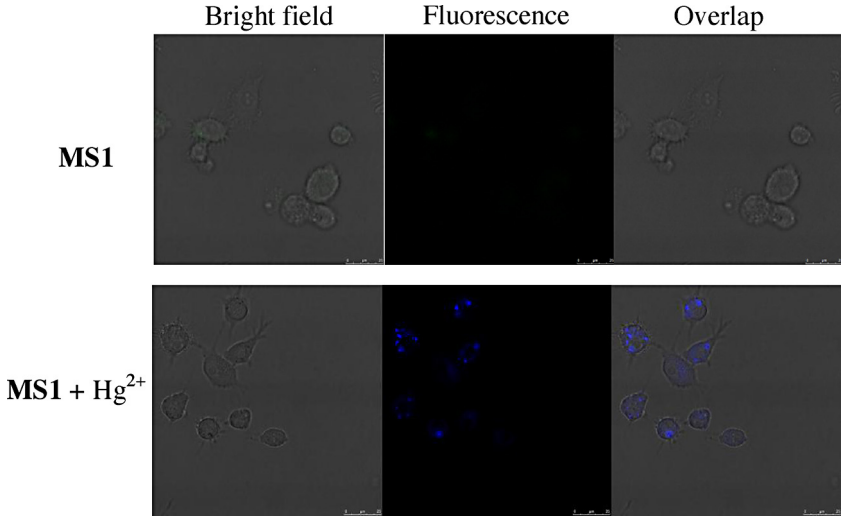


Fig. 10. Fluorescence images of RAW 264.7 cells treated with MS1 and Hg^{2+}. (Left) Bright field image; (middle) fluorescence image; and (right) merged image.

blue fluorescence was observed in the RAW264.7 cells (Fig. 10b). An overlay of fluorescence and bright-field images shows that the fluorescence signals are localized in the intracellular area, indicating a subcellular distribution of Hg^{2+} and good cell-membrane permeability of **MS1**.

4. Conclusion

In conclusion, we developed a coumarin-based fluorescent chemosensor for Hg^{2+} sensing. We observed significant fluorescence enhancement with **MS1** in the presence of Hg^{2+}. However, adding Al^{3+}, Ca^{2+}, Cr^{3+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, or Zn^{2+} to the chemosensor solution caused only minimal changes in fluorescence emission. The optimal pH range for Hg^{2+} detection by **MS1** is 6.5–9.0. In addition, the chemosensor **MS1** has low cytotoxicity and therefore can be applied for detecting Hg^{2+} in living cells.

Supplementary information

^{1}H and ^{13}C NMR spectra of **MS1**, calibration curve of **MS1**–Hg^{2+} in a water–MeOH (v/v = 1/4, 10 mM HEPES, pH 7.0) solutions.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2014.04.077.

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