A Simple and Rapid Fluorescent Probe for Detection of Cr\textsuperscript{3+} Based on a Coumarin Schiff Base in Aqueous Solution

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A new Cr\textsuperscript{3+} probe was synthesized using simple Schiff base reaction, which showed prominent fluorescence increasing switch before and after addition Cr\textsuperscript{3+}. The probe proved to have excellent properties, based on both UV-vis absorption and fluorescence spectra. Those properties included high switching performance, good selectivity, and small interference with other metal ions. The fluorescent change mechanism of the probe was attributed to the combined action between the restricted C=N isomerization and the suppression of highly efficient photo-induced electron transfer (PET) process. Moreover, this fluorescence probe for Cr\textsuperscript{3+} detection also has great potential for bioimaging of cancer cells.

Keywords Fluorescent probe, coumarin Schiff base, Cr\textsuperscript{3+} ions, cell imaging

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Introduction

Cr\textsuperscript{3+} is a necessary trace element in organisms and has important significance in the metabolism of saccharides, lipids, albuminoid and nucleic acids.\textsuperscript{1–3} Chromium deficiency could induce cardiovascular diseases and diabetes.\textsuperscript{4–6} However, high levels of Cr\textsuperscript{3+} not only results in damage to cellular structure and function but also causes genotoxic effects. Due to the biological effect, environmental protection agency has established clear admissible concentration (1.92 \(\mu\)M) of Cr\textsuperscript{3+} ions in natural water.\textsuperscript{11} The World Health Organization has proposed that the maximal permitted intake of Cr\textsuperscript{3+} be limited to amounts of 50 – 200 \(\mu\)g per day.\textsuperscript{12} Therefore, the detection of Cr\textsuperscript{3+} concentration level in circumstances and \textit{in vivo} is a subject of great concern.

The previous detection methods of Cr\textsuperscript{3+} are accurately addressed by ICP-MS and ICP-AES. However, these detection methods come with many inconveniences, including complex sample pretreatment, expensive equipment, and the need for skillful manipulation. Many new sensing technologies of metal ions are being developed, such as fluorescent probes and electrochemical sensors.\textsuperscript{13–15} Fluorescent probes are a very crucial tool on account of their handy operability, low cost, and applicability for \textit{in situ} study and visualization.\textsuperscript{16–19} Recently, some fluorescent probes for Cr\textsuperscript{3+} have been prepared based on different fluorophores, for instance rhodamine, lanthanide complex, anthocyanin and quinoline.\textsuperscript{20–28} Although these probes displayed good performances toward Cr\textsuperscript{3+} ions, some of them only worked in organic medium for water-insoluble or quenching phenomenon in aqueous solutions, others involved high cost because of the rare earth doping, and still others were badly disturbed by Hg\textsuperscript{2+}, Cu\textsuperscript{2+}, Fe\textsuperscript{3+} or Al\textsuperscript{3+} in the sensing procedure of Cr\textsuperscript{3+}. Hence, it is of great significance to develop a novel fluorescent probe for Cr\textsuperscript{3+} with good water solubility, low-cost and less disturbance.

We managed to find our way to the development an excellent fluorescent probe with low-cost and free interference characteristics to recognize Cr\textsuperscript{3+} ions in biological samples.

Experimental

Reagents and apparatus

NMR spectra were obtained on an AVANCE III HD 600 MHz spectrometer in CDCl\(_3\). Melting point was tested using an SGW X-4 melting point instrument. UV-vis spectra were obtained using a Shimadzu UV-2450 spectrometer. Fluorescence data were acquired by an FL-3 spectrometer Horiba Jobin Yvon Inc. Elemental analysis was taken with a Vario EL CHNS elemental analyzer (Elementar, Germany). All experimental procedures were carried out at ambient temperature. Organic solvents were of analytical grade and were purchased from J&K Scientific.

Synthesis

First, 7-diethylamino coumarin (compound 3) and 7-diethylamino coumarin-3-aldehyde (compound 2) were prepared by the known method (Scheme 1).\textsuperscript{20,28} Briefly, 4-N,N-diethylnaminosalicylaldehyde 6.5 g (30 mmol), diethyl malonate 6.0 g (42 mmol), and piperidine 0.5 mL were dissolved in 100 mL C\(_2\)H\(_5\)OH. The mixture was refluxed for 12 h. Then the residue was obtained after evaporation of solvent. A mixture of 2.5 mL POCl\(_3\) and 3.5 mL DMF was added in a
1080 ANALYTICAL SCIENCES SEPTEMBER 2018, VOL. 34

50 mL three neck round-bottom flask. The mixture solution was continuously stirred for 2 h at 60°C in the nitrogen environment. When the solution of compound 3 (2.0 g dissolved in 10 mL DMF) was added slowly, the solution was insulated for another 10 h. Then the dark brown solution was poured into 100 mL of ice-water, and a dark-orange solid was precipitated. The target product of orange needle crystals was obtained after recrystallization from anhydrous C₂H₅OH. Yield: 70%.

1H NMR (CDCl₃, 600 MHz, δ ppm): 10.11 (s, 1H), 8.24 (s, 1H), 7.41, 7.39 (d, 1H, J = 12.0 Hz), 6.65, 6.63 (d, 1H, J = 12.0 Hz), 6.49 (s, 1H), 3.48 – 3.45 (m, 4H), 1.26, 1.25, 1.24 (t, 6H, J = 6.0 Hz).

13C NMR (CDCl₃, 150 MHz, δ ppm): 187.93, 161.84, 158.91, 153.36, 145.34, 132.52, 114.46, 110.31, 108.36, 97.32, 45.37, 12.44.

Compound 2 (0.49 g, 0.2 mmol) and ethylenediamine (0.06 g, 0.1 mmol) were dissolved in anhydrous ethanol (30.0 mL). The reaction mixture was refluxed for 16 h and then the precipitate was filtered off, washed with ethanol two times and dried in a vacuum to give the desired compound 1 as yellow powder. Yield 0.41 g (78.1%). m.p. 213.1 – 214.6°C. 1H NMR (CDCl₃, 600 MHz, δ ppm): 8.50 (s, 2H), 8.31 (s, 2H), 7.27, 7.26 (d, 2H, J = 6.0 Hz), 6.60, 6.58 (d, 2H, J = 6.0 Hz), 6.47 (s, 2H), 3.93 (s, 4H); 3.44 – 3.41 (m, 8H), 1.23, 1.22, 1.21 (t, 12H, J = 6.0 Hz). 13C NMR (CDCl₃, 150 MHz, δ ppm): 162.34, 157.35, 151.55, 140.74, 130.66, 115.09, 109.42, 108.76, 97.11, 61.77, 53.43, 44.96, 12.47. Found, %: C, 70.09; H, 6.72; N, 10.81. Calculated for C₃₀H₃₄N₄O₄: C, 70.02; H, 6.66; N, 10.89.

UV-vis and fluorescence spectroscopic studies
The stock solutions of probe 1 (1.0 mM) was prepared in DMSO. The stock solutions of HEPES (pH = 7.0, 10.0 mM) and of Li⁺, Cu⁺², Ag⁺, Pb⁺², Hg⁺², Ni⁺², Co⁺², Cu⁺², Zn⁺², Cd⁺², Mg⁺², Fe⁺³, Cr⁺⁶, and Al⁺³ (as their nitrate salts, 1.0 mM) were prepared in H₂O/DMSO (9:1, v/v). Then 0.1 mL of the stock solutions of probe 1 and 0.5 mL of the stock solutions of metal ions were transferred to a 10-mL volumetric flask, followed by the addition of HEPES to volume. The testing concentrations of probe 1 and every metal ion were 10.0 and 50.0 μM, respectively. Titration spectra were recorded by adding a corresponding volume of cation solutions to a solution of probe 1. All fluorescence spectra were measured at least three times to get the consistent values. And excitation wavelength was at 395 nm at room temperature.

Results and Discussion
Spectroscopic studies
The metal ion response performance of probe 1 was firstly investigated by fluorescence spectroscopy. Figure 1 shows the emission changes of probe 1 in the presence of 5.0-fold concentration of every ion. Probe 1 exhibited faint fluorescence at 498 nm in H₂O/DMSO (9:1, v/v) solution. Nevertheless, its fluorescence behavior changed dramatically with the addition of 5.0 equiv. of Cr⁺⁶. The maximum emission peak of probe 1 solution shifted to 450 nm from 498 nm with a significant improvement of fluorescence intensity. Moreover,
the quantum yield ($\Phi_F$) of probe 1 strengthened to five times from 1.2 to 6.4%. Although the addition of Hg$^{2+}$ and Al$^{3+}$ to the solution of probe 1 had a slight influence on emission intensity, their fluorescence intensities were far below that of Cr$^{3+}$ ions. Under the same conditions, other metal ions (Ag$^+$, Li$^+$, Ca$^{2+}$, Pb$^{2+}$, Ni$^{2+}$, Co$^{2+}$, Mg$^{2+}$, Zn$^{2+}$, Cd$^{2+}$ and Fe$^{3+}$) did not produce significant fluorescence changes. The remarkable difference of the fluorescent emission along with Cr$^{3+}$ addition was mainly attributed to the PET effect and C =N isomerization.\textsuperscript{31,32} The PET effect from double bond C =N to the coumarin group could lead to fluorescence quenching of compound 1. Then the quenched fluorescence recovered dramatically along with their chelation between Cr$^{3+}$ and N atoms. The fluorescence of compound 1 could be recovered noticeably mainly because of the control of C=N isomerization upon complexation with Cr$^{3+}$,\textsuperscript{33} Simultaneously, the inhibition of the PET process cannot be ruled out, which also can lead to fluorescence enhancement of the probe based on Schiff base compound.\textsuperscript{34} A green color fluorescence emission can be observed after addition of Cr$^{3+}$ to compound 1 solution as shown in Fig. 2. As a result, it can be concluded that compound 1 possesses good responsiveness to Cr$^{3+}$ in water, and can serve as a fluorescence “off-on” probe.

Next, we indicated the anti-jamming capability of probe 1 to decide if it can recognize Cr$^{3+}$ in the presence of other competitive ions. As shown in Fig. 3, there was not any noteworthy disadvantage for detection of Cr$^{3+}$ upon addition of other metal ions. Thus, probe 1 can detect Cr$^{3+}$ with “turn on” emission even when there is competition between Cr$^{3+}$ and competing metal ions was exist.

In order to verify the quantitative analysis possibility of probe 1 for detecting Cr$^{3+}$ ions, a titration experiment of probe 1 with Cr$^{3+}$ was carried out (Fig. 4). Fluorescence intensity (498 nm) of probe 1 showed a gradual increasing trend along with the increase of Cr$^{3+}$ concentration (0 - 2.2 equiv.). The emission peaks of fluorescence spectra vs. the concentration of Cr$^{3+}$ (0 - 16 μM) were in accordance with the Stern-Volmer relationship: $y = 1.67 \times 10^5 + 1.90 \times 10^5 \times [Cr^{3+}]$ ($R^2 = 0.9850$). The detection limit (DL) can be calculated to be 0.79 μM by Eq. (1) when SNR = 3,\textsuperscript{35} which is lower than the EPA’s standard for the acceptable concentration of healthy water range of Cr$^{3+}$ in water (≤1.92 μM).

$$DL = \frac{3\sigma}{\kappa}$$

Here $\sigma$ is RSD of probe 1 with low concentration Cr$^{3+}$ for 10 testings (about 4.9%), $\kappa$ represents the slope of the Stern-Volmer relationship. The association constant ($K_a$) of probe 1 with Cr$^{3+}$ was calculated to be $2.89 \times 10^4$ M$^{-1}$ with an ideal linear relationship: $y = 1.05 \times 10^{-6} + 3.63 \times 10^{-11} \times x$ ($R^2 = 0.9951$) by the Benesi-Hildebrand Eq. (2).\textsuperscript{36}

$$\frac{1}{F - F_0} = \frac{1}{K(F_{\text{max}} - F_0)[Cr^{3+}]} + \frac{1}{F_{\text{max}} - F_0}$$

The variation tendency of UV-vis absorption spectra of probe 1 in the wake of Cr$^{3+}$ counts is displayed in Fig. 5. The absorption peaks of UV-vis absorption spectra of probe 1 without Cr$^{3+}$ were located at 274, 370 and 444 nm, respectively. After addition of 0 - 1.6 equiv. of Cr$^{3+}$ ions into the solution of probe 1 (10 μM), the absorption peaks at 370 nm weaken gradually. In the meantime, the absorption peaks at 274 and 444 nm could be enhanced synchronously. The results are probably due to the restriction of cis versus trans conformation of probe 1 after the formation of a complex between 1 and Cr$^{3+}$. The complex was also demonstrated by means of transient state fluorescence...
(Fig. S7, Supporting Information). The decay curves of probe 1 and 1 + Cr³⁺ are well fitted by a double exponential function. However, they show different lifetimes: 150 and 300 ps, respectively. Thus, probe 1 and Cr³⁺ formed a new complex different from probe 1.

For the purpose of the stoichiometric ratio of probe 1 with Cr³⁺, Job’s plot was adopted as shown in Fig. 6. Fluorescence intensity of probe 1 rises to the peak value when the molar ratio of probe 1 and Cr³⁺ is 1:1, which means probe 1 and Cr³⁺ form 1:1 complexes.

It is also very important to detect Cr³⁺ as soon as possible using the fluorescence probe. The response time of probe 1 was further determined through the kinetics experiment for the fluorescent probe as shown in Fig. 7. When combined with Cr³⁺ ions (10 μM), the emission intensity of probe 1 (10 μM) slowly became higher and reached the maximum in 10 min, and finally became stable from 10 to 30 min. The results show that reaction time of 10 min is sufficient for realizing valid analysis of Cr³⁺ using this probe.

**Effect of pH on the detection performance of probe 1 to Cr³⁺**

The pH value of the testing system is an important factor of fluorescence detection, and can have a significant impact on the analytical result. Consequently, the effect of pH on the detection performance of probe 1 was adopted to identify the appropriate scope of pH values. As shown in Fig. 8, the influence of pH values in the range of (4 – 11) was investigated by the changes of fluorescence intensity (498 nm) of probe 1. The universal fluorescence maximums of probe 1 with Cr³⁺ were still higher than that of probe 1 in the absence of Cr³⁺. More specifically, the maximum of emission intensity of probe 1 in the presence of Cr³⁺ decreased by degrees when pH value was above 8, which can be attributed to the transformation of Cr³⁺ from Cr³⁺ ions to Cr(OH)₃ removing Cr³⁺ from probe 1. Similarly, maximum emission intensity of probe 1 with Cr³⁺ displayed weakening of the situation when pH values were below 7 probably because probe molecules combine with H⁺ preferentially and refuse to combine with Cr³⁺. Probe 1 revealed strong emission in the presence of Cr³⁺ within a pH range of 7 – 8. At the same time, the probe showed weak fluorescence in the absence of Cr³⁺ in the same pH range. This showed that, probe 1 can detect of Cr³⁺ in the pH range of 7 – 8.

**Cell imaging**

In order to certify utilization potentiality of probe 1 in actual samples, microscopy images of probe 1 with Cr³⁺ in living MCF-7 cells were collected by confocal laser scanning microscopy. Figure 9 shows bright-field, fluorescence and overlap images of MCF-7 cells after incubation with probe 1 and Cr³⁺ under the green channel. MCF-7 cells were cultured in culture containing probe 1 and Cr³⁺ in turn, and then emitted a
sensitive detection of Cr^{3+} even in the presence of other fluorescent probe can be applicable for effective, selective and the probe system. Experimental results showed that this probe had significant fluorescence. This experiment showed that probe 1 could be absorbed by living cells and labeled specifically with intracellular Cr^{3+}.

Conclusions

In this paper, a novel fluorescent probe based on coumarin Schiff base with good performance has been designed and synthesized. It displayed a conspicuous “turn on” phenomenon with significant emission enhancement when Cr^{3+} was added to the probe system. Experimental results showed that this fluorescent probe can be applicable for effective, selective and sensitive detection of Cr^{3+} even in the presence of other competing metal ions. With nothing, this probe has a very low detection limit of 0.46 µM, which suggests it would be applicable for effective analysis and fluorescence imaging in living cells.

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Supporting Information

NMR spectra for the detection of Cr^{3+} and other figures are listed in Supporting Information. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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