A Novel Coumarin-based Fluorescent Probe for Recognition of Copper(II) Ions and Its Application in Bioimaging

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Simple, accurate and real-time analytical methods are required for the detection of metal ions in a complex environment. In the present work, a fluorescent probe CPB based on coumarin was designed for recognizing Cu2+ ions. The fluorescence of CPB gradually quenched with increasing concentration of Cu2+ ions, due to the interactions between CPB and Cu2+ ions. With the addition of Cu2+ ions, the emission changes of CPB exhibited a good liner relationship toward the Cu2+ ions content in solution. Additionally, CPB could highly selective recognize Cu2+ ions among other metal ions in solution. Bearing the selectivity and fluorescence property toward Cu2+ ions, CPB was successfully applied to monitoring Cu2+ ions in Hela cells and zebrafish.

Keywords Copper(II) ions recognition, fluorescent probe, coumarin derivative, bioimaging

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Introduction

Copper, as the third most abundant transition metal in the human body, participates in a variety of physiological and pathological processes. Copper(II) ions play an important role in many biological processes, such as hemoglobin synthesis, oxygen binding, nerve function and electron transfer processes. Lack of copper(II) ions would affect the activity of enzymes, the stability of bones, and the development of nerve and immune systems. Excessive intakes of copper(II) ions can cause many diseases, for instance liver cirrhosis and cerebrovascular diseases. Copper(II) ions must be controlled at reasonable concentrations in living cells to maintain the normal functions of enzymes and the balance of intracellular metabolism. It is necessary to develop a highly sensitive and selective copper(II) ions recognition method for environmental analysis and biological applications.

There are many effective methods for detecting metal ions, such as inductively coupled plasma mass spectrometry, and cold vapor atomic absorption spectrometry. Among them, the fluorescence method has the advantages of simple operation, a wide dynamic range and real-time detection. Further, fluorescent probes are widely used for metal ion recognition. After interacting with the metal ions, significant changes in the optical signal response of a fluorescent probe would be of great benefit to quantitative analyte determination. Fluorescent probes are suitable for detecting ions because many excellent optical properties of fluorophores in probes could show the interactions of the probe and analytes based on emission signals. These have attracted constant attentions in the fields of chemistry, materials, biology and environmental science. Coumarin, as one of the most widely used fluorophores, has a large Stokes shift and reasonable photostability. Coumarin derivatives are present in various plants in nature, and have been widely used to manufacture daily products. Due to the advantages of easy modification and good biocompatibility, coumarin derivatives have good versatility in the organic synthesis of biomedicine and molecular imaging materials. Probes based on coumarin have been extensively studied and widely used to detect metal ions.

Herein, a fluorescent probe CPB was designed and synthesized by 7-N,N-diethylamino-3-hydrazide-coumarin and 4-(1H-phenanthro[9,10-d]imidazol-2-yl)benzaldehyde for recognizing Cu2+ ions, of which the proposed recognizing Cu2+ is shown in Scheme 1. A series of fluorescence characteristics of CPB in solution were explored. The fluorescence emission signal showed that the fluorescence quenched linearly with an increased concentration of Cu2+ ions. Intracellular fluorescence imaging was performed with Hela cells and zebrafish, which proved the excellent bio-imaging capability of CPB.

Experimental

Reagents and chemicals

All chemicals were obtained from commercial sources and used without further purification. A stock solution of CPB was prepared in DMSO at a concentration of 5.0 × 10−4 mol/L. The stock perchlorate solutions (including the perchlorate of Ag+, Al3+, Ca2+, Cd2+, Co2+, Cr3+, Cu2+, Fe2+, Fe3+, Mn2+, Ni2+, Zn2+, Hg2+, Pb2+) were freshly prepared in deionized water at a concentration of 10 mM.

1H NMR and 13C NMR spectra were recorded on a Bruker AVANCE III 400 MHz or 500 MHz NMR spectrometer. Mass spectrometry was carried out on a Thermo Scientific-LTQ Orbitrap XL mass spectrometer. Fluorescence spectra were recorded by a FS920 fluorescence spectrometer. Ultraviolet-visible absorption spectra were obtained on a TU 1900 UV-vis
spectrometer. Confocal fluorescence imaging was operated on an OLYMPUS FV1000 confocal microscopy.

Synthesis of CPB

(1) Synthesis of compound 1. 4-Diethylenaminoaldehyde (1.93 g, 10 mmol), diethyl malonate (3.2 g, 20 mmol) and 3 - 5 drops of piperidine were added to 40 mL of absolute ethanol, and the mixture was refluxed for 6 h. After the reaction, the mixture was cooled to room temperature, and the solvent was evaporated. The solid was extracted with ethyl acetate, the organic layer was dried with magnesium sulfate, and the solvent was removed under reduced pressure. The obtained solid was purified by column chromatography (ethyl acetate-petroleum ether = 1:4, v/v) to obtain a yellow product (yield: 63%). 1H NMR (400 MHz, CDCl3) δ 8.42 (s, 1H), 7.36 (d, J = 8.9 Hz, 1H), 6.61 (dd, J = 9.0, 2.4 Hz, 1H), 6.45 (d, J = 2.4 Hz, 1H), 4.37 (q, J = 7.1 Hz, 2H), 3.45 (q, J = 7.1 Hz, 5H), 1.39 (t, J = 7.1 Hz, 3H), 1.23 (t, J = 7.1 Hz, 7H). 13C NMR (126 MHz, CDCl3) δ 164.18, 158.30, 152.89, 149.17, 131.06, 109.58, 108.81, 107.63, 96.63, 61.08, 45.10, 14.39, 12.42.

(2) Synthesis of compound 2. Compound 1 (1.08 g, 3.75 mmol) was dissolved in 25 mL of ethanol, and then 1 mL of 80% hydrazine hydrate was added dropwise. The resulting solution was stirred under room temperature for 12 h. The cooled mixture was filtered under reduced pressure to remove the solid. The solution was washed with 20 mL mixture was filtered under reduced pressure, the filter cake was washed with cold ethanol (20 mL × 3) and dried to obtain an orange product (yield, 40%). 1H NMR (400 MHz, acetic acid-4H) δ 8.09 (d, J = 7.6 Hz, 2H), 7.86 (d, J = 9.2 Hz, 2H), 7.77 (d, J = 8.0 Hz, 2H), 7.55 (s, 1H), 7.50 (t, J = 7.1 Hz, 2H), 7.36 - 7.26 (m, 5H), 6.69 (d, J = 8.7 Hz, 1H), 6.37 (d, J = 9.1 Hz, 1H), 5.80 (s, 1H), 3.27 (s, 4H), 1.15 (t, J = 7.0 Hz, 6H). The high-resolution MS of [CPB + H]+ was measured at 580.2340.

Fluorescence spectroscopic studies

The stock solution of CPB (5.0 × 10^{-4} M/L) was diluted to 2.0 × 10^{-6} M/L with CH3CN/HEPES (4/6, v/v) solution (HEPES 10 mM, pH 7.4). Different metal ions (Ag+, Al3+, Cu2+, Cd2+, Co2+, Cr3+, Zn2+, Fe2+, Fe3+, Hg2+, Mn2+, Ni2+, Pb2+, and Cu2+) perchlorate salt solutions were prepared and added respectively to the solutions of CPB to perform fluorescence scanning. The excitation and emission wavelengths of CPB in fluorescence experiments in solution were 370 and 490 nm at room temperature, respectively.

Fluorescence imaging

CPB was used for the imaging of different concentrations of Cu2+ ions in Hela cells and zebrafish to verify its practical properties in biological samples. Hela cells were cultured in a DMEM medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) for 24 h in a humidified incubator containing 5% CO2 at 37°C. The cells were treated with 1 μM CPB and various concentrations of Cu2+ ions (0 - 20 μM) in growth medium. And then, the dishes were washed three times with PBS and placed on a microscope for confocal microscopic imaging.31-33 The zebrafish were maintained at 28°C and treated with 1 μM CPB and various concentrations of Cu2+ ions (0 - 20 μM). After that, the zebrafish were washed with E3 for fluorescence imaging.

Results and Discussion

Synthesis of CPB

The fluorescent probe CPB was synthesized by four steps. The detailed synthetic steps are shown in Scheme 2. In brief, 7-N,N-diethylenamino-3-hydrazide-coumarin (compound 2) and 4-(1H-phenanthro[9,10-d]imidazol-2-yl)benzaldehyde (compound 3) were synthesized, respectively. The target compound CPB was obtained by a Schiff’s base reaction of compounds 2 and 3. The resulting compounds were characterized by 1H NMR, 13C NMR and ESI-MS (Figs. S1 - S8).

Optical properties of CPB toward Cu2+ ions

The optical properties of CPB were investigated in a solution of CH3CN/HEPES (4/6, v/v). The concentration of CPB in the cuvette was 2 μM, and a solution of Cu2+ ions were added
When excited at 370 nm, CPB displayed strong fluorescence emission at 490 nm (Fig. 1). When an equivalent of Cu$^{2+}$ ions was added to a solution of CPB, the fluorescence was slightly quenched. The fluorescence intensity of CPB gradually decreased with an increased concentration of Cu$^{2+}$ ions, and finally reached to 30% of the original value after 20 equivalents of Cu$^{2+}$ ions were added. The quenching effect also showed a linear relationship between fluorescence intensity of CPB (2 $\mu$M) and the concentration of Cu$^{2+}$ ions (0 – 40 $\mu$M) (Fig. 2).

The recognition capability of probe CPB with different metal ions was investigated in CH$_3$CN/HEPES (4:6, v/v), which is an important indicator to measure the practical applicability of the fluorescent probe. After 20 equivalents of various metal ions (Ag$^+$, Al$^{3+}$, Cu$^{2+}$, Cd$^{2+}$, Co$^{2+}$, Cr$^{3+}$, Cu$^{2+}$, Fe$^{3+}$, Fe$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, Hg$^{2+}$) were added, respectively, the changes of the fluorescence intensity were very weak, and could be ignored (Fig. 3). However, the fluorescence emission at 490 nm showed considerable decreasing after Cu$^{2+}$ ions were added to the solutions, respectively, similar to that of CPB with addition of Cu$^{2+}$ ions. The other metal ions had little interference to the interaction between the CPB and Cu$^{2+}$ ions, which indicated the practical applicability of CPB to detect Cu$^{2+}$ over other metal ions.

The stability of CPB in the presence of Cu$^{2+}$ ions was certified by fluorescence emission spectroscopy (Fig. 4). After adding 20 equivalents of Cu$^{2+}$ ions to the solution of CPB, the fluorescence was significantly quenched to 30% of the original value within 1 min, and exhibited no significant change within 10 min. The probe could be used to quickly detect Cu$^{2+}$ ions with outstanding stability.

CPB as a sensor for Cu$^{2+}$ ions in living cells and zebrafish

The ability of the probe CPB to monitor Cu$^{2+}$ ions in living cells and zebrafish was determined. CPB showed excellent biocompatibility in both living Hela cells and zebrafish (Figs. 5 and 6). When Hela cells and zebrafish were treated only with CPB (1 $\mu$M), strong fluorescence in the cells and zebrafish could be observed. However, when the cells and zebrafish were treated with CPB (1 $\mu$M) and various concentration of Cu$^{2+}$ ions (5, 10, 20 $\mu$M), the fluorescence obviously quenched, which showed a good relevance to the concentration of Cu$^{2+}$ ions.
Fluorescence response of CPB (2 μM) to Cu²⁺ in the presence of various metal ions (including Ag⁺, Al³⁺, Ca²⁺, Cd²⁺, Co²⁺, Cr³⁺, Zn²⁺, Fe²⁺, Fe³⁺, Hg²⁺, Mn²⁺, Ni²⁺, Pb²⁺) in CH₃CN/HEPES (4:6, v/v). (λ_ex = 370 nm, λ_em = 490 nm).

Fluorescence images and bright-field images of Hela cells treated with CPB (1 μM) and various concentration of Cu²⁺ ions (0 - 20 μM) at 37°C.

Fluorescence images and bright-field images of zebrafish treated with CPB (1 μM) and various concentration of Cu²⁺ ions (0 - 20 μM) at 28°C.

Time-dependent changes of CPB (2 μM) (black line) with the addition of Cu²⁺ ions (red line) in CH₃CN/HEPES (4:6, v/v). (λ_ex = 370 nm, λ_em = 490 nm).
Conclusions

In the present work, a novel “turn-off” fluorescent probe CPB based on coumarin was designed and synthesized for the detection of Cu²⁺ in solution. After adding Cu²⁺ ions to a solution of CPB, the peak of fluorescence emission at 490 nm was significantly decreased. With the increase of the concentration of Cu²⁺ ions in the range of 0 - 40 μM, the fluorescence intensity of CPB changed proportionally. CPB was applied to detection of Cu²⁺ ions in HeLa cells and zebrafish. The results of this study suggested the potential value of the probe CPB in practical applications.

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Supporting Information

NMR and mass spectrum of the probe are given as Supporting Information. This material is available free charge on the Web at http://www.jsac.or.jp/analsci/.

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