Fluorescent probe visualization for selective detection of cuprous ion

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Abstract
Cuprous ion is an essential element for human life activities. However, excessive cuprous can cause dysfunction of the human body system. To help explain this evolving biology, we report a fluorescent probe for detecting unstable Cu(I). Cu(I) undergoes a metal complexation reaction with the sulfur element in the probe, resulting in a photoelectron transfer (PET) effect. The probe fluorescence is greatly suppressed, and rapid and selective visual detection of Cu(I) in the inorganic environment is realized. There is also a good linear relationship between the probe fluorescence intensity and the Cu(I) concentration ($R^2 = 0.992$), which can realize the quantitative detection of Cu(I). When the probe concentration is 0.1 μM, the detection limit is 15 nM, and the detection limit of the visual method is as low as 0.1 μM. This work provides a valuable starting point for real-time monitoring of the Cu(I) concentration in a human anaerobic environment based on active probes.

Keywords Probes · Cuprous ion · Visualization · Fast · Sensitive

Introduction
Copper is an essential redox-active nutrient in life [1]. In the process of oxygen transport, respiratory metabolism and cell growth in the human body, proteins contain the necessary effective catalysis and structural cofactors [2–4]. In addition to the copper ions contained in proteins, newly emerging data indicate that there are unstable cuprous ions. Due to their chemical instability, their binding to low molecular weight ligands is relatively weak [5]. However, cuprous ions play a vital role in human activities. It promotes increasing dynamic transition-metal signaling pathways [6], including neural communication [7], odor [8, 9], and lipolysis [10]. However, an unbalanced copper ratio in the human body can lead to a variety of diseases, such as cancer [11], cardiovascular disease, neurodegenerative Alzheimer's disease, Parkinson's disease and Huntington's disease [12], diabetes and obesity, and genetic phyla, as well as Wilson disease [13–15].

Therefore, it is becoming increasingly important to develop a method that can monitor the content of cuprous ions in the human body in realtime. Additional detection methods are invented. Fluorescent lamps [7, 14, 16–19], bioluminescence [20] and magnetic resonance imaging probes [21–23] provide important information for the visual observation of cuprous ions. However, these methods involve a large volume of instruments used, that are inconvenient to carry, involve complicated sample pretreatment processes, and high detection cost. Further on-site detection of cuprous ions cannot be performed. The fluorescent probe detection method is portable and easy to operate, which can solve the above problems. However, the detection probe designed in the early stage is mainly used to detect cuprous ions [24, 25] in the environment, so it is very important to design a fluorescent probe for measuring Cu(I) under physiological reducing conditions.

Herein, we report a fluorescence quenching strategy using a novel fluorescent compound, 2-(4-methylbenzyl) imidazo[1,5-a]pyridine-3(2H)-thione (AHP), to detect Cu(I) in albumin. Due to the optical effects of $\pi-\pi$, p-$\pi$ conjugated systems and imidazole compounds in the visible wavelength range, these compounds have potential
applications in various fields, such as fluorescent probes [26] and organic dyes [27]. The synthesis of an imidazole ring usually requires specific environmental conditions. We now have developed a method to synthesize the fluorescent molecule AHP through simple steps at room temperature. The probe can quickly and highly selectively detect Cu(I) in an oxygen-free environment. The sulfur element in the probe undergoes a metal complex reaction with Cu(I), resulting in a photo-induced electron transfer (PET) effect. The fluorescence of the probe is greatly suppressed, and the visual detection of cuprous ions is realized. In addition, the probe itself has a high quantum fluorescence yield and stable chemical properties. Therefore, a stable fluorescence intensity can be maintained in the air, which is conducive to the development of detection activities and the accuracy of experimental results. The probe has great potential in the detection and application of human cells, paving the way for future analysis and detection.

**Experimental**

**Materials**

(4-Methyl-benzyl)-pyridin-2-ylmethyl-amine (MDPA) was purchased from Sigma Co. Ltd. Magnesium chloride (MgCl₂), cuprous chloride (CuCl), nickel chloride hexahydrate (NiCl₂·6H₂O) were purchased from Aladdin Reagent Co. Ltd. Ethanol (C₂H₆O), methanol (CH₃OH), ferric chloride (FeCl₃), sodium chloride (NaCl), potassium chloride (KCl), calcium chloride (CaCl₂), barium chloride dihydrate (BaCl₂·2H₂O), ammonium chloride (NH₄Cl), copper sulfate pentahydrate (CuSO₄·5H₂O), silver nitrate (AgNO₃), lead nitrate(Pb(NO₃)₂), ferrous chloride tetrahydrate (FeCl₂·4H₂O), manganese chloride tetrahydrate (MnCl₂·4H₂O), 3/2 hydrated chromium chloride (CdCl₂·3/2H₂O), carbon disulfide (CS₂), ammonium hydroxide (NH₃·H₂O), dichloromethane (DCM) were purchased from Sinopharm Chemical Reagent Co. Ltd. All of the chemicals and solvents were used directly without further purification. Ultrapure water (18.25 MΩ cm) was obtained from a Millipore water purification system.

**Apparatus**

Fluorescence spectra were obtained with a HITACHI F-7100 fluorometer. UV absorption spectra captured by Laser Jet Pro UV-2700. Photographs are captured with a portable ultraviolet lamp, ZF-7, and a canon EOS 600D digital camera with 365 nm emission wavelengths. This layer chromatography (TLC) was performed on a Merck F254 silica gel-60 plate, and the TLC plate was observed by UV light.

**Synthesis of AHP**

The synthesis method of the probe AHP has been slightly changed on the basis of previous research [28]. The designed probe AHP synthesized by adding 50 µL of (4-methyl-benzyl)-pyridin-2-ylmethyl-amine (MDPA) and 30 µL of ammonium hydroxide (NH₃·H₂O) to 2 mL of DCM/ethanol = 1:1 mixed solvent. We cooled the compound in an ice bath for 30 min. We then dropwise added 35 µL CS₂ to dissolve in the same solvent mixture (1.0 mL). Stirred at room temperature for 10 h. Producing a yellow solution. The solution was chromatography on a silica-gel column of PET/EAC = 4:1. We then dried in a vacuum to obtain a pure product. The yield was 61.2%. The synthesis process is as shown in Scheme 1. AHP was confirmed by ¹H NMR and high-resolution mass spectrometry (Fig. S1 and S2†). The high-resolution mass spectrum shows peaks at m/z = 255.09 and 277.07, and their isotope distribution is consistent with the simulated isotope distribution of the molecular formula [M+H]⁺ and [M+Na]⁺.

**Procedure for detecting Cu(I) and other metal ions**

Cu(I) ions were made by reacting ferrous ions (Fe(II)) and copper ions (Cu(II)) in the same amount. FeCl₃ was dissolved in ultrapure water to form iron ions (Fe(III)); Ferrous ion (Fe(II)) was obtained by reacting FeCl₂·4H₂O with ultrapure water; Magnesium ions (Mg(II)) was derived from MgCl₂ in ultrapure water; Sodium ion (Na(I)) was produced from NaCl; Potassium ion (K(II)) was prepared by KCl; Manganese ion (Mn(II)) was derived from MnCl₂·4H₂O dissolved in ultrapure water; Cadmium ion (Cd(II)) was obtained by
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Fig. 1 (a) Excitation and emission spectra of the probe before and after adding 10 µL of Cu(I). (b) Record the fluorescence stability of the AHP probes with different amounts of Cu(I) irradiated continuously for 15 min at the excitation wavelength of 420 nm

Scheme 2 Reaction process of AHP and Cu(I). The reaction can be carried out at room temperature

reacting CdCl₂·3/2H₂O with ultrapure water; CaCl₂ is dissolved in ultrapure water to produce calcium ions (Ca(II)); Helium ion (Ba(II)) was derived from BaCl₂·2H₂O dissolved in ultrapure water; Ammonium ion (NH₄(I)) was obtained by reacting NH₄Cl with ultrapure water; CuSO₄·5H₂O was dissolved in ultrapure water to form copper ions (Cu(II)); Silver ion (Ag(I)) was derived from AgNO₃ in ultrapure water; Nickel ion (Ni(II)) was obtained by reacting NiCl₂·6H₂O with ultrapure water and lead ion (Pb(II)) was produced from Pb(NO₃)₂ dissolved in ultrapure water.

Results and discussion

Probe chemistry and light stability

To determine the best detection environment for the probe, we placed it in solutions with different pH values and measured the effect of Cu(I) on the probe’s fluorescence intensity. It was found that under an environment of pH 7–9, the fluorescence intensity of the probe changed significantly (as shown in Fig. S3), and the optimal reaction conditions were obtained. Therefore, the storage and detection of the probe were performed at pH = 8; that is, 50 μL of a 0.4 M NaOH solution was added to 5 mL of a 10 mM probe solution. To prepare the most suitable reaction conditions, the maximum fluorescence response of the probe could be obtained.

To verify the photostability of the probe, the probe was studied as follows. The excitation and emission spectra of the probe before and after the addition of Cu(I) and the change in fluorescence intensity of the probe itself were measured with the addition of Cu(I). The results are shown in Fig. 1. The fluorescence intensity of the probe AHP after adding Cu(I) was greatly reduced, which was 1/3 of the previous intensity. Therefore, the probe can be an excellent “quenching” detector. The detection mechanism is shown in Scheme 2. The photostability of AHP before and after the reaction with Cu(I) was evaluated by continuous irradiation for 1 h, and the fluorescence intensity was recorded at 490 nm. As shown in Fig. 1(b), the light stability was maintained for at least 1 h, so the light stability of the probe would be good, and it could be applied to environmental detection.
Fluorescence reaction of AHP on Cu(I)

The UV–vis absorption spectra of the probe AHP were tested in the presence and absence of Cu(I), and the results are shown in Fig S4. Obviously, the absorption peak at 282 nm of the probe AHP after adding Cu(I) increased and blue shifted to 300 nm. It further confirms the complexation reaction between the probe and Cu(I).

As expected, as the Cu(I) concentration continued to increase and the fluorescence intensity gradually decreased, as shown in Fig. 2(a). Figure 2(b) shows that a good linear relationship is present with a correlation coefficient of 0.992 in the range of 0–2.8 µM. It can be used to quantify the lower concentration of Cu(I). The detection limit was 15 nM.

Fluorescence probe selectivity and immunity

Detection of probe selectivity in the presence of Ni(II), Fe(III), Fe(II), Mg(II), Na(I), K(I), Mn(II), Cd(II), Ca(II), Ba(II), NH₄(I), Cu(II), Ag(I), Pb(II) metal ions. As shown in Fig. 3(a), the probe AHP has a strong fluorescence quenching effect on Cu(I), but caused no obvious change to other metal ions, so it has high selectivity. To verify the method, we added two times the amount of other metal ions mixed with Cu(I), and the anti-interference effect of AHP on Cu(I) was studied, as shown in Fig. 3(b). As can be seen from the above two figures, even if an excessive amount of interfering metal ions was added, there was no significant influence on the fluorescence of the probe. The probe exhibited high selectivity for Cu(I) compared to other metal ions.

Binding constant

The binding constant, $K$, of the complex was calculated by the Benesi-Hildebrand method.

$$\frac{1}{F - F_{\text{min}}} = \frac{1}{F_{\text{max}} - F_{\text{min}}} K [\text{Cu}^+] + \frac{1}{F_{\text{max}} - F_{\text{min}}}.$$  

$F_{\text{max}}$ is the fluorescence intensity of AHP, $F_{\text{min}}$ is the probe fluorescence intensity after adding excess Cu$, F$ is the intensity within the linear relationship, and $K$ is the binding constant. The $K$ value is obtained from the plot of $F_{\text{max}}/F$ and Cu$^+$, where $K$ is equal to intercept/slope [27].

Then, the binding constant, $K$, was obtained from the actual results of fluorescence detection and the related Benesi-Hildebrand method through the binding constant of AHP and Cu$^+$ ions to verify the accuracy of the $K$ value, which was calculated from the slope by plotting and fitting the data obtained. More importantly, within this range, there is a linear relationship between $F_0/F$ and Cu$^+$ ($R^2 = 0.992$), indicating that AHP is very useful for determining the Cu$^+$ concentration. The binding constant ($K$) calculated from the fluorescence titration data of Cu$^+$ in solution by AHP = 5.16 × 10^5 M$^{-1}$ (Fig. 2b), indicating that AHP has a good binding ability to Cu$^+$.

Molecular mechanism of AHP recognition probe

AHP is a highly fluorescent organic molecule that exhibits excellent optical properties, such as a stable fluorescence intensity and a high quantum fluorescence yield. When cuprous ions are present, they undergo a metal complexation reaction with the sulfur element in the probe, resulting in a photo-induced electron transfer (PET) effect. The fluorescence intensity of the probe is suppressed to a great extent, so as to realize a visual detection of Cu(I). ESI–MS spectra proved the reaction mechanism of AHP and Cu(I) (Fig. S5).

Detection of Cu(I) in serum protein

To realize the application of the probe in the biological field, we explored the ability of the probe to detect Cu(I) in serum.
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**Conclusions**

By developing a quenched fluorescent probe, a method for the selective detection of cuprous ions was developed. During the analysis process, the cuprous ion destroyed the balance of the electron donating and withdrawing ability of the C=S double bond of the probe, itself, resulting in that the electron-donating ability of the S–Cu bond after the reaction became lower than that of the C=S double bond, and the electron balance of the probe was destroyed, and the fluorescence intensity was obviously quenched. In addition, we tested different environmental samples and obtained satisfactory results. The developed method does not require expensive equipment and can be implemented with a small sample of only a few milliliters. Therefore, it is very suitable for the detection of cuprous ions.

**Supplementary Information** The online version contains supplementary material available at [https://doi.org/10.2116/analsci.21P218](https://doi.org/10.2116/analsci.21P218).

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