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Retinal-based polyene fluorescent probe for selectively detection of Cu^{2+} in physiological saline and serum

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ABSTRACT

Retinal is a flexible natural chromophore and widely present in organisms. The slender conjugated polyene structure retinal is conducive to entering protein structure. In this work, a novel turn-on fluorescent probe for Cu^{2+} based on retinal and phenylenediamine was designed and synthesized. The probe achieved recognition of copper ions in human serum complex protein environment. Furthermore, the high sensitivity, selectivity for Cu^{2+} and the sensing mechanism was also investigated.

1. Introduction

Retinal is a natural biochemical chromophore with conjugated polyene. There are more than 300 photochemically active proteins in the living system with retinal as their photochromic chromophore [1]. Retinal possessed innate biocompatibility, which can be widely used as the chromophore of fluorescent probes from organic solvent to a physiological environment. In recent years, significant emphasis has been placed on exploring or mimicking retinal photochemistry and photophysics in unique steric and dielectric protein environment [2–4]. However, the retinal derivatives were rarely used as a fluorescent probe due to their poor fluorescence performance [5]. In order to make full use of the fluorophore biocompatibility advantage and improve its fluorescent performance, the conjugated polyene structure of retinal was appropriately modified in this work. The modifications based on fluorescence properties have been reported [6], here we focus on the development of retinol-based copper ion fluorescent probe.

Copper ions play a critical role in the area of biological systems. As a cofactor for the enzyme, only when copper ions are bonded to proteins can they participate in physiological processes, such as cell respiration, neurotransmission, iron uptake and defense against oxidative stresses [7]. Copper ions have also been found to be associated with many diseases, such as Alzheimer's disease [8], Parkinson's disease [9,10], prion disease [11], Wilson's disease [12] and familial amyotrophic lateral sclerosis [13]. Therefore, it is very important to develop highly selective, sensitive and rapid probe for Cu^{2+}. However, the protein environment in which copper ions are located increases the difficulty of detecting copper ions. Generally, fluorescent probes employ larger conjugated chromophores, which are not conducive to entering protein structure. Comparatively, the flexible conjugated polyene structure retinal chromophore provides a possibility to solve the problem.

Fluorescent 'turn-on' probes have been highly regarded due to their high sensitivity, selectivity and ease of observation [14–18]. Among many kinds of signaling mechanisms for optical detection of Cu^{2+}, the organic fluorescent molecules with Schiff base structure was selected in this work due to their rapid response, high sensitivity, and excellent selectivity [19–23]. However, conjugated structures with multiple aromatic rings are inherently poorly soluble, the probes will become less soluble when coupled with the Schiff base structure [24]. The construction of probes based on flexible fluorophores is an ideal choice. The luminescent properties of the flexible fluorescent probe before the binding of copper ions are poor. Once the copper ion and the probe are bound, the probe structure becomes rigid, the conjugation is stable and the luminescent properties are greatly enhanced. Among many fluorophores, retinal is an ideal candidate [25].

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The soft retinal chromophore backbone without functional substituents is not suitable for binding copper ions [26]. Previously, we developed a fluorescent probe for Cu^{2+} based on o-phenylenediamino [24] as chelator, which exhibited excellent selectivity. In this work, a novel fluorescent probe was designed and synthesized, the o-phenylenediamino as a Schiff base chelator, the retinal as the reporter. The probe with Schiff base actually still has good solubility, due to the soft chromophore retinal. As expected, the soft probe becomes rigid in the process of sensing copper ions, promoting significant changes in the fluorescence.

2. Experimental

2.1. Reagents

Retinol acetate, MnO_2, o-Phenylenediamine were purchased from J&K Chemical Ltd. Tetrahydrofuran, ethyl acetate, acetonitrile, metal ions purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai). Human serum was from Solarbio. Other chemicals were provided from Sigma-Aldrich. All commercially purchased chemical reagent materials were used without further purification.

2.2. General method

^{1}H NMR (400MHZ) and ^{13}C NMR (100MHZ) spectra data were recorded on a Bruker UltrashiedTM 400MHZ Plus nuclear magnetic resonance spectrometer using DMSO-d_6/$CDCl_3$ as solvent and tetramethylsilane as an internal standard. High-resolution mass spectra were carried out on a Bruker Micro TOF II 10257 instrument. Fourier transform infrared (FT-IR) spectra were performed by using NICOLET NEXUS 470 spectrometer (KBr discs) in the 4000-400 cm^{-1} region. UV–visible spectra were recorded on a Shimadzu UV-2600 spectrometer. Steady-state fluorescence spectra were recorded on Hitachi F-4600 spectrophotometers. The pH of buffed solution was determined using a PHS-3C digital pH-meter (Leici, Shanghai, China).

2.3. Synthesis

The synthesis route of **V1** is shown in Scheme 1.

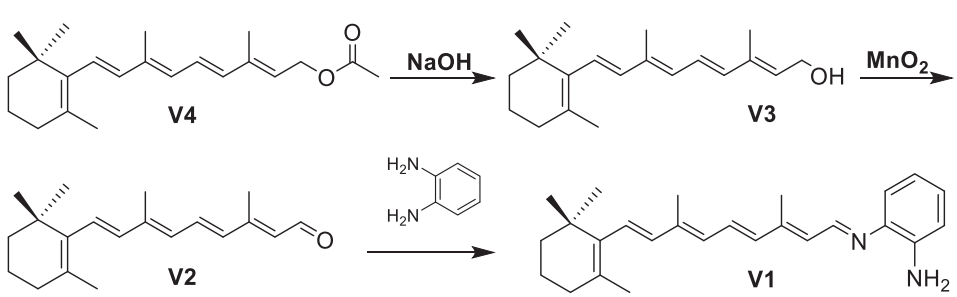
Synthesis of **V3**: The commercial retinol acetate **V4** (10.0 g) was dissolved in 75.0 mL NaOH solution (1 M) and 75.0 mL THF. Then the mixture is stirred at the room temperature for 12 h under nitrogen atmosphere. After the reaction finished, the reaction mixture was transferred to separating funnel, standing and separation of organic and aqueous phases. The aqueous phase was washed by THF and ethyl acetate three times. The organic layer was combined and dried by Na_2SO_4. The organic solvent was evaporated by rotary evaporator, obtaining 4.5 g light yellow oil, yield 60%. The crud product was used in the following reaction without purification [27].

Synthesis of **V2**: The retinol (2.1 g, 7.34 mmol) and Na_2CO_3 (11.66 g, 0.11 mol) was dissolved in 130 mL THF and cooled to 0 °C in ice bar. The MnO_2 (9.57 g, 0.11 mol) was slowly added into above cooling mixture. The reaction mixture was continually stirred for 1 h after removing the ice bar. The reaction residue was separated by vacuum filtration, retaining filtrate [27]. The crud product was concentrated and further purified by silica gel column chromatography, obtaining light yellow waxy solid 0.6 g, yield 29%.^{1}H NMR (400 MHz, $CDCl_3$) δ 10.11 (d, J = 8.2 Hz, 1H), 7.14 (dd, J = 15.1, 11.5 Hz, 1H), 6.36 (dd, J = 15.5, 11.5 Hz, 2H), 6.22–6.13 (m, 2H), 5.97 (d, J = 8.2 Hz, 1H), 2.33 (d, J = 0.9 Hz, 3H), 2.07–1.98 (m, 5H), 1.72 (s, 3H), 1.65–1.60 (m, 3H), 1.49–1.45 (m, 2H), 1.04 (s, 6H).

Synthesis of **V1**: The retinal (0.30 g, 1.06 mmol) and o-Phenylenediamine (0.34 g, 3.18 mmol) was refluxed in 35 mL acetonitril for 2 h. The solvent was evaporated by rotary evaporator, getting crud product. The crud product was recrystallized in 20 mL petroleum ether and 2 mL ethanol [24], cooling and vacuum filtration, obtaining orange solid 0.35 g, yield 87.5%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.64 (d, J = 9.7 Hz, 1H), 7.08 (dd, J = 8.0, 1.2 Hz, 1H), 7.02–6.88 (m, 2H), 6.67 (dd, J = 8.0, 1.3 Hz, 1H), 6.60–6.45 (m, 2H), 6.44–6.33 (m, 1H), 6.24 (dt, J = 29.7, 11.7 Hz, 3H), 5.06 (s, 2H), 2.20 (s, 3H), 2.06–1.94 (m, 5H), 1.70 (s, 3H), 1.58 (dt, J = 8.5, 4.7 Hz, 2H), 1.49–1.39 (m, 2H), 1.02 (s, 6H). ^{13}CNMR (101 MHz, $CDCl_3$) δ 155.25, 154.42, 143.70, 137.63, 136.96, 135.45, 134.27, 131.43, 131.22, 129.85, 124.84, 114.79, 112.57, 79.28, 39.65, 34.33, 33.29, 29.02, 21.87, 19.17, 14.05, 13.18. HR-MS: calcd for $C_{26}H_{36}N_2^+$ $[M+H]^+$, 375.2800; found: 375.2803.

2.4. Procedures of the metal ion sensing

The solutions of metal ions (10.0 mM) were prepared in doubly distilled water by the dissolution of their corresponding nitrate salts, including Al^{3+}, Ca^{2+}, Cr^{3+}, Mn^{2+}, Fe^{3+}, Be^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Ag^+, Cd^{2+}, Ba^{2+}, Hg^{2+}, Pb^{2+}, K^+, Na^{2+}, Mg^{2+}. Stock solution of **V1** (1.0×10^{-3} mol/L) was prepared in acetonitrile or DMSO by dissolving the corresponding amount of **V1** powder and then diluted to 1.0×10^{-5} mol/L with acetonitrile, DMSO/stroke-physiological saline solution (1:1000, v/v; pH = 6.5). Human serum was added into the test solution according to the mass ratio. In titration experiments, test solution of **V1** (2 mL) was filled in a quartz optical cell of 1 cm optical path length, and then aliquots of 2 μL of $Cu(NO_3)_2$ solution (1.0×10^{-2} mol/L) were gradually added by using a micro-pipette. Spectral data were recorded for 5 min after addition of the metal ions at steady temperature (298 K). For fluorescence measurements, the excitation wavelength was at 400 nm using Xe lamp as an excitation source, the fluorescence emission channel was from 410 to 800 nm. In pH titration experiments, stock solution of **V1** was added to the sodium phosphate buffer aqueous solution with different pH values. The **V1**–$\mathbf{Cu^{2+}}$ complex reparation, 1.12 mg (3 mmol) **V1** and 0.6 mg (3.6 mmol) $Cu(NO_3)_2$ was dissolved in 5 ml acetonitrile and stirred for 5 mints, and then the



Scheme 1. Synthesis of compound **V1**.

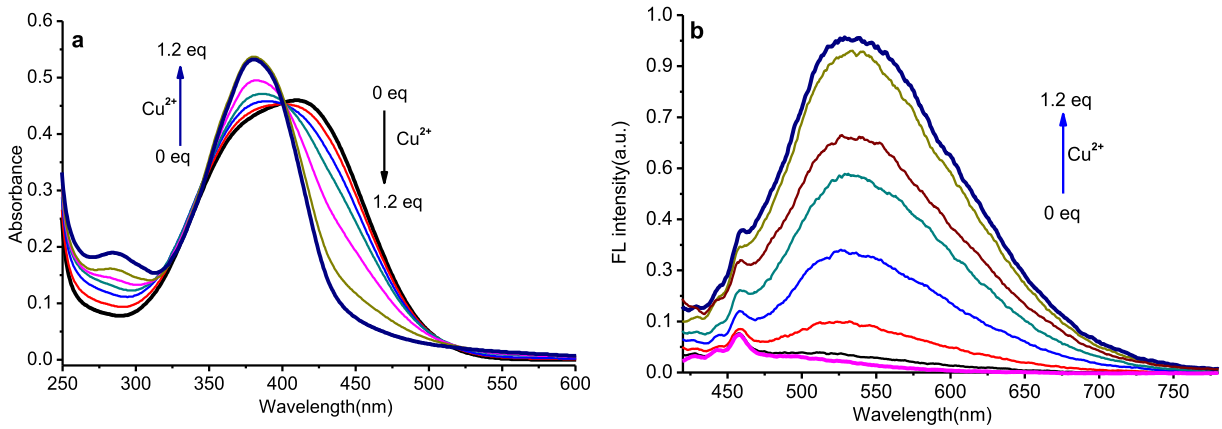


Fig. 1. Absorption spectra (a) and fluorescence spectra (b) of **V1** (10 μmol/L) with the addition of increasing concentrations of Cu^{2+} (0–12 μmol/L) in acetonitrile (Ex = 400 nm).

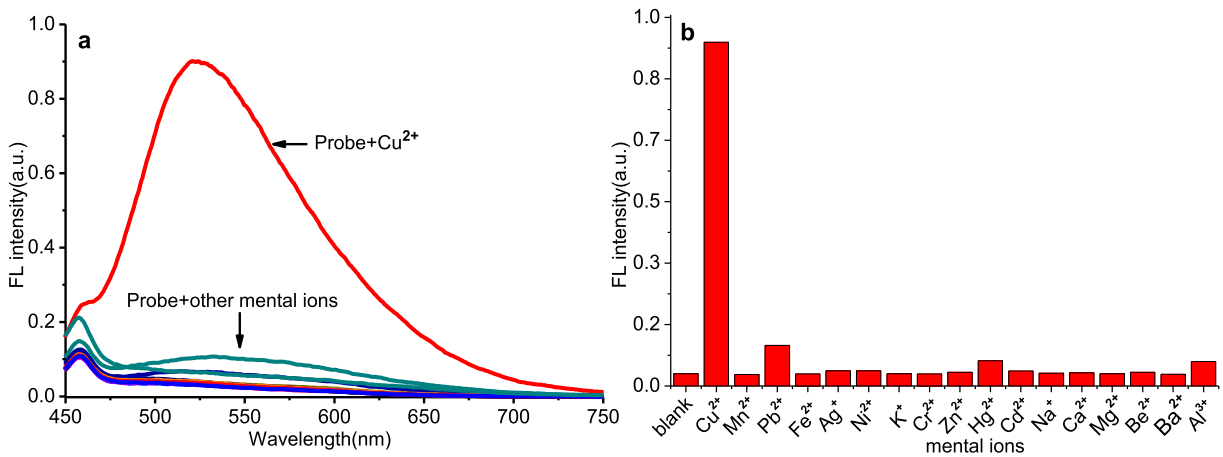


Fig. 2. The fluorescence spectra (a) and histogram (b) at 525 nm of probe **V1** (10 μmol/L) upon the addition of various metal ions (10 μmol/L) in the presence of Cu^{2+} (10 μmol/L) as background in acetonitrile.

solvent was evaporated by rotary evaporator, getting powder product **V1**–$\mathbf{Cu^{2+}}$complex. The sample was used directly for infrared (FT-IR) spectra testing without purification.

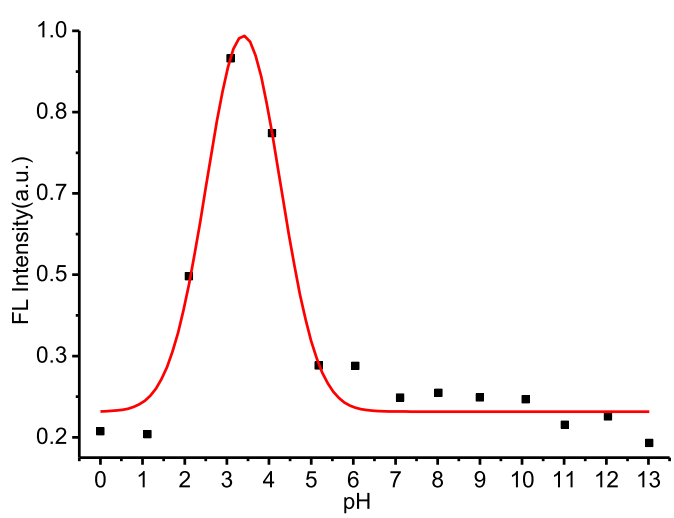


Fig. 3. The fluorescence intensity at 525 nm of probe V1 (10 μM) in PBS solution at different pH values (λex = 380 nm).

3. Results and discussion

3.1. Spectral characteristics

The absorption and fluorescence spectra of **V1** (1.0×10^{-5} M) in acetonitrile was explored. As shown in Fig. 1a, there was a wide absorption band centered at 415 nm in the range 300–500 nm.

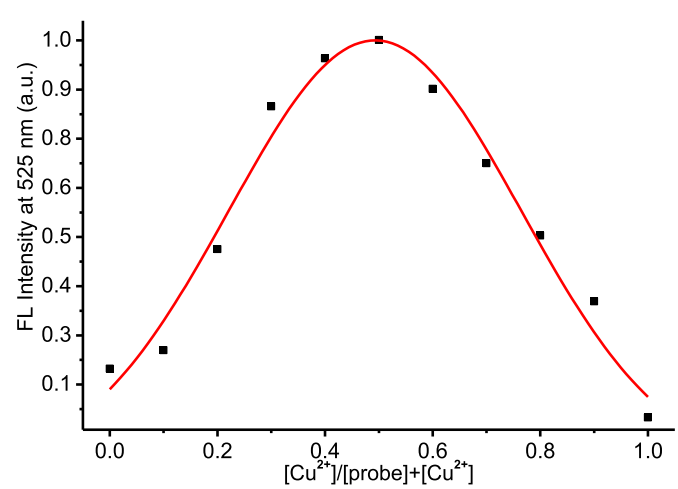


Fig. 4. Job's plot of the binding between probe and Cu^{2+}. Fluorescence at 525 nm (λex = 380 nm) was plotted as a function of the molar ratio $[Cu^{2+}]/([probe]+[Cu^{2+}])$ (the total concentration of probe and Cu^{2+} were 20 μmol/L).

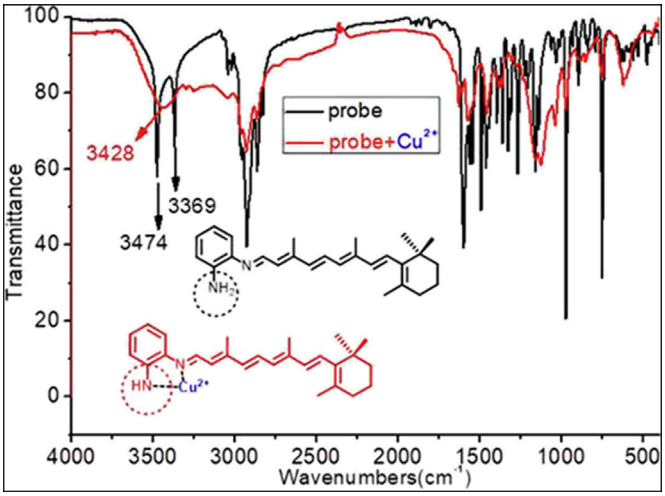
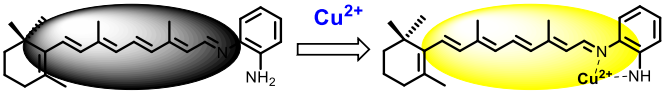


Fig. 5. Infrared spectra of compound **V1** (black) and **V1**–**Cu**$^{2+}$ (red).



Scheme 2. The proposed binding mechanism of compound **V1** with Cu^{2+}.

Under the excitation of 400 nm light, no fluorescence emission peak of **V1** was detected within 425–750 nm (Fig. 2b). And then, the quantitative copper ions were gradually added to V1 solution. Correspondingly, the original absorption peak at 415 nm regularly blue shift to 380 nm. The isosbestic point at 400 nm indicated the existence of an equilibrium between **V1** and **V1**–**Cu**$^{2+}$ complex (Fig. 1a). The fluorescence emission peak at 525 nm gradually increased with the increasing concentrations of Cu^{2+}. After 1.2 equivalents of copper ions were added to the **V1** solution, the intensity of the fluorescence was significantly enhanced by 50 times (Fig. 1b). The change of UV–visible spectrum is attributed to the change of charge distribution of the probe due to the formation of complexes **V1**–**Cu**$^{2+}$. The appearance of a fluorescent emission peak may involve the blocking of the photo-induced electron transfer when imine and amine groups coordinated to the copper ion [28].

3.2. Selectivity

As the recognition group, Schiff base structure has excellent selectivity to copper ion [24,28]. The fluorescent probe **V1** based on Schiff base structure of retinal and o-phenylenediamine inherit the characteristics of excellent selectivity. The experimental data also demonstrates that the probe **V1** has a high selectivity for copper ions. After the 16 different metal ions (Mn^{2+}, pb^{2+}, Fe^{2+}, Ag^{+}, Ni^{2+}, K^{+}, Cr^{3+}, Zn^{2+}, Hg^{2+}, Cd^{2+}, Na^{+}, Ca^{2+}, Mg^{2+}, Be^{2+}, Ba^{2+} and Al^{3+}) were separately added into **V1** solution, only slight changes were observed in its fluorescence spectra. The fluorescence enhancement process happened only in the presence of Cu^{2+} compared to the blank (Fig. 2). The corresponding absorption spectra changes of **V1** were shown in Fig. S5.

3.3. Effects of pH

The nitrogen-containing chelator is susceptible to acid-base effects [29,30]. Therefore, it is necessary to check the fluorescence properties of **V1** in solution at different pH values. The acid–base titration experiments were carried out by adjusting the pH with an aqueous solution of NaOH and HCl of phosphate-buffered saline (PBS) (Fig. 3) As evidenced in Fig. 5, these results showed that the fluorescence at 525 nm of free **V1** was low and varied little in a wide range (pH = 6.0–13.0). The probe showed the increased fluorescence intensity at 525 nm in the acidic medium (pH = 1.0–5.0). It can be predicted that probe **V1** could be used in drinking water and also sense the Cu^{2+} concentration under physiological conditions for biological applications. The proposed mechanism for the fluorescence of **V1** affected by pH may be that the protonated nitrogen atom inhibited the quenching process via the photo-induced electron transfer (PET) from o-phenylenediamino to the excited fluorophore retinal [31].

3.4. Sensing mechanism

Theoretically, probes and analytes have specific stoichiometric ratios, using coordination as sensing mechanism [32]. Therefore the binding stoichiometry between **V1** and Cu^{2+} was investigated through Job's plot experiments (Fig. 4). The fluorescence emission intensities of **V1** and Cu^{2+} mixture at 525 nm were recorded. The molar fraction of Cu^{2+} with a constant total concentration of **V1** and Cu^{2+} (20 μmol) varied from 0 to 1. The fluorescence emission intensities at 525 nm reached maximum value, when the molar fraction was 0.5 for **V1**, indicating the formation of 1 : 1 complexes between the **V1** and Cu^{2+} (Scheme 2).

To further explore the binding mechanism in detail, the infrared spectra of compound **V1** and its coordination complex **V1**–**Cu**$^{2+}$ were measured. The infrared spectrum of **V1** and **V1**–**Cu**$^{2+}$ were overlaid and compared (Fig. 5). The significant differences in spectra can lead to important inferences. Firstly, two N–H stretching vibration peaks of the N–H_2 group at 3369 cm^{-1} and

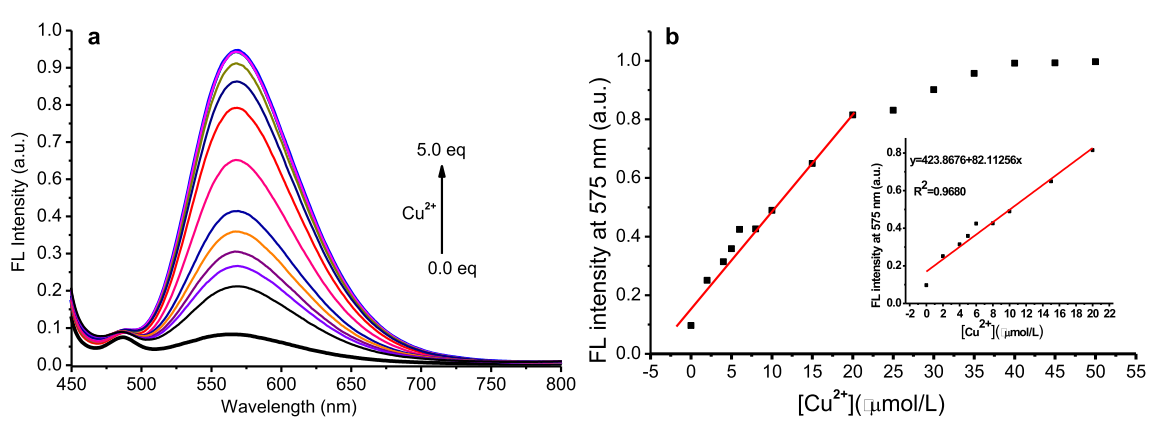


Fig. 6. (a) Fluorescence specta of **V1** (10 μmol/L) with the addition of increasing concentrations of Cu^{2+} (0–50 μmol/L) in physiological saline (DMSO/physiological saline (V/V) = 1:1000, pH = 6.5, Ex = 400 nm). (b) The curves of the fluorescence emission intensity at 575 nm versus the concentration of Cu^{2+}. Inset: Calibrations curve in the concentrations range of 0–20 μmol/L of Cu^{2+}.

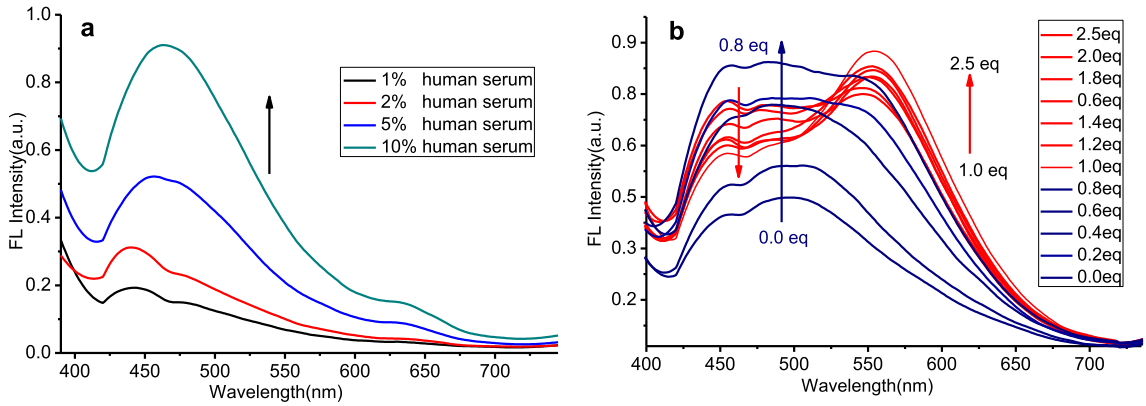


Fig. 7. (a) Fluorescence spectra of **V1** (10 μM) in the presence of different human serum solution (1%, 2%, 5%, 10% human serum); (b) Fluorescence spectra of **V1** (10 μmol/L) with the addition of increasing concentrations of Cu^{2+} (0–25 μmol/L) in physiological saline containing 2% human serum (λex = 380 nm).

3474 cm^{-1} was obvious recorded in the free **V1** molecular. Secondly, after the N–H_2 group on phenylenediamino binding with Cu^{2+}, the two stretching vibration peaks of N–H_2 group disappeared. Instead, the single stretching vibration of the N–H at 3428 cm^{-1} appeared in the coordination complex **V1**–$\mathbf{Cu^{2+}}$ (Fig. 5). According to the Job's plot and infrared spectra, a proposed binding mechanism of **V1** with Cu^{2+} was shown in Scheme 2. Two nitrogen atoms on the phenylenediamino can cooperatively participate in the binding with Cu^{2+}.

3.5. Sensing of Cu^{2+} in physiological saline and human serum

In order to assess the practical application of the probe **V1**, the titration experiment for Cu^{2+} were carried out in physiological saline (Fig. 6a). As shown in Fig. 6a, the emission intensity at 575 nm gradually increased with the increasing concentrations of Cu^{2+} until 50 μmol/L Cu^{2+}. Due to the strong polarity of physiological saline, the fluorescence emission of **V1** shifted from 525 nm (in acetonitrile) to 575 nm [33]. Furthermore, the curve of the fluorescence emission intensity at 575 nm versus the concentration of Cu^{2+} appeared a good linear relationship in the range of 0–20 μmol/L of Cu^{2+} (Fig. 6b). The linear equation was $y = 82.1125\,x + 423.8676$ ($R^2 = 0.9680$) and the detection limit of **V1** for Cu^{2+} was 0.05 μmol/L [34]. Therefore, the probe **V1** is fully capable of detecting copper ions in drinking water (ca. 20.0 mol/L) [35] or blood (ca.15.7–23.6 mol/L) [36]. These basic experimental data will provide an important reference for the probe application in the actual physiological environment.

In order to give insight to the advantages of retinal compatibility with the protein environment, the titration experiment for Cu^{2+} were carried out in human serum solution. The residues of proteins in serum have strong chelation to copper ions and compete with probes. In addition, protein structure and probe interactions in serum may affect probe's fluorescence emission [37]. Both of these aspects will increase the difficulty of Cu^{2+}detection in serum solution. It was exciting that probe **V1** was able to differentially respond to serum proteins and copper ions, exhibiting different fluorescence emissions. As shown in Fig. 7a, owing to the slender conjugated polyene structure retinal of **V1** was conducive to entering protein structure, the emission intensity at 460 nm of **V1** gradually increased with the increasing concentrations of human serum. However, the fluorescence emission peaks at 460 nm and 550 nm both gradually increased in the range of 0–8 μmol/L of Cu^{2+} in human serum (Fig. 7b). When the Cu^{2+} concentration from 10 μmol/L increasing to 25 μmol/L, the fluorescence emission peak at 460 nm gradually decreased and the peak at 550 nm continuously increased (Fig. 7b). Although most of the copper ions may be sequestered by serum proteins in the low Cu^{2+} concentration range (0–8 μmol/L), the probe **V1** was still able to respond to chelated copper ions. This fully reflected the advantages of retinal chromophores in congenital compatibility with protein environment.

4. Conclusion

In summary, we have synthesized a novel retinal-based fluorescence probe **V1** that can be utilized as an excellent selectivity and sensitivity sensor for Cu^{2+}. This work opens up the use of flexible fluorophore retinal in fluorescent probes. In addition, the probe **V1** can work in physiological saline and serum which is important for possible use in a practical view on selective requirements for biomedical and environmental application. Further studies to develop fluorescent probe based on retinal are underway.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2019.117565.

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