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Short communication

A novel pyrazoline-based fluorescent probe for Cu^{2+} in aqueous solution and imaging in live cell

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ABSTRACT

In this study, a new pyrazoline derivate (1-acetyl-3- (2-hydroxy-1-naphthyl)-5-(2-phenyl-2H-chromene)-2-pyrazoline, **N**) is described as fluorescent probe for fluorometric detection of Cu^{2+} . This fluorescence of probe has a high selectivity only toward Cu^{2+} ion in the other metal ions. It is remarkably observed that the fluorescence intensity of **N** decreased in the presence of Cu^{2+} ions with a strong capability to resist perturbations. The Limit of detection (LOD) value is calculated as 8.85×10^{-7} M for Cu^{2+} while the complexation constant is calculated as 3.14×10^5 M⁻² via fluorescence measurements. The interaction between of **N** and Cu^{2+} is reversible after adding **PPi**. The optimized calculation of the probe for Cu^{2+} is confirmed by TD-DFT calculations. **N-Cu**²⁺ is successfully applied in the intracellular imaging in living **PK-21** cells that will have a great chance for wide application.

1. Introduction

 Cu^{2+} plays critical function in living organisms, especially in the process of cellular energy generation, free radical detocification, oxygenic photosynthesis and signal transduction [1–4]. It plays an indispensable role in the physiology and pathology process, since the Cu^{2+} ion participates in various complexation of amino acids, enzyme reactions and electron transduction as the co-factor of related enzymes [5]. However, its insufficiency or excess load in the human body will cause negative effects on human body, such as Alzheimer's disease, gastrointestinal disturbances, neurological disorders, and progression of cancer [6–9]. So, selective and sensitive detection studies for Cu^{2+} are quite important.

In recent years, the detection of Cu^{2+} ions using a new fluorescence probe is a greatly attractive research area due to fluorometric detection is highly sensitive, the economy in addition to getting the response rapidly and compared to traditional methods including atomic absorption spectrometry, chromatography, and plasma mass spectroscopy [10–12]. Many fluorescence probes based on rhodamine, naphthalimide, coumarin, and tetraphenylethylene are widely used in the detection of Cu^{2+} ion [13–18]. However, they have limited application in the detection and real-time monitoring of copper due to they are typically irreversible and tend to be affected by many factors such as environmental conditions, instrumental efficiency, and probe concentrations [19]. Beyond that, among the various chemosensing techniques, sensors based on a naked-eye response (colorimetric) have great attention since they do not depend on any expensive and specific instrumentation and can provide a selective, precise and sensitive method for the detection of a target analyte [20,21]. So, it is an attractive and challenging field development of new fluorometric sensors for the detection of Cu^{2+} ion.

Pyrazoline derivatives are five-membered heterocyclic compounds with excellent non-toxic bioactive compounds [22,23]. Therefore, they have become a popular and important pharma core in medicinal chemistry such as antibacterial, anticancer, antitumor, antiinflammatory and many other activities [24–28]. In addition to these compounds have widely been used as fluorometric ion sensor, holetransport material in optoelectronic application with special fluorescence properties [29–31]. Recently, Li reported a dicyanomethylene-4*H*pyran-based fluorescence probe was designed for Cu^{2+} detection [4]. It exhibited remarkable sensitivity and selectivity. Importantly, DCM-Cu was successfully applied in the detection of Cu^{2+} in living cells with

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Scheme 1. The synthetic route of Compound (N).

low cytotoxicity. While preparing the manuscript, Qian had synthesized a fluorescent Au nanocluster LRL-AuNCs with RGDK-containing peptide [32]. The LRL-AuNCs probe was used to detect copper ions in water samples. Moreover, it has the advantages of low cytotoxicity and good biocompatibility. These two pioneering research reports were particularly encouraging for our work. However, these two probes are easily affected by the surrounding environment and can't do reversible recognition, naked-eye recognition.

In the present study, we have been investigated the influences of many kinds of metal ions on absorption and fluorescence properties of the novel pyrazoline derivative compound N (Scheme 1) having the chemical structure(1-acetyl-3- (2-hydroxy-1-naphthyl)-5-(2-phenyl-2Hchromene)-2-pyrazoline) by using UV-Vis absorption and steady-state fluorescence measurements. These obtained results exhibited that N is a sensor with highly selective recognition behavior for the detection of Cu^{2+} compared to the other relevant metal ions. As a Cu^{2+} selective probe, this quenched fluorescence intensity can be used for the detection of analytes [33,34]. Besides, the resulting N-Cu²⁺ compound reacts specifically with pyrophosphate (PPi) anion through fluorescence quenching. Furthermore, this probe was successfully applied for fluorescent Cu²⁺ imaging in living cells and the color of compound N changed significantly after interaction with Cu²⁺ from colorless to pale yellow by naked-eye observation, indicating its value of practical application in environment and biology.

2. Experimental

2.1. Chemicals and reagents

The details are in supporting information

2.2. Synthesis of compound N

The synthesis of compound N is as follows: 2-hydroxy-1-acetonaphthone is purchased from commercial suppliers. Compound 3 was prepared according to literature method [36,37]. The details are in supporting information.

2.3. Spectral test

The details are in supporting information

2.4. Cytotoxicity assay

The details are in supporting information

3. Results and discussion

3.1. Identification of copper ions by fluorescence and UV

In the CH3CH2OH/H2O system, under the excitation wavelength of



Fig. 1. The fluorescence spectra of N in CH₃CH₂OH/H₂O (1:1(v/v), HEPES = 10 mM, pH = 7.4) solution in the presence of various metal ions. (λ ex = 350 nm).). Insert: Photos of N (10 μ M) in ethanol–water with and without addition of Cu²⁺ (5 equiv.)

275 nm, the probe clearly showed strong fluorescence emission at 417 nm (Fig. 1). This strong fluorescence of probe N may be due to the C = N - N isomerization with the pyrazoline fluorescent group. The experimental results show that upon the addition of Cu^{2+} to the solution of probe N, an obvious decrease in fluorescence intensity was observed. Whereas, other metal ions have little effect on the fluorescence of the system or have a very small fluorescence quenching effect.

The concentration of probe N was kept at 1.0 \times 10^{-5} M, and the concentration of Cu^{2+} varied from 0 to 15.0×10^{-5} M, and the UV absorption curve was measured. As can be seen from Fig. 2, with the increasing concentration of Cu^{2+} , the absorbance in the range of 255-330 nm and 390-450 nm gradually increased and remained stable, This may be due to the complexity of probe N with Cu²⁺ which affected the structural change of probe molecule, resulting in the change of absorption peak in the UV absorption spectrum. There was a good linear relationship between probe N absorbance and Cu²⁺ concentration (Fig. 2(a)) at 425 nm. The linear regression equation y = 0.150x-0.0156, $R^2 = 0.989$ was obtained by fitting, as shown in Fig. 2(b). Moreover, large changes in fluorescence intensity and obvious changes in fluorescence color during recognition make it possible to detect Cu²⁺ visually (as shown in Fig. 2(c)). It showed that the color contrast of probe N and probe N-Cu²⁺ in the solution under visible light, indicating that the naked eye recognition effect can be realized during the interaction between probe N and probe N-Cu²⁺.

3.2. Fluorometric titration test of copper ion by probe

In order to solve the sensitivity of L to the Cu²⁺, the fluorescence



Fig. 2. The UV absorption spectra of titration curve of probe N to Cu^{2+} . (a) The change of absorbance of probe N (1.0×10^{-5} M) with Cu^{2+} ; Inset: the nonlinear fitting curve of N to Cu^{2+} at 425 nm and color responses in visible light. (b) A linear fitting plot of absorbance of probe N with Cu^{2+} at 425 nm.



Fig. 3. The fluorescence spectra titration curve of probe N. (a) Fluorescence changes of N with different concentrations of Cu^{2+} in CH_3CH_2OH/H_2O (1:1(v/v), HEPES = 10 mM, pH = 7.4); (b) Fluorescence spectra of the probe N as a function of Cu^{2+} concentration.



Fig. 4. The fluorescence intensity of probe N was changed due to the change of Cu^{2+} concentration at 417 nm.



Fig. 5. Fluorescence intensities of N in CH_3CH_2OH/H_2O (1:1(v/v), HEPES = 10 mM, pH = 7.4) in the presence of various metal ions and further addition of Cu^{2+} .



Fig. 6. Fluorescence spectra of N in CH_3CH_2OH/H_2O (1:1(v/v), HEPES = 10 mM, pH = 7.4) in the presence of different copper salts.

titration of N (10 μ M) was performed with Cu²⁺ in ethanol–water (1:1 (v/v)) solution (Fig. 3a). The Cu²⁺ ions concentration as the factor increases, the fluorescence intensity of probe N decreases continuously until it reaches saturation. The quantitative response of probe N to Cu²⁺ has a good linear relationship. The Benesi-Hildebrand [39] nonlinear curve fits a straight line, showing a linear fit, and confirms that the stoichiometric relationship between Cu²⁺and probe N is 1:1. From Fig. 3b, we can conclude that the binding constant of the M–Cu²⁺complex is 3.14 × 10⁴ M⁻¹. As shown in Fig. 4, the linear regression equation is y = 1.59879 + 0.26413x, R² = 0.9298. The Benesi-Hildebrand nonlinear curve fits a straight line, showing a linear fit, and confirms that the stoichiometric relationship between Cu²⁺and probe N is 1:1. The minimum detection limit is 8.85 × 10⁻⁷ M (probe of concentration 1 × 10⁻⁵ M) according to the formula LOD = 3\sigma/k (σ denotes the 10 relative standard deviation fluorescence intensity of N).

3.3. Competitive and selective testings

The interference of coexisting ions is an important indicator of the recognition performance of the probe. Therefore, the anti-interference experiment was carried out. As shown in Fig. 5, the fluorescence intensity of probe N was significantly quenched when various ions

coexisted with Cu²⁺. This shows that probe N has a high antiinterference capability without affecting the fluorescence recognition of Cu²⁺ under the interference of other metal cations. In addition, in order to explore the influence of anions on the process of recognition of Cu²⁺ by probe N, fluorescence spectrum experiments were conducted on copper salts of sulfate, acetic acid, chloride and nitrate in CH₃CH₂OH/ H₂O(1:1(V/V), HEPES = 10 mM, pH = 7.4) solution by probe N. The concentration of various metal ions is 1.0×10^{-2} mM. As can be seen from Fig. 6, there was no significant change in fluorescence intensity in the process of probe N identifying CuSO₄, Cu(CH₃COO)₂·H₂O, CuCl₂ and Cu(NO₃)₂.

In order to explore the practical application of probe N in a biological environment at room temperature, the absorption spectrum characteristics of probe N in a mixed solvent containing CH_3CH_2OH/H_2O ((1:1, v/ v) for various metal ions were studied. As shown in Fig. 7a, without adding Cu^{2+} ions, probe N showed a maximum absorption peak at 320 nm. Then, Cu^{2+} ions were added to the probe N. Cu^{2+} complexed with probe N, causing the absorption peak to appear at 325 nm with a slight redshift, and a new strong absorption peak appeared within the range of 390–450 nm It can be clearly seen from Fig. 7b that when adding Cu^{2+} and other metal ions (Ag⁺, Al³⁺, Fe³⁺, Co²⁺, Ni²⁺, Ba²⁺, Ca²⁺, Cu²⁺, Cd²⁺, K⁺, Mg²⁺, Na⁺, Hg²⁺, Zn²⁺, Pb²⁺, Li⁺, Mn²⁺), only Cu²⁺ absorption peak has obvious changes. The addition of Fe³⁺ and Fe²⁺



Fig. 9. Job's plot of N and $\rm Cu^{2+}$ complexation in $\rm CH_3CH_2OH/H_2O(1:1(v/v),$ $\rm HEPES=10$ mM, pH=7.4).



Fig. 7. The UV absorption spectra of probe N. (a) The UV absorption spectra of N $(1.0 \times 10^{-5} \text{ M})$ and N-Cu²⁺ in CH₃CH₂OH/H₂O(1:1(v/v), HEPES = 10 mM, pH = 7.4) solution. (b) The UV absorption spectra of N in CH₃CH₂OH/H₂O(1:1(v/v), HEPES = 10 mM, pH = 7.4) solution in the presence of various metal ions(1.0 × 10^{-2} mM).



Fig. 10. ¹H NMR spectra of N with Cu^{2+} in d-DMSO. a) Free probe N; b) Upon addition of 1.0 equiv. of Cu^{2+} to probe N; c) PPi to the mixed solution of N and Cu^{2+} ;d).



Fig. 11. Possible complexation of probe N with Cu^{2+} .

enhanced the overall absorption strength of probe N.

3.4. Reversibility and the proposed sensing mechanism

3.4.1. Possible complexation mechanism between probe N and Cu^{2+}

In order to explore the stoichiometric ratio of complexation between probe N and Cu²⁺, we carried out the Job's Plot experiment. The total concentration of probe N and Cu^{2+} was maintained at $1.0\times 10^{-5}\text{m},$ the concentration ratio of the two was changed, and the fluorescence intensity at 417 nm was measured respectively. It is concluded that when the concentration ratio of Cu^{2+} in probe N and Cu^{2+} is 0.5 in the whole system, the minimum fluorescence intensity occurs (Fig. 9). It also indicates that the complexation ratio of probe N and Cu^{2+} is 1:1. In summary, combined with the recognition mechanism of some pyrazoline based fluorescent probes reported currently [40-44], it may be that Cu²⁺ coordinates with nitrogen atoms and oxygen atoms on the corresponding pyrazoline structure and oxygen atoms on the hydroxyl group on the naphthene structure, leading to fluorescence quenching. In order to further explore the coordination mode between probe and N and Cu²⁺, ¹H NMR titration experiment was conducted (Fig. 10). It was found that when Cu^{2+} was added to probe N, pyrazoline and the protons on naphthalene showed the displacement of high or low field, and the hydroxyl proton peak at 10.29 PPM decreased during the addition of Cu^{2+} . It almost disappears when adding 1.0 equivalent Cu^{2+} . Thus, the



Fig. 12. The change of fluorescence spectra of N-Cu²⁺ in CH₃CH₂OH/H₂O(1:1 (v/v), HEPES = 10 mM, pH = 7.4) upon addition of PPi.

hydroxyl group was involved in the reaction of probe N and Cu^{2+} in the binding process, and the complexation ratio was 1:1. While the PPi was added, the probe N could be reused. We could infer the possible complexation mode between probe N and Cu^{2+} , as shown in Fig. 11.

3.4.2. PPI test fluorescence probe N detection Cu^{2+} reversibility experiment

In order to further understand the complexation behavior between probe N and Cu^{2+} , PPi(pyrophosphate) was added to the N- Cu^{2+} system to study the effect of PPi on fluorescence signals. As the amount of PPi was continuously added to the N- Cu^{2+} system, the fluorescence intensity of probe N was restored (Fig. 12). The added PPi can eliminate Cu^{2+} from the complexed system with probe N. Because the complexation of PPi and Cu^{2+} is much larger than that of probe N and Cu^{2+} , thus making



Fig. 13. Optimized structures of N and N-Cu²⁺.



Fig. 14. Frontier molecular orbital energy of N and N-Cu²⁺ at the excited state.

probe N free to recover its fluorescence intensity. This indicates that the fluorescence attenuation of the N-Cu²⁺ system is caused by the complexation of probe N and Cu²⁺, rather than the change of its structure.

3.4.3. Optimized calculation

The Gaussian 09 package of quantum chemistry [45] was used to optimize the structure of N and N-Cu²⁺ with B3LYP/ 6-31g(d) [46–48], and the structure of the probe molecule with the minimum energy was optimized as shown in Fig. 13. TD-DET was used to calculate the excited states of the above-optimized probe molecules, and their front-line orbital energy and molecular transition energy were obtained. As shown in Fig. 14, the energy difference of electrons from HOMO to LUMO is 0.0764 eV after probe N is excited. In the N-Cu²⁺ molecule, it was found that the addition of Cu²⁺ resulted in a reduction in the HOMO-LUMO energy gap of the probe, and the energy level difference was 0.0172 eV when the HOMO jumped to the LUMO orbital. By comparison, the reduction was 0.0592 eV. This is due to the hydrogen

bonding interaction between C=N and C=O. Due to the addition of Cu^{2+} in pyrazoline, the HOMO of $N+Cu^{2+}$ is limited to this fragment, and the CT from the electron donor to the electron acceptor group is disturbed in $N+Cu^{2+}$, leading to a decrease in fluorescence.

3.5. Imaging of intracellular Cu^{2+} and cell viability of the probe

The sensitivity of **N** for Cu^{2+} in living PK-21 cells was measured by fluorescence microscopy. Fluorescence images of cells were obtained by fluorescence microscope at 425 nm excitation wavelength and 5.0 nm emission band. First, PK-21 cells incubated with the probe **L** indicated a blue fluorescence image (Fig. 15(a)). The blue fluorescence was disappeared after incubation of the probe **N** treated cells with Cu^{2+} (Fig. 15 (b)). The fluorescence images showed that the fluorescence signals were located in the intracellular region which indicated that probe **N** had great cell membrane permeability. After adding Cu^{2+} , it was found that the fluorescence intensity of the PK-21 cell nucleus decreased significantly over time. Therefore, the fluorescence intensity signals were



Fig. 15. Luminescence images of PK-21 cells. (a) PK-21 cells incubated with probe N (20 μ mol/L) for 30 min at 37 °C; (b) PK-21 cells incubated with probe N for 30 min and then further incubated with 30 μ L Cu²⁺ for 30 min at 37 °C.



Fig. 16. Cell viability graph of probe L using BK-21 cells by MTS assay.

changed the probe N which could be used to detect intracellular Cu²⁺ and sense the change of its concentration. It also provided a feasible method and means to detect Cu²⁺ in the biological system in the future. An MTS assay was used to detect cell viability. The viability rate of cells treated with the range of concentration 0–100 μ M of N for 24 h was shown in Fig. 16. The probe N was found to be the least toxic to the cells.

4. Conclusion

In summary, a novel fluorescent probe based on naphthyl-pyrazoline for Cu (II) ions recognition was designed and synthesized. It showed that the probe N in the mixed solution has high selectivity, sensitivity and anti-interference abilities which were observed by the ultraviolet absorption spectrum and fluorescence spectrum in the ethanol–water system. The complexation of probe N and Cu²⁺ is a reversible process and can be recognized by naked eyes. The blinding constant of the N-Cu²⁺ complex was found to be Ka = 3.14×10^4 M⁻¹ and the limit of detection is 8.85×10^{-7} M. The probe N has been used for imaging Cu²⁺

in cells under physiological condition and shows low toxicity. Moreover, the binding mode and sensing mechanism of N with Cu^{2+} was proved by theoretical calculations using Gaussian 09based on B3LYP/6-31G(d) basis.

CRediT authorship contribution statement

Ying-peng Zhang: Conceptualization, Methodology, Supervision. Yu-chen Zhao: Data curation, Writing - original draft. Qing-hua Xue: Investigation. Yun-shang Yang: Investigation. Hui-Chen Guo: . Ji-Jun Xue: Software.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.inoche.2021.108612.

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