A novel approach to study the structure-property relationships and applications in living systems of modular Cu²⁺ fluorescent probes

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A series of Cu²⁺ probe which contains 9 probes have been synthesized and established. All the probes were synthesized using Rhodamine B as the fluorophore, conjugated to various differently substituted cinnamyl aldehyde with C=N Schiff base structural motif as their core moiety. The structure-property relationships of these probes have been investigated. The change of optical properties, caused by different electronic effect and steric effect of the recognition group, has been analyzed systematically. DFT calculation simulation of the Ring-Close and Ring-Open form of all the probes have been employed to illuminate, summarize and confirm these correlations between optical properties and molecular structures. In addition, biological experiment demonstrated that all the probes have a high potential for both sensitive and selective detection, mapping of adsorbed Cu²⁺ both in vivo and environmental microbial systems. This approach provides a significant strategy for studying structure-property relationships and guiding the synthesis of probes with various optical properties.

Selective recognition and detection of cations by receptors have attracted a significant amount of attention in chemistry, biology, and environmental science⁴⁻⁷. Among various detection receptors, fluorescent probe, based ion-induced changes in absorption or fluorescence spectra, appears to be particularly attractive due to their simplicity, high sensitivity, excellent selectivity, and instantaneous response⁵⁻⁷. However, properties of a fluorescent probe depend strongly on the binding properties of the target cation and the recognizing site in probe molecular³⁻⁴. Therefore, the binding properties and the geometrical configuration of the coordination sites in the corresponding probes are the most important parameters to consider during design and synthesis fluorescent probes. A commonly strategy is to attach a ligand in fluorophore to form the target recognizing site in the design of fluorescent probes¹⁰⁻¹². High sensitivity and selectivity are fundamental goals and core design principles for an excellent probe. Though a large amount of researches had reported in the past decades, these researches did not mention electronic and steric effects that can regulate the performance in the recognition process¹³⁻¹⁶.

Copper, as the third most abundant essential trace element in human body, plays a crucial role for a broad range of biological processes¹⁷⁻²⁰. However, copper excess cause toxicity to most living organisms, and copper release can eventually lead to serious environmental contamination²¹⁻²³. Therefore, a convenient and rapid method for the detection of copper became increasingly important in biological and environmental concerns. Though chelating of Cu²⁺ with chemo sensors is well known to induce intrinsic fluorescence quenching due to the paramagnetic nature of Cu²⁺²⁴, hundreds of fluorescent probes have been developed for varying purposes within the last few decades²⁵⁻²⁷. Systematic studies of these effects thus have expected to get great significance for the further development of Cu²⁺ fluorescent probes. Evermore, selection of an adequate probe for CLSM studies in bio-, geo-, and environmental sciences became more and more challenging²⁸.

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In order to investigate the effect of structure on performance for a probe, we synthesized and established a series of Cu²⁺ probe which contains 9 probes 1a-1i based on our previous work (Fig. 1). Each of them is synthesized using rhodamine B as the fluorophore, conjugated to various differently substituted cinnamyl aldehyde with C=N Schiff base structural motif as its core segment. These probes show superior stable optical properties for the detection of Cu²⁺ which are not affected by fluorescence quenching. All these probes are synthesized easily under chemically mild conditions with a high yield (>80%) through a two-step reaction via Rhodamine B with substituted cinnamyl aldehyde. To our surprise, all probes showed different absorption and fluorescence response intensity to Cu²⁺.

In this work, we focused on the structure-property relationships of this probe model, investigated the change of optical properties caused by different electronic effect and steric effect of the recognition group, and also employed DFT calculation simulate the Ring-Close and Ring-Open form of all the probes (Fig. 2). Comprehensive analyses on the regulatory effects of substituent group about this probe model have been elaborated using frontier molecular orbital theory, NBO charge population and Fukui functional analysis.

Results and Discussion

UV-vis properties. Binding affinities of each probe toward various metal ions, namely the chloride salts of Li⁺, K⁺, Ba²⁺, Ca²⁺, Cd²⁺, Mg²⁺, Co²⁺, Mn²⁺, Zn²⁺, Pb²⁺, Ni²⁺, Fe²⁺, Hg²⁺, Fe³⁺, Al³⁺, Cr³⁺, Cu²⁺ and the nitrate salt of Ag⁺ were evaluated by UV-vis spectroscopy. Upon addition of the respective metal ions, the absorption spectrum of the individual probes changed in different manners as shown in Figure S9. However, all tested metal ions except Cu²⁺ showed only minor spectral or color changes. Similar responses were observed when Cu²⁺ ions were added to the probe solutions in the presence of other metal ions. The absorption spectrum of probe 1a-1i
exhibited a broad band at 556 nm at room temperature in the PBS buffer solution (pH = 7.4, 50% (v/v) ethanol). Since the closed form of rhodamine did not show an intense absorption band in the visible region, this result suggested that probe Cu2+ complexes were not present in the form of closed spirolactone, but in an opened quinoid configuration at neutral pH. Furthermore, the absorption response all individual probes toward Cu2+ showed linear relationships in the range 0–50 μM of Cu2+ (all the graphs are showed in Supplementary Information).

More importantly, the UV absorbance of these probes was shown to be adjusted by the different substituent groups. As shown in (Fig. 3a), the comparison of probe 1a, 1b, 1c indicates that with a volume increases of R1, the strength of UV absorbance decreases; the reasons for this phenomenon may be caused by the increase of steric hindrance effects that obstruct the combination of probe with Cu2+. In other words, R1 plays a negative, volume-dependent role in the detection of Cu2+. When other heavy elements used as substituent at position R1 instead, the UV absorption spectrum in presence of Cu2+ showed a strong absorption band. In this study, we used Br as a substituent for instance. Moreover, we recognized visually that electronenagative groups in the specific recognition unit of probe had a positive effect on the UV absorbance whereas electron-donating groups have a negative effect (probe 1e: R1=NO2, probe 1f: R1=OCH3). In addition, the absorbance of the probe + Cu2+ complexes were reduced gradually (1g > 1h > 1l), when methyl was introduced into different positions of benzene ring, whereas in ortho-, meta-, para-position it had scarcely any effect on the UV absorbance response in this series of probes.

Fluorescence properties. The fluorescence properties of the probes and their respective responses to Cu2+ was evaluated in ethanol-PBS (5/5, v/v, pH 7.4) solution. The remarkable orange fluorescence emission of our probes increase that is induced by Cu2+ can interpreted as the opening of the spiral ring at appropriate pH; the emission band of this probe series appeared at 576 nm. The changes of 1a-1l (10 μM) upon addition of Cu2+ (0–50 μM) have been studied (Figure S9). Fluorescence titration spectra of 1a-1l with Cu2+ from 0 μM to 50 μM showed that these spectroscopic responses increased with increasing concentration of Cu2+ for each probe in this series. We also performed competition experiments with metal ions other than Cu2+ as for the absorbance study. The result revealed that this series of probes has significant anti-jamming capabilities in the presence of 5.0 equiv. of other ions (Figure S5). Furthermore, concentrations of Cu2+ and fluorescence intensities have a good linear relationship as shown in Figure S6. The coordination also shown to be a reversible process. When excess ethylenediamine was added to the colored solution of the complex, the fluorescence disappeared and the pink solution turned back to colorless owing to the de-coordination of Cu2+ (all the graphs are showed in Supplementary Information).

As show in (Fig. 3b), fluorescence intensity of probe 1a-1c was reduced by increasing steric hindrance of substituent group R1. In addition, heavy elements substituting for R1 (e.g. Br) enhanced the fluorescence response significantly. For comparison, we have synthetized a congeneric probe 1j which is substituted by Cl at the same position of Br and exhibited a lower fluorescence intensity than that of 1d. Similarly, we introduced -NO2 and -OCH3 substituents to the recognition unit of the molecule to analyze the relationship between fluorescence intensity and electronic effects. The result suggested that electron-drawing groups promote fluorescence enhancement to a higher extent as compared to electron-donating groups. Substitutions in the ortho-position of the benzene ring increased the fluorescence intensity, whereas this effect was not observed when the substitution was inserted in meta- or para-position.

Theoretical calculation analysis. To better understand the structure-property relationships of these 9 probes, the mode of ring-close and ring-open were studied by DFT calculations, all the calculations were performed with B3LYP functional with a combination of basis of double-ζ quality consisting of 6-31G** for C, H elements and 6-31+G* for N, O, Br elements on Gaussian 09 Program. (Full citations are given in supporting information). The optimized structures were confirmed to be local minima due to the non-existence of imaginary frequency. The environmental effect was included via PCM model with ethanol as the solvent molecule.
Table 1. The calculated energy(E) of all structures.

| No. | Ring-close form (C) | Ring-open form (O) | ΔE (Kcal/mol) |
|-----|---------------------|--------------------|--------------|
| 1   | −1802.3177          | −1802.2962         | 13.5098      |
| 2   | −1841.6321          | −1841.6095         | 14.2351      |
| 3   | −1998.8926          | −1998.8673         | 15.8898      |
| 4   | −4373.1169          | −4373.0969         | 12.5093      |
| 5   | −2006.8251          | −2006.8018         | 14.6411      |
| 6   | −1916.8454          | −1916.8230         | 14.0357      |
| 7   | −1841.6329          | −1841.6110         | 13.7240      |
| 8   | −1841.6377          | −1841.6160         | 13.6003      |
| 9   | −1841.6383          | −1841.6162         | 13.8344      |

As shown in Table 1, all the Ring-Open forms possess higher energy than that of Ring-Close form over 12 Kcal/mol. These results indicated that these probes prefer to present in a lactam form without environmental disturbance. Especially, probe 1a-1c show gradualness energy increase, which may be attributed to the block transition process by the growing steric hindrance. Extraordinary for probe 1d, the exist of heavy atom Br could promote the equalization of electronic cloud distribution and reduce energy accordingly, and the energy required for the formation of Ring-Open structure is less than any other probe, which indicated that probe 1d may show a better optical property because of its easy changed structure. The molecule energy seems to be insensitive to the Push-pull electronic effect and spatial effect on the benzene ring of recognition group.

The results of NBO analysis demonstrate the polarization of Charge distribution (Fig. 5), the electron was flowing from xanthene moiety to the recognized moiety during the process of transform, the charge is which was consistent with reported literature. The larger density of electron cloud represents the higher electron donating ability.

On the other hand, has been successfully used to describe the reactivity concerning electrophilic attack, CuCl₂ attack in this model of probe. The condensed Fukui function of individual atom is obtained from NBO analysis. As shown in Fig. 5 the 3D representation of the clearly demonstrates that the region around carbonyl O atom and hydrazide N atom possesses higher reactivity than other parts of the Ring-Open form and there is no obvious electrophilic site in the recognized moiety of Ring-Close form. These data illustrate that all the probes have the tendency to be opened rather than closed under the entrainment and coordination of Cu²⁺.

CLSM bioimaging applications. These probes were selected for Cu²⁺-localization studies in microbial cell-extracellular polymeric substances (EPS)-mineral aggregates. The photosynthetic Fe(II)-oxidizing Rhodobacter sp. strain SW2 was selected as a model organism because SW2 is known to form such aggregates and for being highly resistant to heavy metals during its Fe(II) oxidization. It was incubated under permanent illumination in fresh water mineral medium for 1 week. The resulting suspension of aggregates that consisted of microbial cells, extracellular polymers and Fe(III)-minerals was equilibrated with 50 μM of CuCl₂ for 30 min. Probe 1a-1i, SYTO 9 green fluorescent nucleic acid stain (1:500) and polysaccharide-specific fluorescent dyes, they further provide the possibility to explore the correlation between Cu²⁺ and organic components in this system on the single cell level under natural and hydrated conditions. Our approach also provides a successful case wherein it is shown how to design, synthesize and improve other metal fluorescent probes with high sensitivity and selectivity. Finally, our attempt also revealed that the library-based strategy for imaging-based fluorescence screening is a powerful tool to select the most appropriate fluorescent metal probes for CLSM studies.

Living cells bioimaging applications. Next, we proceeded to investigate the utility of probe 1a-1i in intracellular imaging with L929 to examine whether it can work in biological systems, the distribution of the probe within the cells was observed by laser scanning confocal microscopy following excitation at 543 nm. As shown in Fig. 7, all the probes can permeate cellular membrane due to the molecule as well hydrophobicity, following
addition of exogenous Cu^{2+}, show relatively obvious intracellular fluorescence with excitation. Bright-field transmission measurements after the probe incubation confirm that the cells are viable. The overlay of fluorescence and bright-field images revealed that the fluorescence signals were localized in the perinuclear region of the cytosol, indicating the subcellular distribution of Cu^{2+} which was internalized into the living cells from the growth medium. We anticipate that these probes will be of great benefit for biomedical researchers investigating the effects of Cu^{2+} in biological systems.

Methods

Materials and Methods. All the reagent grade chemicals consumed in this work were procured from commercial sources and used as received. Cu^{2+} stock solution is prepared to be 200 μmol/L with ethanol using CuCl_2 as the source, and probe 1a-1i stock solution (50 ml) were prepared to be 200 μmol/L with ethanol and solubilized by little acetone (0.1 ml). The solutions of metal ions were prepared in ethanol for the selectivity and competitive
study with chloride salts of Li\(^{+}\), Na\(^{+}\), K\(^{+}\), Ba\(^{2+}\), Ca\(^{2+}\), Cd\(^{2+}\), Mg\(^{2+}\), Co\(^{2+}\), Mn\(^{2+}\), Zn\(^{2+}\), Pb\(^{2+}\), Ni\(^{2+}\), Fe\(^{2+}\), Hg\(^{2+}\), Fe\(^{3+}\), Al\(^{3+}\), Cr\(^{3+}\), Cu\(^{2+}\) and the nitrate salt of Ag\(^{+}\).

To a 10 mL volumetric tube, different concentration of Cu\(^{2+}\), 5.0 mL PBS and 0.50 mL of 200 \(\mu\)M probes were added. The mixtures were diluted to 10 mL with ethanol. Then, 3.0 mL of each solution were transferred to a 1 cm cuvette. The absorbance was recorded at 556 nm and the fluorescence intensity was recorded at 576 nm. The

Figure 6. Single cell scale maps showing the sorption of Cu\(^{2+}\) to cell-EPS-mineral aggregates formed by Fe(II)-oxidizing Rhodobacter sp. strain SW2 incubated with 50 \(\mu\)M CuCl\(_2\) for 1 h at 25 \(^\circ\)C. The aggregate was simultaneously incubated with 50 \(\mu\)M of probes 1a-1i (2) and 20% C\(_2\)H\(_5\)OH, fluorescent nucleic acid stain (1) and lectin conjugate (4) for 1 h at 25 \(^\circ\)C, minerals of biogenic origin were visualized employing their reflection signal (5) (\(\lambda_{ex}\) = 488 nm, 561 nm, 635 nm). The overlay image of (1) (2) and (4) is shown in (3); the overlay image of all four signals is shown in (6). Brighter colour indicates a higher metal concentration.

Figure 7. Fluorescent images of L929 cells incubated with 10 \(\mu\)M probes for 30 min (1) and then further incubated with 50 \(\mu\)M Cu\(^{2+}\) for 30 min (2). (3) Bright field image of cells. The overlay image of (2) and (3) is shown in (4) (\(\lambda_{ex}\) = 543 nm).
excitation and emission wavelength bandpasses were both set as 5.0 nm and the excitation wavelength was set at 550 nm.

A Beijing TAIKE XT-4 microscopy melting point apparatus was used to measure the melting points of the compounds. Mass spectrometry was performed on a BRUKER microOTOF-Q II ESI-Q-TOF LC/MS/MS Spectroscopy. NMR spectra were recorded on a VARIAN INOVA-400 spectrometer, using TMS (TMS trimethylsilyl) as an internal standard. The absorbance spectra were recorded on a SHIMADZU UV-1700 spectrophotometer. A HITACHI F-4500 FL spectrophotometer employed to record the fluorescence spectra. A VARIO EL analyzer was used for performing the elemental analyses. Samples for mapping Cu²⁺ were analyzed using an upright confocal laser scanning microscope (LEICA SPE, Wetzlar, Germany), equipped with 4 lasers (405 nm, 488 nm, 561 nm, 635 nm) and a 63x water immersion objective (ACS APO 63x, NA = 1.15).

Conclusions

In summary, we have synthesized and evaluated a series of Cu²⁺ probes which contains 9 probes, all the probes are based on the core segment of C=N Schiff base structural motif. This study revealed the relation between the structure and the optical properties of nine probes focusing on the influence of various substituents and provided a significant strategy for studying structure-property relationships which were illuminated, summarized and confirmed by DFT calculation simulation on the Ring-Close and Ring-Open form of this modular Schiff base Cu²⁺ fluorescent probes. We expect that these results of our research would help to guide the synthesis of high performance metal fluorescence probes with various properties. On the other hand, all the probes could permeate cellular membrane to investigating the effects of Cu²⁺ in living cells. Furthermore, CLSM experiments suggested that all the probes would be a powerful tool for sensitive and selective detection and mapping of Cu²⁺ adsorbed in environmental microbial systems.

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Author Contributions
M.S. designed the study, theoretical calculation analysis and wrote the manuscript. Z.Y. performed the probes characterization, cell assays and edited the manuscript. Z.W. and T.L. did the synthesis of these fluorescent probes. L.H. and M.O. performed the CLSM bioimaging applications. P.L. and Y.S. edited the manuscript, S.Z. and J.L. supervised the project. All authors reviewed the manuscript.

Additional Information
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