Molecular design for novel sensing materials with self-screening interference effect (SSIE): Reversible recognizing Cu$^{2+}$ in aqueous and biologic samples

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A B S T R A C T

In the work, self-screening interference effect (SSIE) was proposed for sensing trace Cu$^{2+}$ by simply thermodynamic control reactions, using dipyridine as self-screening interference group, rhodamine as mother chromophore and cyanuric chloride as connecting bridge. After its UV-vis and fluorescent spectral properties were optimized in detail, it was noted that the present sensing material (RACD) could selectively and reversibly react Cu$^{2+}$ with obvious colorimetric or fluorescent spectral and color changes from colorless to pink or orange-red. Some other concomitant ions, even trivalent Fe$^{3+}$ or Al$^{3+}$, had no interferences on it. Under the optimized conditions, RACD could multiple-mode sense trace Cu$^{2+}$ in aqueous with a detection limit as low as 11.0 nmol/L. Especially with low toxicity, RACD was successfully applied for quantitatively monitoring Cu$^{2+}$ and evaluating its toxicity in living cells and bio-tissues. RACD-functionalized paper-strips were also prepared to visibly recognize Cu$^{2+}$ more conveniently. The selective action mechanism for RACD to Cu$^{2+}$ was to form some stable 5-membered and 5-membered condensed rings between Cu$^{2+}$ and O or N atoms.

1. Introduction

Optical functional materials with special structures and eminent properties have drawn considerable attention with the development of modern science and technology in recent years, which have been widely applied in the fields of photoelectric conversion [1–3], photodynamic therapy [4,5], laser protection [6,7], chemo-sensors [8–11], etc. For constructing an efficient sensing molecule, a generic way is to choose excellent chromophores accompanying with exclusive receptors connecting by suitable conjugated bridging-groups [12–15]. A plethora of functional chromophores have been developed with excellent sensing properties, i.e., nano materials [16–18], phthalocyanine [19–22], rhodamine [23–26], azo compounds [27–29], Schiff base [30–32], squaraine [33–35] and their derivatives. Among all the chromophores above, rhodamine is one of the most popular to be employed as dual-mode colorimetric and turn-on fluorescent application [8,36–41]. For example, Liu, et al. [42], presented a modular approach to constructing a fluorescent dimeric peptide grafting rhodamine chromophore. The work successfully solved the discrepancy in detection limits between PET and fluorescence by improving molar quantum yields, camera hardware, and analyte uptake. Recently, our group [43] also developed a multifunctional rhodamine derivative combining triazine, Schiff-base and phenolic hydroxyl group for turn-on fluorescent responses to Zn$^{2+}$ and Bi$^{3+}$ in CH$_3$CN-water (99/1, V/V), with a detection limit of 3.0 nmol·L$^{-1}$ for Zn$^{2+}$ and 8.6 nmol·L$^{-1}$ for Bi$^{3+}$. All the results suggested that reasonably modifying optical chromophore would be a promising way to improve the resultant sensing properties. However, some of the developed sensors often needed some additional masking reagents to overcome the influence of coexisting interfering substances. For instance, tartaric acid was utilized as a masking agent to selectively determine manganese(II) [44], bisdiglycolamide was selected for detecting americium(III) from europium(III) [45], S$_2$O$_8^{2-}$ and H$_2$O$_2$ were identified for highly selective ‘turn-on’ fluorescent switching recognition of CN$^-$ and I$^-$ [46], and so on [47–49]. Especially for Cu$^{2+}$, the third most abundant metal, which possesses double-edged, i.e., either boosting or damaging human being healthy [50–52], few efficient rhodamine-based sensors have been reported without extra masking agents so far, owing the serious interferences from high-valence ions Fe$^{3+}$ and Al$^{3+}$ [53].

To elaborately illustrate the influence of molecular structure,
especially receptors and bridge groups on selectively sensing performance, in this work, we will take Cu^{2+} as an example to explore a generic approach for constructing a multiple-mode, reversible and visual sensor to meet its safe limit for Cu^{2+} in drinking water (31.5 μM) set by the World Health Organization (WHO) [54]. What’s more, the target sensing molecule, without addition of any extra masking agents, was expected to possess self-screening the interference effect (SSIE) from high-valence ions Fe^{3+} and Al^{3+} by virtue of the synergistic effect of dipyridine groups, i.e., coordination recognition of the target Cu^{2+} and masking the influence of coexisting Fe^{3+} and Al^{3+} as well [55,56]. The action mechanism will be investigated in detail, which was expected to pave a theoretical and experimental basis for designing optical functional sensors in the future.

2. Experimental

2.1. Materials and measurements

All the chemical reagents in the work were analytical and purchased from Shanghai Chemical Reagent Company. They were used directly without any further purification except that tetrahydrofuran was re-distilled by sodium and benzophenone. HeLa cells were obtained from Institute of Biochemistry and Cell Biology (the Chinese academy of Sciences, Shanghai, China). The bearing-tumor nude mice were purchased from Institute of Burn Research South-West Hospital, AMU (Chongqing, China) for in vivo imaging investigation. All animal experiments were performed in compliance with the Animal Management Rules of the Ministry of Health of the People’s Republic of China (Document no. 55. 2001) and the guidelines for the Care and Use of Laboratory Animals of China Pharmaceutical University. Water used throughout was doubly deionized.

FTIR spectra of KBr disks were acquired using a Nicolet NEXUS 870 FTIR spectrophotometer at room temperature; 32 scans were collected at a resolution of 1 cm⁻¹ scanning from 4000-500 cm⁻¹. ¹H NMR and ¹³C NMR spectra were recorded on Bruker AVANCE 600 using DMSO-d₆, or CDCl₃ as the solvents and tetramethylsilane (TMS) as an internal standard. Elemental analysis was obtained using an ElementalVario EL II apparatus. UV–vis spectra and fluorescent spectra were recorded on a Lambda 35 UV/Vis spectrometer (Perkin Elmer Precisely) and LS 55 fluorescent spectrometer (PerkinElmer) respectively, using a 1-cm square quartz cell. The fluorescence imaging of HeLa cells was observed under an Olympus IX71 inverted fluorescence microscope with 20 × objective lens. All pH measurements were made with a PHS-25 pH meter.

2.2. Synthesis of the target fluorescent sensor RACD

According to Scheme 1, the intermediates Rhodamine B hydrazide (1) and rhodamine B hydrazide cyanuric chloride (2) were prepared according to the documental procedures [57,58]. For target RACD, into a 250 mL round-bottom flask, a mixture of 0.15 g (0.6 mmol) N,N-dimethylpyridine amine, 0.17 g (2.1 mmol) anhydrous K₂CO₃ and 10.0 mL dry THF was added and dissolved under N₂ atmosphere. After the mixture was cooled to 0 °C, 0.3 g (0.57 mmol) compound 2 dissolved in 10.0 mL dry THF was added dropwise into and stirred for 24 h at 60 °C. Then the solvent was distilled off under reduced pressure to obtain a residue, which was washed thoroughly with water for 24 h at 60 °C.

To investigate the interaction between RACD and Cu^{2+}, the UV–vis and fluorescent spectra of RACD were recorded in the absence and presence of Cu^{2+} first as shown in Fig. 1. Without Cu^{2+}, there was no absorption at 553 nm for RACD in CH₃CN/H₂O (v/v = 1:1). The structure of RACD was confirmed by FTIR, H/¹³C NMR, Elemental analysis and ESI-MS with satisfactory analytic data corresponding to their molecular structures (see in experimental section 2.2).

2.3. UV–vis and fluorescent spectral properties

To investigate the interaction between RACD and Cu^{2+}, the UV–vis and fluorescent spectra of RACD were recorded in the absence and presence of Cu^{2+} first as shown in Fig. 1. Without Cu^{2+}, there was no absorption at 553 nm for RACD in CH₃CN/H₂O (v/v = 1:1). Upon addition of Cu^{2+} (2.0 equiv.), a new absorption peak emerged at 553 nm with Ex = 8.5 × 10⁵ L mol⁻¹ cm⁻¹, which resulted from opening-ring of rhodamine spirolactam. What's
more, the color of RACD solution changed into pink from colorless in the presence of Cu²⁺, making it possible to sense Cu²⁺ by naked-eye (Seeing Inert in Fig. 1a). For fluorescent spectra (Fig. 1b), the fluorescent intensity (F) at 576 nm was quite weak (F = 9.7 a.u.) and colorless without Cu²⁺ in CH₃CN-H₂O. While in the presence of Cu²⁺ (2.0 equiv.), the fluorescent intensity increased greatly to 638.2 a.u., with the fluorescent color from colorless to orange-red. The quantum yield of RACD in the absence or presence of Cu²⁺ was calculated to be 0.005 and 0.57 respectively, a 66-fold enhancement just as its fluorescent intensity, suggesting that the rhodamine spirolactam was opened upon addition of Cu²⁺.

As an organic molecule with multiple N and O atoms, pH will greatly influence its existing form, which alternates its chelating ability to Cu²⁺ accordingly. Here the effect of pH (2.0~12.0) on the fluorescent intensity of RACD at 576 nm (10.0 μM) was investigated in the absence or presence of Cu²⁺ (10.0 μM) in CH₃CN-H₂O (v/v = 1:1, Tris-HCl, 50.0 mM, pH 7.0, λₑₓ = 553 nm) and the results were given in Fig. S1a. On the acidic conditions (pH < 7.0), high concentration H⁺ would make N and O atoms in RACD protonated, which results in great decrease of its chelating ability to Cu²⁺. While pH is more than 10.0, high concentration OH⁻ would lead to most of Cu²⁺ precipitated in aqueous solution. Both above factors will greatly weaken the interaction between RACD and Cu²⁺, so the fluorescent intensity of the sensing system at 576 nm deduces accordingly. RACD could be applied under physiological condition of pH 7.0 for Cu²⁺ sensing.

To make sure RACD could be applied in practice, the influence of water-content on the fluorescent intensity of RACD at 576 nm was also investigated with and without Cu²⁺ (Fig. S1b). From Fig. S1b, we could find that the fluorescent intensity at 576 nm reaches the maximum when water content is 50% (v/v). The reason perhaps results from the fact that Cu²⁺ could coordinate with CN group of CH₃CN, leading to few free Cu²⁺ in pure CH₃CN and weak chelating ability between Cu²⁺ and RACD [59]. With increase H₂O content from 0 to 50% v/v, increasing free Cu²⁺ will come into being and reach the maximum, i.e., the chelation between Cu²⁺ and RACD gradually formed and the fluorescent intensity improved accordingly. However, once H₂O content is more than 50% (v/v), the solubility of RACD will deduce and some RACD molecules tend to aggregate. Accordingly, pH 7.0 and

**Scheme 1.** Synthesis route of the target organic chromophore RACD and a possible bonding mode deduced between RACD and Cu²⁺.
CH$_3$CN-H$_2$O (v/v = 1:1) were selected for all subsequent experiments. Also, the fluorescent intensity at 576 nm was recorded to illustrate the response rate and stability of RACD to Cu$^{2+}$ upon addition of 2.0 equiv. of Cu$^{2+}$ in CH$_3$CN-H$_2$O (v/v = 1:1). As shown in Fig. S2, the fluorescent intensity increases from 0 to 12 min and keeps unchangeable during the following testing time, suggesting that the response of RACD to Cu$^{2+}$ is quick and stable. RACD would be a potential sensor for Cu$^{2+}$ analysis in practice.

3.3. Special response to Cu$^{2+}$

To illustrate the self-screening interference effect from high-valence ions Fe$^{3+}$ and Al$^{3+}$, and the selectivity to Cu$^{2+}$, the corresponding UV–vis absorption and fluorescence spectra of RACD accompanying with the color changes were recorded in the presence of some common environmental metal ions, i.e., Zn$^{2+}$, Cd$^{2+}$, Mg$^{2+}$, Ni$^{2+}$, Hg$^{2+}$, Co$^{2+}$, Ba$^{2+}$, Pb$^{2+}$, Cr$^{3+}$, Sn$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Fe$^{3+}$, and Cu$^{2+}$ in CH$_3$CN-H$_2$O (v/v = 1:1). As shown in Fig. S2, the fluorescent intensity increases from 0 to 12 min and keeps unchangeable during the following testing time, suggesting that the response of RACD to Cu$^{2+}$ is quick and stable. RACD would be a potential sensor for Cu$^{2+}$ analysis in practice.

3.4. Reversible response of RACD to Cu$^{2+}$

To check the reversibility, the fluorescent intensity of RACD at 576 nm was recorded with addition of 2.0 equiv. ethylenediaminetetraacetic acid (EDTA) repeatedly (Fig. 3). After adding 2.0 equiv. EDTA to the resultant RACD-Cu$^{2+}$ system, the color returns to colorless and the fluorescence at 576 nm disappears accordingly. Once addition of 2.0 equiv. Cu$^{2+}$ again, both the orange-red color and the fluorescence at 576 nm recover. Even after the above process has been repeated 4 times, the fluorescence intensity at 576 nm could remain more than 94.6%, illustrating an excellent reversibility for RACD to sense Cu$^{2+}$.

3.5. Colorimetric and turn-on fluorescent analytical parameters

To discover the rule of RACD to sense Cu$^{2+}$, both UV–vis and...
fluorescent spectral titrations were conducted accompanying with the corresponding visual and fluorescent color changes. For colorimetric analysis (Fig. 4a, b), the absorption intensity ($A$) at 553 nm decreased with $c_{Cu^{2+}}$. The linear relationship of $A$ to $c_{Cu^{2+}}$ was between $0.1 \sim 0.8 \times 10^{-5}$ mol·L$^{-1}$ with $R^2 = 0.990$ and the regression equation was $A = -0.04 + 0.47 c_{(10^{-5} \text{ mol·L}^{-1})}$. For fluorescent detection (Fig. 4c and d), the linear relationship between $F/F_0$ and $c_{Cu^{2+}}$ was exhibited in the range of $0 \sim 10.0 \mu$mol L$^{-1}$ with $R^2 = 0.995$ and the

<table>
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<th>Samples</th>
<th>$Cu^{2+}$ Spiked ($\mu$M)</th>
<th>$Cu^{2+}$ Found ($\mu$M)</th>
<th>Recovery (%)</th>
<th>R.S.D. (%) (n = 5)</th>
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<tbody>
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<td>River water 1</td>
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<td>4.76</td>
<td>95.3</td>
<td>5.1</td>
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<tr>
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<td>89.1</td>
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<td>6.34</td>
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<td>6.59</td>
<td>95.5</td>
<td>3.4</td>
</tr>
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</table>

Table 1: Analytical results of $Cu^{2+}$ in different water samples (n = 5).

$e_{RACD} = 10.0 \mu$M, CH$_3$CN:H$_2$O = 1:1 (v/v = 1:1, Tris-HCl, 50.0 mM, pH 7.0).

fluorescent spectral titrations were conducted accompanying with the corresponding visual and fluorescent color changes. For colorimetric analysis (Fig. 4a, b), the absorption intensity ($A$) at 553 nm decreased with $c_{Cu^{2+}}$. The linear relationship of $A$ to $c_{Cu^{2+}}$ was between $0.1 \sim 0.8 \times 10^{-5}$ mol·L$^{-1}$ with $R^2 = 0.990$ and the regression equation was $A = -0.04 + 0.47 c_{(10^{-5} \text{ mol·L}^{-1})}$. For fluorescent detection (Fig. 4c and d), the linear relationship between $F/F_0$ and $c_{Cu^{2+}}$ was exhibited in the range of $0 \sim 10.0 \mu$mol L$^{-1}$ with $R^2 = 0.995$ and the

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regression equation was \( F/F_0 = -13.96 + 81.76 \times (10^{-5} \text{ molL}^{-1}) \).

Based on the definition of detection limit [60,61], three times of average deviation of A at 553 nm and \( F/F_0 \) at 576 nm in 20 blank samples without Cu\(^{2+}\) used here, the limits of colorimetric and fluorescent detection (LOD) for Cu\(^{2+}\) were 0.21 \( \mu \text{mol L}^{-1} \) and 11.0 \( \text{nmol L}^{-1} \), respectively. Importantly, the visual color of RACD solution could change gradually from colorless to pink and the fluorescent color excited at 553 nm changed from colorless to orange with the increase of Cu\(^{2+}\), which could be detected by naked eye (Fig. 4e and f).

### 3.6. Application for tracing Cu\(^{2+}\) in aqueous and biologic samples

To make sure of its practical application, the proposed RACD was first applied as a fluorescent probe for sensing Cu\(^{2+}\) in some natural water samples. As shown in Table 1, the recoveries of spiked amounts of Cu\(^{2+}\) are calculated from 89.1% to 100.3% with relative standard deviations (R.S.D.) \( \leq 6.0\% \), showing the reliability and practicality for RACD as a fluorescent sensor to detect Cu\(^{2+}\) in aqueous.

For its potential application in biological assay, the toxicity of RACD on HeLa cells and living tissues (bowel, lung, spleen, kidney, stomach, heart, swollen) were evaluated first through the MTT assay with \( c_{\text{RACD}} \) from 0 to 20 \( \mu \text{mol L}^{-1} \). As shown in Fig. S3, more than 80% HeLa cells could remain alive after they had been exposed to RACD for 24 h continuously even at the highest \( c_{\text{RACD}} \) of 20 \( \mu \text{mol L}^{-1} \), i.e., the cytotoxicity of RACD was negligible to HeLa cells at the concentration for analytic application. Further from Fig. 5, we could find that all the living tissues (bowel, lung, liver, spleen, kidney, stomach, heart, swollen) treated by RACD and RACD-Cu\(^{2+}\) with high concentration of 10 \( \mu \text{mol L}^{-1} \) all exhibit normal tissue morphologies, hinting both RACD and RACD-Cu\(^{2+}\) have no influence for various tissues in vivo.

Accordingly, a series of cell imaging experiments with different \( c_{\text{Cu}^{2+}} \) were carried out. From Fig. 6, we could find that not only RACD could permeate through the cell membrane as a fluorescent imaging for monitoring Cu\(^{2+}\) in living cell samples, it could also be utilized as an image indicator to trace cell deformation and decay. The cell morphology becomes deformed when \( c_{\text{Cu}^{2+}} \) increases to 1.0 \( \times 10^{-5} \text{ M} \), and to die at the minimum death concentration of 5.0 \( \times 10^{-3} \text{ M} \). The resultant fluorescent signals increase with Cu\(^{2+}\) concentration, suggesting that RACD would be a useful chemical sensor for real-time monitoring Cu\(^{2+}\) in living mice.

Further to make sure its possibility for more convenient colorimetric application, RACD functionalized test paper-strips were prepared by dipping pieces of filter paper into RACD solution in \( \text{CH}_3\text{CN}-\text{H}_2\text{O} \) (v/v = 1:1, Tris-HCl, 50.0 mM, pH 7.0) and then dried in air. As shown in Fig. 5, when these strips are immersed Cu\(^{2+}\) solutions with different concentrations from 0 to 1 \( \times 10^{-3} \text{ M} \), their color would gradually change from colorless to purple under natural light (Fig. S4a) or from blue to brown-yellow excited at 365 nm (Fig. S4b). The proposed RACD functionalized paper-strips possess a potential for on-sited colorimetric sensing Cu\(^{2+}\).

### 3.7. Interaction mechanism between RACD and Cu\(^{2+}\)

To confirm the selective reaction mechanism between RACD and Cu\(^{2+}\), UV-vis and fluorescent spectral titration experiments have been conducted first as shown in Fig. 4a and c. With addition of Cu\(^{2+}\), both \( A_{532}\) and \( F_{576}\) increased gradually and reached a platform when \( c_{\text{Cu}^{2+}} \) = 2.1, hinting RACD perhaps bound to Cu\(^{2+}\) with 1:2 stoichiometric ratio. The binding constants for Cu\(^{2+}\) with the spirilactam of Rhodamine in RACD were calculated to be \( K_a = 7.4 \times 10^4 \text{ M}^{-1} \) and \( 5.3 \times 10^4 \text{ M}^{-1} \) with linear equations of 1/[\( A_{532}\)] = 0.6730 + 9.066 \( \times 10^{-5}/[\text{Cu}^{2+}] \) (R = 0.9935) and 1/[\( F_{576}\)] = 0.0145 + 2.6924 \( \times 10^{-7}/[\text{Cu}^{2+}] \) (R = 0.9978) from UV-vis and fluorescent spectral titration using Tsien equation (Fig. 8a, b). While for Cu\(^{2+}\) with the bipyridine in RACD, \( K_a \) was improved to 7.9 \( \times 10^5 \text{ M}^{-1} \) and 1.8 \( \times 10^5 \text{ M}^{-1} \) with 1/[\( A_{532}\)] = 0.8863 + 1.108 \( \times 10^{-5}/[\text{Cu}^{2+}] \) (R = 0.9911) and 1/[\( F_{576}\)] = 0.1233 + 6.6900 \( \times 10^{-7}/[\text{Cu}^{2+}] \) (R = 0.9957). Large \( K_a \) well illustrates why RACD could selectively recognize Cu\(^{2+}\).

Based on the conclusions above, Job’s plots, one of the most popular methods for determining stoichiometry were drawn. For these, both the alteration in UV–vis absorption intensity at 553 nm (\( A_{532} \)) and fluorescence intensity at 576 nm (\( F_{576} \)) was plotted against changing the molar fraction of RACD to Cu\(^{2+}\) but keeping the total concentration of RACD and Cu\(^{2+}\) at 50.0 \( \mu \text{mol L}^{-1} \). As shown in Fig. 9a and b, either the highest \( A_{532} \) or the highest \( F_{576} \) is obtained at a molar fraction of 0.6, suggesting that RACD binds to Cu\(^{2+}\) with 1:2 stoichiometry. Accordingly, a possible bonding mode for RACD and Cu\(^{2+}\) is proposed as shown in Scheme 1, during which the configuration will transfer from the closed spirilactam to the opened-ring amide with strong fluorescence.

Further, FTIR and \(^1\)H NMR of RACD were recorded before and after their reacting with Cu\(^{2+}\). For FTIR spectra as shown in Fig. 10a, the peaks at 1720, 1589 \( \text{cm}^{-1} \), ascribed to the characteristic amide carbonyl and pyridine ring, shift to a lower frequency of 1680 \( \text{cm}^{-1} \) and 1571 \( \text{cm}^{-1} \) upon addition of Cu\(^{2+}\). The reason maybe results from the polarization of C=O and the cleavage of N–C bond in the opened-ring spirilactam once RACD chelating with Cu\(^{2+}\). In addition, the stretching peaks corresponding to C=O and aromatic C=O in RACD are also shifted to lower wave number by 10 and 20 \( \text{cm}^{-1} \), respectively. Interestingly, the pyridine ring peak (1589 \( \text{cm}^{-1} \)) do not shift any more upon addition of less than 0.5 equiv. Cu\(^{2+}\), while shifts to a lower frequency by 40 \( \text{cm}^{-1} \) (1720 \( \text{cm}^{-1} \)) upon addition of 2.0 equiv. Cu\(^{2+}\), hinting that Cu\(^{2+}\) first combines with the spirilactam of rhodamine and then chelates with bipyridine as illustrated in Scheme 1.

![Fig. 5. Histological H&E staining for main organs (bowel, lung, liver, spleen, kidney, stomach, heart, swollen) of the mice intravenously administrated with sensing materials RACD and RACD-Cu\(^{2+}\) for 2 h. The scale bar indicates 20 \( \mu \text{m} \).](attachment:image)
$^1$H NMR spectra of RACD in the presence of 0.00, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 2.00, 2.50 equiv. Cu$^{2+}$ were given in Fig. 10b. Upon addition of Cu$^{2+}$ less than 0.5 equiv., the proton peaks ($H_{\delta} 3.33$, $H_{\delta} 6.38$, $H_{\delta} 7.20$, $H_{\delta} 7.63$ and $H_{\delta} 7.83$) from the rhodamine moiety shift downfield and broadened, owing to the decreasing electron density upon coordination with Cu$^{2+}$. While protons peak at $H_{\delta} 8.34$ ppm ascribed to pyridine ring do not change, suggesting pyridine ring don’t chelate with Cu$^{2+}$. However, with $c_{Cu}^{2+}$ increasing more than 0.5 equiv., the protons peak $H_{sa}$ at 8.34 gradually shifts to $H_{sa}$ 8.47 and broadens. The results further confirm that Cu$^{2+}$ first chelates to the spirocyclic of rhodamine, and then binds to the bipyridine, which is well consistent with the deduce from FTIR and spectral titrations.

The conclusion was also confirmed by ESI-MS data of RACD and RACD-Cu$^{2+}$. The peak for RACD (Fig. S5) locates at $m/z = 789.9$ corresponding to the species [RACD + Na]$^+$, and the one for RACD-Cu$^{2+}$ locates at 966.3245 corresponding to [RACD + 2Cu$^{2+}$ + 2Cl$^-$], respectively. The resultant RACD: Cu$^{2+}$ complex with $m/z = 966.3245$ (Fig. S6) completely confirms that RACD chelates to Cu$^{2+}$ by both the spirocyclic of rhodamine and pyridine with 1:2 stoichiometry.

Density functional theory was further performed to understand the chelating mode of RACD with Cu$^{2+}$ using the B3LYP level at the Gaussian 09 suite of program (Fig. 11). In the optimized structure of RACD-Cu$^{2+}$, the distances of Cu$^{2+}$ to N atoms in pyridine, imine and $NH-C-N$ groups, and to O atom in $C=O$ group are 1.9211 Å, 1.9199 Å, 2.1966 Å, 1.8759 Å and 1.9964 Å, respectively, all which meet the prerequisite to form chelating bonds. Without Cu$^{2+}$, RACD is spread spirolactam ring, whose electronic cloud in LUMO mainly distributes on the pyridine moiety. Once addition of Cu$^{2+}$, the spirocyclic C-N bond breaks and the electronic cloud in LUMO is distributed on the xanthene moiety. The energy gaps ($\Delta E$) between HOMO and LUMO for RACD and
RACD-Cu$^{2+}$ are 4.173 eV and 0.438 eV respectively. A low $\Delta E$ well explains why there are strong spectral absorption and fluorescent emission at long-wavelength for RACD in the presence of Cu$^{2+}$.

4. Conclusion

In conclusion, a self-screening interference effect (SSIE) with improved optical and chelating properties was developed for tracing Cu$^{2+}$ in natural aqueous and living biological samples. By combining dipyridine as a self-screening group, rhodamine as mother chromophore, spirocycle as multiple receptors, and cyanuric chloride as connecting bridge, RACD could sense Cu$^{2+}$ selectively, reversibly, and conveniently, accompanying with obvious color changes from colorless to purple under nature light and fluorescent color from colorless to...
orange-red excited at 553 nm in CH$_3$CN-H$_2$O (v/v = 1:1, Tris-HCl, 50.0 mM, pH 7.0). Some other coexisted metal ions tested, even high-valence Fe$^{3+}$ and Al$^{3+}$, had no influence by virtues of RACD's self-screening interference effect. The detection linear relationship of A to $c_{\text{Cu}^{2+}}$ was between 0.0$\times$0.8$ \times 10^{-5}$ mol·L$^{-1}$ with $R^2$ = 0.990 and the one for $F/F_0$ and $c_{\text{Cu}^{2+}}$ was between 0$\times$10.0 μmol·L$^{-1}$ with $R^2$ = 0.995. When applied for detecting Cu$^{2+}$ in natural aqueous samples, the limits of colorimetric and fluorescent detection (LOD) for Cu$^{2+}$ were 0.21 μmol·L$^{-1}$ and 1.10 nmol·L$^{-1}$, respectively. Importantly, low toxic RACD could trace Cu$^{2+}$ in living cells and biological tissue samples and its functionalized paper-strips were successfully applied to on-site recognize Cu$^{2+}$. The action mechanism for RACD to Cu$^{2+}$ was confirmed to form some stable 5-membered and 5-membered condensed rings between Cu$^{2+}$ and O or N atoms with a 1:2 binding ratio and high binding constant by means of spectral titration, Job’s plot, FTIR, $^1$H NMR, ESI-MS and theoretical simulation. This work will pave a promising in-sight for sensitive, multiple-mode sensing molecular design based on self-screening interference effect (SSIE) in the future.

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Appendix A. Supplementary data

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