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Synthesis and Application of a Full Water-Soluble and Red-Emitting Chemosensor Based on Phenoxazinium for Copper(II) Ions

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A phenoxazinium-based chemosensor (**1**) bearing di(2-picolyl)amine unit was successfully synthesized. The result shows that it is a red-emitting and full water-soluble chemosensor for the selective detection of Cu^{2+} in pure water. The fluorescence on-off mechanism was studied by *ab initio* calculations. To confirm the suitability of **1** for biological applications, it was employed in the fluorescence detection of the intracellular Cu^{2+} with cultured KB cells. The results indicated that **1** had good membrane permeability and could be useful for the fluorescence microscopic imaging.

Keywords chemosensor, phenoxazinium, water-soluble, copper

Introduction

Recently, fluorescent probes have become the hot research topics. The long wavelength probes (650—900 nm) are highly preferable for applications in biological systems due to their several advantages:[1] The auto-fluorescence and photo-damage to living cells are reduced; the low absorption and light scattering of biological samples facilitate deeper penetration of long wavelength radiation into biological matrices, which can help to obtain information from the inner regions of tissues. Therefore, a pressing need exists to develop fluorescent dyes satisfying the multiple criteria such as long-wavelength excitation/emission, high selectivity and live-cell permeability.[1b-1d]

Copper ions play an important role in various biological processes ranging from bacteria to mammals.[2] The abnormal concentration of Cu^{2+} can cause neurodegenerative diseases such as Menkes disease, Wilson disease, and Alzheimer's disease.[3] Long-term exposure can cause liver or kidney damage. Even a short period of exposure to a high level of copper can cause gastrointestinal disturbance. The US Environmental Protection Agency has set the limit of copper in drinking water to 1.3 ppm (≈20 μmol/L). Considerable efforts have been made to synthesize fluorescent chemosensors, such as coumarin, rhodamine derivatives and so on, for selective, sensitive detection of Cu^{2+}.[4,5] Water-compatible fluorescent chemosensors for Cu^{2+}-selective detection were recently reported and used with some progress in biological applications. However, most of them were neither long-wavelength excitation/emission probes,[4,5b-5f] nor full water soluble compounds.[4b-4e,5] Hence, auto-fluorescence and photo-damage to living cells would emerge in more complicated biological system; and the membrane permeability would be a problem *in vitro* test. Therefore, development of new full water soluble Cu^{2+}-selective probe with good membrane permeability, which has long-wavelength excitation or emission, is important and necessary.

Symmetric and unsymmetric 3,7-bis(dialkylamino)-phenoxazin-5-ium type derivatives contain a phenoxazinium cation with absorption in the 640—650 nm region and have corresponding emission bands between 650 and 670 nm.[6] It is also known that phenoxazinium type compounds have good membrane permeability and comparatively low toxicity according to previous *in vitro* and *in vivo* assay results.[7] A red-emitting phenoxazinium based probe,[8a] 3-(diethylamino)-7-(1,4-dioxa-7,13-dithia-10-azacyclo-pentadecan-10-yl)phe-

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noxa-zin-5-ium chloride, has been reported as a low in cost, and open to real-time monitoring probe for selective ratiometric detection of Hg^{2+} in pure water, and a benzo[a]phenoxazinium-based red-emitting chemosensor[8b] for Zn^{2+} in PBS buffer (pH＝7.4) and in cultured KB cells has also been reported.

We assume that 3,7-bis(dialkylamino)phenoxazin-5-ium derivatives appended with a di(2-picolyl) amine (DPA) unit will coordinate with Cu^{2+}, and the absorption and emission properties will be modulated due to the extended p-π conjugation between the nitrogen atom and phenoxazinium. The fluorescent sensor (**1**) was designed based on the above assumption. The synthesis and application of the red-emitting chemosensor based on phenoxazinium for Cu^{2+} in pure water and cultured KB cells are discussed as follows in details.

Experimental

General

Starting materials were purchased from TCI (Shanghai) Development Co., Ltd. or Sinogharm Chemical Reagent Co., Ltd. All analytic grade solvents (A.R.) were obtained from commercial suppliers and used directly. Flash chromatography was performed with silica gel (300—400 mesh). Nuclear magnetic resonance spectra were recorded on Varian Inova NMR spectrometers (400 or 300 MHz). TMS was used as an internal standard for ^{1}H NMR and solvent peak used as an internal standard for ^{13}C NMR. High resolution mass spectra were obtained on a GC-TOF or a Saturan2200 LC-MS instrument. UV/vis spectrum experiments were recorded on a U-3900 spectrometer. Fluorescence measurements were performed on a HORIBA JobinYvon's fluorescence spectrofluorometers at room temperature. Metaling points were determined on an X-4 microscope electron thermal apparatus (Taike, China). All pH measurements were made with a Model PHS-3C meter (Jingke, China). Elementary analyses were conducted on a Carlo Erba-MOD1106 elementary analysis apparatus. IR spectra were recorded on a Nicolet 5200 FT-IR instrument using solid samples dispersed in KBr pellets. A Shimadzu LC-20A was used for HPLC analysis.

Synthesis

3-Methoxy-*N*,*N*-bis(2-picolyl)aniline (3) To a solution of 2-chloromethyl -prydine hydrochloride (3.78 g, 23.0 mmol) in H_2O (1 mL), 3-methoxyaniline (1.42 g, 11.5 mmol), 5 mol/L NaOH (5.8 mL), and hexadecytrimethylammonium chloride (40 mg) were added under N_2 protection. The mixture was stirred vigorously for 48 h at room temperature. The mixture was extracted with CH_2Cl_2 (50 mL×3), and the extract was washed with H_2O and dried with $MgSO_4$. After evaporation of the solvent, the desired product **3** (1.2 g) was obtained as yellow oil in 34.2% yield via column chromatography (silica, from petroleum ether∶AcOEt (1∶1＝*V*/*V*) to AcOEt). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.68 (s, 3H, OCH_3), 4.81 (s, 4H, 2×NCH_2), 6.48—6.13 (m, 3H, 3×ArH), 7.07 (t, *J*＝8.2 Hz, 1H, ArH), 7.16 (td, *J*＝7.0, 1.6 Hz, 2H, 2×ArH), 7.27 (d, *J*＝7.5 Hz, 2H, 2×ArH), 7.62 (td, *J*＝7.7, 1.4 Hz, 2H, 2×ArH), 8.58 (d, *J*＝4.8 Hz, 2H, 2×ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 55.3 (OCH_3), 57.5 (2×NCH_2), 99.3 (ArCH), 102.4 (ArCH), 105.8 (ArCH), 121.0 (2×ArCH), 122.2 (2×ArCH), 130.3 (ArC), 137.1 (2×ArCH), 149.9 (3×ArCH), 159.0 (ArCH), 161.0 (ArCH); IR (neat) ν_{max}: 2934, 2837 (CH_2), 1731, 1505, 1176, 893, 819 (ArH), 1348 (Aryl-N, Tertiary amine), 1265, 1035 (Aryl-O-C) cm^{-1}; HRMS (EI-TOF) calcd for $C_{19}H_{19}N_3O$: 305.1528, found 305.1526.

3-Methoxy-4-nitroso-*N*,*N*-bis(2-picolyl) aniline (4) To a stirred solution of **3** (1900.0 mg, 6.22 mmol) and 6 mol•L^{-1} aqueous hydrochloride solution (25 mL) in an ice-water bath, a solution of $NaNO_2$ (472.2 mg, 6.84 mmol) in 2 mL water was added dropwise in 10 min. After the addition, the reaction was stirred for 1 h. Then, aqueous NaOH solution (10%) was added until the solution turned dark green and solid precipitated (pH≈10). The mixture was extracted with CH_2Cl_2 (50 mL×3), and combined organic phase was dried over anhydrous $MgSO_4$. The deep green solid, which was obtained by evaporation, was purified by chromatography with silica gel eluting with CH_2Cl_2. **4** (1768.0 mg, 85%) was obtained. m.p. 99.2—100.1 ℃; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.00 (s, 3H, OCH_3), 4.96 (s, 4H, 2×NCH_2), 6.22 (d, *J*＝8.8 Hz, 1H, ArH), 6.40 (s, 1H, ArH), 6.56 (d, *J*＝9.0 Hz, 1H, ArH), 7.39—7.11 (m, 4H, 4×ArH), 7.69 (t, *J*＝7.4 Hz, 2H, 2×ArH), 8.62 (s, 2H, 2×ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 56.2 (OCH_3), 57.7 (2×NCH_2), 94.7 (ArCH), 104.7 (ArCH), 112.9 (ArCH), 121.0 (2×ArCH), 122.8 (2×ArCH), 137.2 (2×ArCH), 150.0 (2×ArCH), 156.2 (ArCH), 156.4 (ArCH), 157.1 (ArCH), 164.4 (ArCH); IR (neat) ν_{max}: 2932, 2838 (CH_2), 1728, 894, 818, (Ar-H) 1340 (Aryl-N, Tertiary amine), 1253, 1047 (Aryl-O-C), 1515 (Aryl-NO) cm^{-1}; HRMS (EI-TOF) calcd for $C_{19}H_{18}N_4O_2$: 334.1430, found 334.1436.

3-(Diethylamino)-7-(di(2-picolyl)amino)phenoxazin-5-ium chloride (1) 3-(Diethylamino)phenol (395.3 mg, 2.39 mmol) and 90% *i*-PrOH (30 mL) were stirred at 70 ℃ in a 100 mL two-neck bottle with distilling apparatus filled with N_2. A suspended solution, which was obtained by the mixture of **4** (800.0 mg, 2.39 mmol), 1 mol•L^{-1} HCl (2.54 mL, 2.54 mmol), water (8 mL) and *i*-PrOH (30 mL), was injected with a syringe into the above mixture in four portions during 1.5 h. After 2 h at 70 ℃, the temperature rose to reflux. When about 30 mL of the solvent was distilled out, 30 mL of 90% *i*-PrOH was added to the reaction mixture. This procedure was repeated three times during 5 h. The dark-blue solution was evaporated, and the residue was purified by column chromatography with silica gel, eluting with CH_2Cl_2∶MeOH from 15∶1 to 10∶1 (*V*/*V*). The deep-blue solution was evaporated. The powder was dried in vacuum. Green solid **1** (290.4 mg,

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28%) with metallic luster was obtained, purity＝99.3 %; m.p.＞200 ℃; ^{1}H NMR (400 MHz, CD_3OD) δ: 1.36 (t, J＝7.1 Hz, 6H, 2×CH_3), 3.98—3.66 (m, 4H, 2×$ArCH_2$), 5.22 (s, 4H, 2×NCH_2Ar), 6.99 (s, 2H, 2×ArH), 7.45—7.29 (m, 8H, 8×ArH), 7.83 (td, J＝7.8, 1.4 Hz, 2H, 2×ArH), 8.56 (d, J＝4.5 Hz, 2H, 2×ArH); ^{13}C NMR (75 MHz, CD_3OD) δ: 11.9 (2×CH_3), 47.0 (2×$ArNCH_2$), 57.3 (2×NCH_2Ar), 96.5 (ArCH), 97.7 (ArCH), 117.0 (ArCH), 119.1 (ArCH), 122.2 (2×ArCH), 123.3 (2×ArCH), 133.6 (ArCH), 135.0 (ArCH), 136.8 (ArCH), 137.9 (2×ArCH), 148.7 (ArCH), 149.5 (2×ArCH), 150.0 (ArCH), 155.8 (ArCH), 157.6 (2×ArCH); IR (neat) ν_{max}: 2930, 2850 (CH_2), 857, 820, (ArH), 1337 (Aryl-N, Tertiary amine) cm^{-1}; HRMS (ESI^+) calcd for $C_{28}H_{28}N_5O^+$: 450.2294, found [M—Cl^-]$^+$ 450.2276.

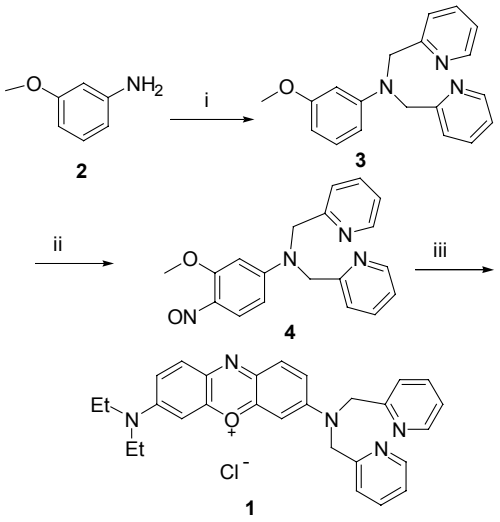
Cell incubation

KB cells were cultured in Roswell Park Memorial Institute (RPMI-1640), supplemented with 10% calfserum, 1% penicillin and 1% streptomycin at 37 ℃ in a 5∶95 CO_2-air incubator. The cells were cultured for 2 d then loaded onto a 35 mm diameter glass-bottomed cover slip. An Nikon AY laser scanning microscopy system equipped with a 630 nm laser head was applied to confocal image KB cells stained with **1**. The emission was collected at 650—750 nm. All of the images were gathered at the same confocal microscope settings and processed with Nikon AY software.

Results and Discussion

3-(Diethylamino)-7-(di(2-picolyl)amino)phenoxazin-5-iumchl-oride (**1**) was obtained by reacting 3-(diethylamino)phenol with DPA derivative (**4**), which was synthesized from 3-methoxyaniline with two steps (Scheme 1). Compound **1** exhibits good solubility in water and polar solvents such as methanol and chloroform.

Scheme 1 Synthesis of phenoxazinium **1**



(i) 2-chloromethylpyridine hydrochloride, 34.2%; (ii) (1) $NaNO_2$, HCl; (2) NaOH, 85%; (iii) 3-(diethylamino)phenol, 28%.

Figure 1 shows the changes in the absorption spectrum of **1** as a function of Cu^{2+} concentration in water at room temperature. The UV-vis spectrum of **1** in water is characterized by a very intense band centered at 632 nm (ε＝42000 mol^{-1}•L•cm^{-1}). This is responsible for the light blue color of the solution. During photometric titrations of **1** with Cu^{2+}, a blue shift of the long-wavelength absorption maximum from 632 nm to 582 nm (ε＝18500 mol^{-1}•L•cm^{-1}) (with an isosbestic point at 560 nm) was observed. This indicates the complexity of Cu^{2+} with the DPA unit. As it is reported, the difference in spatial configuration (Figure S1) and the change in electric charge distribution, (Table 1) will affect the frontier molecular orbitals,[4c] then the absorption spectrum of phenoxazinium cation (**1**$^+$) and **1**$^+$•Cu^{2+}. Decreases in the electron donating capability of nitrogen, proven by density functional theory (DFT) calculations with B3LYP at the level of 6-31G* for C, H, N, O atoms and LANL2DZ for Cu atom, indicate the changes of electric charge distribution (Table 1).

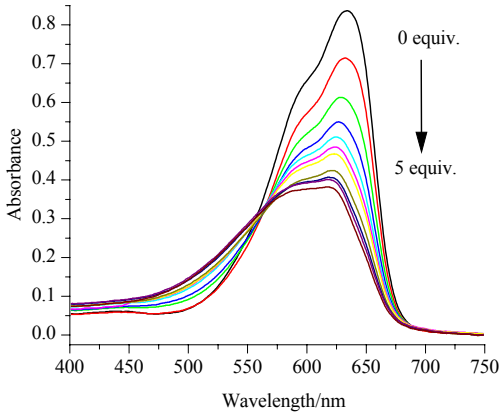


Figure 1 Changes in the UV-vis spectra of **1** (20 μmol/L in water) upon titration by $CuCl_2$ from 5 to 100 μmol/L.

Table 1 Calculated NBO charges (electrons) of **1**$^+$and **1**$^+$•Cu^{2+}

	N(1)	N(2)	O(1)	N(3)
1$^+$	−0.381	−0.334	−0.438	−0.354
1$^+$•Cu^{2+}	−0.290	−0.313	−0.436	−0.522

The measured absorbance [1/(A−A_0)] at 632 nm varied as a function of 1/[Cu^{2+}] in a linear relationship, indicating the 1∶1 stoichiometry between Cu^{2+} and **1** (Figure 2). This is according to the linear Benesi-Hildebr and expression.[9] On the basis of the 1∶1 stoichiometry and UV-vis titration data in Figure 1, the association constant of **1** with Cu^{2+} in water was found to be (5.60±0.08)×10^4 mol^{-1}•L. Job's plot analysis[5d] of the UV-vis titrations carried out in water revealed a maximum of 50% mole fraction in accordance with the proposed 1∶1 binding stoichiometry (Figure 3).

The fluorescence titration of **1** (20 μmol/L) in the presence of different Cu^{2+} concentrations was then

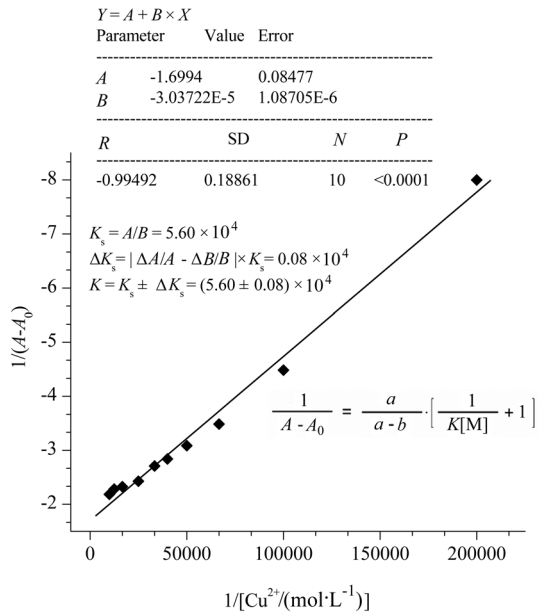


Figure 2 Benesi-Hilderbrand plot of **1** with Cu^{2+}.

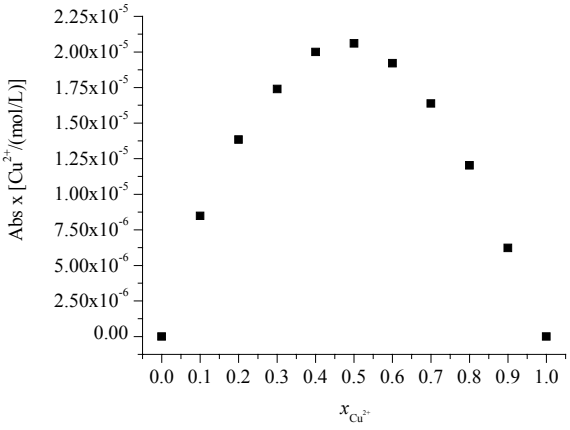


Figure 3 Job's plot analysis of **1** and Cu^{2+} in water; the total molar concentration of **1** and Cu^{2+} is 40 μmol/L. The absorbance was employed at 632 nm.

performed. In Figure 4, sensor **1** showed a strong fluorescence emission at 673 nm. This emission intensity was gradually quenched upon increasing the concentration of Cu^{2+} from 5 to 100 μmol/L. At the employed concentration of the probe (20 μmol/L), we can detect Cu^{2+} in the range of 5—100 μmol/L (Figure 4 Inset). The fluorescence quenching of the ligand may occur through excitation energy transfer from the ligand to the metal d-orbital and/or ligand to metal charge transfer (LMCT) as reported.[10]

To demonstrate the fluorescence on-off mechanism, the excited states of phenoxazinium cation ($\mathbf{1}^+$) and $\mathbf{1}^+$•Cu^{2+}were obtained with the CIS method using Hartree-Fock wave functions (Figure 5, Table 2). The main mode of electron transition for $\mathbf{1}^+$ is from HOMO to LUMO (93.0%). According to ligand to ligand charge transfer (LLCT), the fluorescence intensity does not

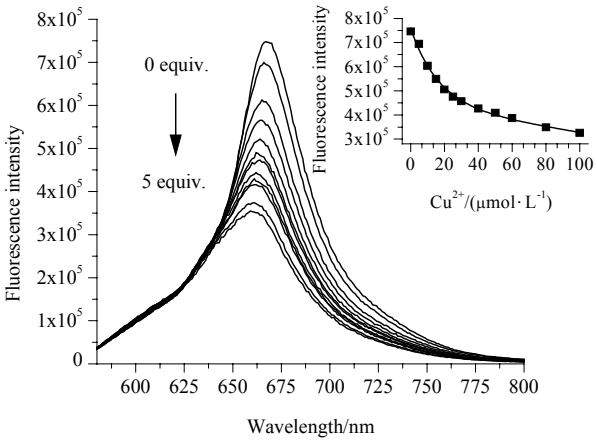


Figure 4 Emission spectra of compound **1** in the presence of an increasing Cu^{2+} concentration (from 5 to 100 μmol/L) in water. The excitation wavelength was 560 nm with 5 nm slit widths. The concentration of chemosensor **1** was 20 μmol/L. Inset: Fluorescence intensity of **1** versus the Cu^{2+} ×concentration at 673 nm.

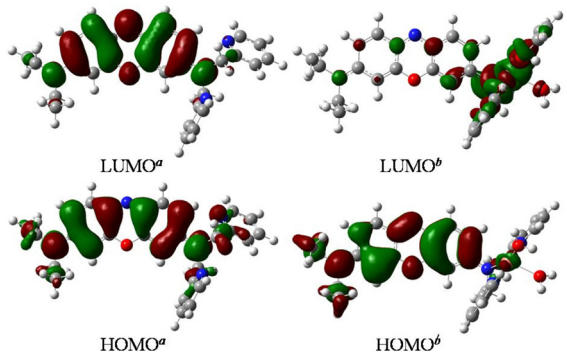


Figure 5 Frontier molecular orbitals of $\mathbf{1}^+$ and $\mathbf{1}^+$•Cu^{2+} (a: $\mathbf{1}^+$; b: $\mathbf{1}^+$•Cu^{2+}).

Table 2 The contributions of each electronic oscillator (orbital transition) to the lowest energy transition of $\mathbf{1}^+$ and $\mathbf{1}^+$•Cu^{2+} (for orbitals) in gas phase by CIS//TDDFT

Structure	Character
$\mathbf{1}^+$	HOMO−1 → LUMO (7.0%, LLCT)
	HOMO → LUMO (93.0%, LLCT)
$\mathbf{1}^+$·Cu^{2+}	HOMO−12 → LUMO (6.6%, LMCT/LLCT)
	HOMO−6 → LUMO (4.3%, LMCT/LLCT)
	HOMO−4 → LUMO (3.0%, LMCT/LLCT)
	HOMO →LUMO (84.3%, LMCT/LLCT)
	HOMO → LUMO+2 (1.8%, LLCT)

weaken. For $\mathbf{1}^+$•Cu^{2+}, the electronic oscillators corresponding to the transitions from HOMO(β) to LUMO(β) are calculated to be 84.3%. The occupied orbital of HOMO(β) is essentially localized on the phenoxazinium ring, however, the LUMO(β) is occupied on N(3), N(4), and N(5) atoms, Cu^{2+}, and two water molecules. Therefore, the main charge displacement takes place

from the phenoxazinium ring to Cu^{2+}. In simple terms, the best description of the emitting state is that of LMCT. This provides a pathway for the nonradioactive deactivation of the excited state, resulting in fluorescence intensity reduction and even fluorescence quenching.[11,4c]

Control experiments were performed to evaluate the selectivity of the fluorescent probe **1** toward Cu^{2+} ions (Figure 6). The influence of the addition of Hg^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}, Pb^{2+}, Co^{2+}, Fe^{3+}, Fe^{2+}, Ag^{+}, Mn^{2+}, K^{+}, Na^{+}, Mg^{2+}, and Ca^{2+} on the emission properties of **1** was studied, respectively. The concentration of the metal ions was 100 μmol/L except for K^{+}, Na^{+}, Mg^{2+}, and Ca^{2+}(100 mmol/L) because these ions may have a similar concentration in the real samples. Note that only the additions of $Cu^{2+}$and Fe^{3+} ions lead to significant changes in the fluorescence of **1**. In the case of Fe^{3+}, the emission maximum was slightly quenched. The introduction of Fe^{2+} results in about 10 nm blue-shift in the fluorescence spectrum without changes in the emission intensity .

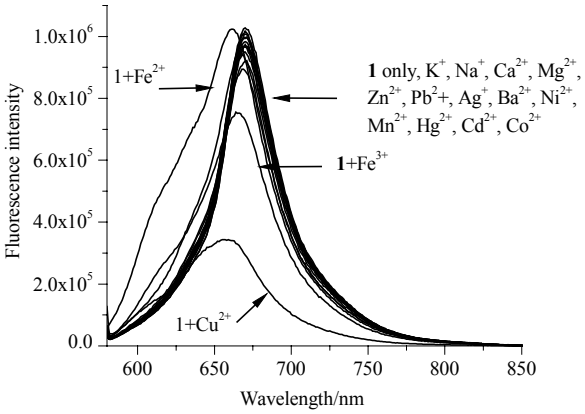


Figure 6 Fluorescence spectra change of **1** (20 μmol/L) upon addition of different metal cations (100 μmol/L: Hg^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}, Pb^{2+}, Co^{2+}, Fe^{3+}, Fe^{2+}, Ag^{+}, Mn^{2+}; 100 mmol/L: K^{+}, Na^{+}, Mg^{2+}, Ca^{2+}) in water. Excitation was at 560 nm.

The interference of other cations to Cu^{2+} is also carried out. As shown in Figure 7, the titration of Cu^{2+} to **1** in the presence of the screened metal cations (Hg^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}, Pb^{2+}, Co^{2+}, Fe^{3+}, Fe^{2+}, Ag^{+}, and Mn^{2+}) did not lead to significant changes in fluorescence output compared with that in the absence of other metal cations. The fluorescence of **1**•Cu^{2+}has slightly changes in the presence of a large excess of K^{+}, Na^{+}, Mg^{2+}, and Ca^{2+}(100 mmol/L) (Figure 7), presumably resulting from the large ionic strength.[12]

The fluorescence intensity changes at 673 nm with pH ranging from 5.90 to 8.10 were also discussed (Figure S3). The fluorescence intensity of **1** was relatively unaffected by protons. With the introduction of Cu^{2+}, the intensity of **1** was obviously quenched under acid condition, and the differential of emission intensity around pH ~7.4 did not have much effect, which

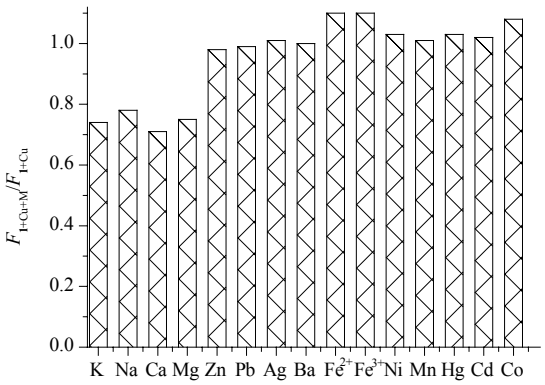


Figure 7 Fluorescence response of **1** (20 μmol/L) containing 100 μmol/L Cu^{2+} to the selected metal ions (100 μmol/L: Hg^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+}, Pb^{2+}, Co^{2+}, Fe^{3+}, Fe^{2+}, Ag^{+}, Mn^{2+}; 100 mmol/L: K^{+}, Na^{+}, Mg^{2+}, Ca^{2+}) in water. $F_{\mathbf{1}+Cu}$ indicates the fluorescence signals of **1** in the presence of Cu^{2+} and $F_{\mathbf{1}+Cu+M}$ denotes the fluorescence signals of **1** in the presence of Cu^{2+} and competing ions. Excitation was at 560 nm and emission was at 673 nm.

means **1** can be used to analyze intracellular Cu^{2+} ions in biological samples.

Chemosensor **1** is also well-suited to fluorescence imaging in living cells. Human oral epidermoid carcinoma cells (KB cell) were incubated with 20 μmol/L **1** for 20 min and a strong fluorescence emission was observed, as shown in Figure 8b, then washed with physiological saline to remove excess **1**. After it had been treated with 5 equiv. of copper ions for 20 min, intracellular fluorescence was almost completely suppressed, as shown in Figure 8c. Therefore, **1** is cell-permeable and can respond to copper ions within living cells.

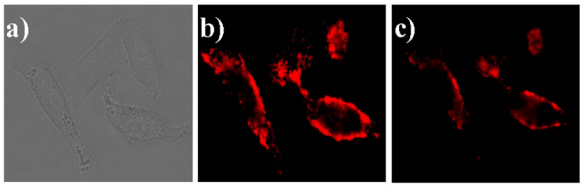


Figure 8 (a) Bright-field of the KB cell; (b) the cells were incubated with **1** (20 μmol/L) for 20 min then washed with physiological saline; (c) the cells were then incubated with $CuCl_2$ (100 μmol/L) for 20 min. Excitation was at 633 nm.

Conclusions

In conclusion, a selection of the suitable fluorophore for ion probes from existing or potential drug molecules was presented. A phenoxazinium-based chemosensor bearing di(2-picolyl) amine unit (**1**) with long-wavelength excitation and emission was used as a promising analytical tool for fluorometric detecting copper ions in pure water. Notably, the selectivity of the probe for Cu^{2+} over other metal ions is relatively high. The corresponding quenching mechanism was explained by *ab*

initio calculations. In addition, **1** can be one of the most promising probes for the detection of Cu^{2+} in living cells due to its good membrane permeability.

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