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A Bis(pyridine-2-ylmethyl)amine-based Selective and Sensitive Colorimetric and Fluorescent Chemosensor for Cu²⁺

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ABSTRACT

A new bis(pyridine-2-ylmethyl)amine derivative (**1**) was synthesized as a colorimetric and fluorescent chemosensor for metal ions. Experimental results indicate that **1** displays significant colorimetric and fluorescent changes upon binding of Cu²⁺. Since **1** has a high water solubility as well as good cell-permeability, it successfully applied to detect the presence of Cu²⁺ ion in HepG2 cells in culture medium. Thus, **1** can be used as a potential Cu²⁺ chemosensor in aqueous solution and mammalian cells. Further, the fluorescence behavior of **1** upon Cu²⁺ binding was well explained by our quantum calculation based on electronic structure.

Keywords: Colorimetric; Fluorescent; Cu²⁺; DPA; chemosensor

1. Introduction

New colorimetric and fluorescent chemosensors for the detection of metal ions have been developed and thus widely exploited in diverse applications [1-6]. Copper, one of the important trace elements, is involved in many essential biological processes, while high concentration of Cu^{2+} can also cause serious health problems such as nausea, vomiting and diarrhea [7, 8]. It is also well known that Cu^{2+} has been identified as a major environmental pollutant [9]. Thus, detection and monitoring of Cu^{2+} is extremely important in biological and environmental sciences.

Di(2-pyridylmethyl)amine (DPA) derivatives have been used as both colorimetric and fluorescent chemosensors for a variety of analytes [10]. For example, DPA-based colorimetric and fluorescent chemosensors for detecting Zn^{2+} and Cu^{2+} have been reported by various groups [11-16]. However, their selective binding capabilities with metal ions need to be improved. Herein, a new efficient colorimetric and fluorescent chemosensor (**1**) for Cu^{2+} ion was synthesized based on DPA. **1** is a donor- π -accepter (D- π -A) type compound, in which DPA and 3-dimethylbenzothiazol-3-ium moieties act as an electron donating and accepting group, respectively [17-19]. In **1**, DPA also functions as a binding site for metal ions. The electron donating nature of DPA would be influenced by metal ion binding, hence optical properties would change. Based on this strategy, our study demonstrates **1** has a highly selective binding property for Cu^{2+} accompanying with significant colorimetric and fluorescent changes. In addition, **1** can be used for monitoring Cu^{2+} within living HepG2 cells. Finally, our quantum calculation shows a good agreement with the fluorescence behaviour of **1** upon Cu^{2+} binding.

2. Experimental

2.1. Synthesis

2.1.1. Materials and instruments

Unless stated otherwise, reactions were carried out under a dry nitrogen atmosphere in vacuum-flame dried glassware. Thin-layer chromatography (TLC) was performed on Merck silica gel 60 F254. All fluorescence and UV-vis absorption spectra were recorded with a FP-6200 spectrofluorometer (JASCO) and UV-1650PC spectrophotometer (SHIMADZU, Japan), respectively. ^1H NMR spectrum were recorded on a Varian spectrometer at 300 MHz in CDCl_3 (δ 7.26 ppm) or $\text{DMSO-}d_6$ (δ 2.50 ppm), ^{13}C NMR spectral measurements were performed at 75 MHz using CDCl_3 (δ 77.16 ppm) or $\text{DMSO-}d_6$ (δ 39.52 ppm). The terms m, s, d, t, q, quint., and sept, represent multiplet, singlet, doublet, triplet, quadruplet, quintuplet, and septet, respectively. Infrared spectra were recorded on a Bruker Vertex 70.

2.1.2. Synthesis of **1**

4-[bis(pyridin-2-ylmethyl)amino]benzaldehyde **2** (0.60 g, 1.98 mmol), 3-methylbenzothiazol-3-ium (0.64 g, 2.18 mmol), and piperidine (0.25 g, 2.97 mmol) were stirred in 20 ml of dry ethanol over 4-Å molecular sieves. The mixture was refluxed for 12 hours and then filtered off to give a dark red solid **1** (0.54 g, 61%), which was crystallized from CH_2Cl_2 : hexane=1:9, v/v. ^1H NMR (300 MHz, $\text{DMSO-}d_6$) δ 8.59-8.55 (m, 2H), 8.30 (d, J = 8.1Hz, 1H), 8.09 (d, J = 8.4Hz, 1H), 8.00 (d, J = 15.3Hz, 1H), 7.83-7.74 (m, 5H), 7.70-7.56 (m, 2H), 7.37-7.28 (m, 4H), 6.84 (d, J = 9.4Hz, 2H), 5.00 (s, 4H), 4.20 (s, 3H);

^{13}C NMR (75 MHz, DMSO- d_6); δ 36.16, 57.13, 107.68, 113.16, 116.53, 121.86, 122.78, 122.96, 124.29, 127.33, 128.02, 129.37, 132.92, 137.43, 142.33, 149.89, 150.04, 152.61, 158.08, 171.80. IR (film) ν_{max} 3006, 1572, 1519, 1400, 1339, 1276, 1182, 1162, 822, 755 cm^{-1} ; HRMS (FAB) ($[M - I]^+$) m/z calculated for $\text{C}_{28}\text{H}_{25}\text{N}_4\text{S}^+$: 449.1800. Found 449.1805; m.p. 240-242 °C

2.2. General methods for UV-vis and fluorescence titration of **1** with metal ions

The stock solution of **1** (3×10^{-4} M) was prepared by directly dissolving in DMSO. For the spectroscopic determination, the stock solution was diluted with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , pH 7.3) to the desired concentration (3×10^{-5} M). The final experimental buffer condition is set to DMSO: H_2O = 1:9 (v/v) and pH = 7.3. Stock solutions (1×10^{-2} M) of the chloride salts of Cu^{2+} , Hg^{2+} , Mg^{2+} , Co^{2+} , Ca^{2+} , Fe^{2+} , Mn^{2+} , Zn^{2+} , and Pb^{2+} were prepared in water and diluted to the desired concentrations (1×10^{-3} M). For titration of metal ions, aliquots of 3 μL aqueous metal ion solutions (1×10^{-3} M) were added to 1 mL diluted **1** (3×10^{-5} M) solution (DMSO: H_2O = 1:9, v/v, pH = 7.3). The measurements were carried out within 1 min after the addition. All experiments were carried out at 298 K. To ensure the reproducibility and stability of experimental data, all experiments were carried out at least three times.

2.3. Cell imaging by confocal fluorescence microscopy

HepG2 cells (American Type Culture Collection HB-8065) were cultured in Dulbecco's modified essential medium supplemented with 10% fetal calf serum and 1% of penicillin

(100 units/ml) and streptomycin (100 $\mu\text{g/ml}$) at 37 °C in CO₂-air incubator. 3×10^5 cells were loaded onto a 35 mm diameter coverglass bottom dishes (SPL 200350) 1 day before experiment. The cells were incubated with 30 μM of **1** for 20 min and washed with 1 \times PBS-A (PBS without Ca²⁺, Mg²⁺) three times. After that, cells were incubated with Cu²⁺ for 30 min and washed with 1 \times PBS-A (PBS without Ca²⁺, Mg²⁺) three times. All cells were fixed with ice-cold methanol for 5 min before imaging. ZEISS LSM700 Confocal Laser Scanning Microscopy was applied to image HepG2 cells stained with **1** with excitation wavelength at 488 nm, and confocal images were collected at 560-610 nm.

3. Results and discussion

3.1. Synthesis

1 was synthesized by a 4-step synthetic route as depicted in **Scheme 1**. Vilsmeier formylation of N,N-bis(pyridin-2-ylmethyl)aniline **3** [20] with POCl₃ in DMF afforded intermediate **2** [21] in 68% yield. Subsequent Knoevnagel condensation of **2** with 2,3-dimethylbenzo[d]thiazol-3-ium iodide [22] were carried out by stirring in dry ethanol over 4-Å molecular sieves. The mixture was refluxed for 12 hours and then filtered off to give **1** in 61% yield. The newly synthesized compound **1** was fully characterized by NMR and high resolution FAB mass spectroscopy.

3.2. Optical properties of **1**

To investigate the metal ion binding properties of **1**, various cations (Cu²⁺, Hg²⁺, Mg²⁺,

Co^{2+} , Ca^{2+} , Fe^{2+} , Mn^{2+} , Zn^{2+} , and Pb^{2+}) were tested in PBS buffer solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4). The absorption spectrum of **1** showed the maximum peak at 482 nm and its colour took on reddish orange colour. In the presence of Cu^{2+} , the maximum absorption was blue-shifted to 382 nm and the colour changed to yellow, while no noticeable change for the other aforementioned metal ions was observed (**Figs. 1a and S4**). Thus, **1** can be used as a highly selective naked-eye colorimetric chemosensor (**Fig. 1b**). **Fig. 2a** shows the emission spectra of **1** upon excitation at 400 nm in the presence of metal ions. **1** displayed a broad emission spectrum with the maximum peak at 567 nm. The addition of 4 equiv. of Cu^{2+} caused a dramatic fluorescence quenching to 93%. This fluorescence quenching was most probably attributed to intramolecular charge transfer (ICT) with binding of Cu^{2+} at the DPA moiety of **1** [23-25]. The strong shift of the fluorescence intensity depending on Cu^{2+} can provide a basis of the ratiometric analysis by **1** to calculate the amount of Cu^{2+} . In addition, **1** showed complete reversibility in the associated absorption (**Fig. S5**) and emission signals (**Fig. S6**). Therefore, **1** can be a good candidate for a Cu^{2+} selective fluorescent chemosensor as well since the other metal ions tested gave no significant changes (**Fig. 2b**).

3.3. Characterization of **1**

To characterize the capability of **1**/ Cu^{2+} complex, we firstly calculated the limit of detection (LOD) of **1** by the fluorescence titration. The linear relationship between the fluorescence intensity and the Cu^{2+} concentration responds to the range 0.1-7.5 μM ($R^2 = 0.996$), as shown in **Fig. S7**. The detection limit was determined with the following

equation: $LOD = 3\sigma/k$, where σ is the standard deviation of blank measurement, and k is the slope between the ratio of fluorescence intensity versus Cu^{2+} concentration [26, 27]. The limit of detection was calculated to be 1.89×10^{-7} M for **1**, which is able to sufficiently sense the Cu^{2+} concentration in the blood system but also suitable for the Cu^{2+} detection in drinking water within U.S. EPA limit (*ca.* 2.0×10^{-5} M) [26, 27].

Furthermore, dose-dependent fluorescence quenching of **1** was demonstrated by titration of Cu^{2+} (**Fig. S8**). From the job-plot analysis, **1** forms a 1:1 complex with Cu^{2+} (**Fig. S9**). The association constant was calculated as $1.5 \times 10^6 M^{-1}$ based on fluorescence titrations of **1** with Cu^{2+} (**Fig. S10**) [28, 29].

Finally, to exam the anti-interference performance of **1** to common coexisting metal ions, the fluorescence intensity of **1** was evaluated in excess of various metal ions (**Fig. S11**). The fluorescence intensity of **1** in the presence of varying amounts of metal ion mixture (20, 50 and 100 equiv. of Mg^{2+} , Ca^{2+} , Co^{2+} , Zn^{2+} and Mn^{2+} , respectively) were measured after treatment with 0.6, 1.0 and 2.0 equiv. of Cu^{2+} . Thus, this result indicated that **1** is highly selective for Cu^{2+} over other interfering metal ions.

3.4. Cell imaging

Since **1** can penetrate into cells, we carried out the fluorescence imaging of human hepatoma HepG2 cells in the presence of **1** for the direct detection of Cu^{2+} ion. As determined by MTT assay, **1** does not show any significant cytotoxicity against cells within the range of concentrations used in cell imaging (**Fig. S12**). The cells were incubated with 30 μM of **1** for 20 min and washed three times with PBS to remove residual

1. As shown in **Fig. 3a**, a strong fluorescence was appeared in the filter of 570–610 nm due to the uptake of **1** to the cells. Subsequently, cells were treated with different amounts of Cu^{2+} (1, 2, 5 and 10 equiv.) for 20 min at 37 °C. The fluorescence imaging and the quantification of fluorescence intensity from **1** was determined by ZEISS LSM700 Confocal Laser Scanning Microscopy (**Figs. 3a and 3b**). As expected, fluorescence of **1** was reduced significantly as increasing the amount of Cu^{2+} . This result indicated that **1** as a chemosensor can be effectively applied for cell imaging and to detect the presence of intracellular Cu^{2+} *in vivo*.

3.5. Theoretical calculations

3.5.1. Calculation methods

To provide a theoretical basis on the binding mode between **1** and Cu^{2+} and the fluorescence behavior of **1** upon complexation with Cu^{2+} , we have carried out density functional theory calculations, using the Becke's three parameterized Lee-Yang-Parr exchange functional (B3LYP) with 6-31G* basis sets by employing a suite of Gaussian 09 programs [30].

3.5.2. Structures

The incorporation of Cu^{2+} brings significant changes in the geometry of **1**. As can be seen from **Figure 4**, in the presence of Cu^{2+} , three coordination bonds are formed between a Cu^{2+} and the lone pairs from the nearby N atoms. Through the formation of coordination bonds, all three N atoms and the Cu^{2+} ion are pulled in the same plane. Further, it is worthy

to note, the co-planarity of backbone in **1** is broken due to the addition of Cu^{2+} . The dihedral angle between the benzothiazole moiety and the phenylethene moiety is estimated to be 19.5° . The existence of the torsional angle can effectively shorten the conjugation length and thereby may lead to significant optical property change, such as fluorescence quenching. Since the coordination number of the Cu^{2+} ion in the **1**- Cu^{2+} complex is 3 (generally, the optimum coordination number for Cu^{2+} is 4 or 6), it is possible for the Cu^{2+} ion to interact with other electron lone pairs from water molecules under this experimental condition. **Figs. 4c and 4d** display the optimized geometries of **1**- Cu^{2+} complex considering the solvent water molecules. The corresponding structural details regarding the coordinated atoms are listed in **Table S1**. It is interesting to point out, by addition of water molecules, the torsional angle increases from 19.5° to 20.6° (**1**- Cu^{2+} - H_2O) and 20.2° (**1**- Cu^{2+} - $(\text{H}_2\text{O})_2$), respectively. This indicates that the coordination of water molecules further breaks the co-planarity which may consequently influence the system optical property.

3.5.3. Fluorescent sensing mechanism

To obtain more insights regarding the fluorescence change of **1** upon interaction with a Cu^{2+} ion and water molecules, we performed time-dependent density functional theory (TDDFT) calculations at the optimized geometries. As listed in **Table S2**, the calculated excitation wavelength for **1** is 471 nm; and with subsequent addition of Cu^{2+} and H_2O molecules, the excitation wavelength decreases to 403 nm, 397 nm, and 393 nm, respectively. The calculated excitation wavelength of each complex agrees quite well with the experimental observation. That is, without adding Cu^{2+} , the absorption maximum

occurs at around 475 nm; and after adding Cu^{2+} , the absorption maximum shifts to 380 nm. Further, the calculated oscillator strengths regarding specific excitations are exclusively much higher than those of the other possible excitations, indicating the corresponding transitions are more likely to occur. For the excitation of **1**, HOMO→LUMO orbital transition solely (100%) contributed. Whereas, HOMO→LUMO and HOMO-1→LUMO orbital transitions contributed to 80 and 20 % for **1**- Cu^{2+} , 86 and 14 % for **1**- Cu^{2+} - H_2O , and 87 and 13 % for **1**- Cu^{2+} - $(\text{H}_2\text{O})_2$, respectively (**Table S2**). We compared the frontier orbital shapes of the most responsible excitations. As shown in **Fig. 5**, for the complexes **1**- Cu^{2+} , **1**- Cu^{2+} (H_2O) and **1**- Cu^{2+} (H_2O)₂, the HOMO-1→LUMO excitation moves the electron density from the benzothiazole moiety to the phenylethene plane, which leads to fluorescence quenching by ICT.

In contrast, in the case of **1**, HOMO→LUMO excitation involves no such movement of the electron density; thus, the excited electrons would decay by emitting strong fluorescence. Moreover, the oscillator strengths of Cu^{2+} coordinated complexes were greatly declined compared to that of **1**, which is another factor for the fluorescence quenching. Thus, the calculated electronic structure properties are in excellent agreement with the experimental results.

4. Conclusions

In conclusion we designed and synthesized a novel DPA-based colorimetric and fluorescent chemosensor **1** that can be utilized as an excellent selective and sensitive colorimetric and fluorescent chemosensor to detect Cu^{2+} ion comparing with previously

reported probes [31-34]. Confocal microscopy experiment also showed that the **1** can be used for monitoring Cu^{2+} within living HepG2 cells. Moreover, quantum calculations well explained that the fluorescence quenching of **1** upon Cu^{2+} binding was attributed to intramolecular charge transfer.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at xxxxxx.

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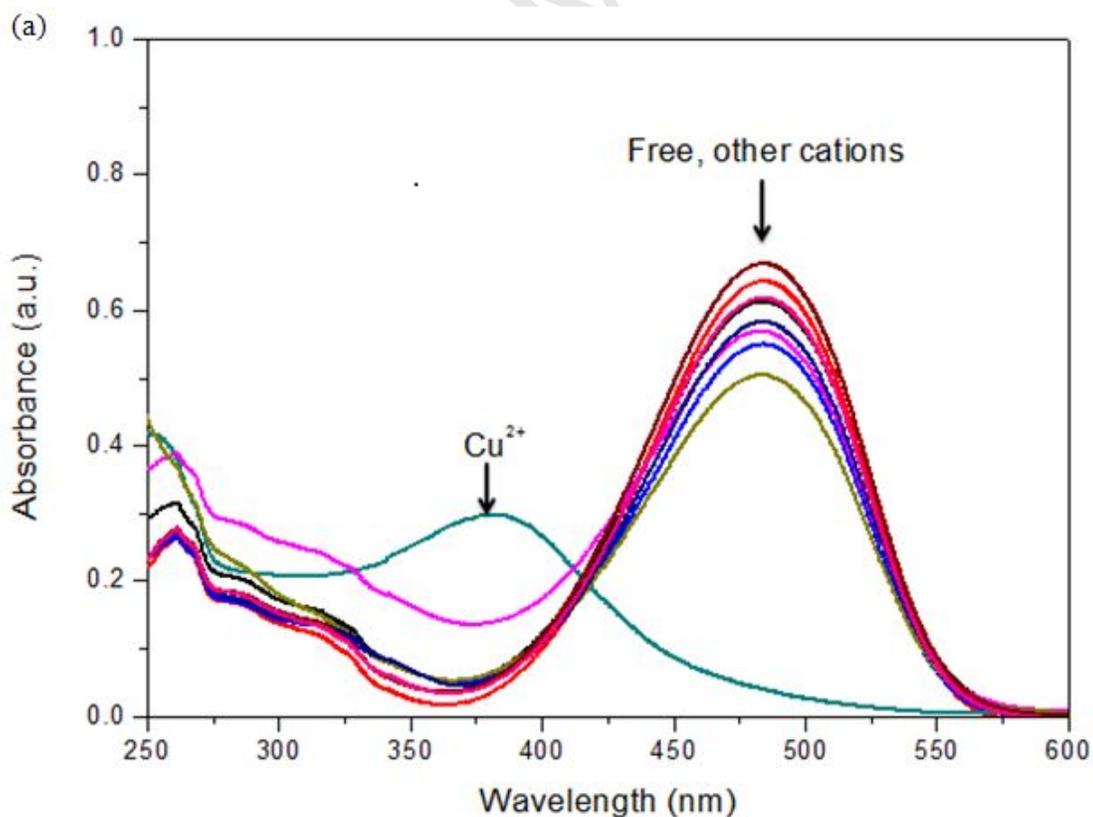
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Figure Legends

Scheme 1. Synthesis of chemosensor **1**. Reagents and conditions: (i) NaOH, CH₂Cl₂, 25 °C, 76%; (ii) POCl₃, DMF, 70 °C, N₂ (g), 58%; (iii) CH₃I, 90 °C, 90%; (iv) Piperidine, dry EtOH, 110 °C, N₂ (g), 61%. Steps of i and iii indicate the synthesis of DPA (electron donating) and 3-dimethylbenzothiazole-3-ium moieties (accepting group), respectively.

Figure 1. (a) Absorption spectrum of **1** (3.0×10^{-5} M) obtained in PBS buffer (DMSO:H₂O = 1:9, v/v, pH = 7.3) when titrated with Cu²⁺, Hg²⁺, Mg²⁺, Co²⁺, Ca²⁺, Fe²⁺, Mn²⁺, Zn²⁺, and Pb²⁺ (2 equiv. of metal ions). (b) The colour change of **1** with various metal ions.

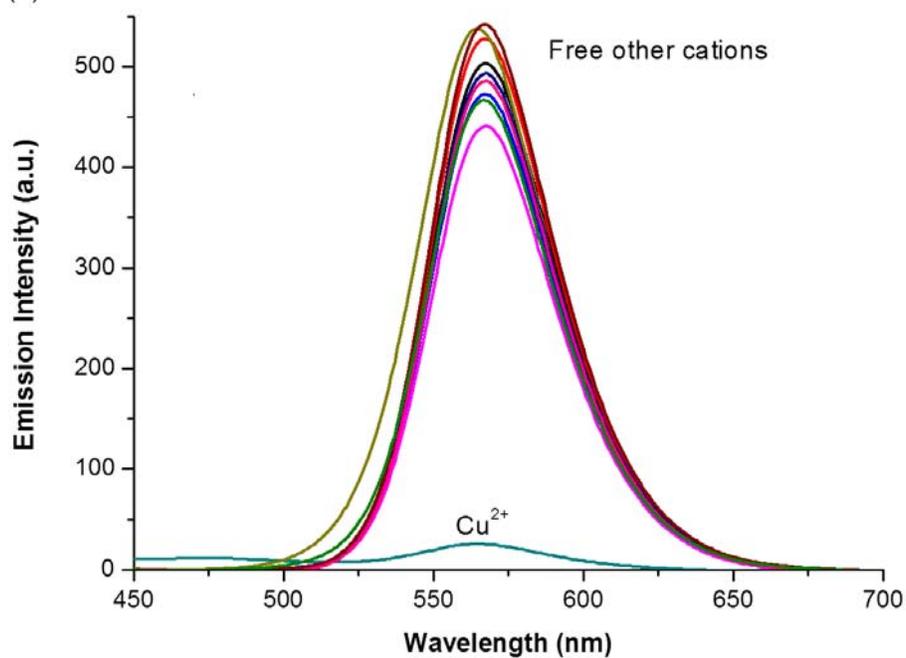


(b)



Figure 2. (a) Emission spectrum of chemosensor **1** (3.0×10^{-5} M) obtained in PBS buffer (DMSO:H₂O = 1:9, v/v, pH = 7.3) upon titrated with Cu²⁺, Hg²⁺, Mg²⁺, Co²⁺, Ca²⁺, Fe²⁺, Mn²⁺, Zn²⁺, and Pb²⁺. Excitation wavelength is 400 nm. Emission wavelength is 567 nm. (b) The Emission intensity enhanced index (F/F_0) in the presence of 4 equiv. of various metal ions.

(a)



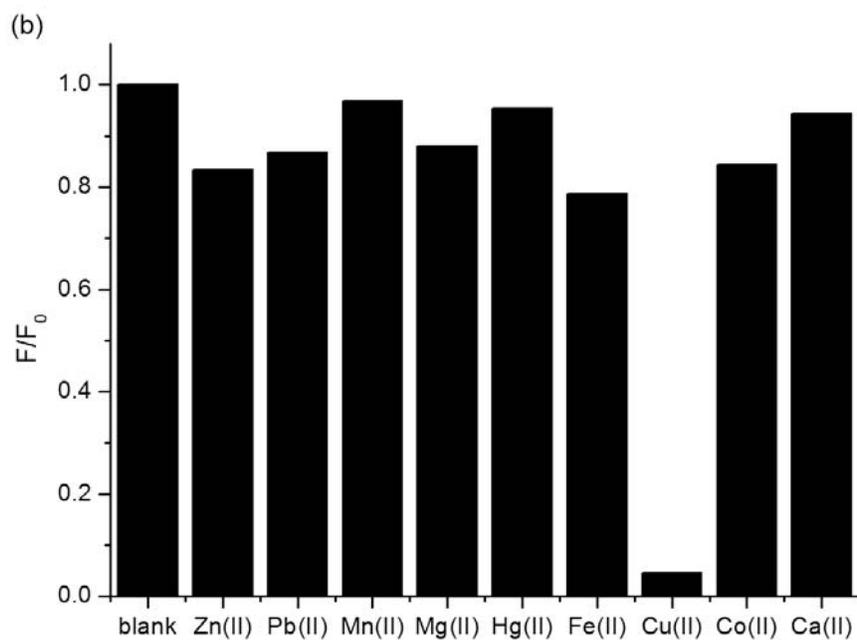
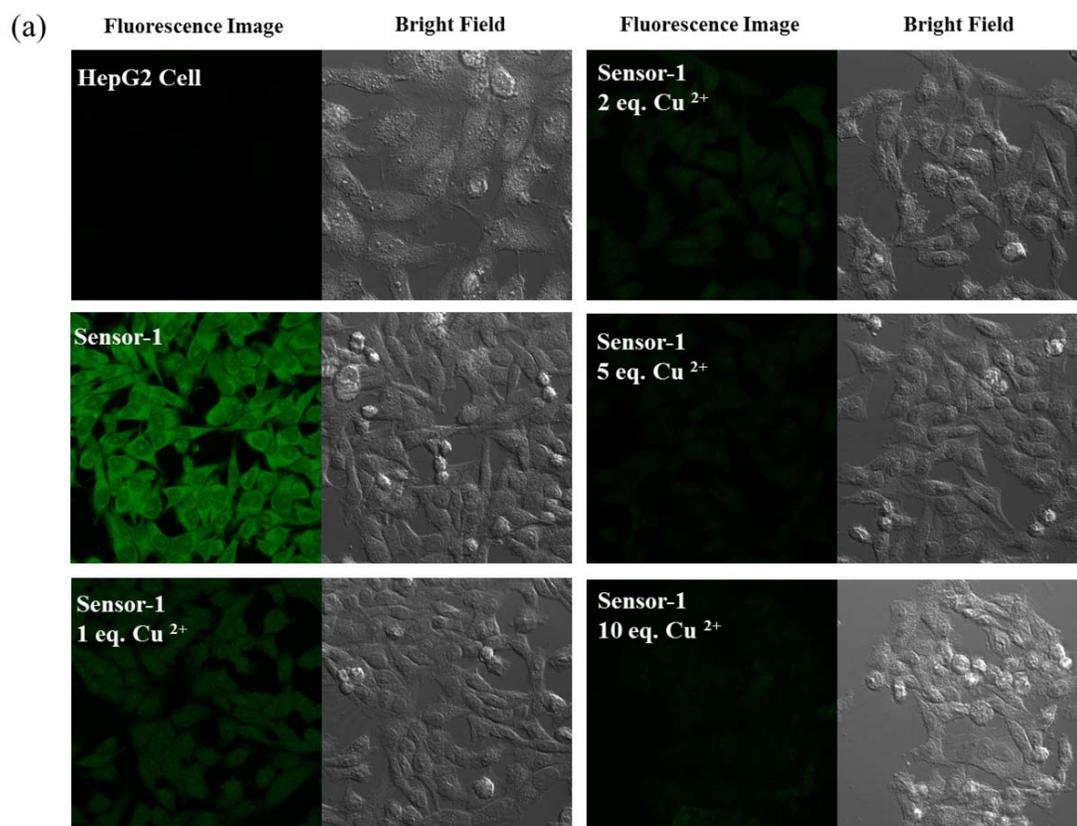


Figure 3. Confocal fluorescence images of human hepatoma HepG2 cells. (a) Confocal fluorescence images of **1** in the presence of different amounts of Cu^{2+} ion (1, 2, 5 and 10 equiv.). (b) Quantitation of intensity of fluorescence signal.



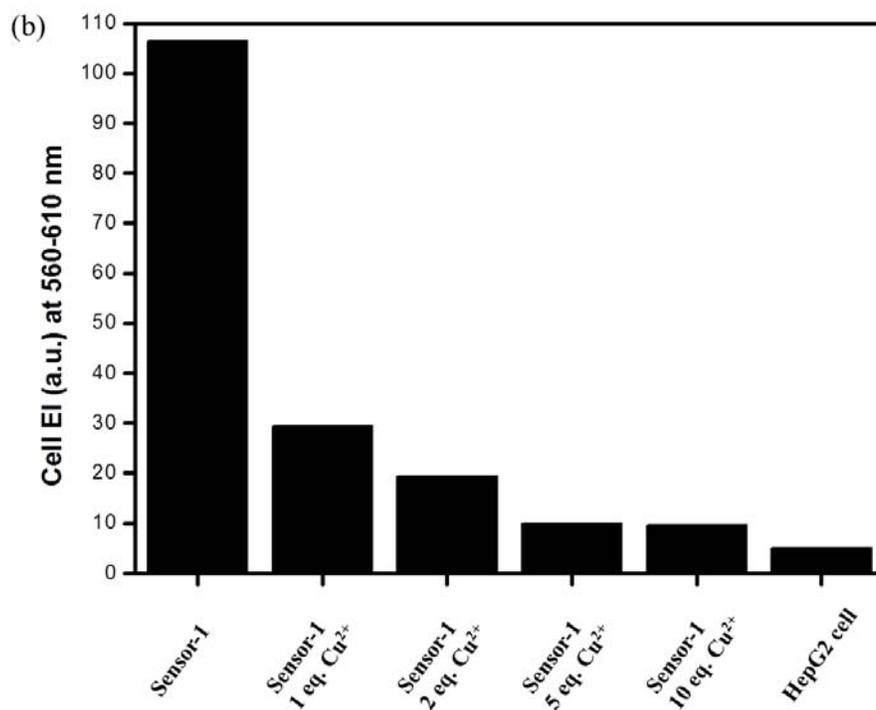


Figure 4. Optimized structures of (a) **1**, (b) **1-Cu²⁺**, (c) **1-Cu²⁺-H₂O**, and (d) **1-Cu²⁺-(H₂O)₂** complexes.

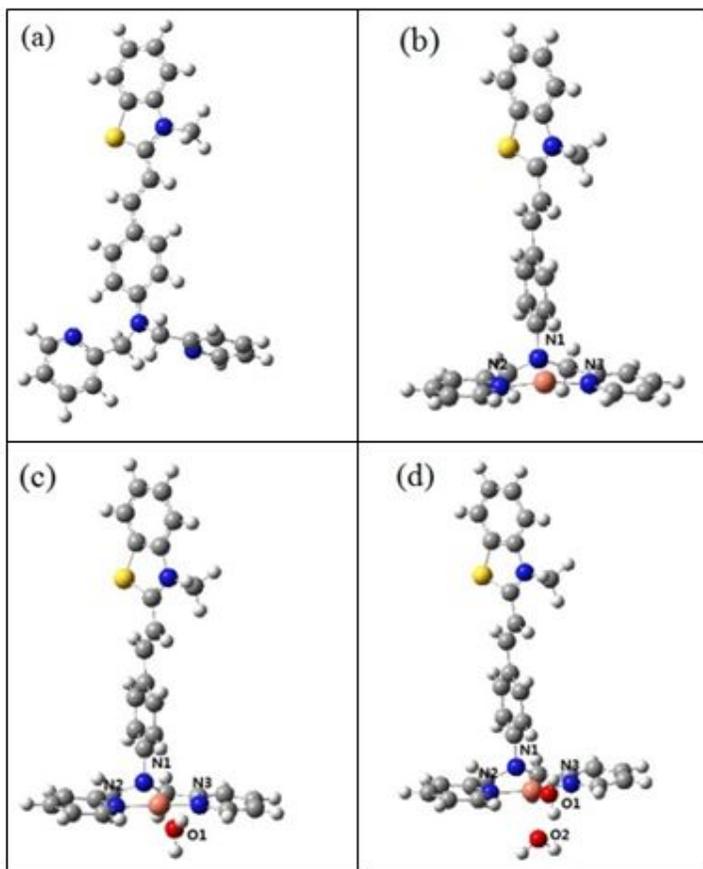
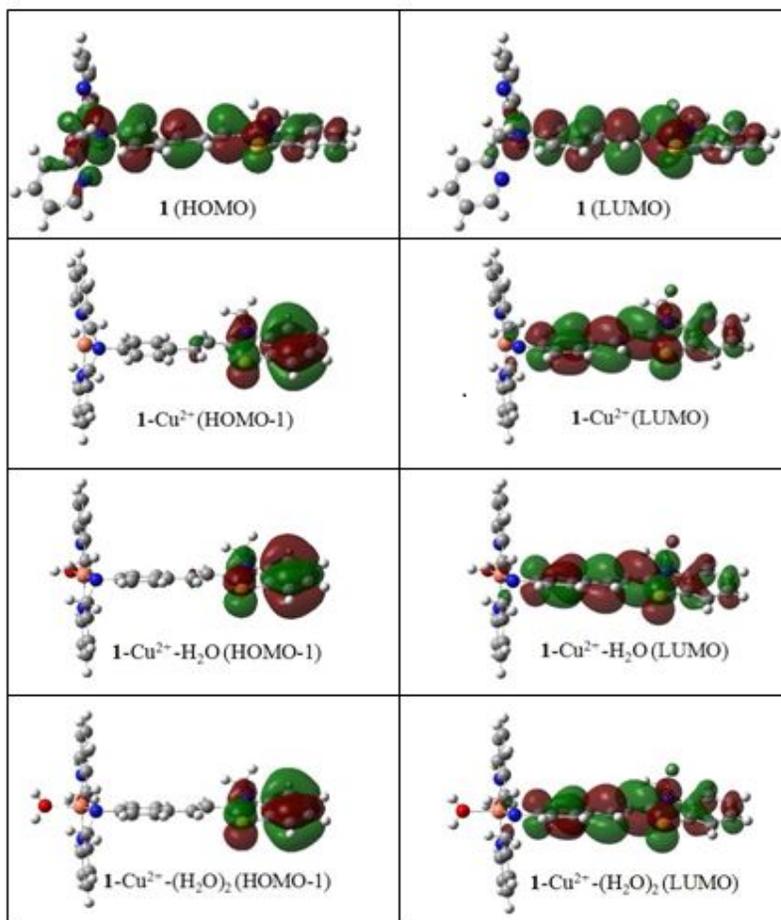


Figure 5. Relevant frontier molecular orbitals to the electronic transition resulting in the fluorescence.



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