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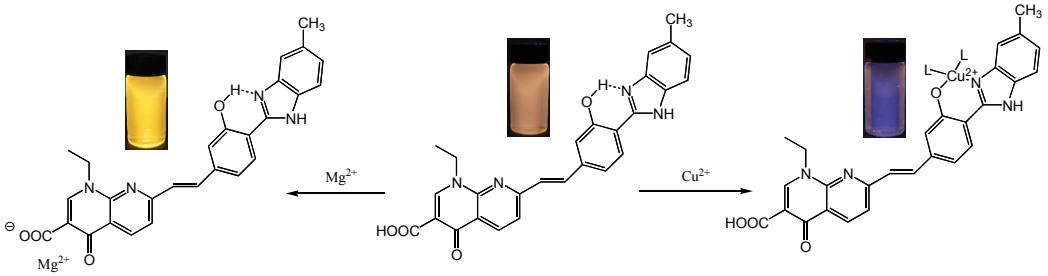

A fluorescent probe for the detection of Mg(II) and Cu(II) and its application of imaging in living cells

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Abstract: A novel fluorescent probe 7-[5′-hydroxy-4′-(5′′-methyl-*1H*-benzo[*d*]imidazole-2-yl)styryl]nalidixic acid (HBIN) was synthesized that contains two independent fluorophores and acts as a very sensitive and selective probe for Mg^{2+} and Cu^{2+} ions. Upon addition of Mg^{2+} and Cu^{2+} ions to the solution of HBIN in MeOH-water (9 : 1, v/v), HBIN exhibited a considerable red-shift in its absorption spectrum. Importantly, this novel fluorescent probe exhibited fluorescence enhancement toward Mg^{2+} ion and fluorescence quenching toward Cu^{2+} ion. These changes result from Mg^{2+} and Cu^{2+} ions binding to HBIN at different site, and undergoing ICT (intramolecular charge transfer) process and ESIPT (excited-state intramolecular proton transfer) process. Its capability of biological application was also evaluated and the results indicate that HBIN could be successfully employed as a Mg^{2+} and Cu^{2+} ions fluorescent probe in living Hela cells.

Keywords: fluorescent probe; metal ions; nalidixic acid; benzimidazole; imaging.

1. Introduction

The past few years have witnessed a large number of reports on the design of fluorescent probes for the detection of metal ions found in environmental and biological processes.[1] Mg^{2+} ion is one of the most abundant divalent ions and plays a crucial role in biology, such as cell proliferation, DNA synthesis,[2] protein phosphorylation,[3] various transporters.[4] Another cation, Cu^{2+} ion, is an essential trace metal ions in human body and plays a critical role in various physiological processes such as biosynthetic, cellular respiration and transport processes within the cell.[5] The alteration of its cellular level is connected to coronary heart disease, and serious neurodegenerative.[6] Therefore, the effective and selective detection of Mg^{2+} and Cu^{2+} ions are of great significance for biochemistry, environmental science and medicine. As well as known, fluorescent probe do offer the advantages of operation simplicity, adaptability, high detection sensitivity and selectivity has drawn much attention. Although some progress has been made on the detection of Mg^{2+} ion and Cu^{2+} ion respectively,[7] researchers have not yet developed any fluorescent probes that can recognize both of two metal ions and give different response signals.

Herein, we describe the synthesis and optical properties of a novel chemosensor **4** for Mg^{2+} and Cu^{2+} ions, in which nalidixic acid (NA) bridged to 2-(2′-Hydroxyphenyl)benzimidazole (HPBI) via ethenyl spacer. As a representative of fluoroquinolone-based antibiotic drug, NA has been widely used in aquiculture, livestock husbandry and human prescription.[8] The structure of NA with a carbonyl adjacent to the carboxyl group allows for proposing the formation of a complex

between Mg^{2+} ion and NA.[9] This interaction can be studied using fluorescence and UV-vis spectrophotometry.

2-(2′-Hydroxyphenyl)benzimidazole (HPBI) and its derivatives show intense fluorescent emission via excited-state intramolecular proton transfer (ESIPT).[10] The HPBI based derivatives have been chosen to construct fluorescent probes.[11] Our choice of HPBI as the Cu^{2+} ion detector comes from the fact that HPBI afforded a hydroxyl and the adjacent nitrogen atom binding site cooperatively with Cu^{2+} ion. When HPBI moiety forms complexes with Cu^{2+} ion, the ESIPT process was efficiently disrupted and a fluorescence quenching was observed.

In this study we focused our attention on compound **4** shown in Scheme 1. We studied its spectroscopic characteristics, selectivity and sensitivity for Mg^{2+} and Cu^{2+} ions in MeOH-water (9 : 1, v/v) solution and its possible utilization as intracellular chemosensor of Mg^{2+} and Cu^{2+} ions by confocal fluorescence microscopy.

2. Results and discussion

2.1 UV-vis spectra

The interaction between **4** and cations was investigated by using UV-Vis and fluorescence spectrometries. The detection ability of **4** in MeOH-water solution (1×10^{-5} mol. dm^{-3}) with cations, such as Ca^{2+}, Cd^{2+}, Hg^{2+}, Co^{2+}, K^{+}, Al^{3+}, Mg^{2+}, Mn^{2+}, Na^{+}, Fe^{3+}, Cu^{2+}, Sn^{4+}, Zn^{2+} and Pb^{2+} showed selective response toward Mg^{2+} and Cu^{2+} ions. As shown in Fig. 1, addition of 1 equiv. Mg^{2+} and Cu^{2+} ions resulted in an obvious change indicating compound **4** had higher binding affinity toward Mg^{2+} and Cu^{2+} than other surveyed metal ions. The observed absorption band of compound **4**

decreased at 383 nm and the other band around 460 nm showed an increased in absorbance. The phenomenon maybe suggest that the complex of **4** - Mg^{2+} and **4** - Cu^{2+} were formed in MeOH-water solution.

Spectrometric titration experiments were performed in the presence of Mg^{2+} and Cu^{2+} ions respectively. Fig. 2 displays the change in the absorption spectrum of **4** upon Mg^{2+} ion addition. Addition of Mg^{2+} ion to a solution of compound **4** in MeOH-water (9:1, v/v) showed gradual decrease at 383 nm and increase around 460nm. An isosbestic point is observed at 413 nm with varying concentrations of Mg^{2+} ion. The same trend was found by adding Cu^{2+} ion to a solution of compound **4** (Fig. 3). These results indicate that **4** - Mg^{2+} and **4** - Cu^{2+}complex coexist with **4** in MeOH-water solution.

2.2 Emission spectra

The effect of Mg^{2+} and Cu^{2+} ions on the fluorescence properties of compound **4** in the MeOH-water solution was investigated. Fig. 4 showed that upon the continuous addition of Mg^{2+} ion from 0 to 1×10^{-5} mol. dm^{-3}, the fluorescent intensities of HBIN centered at 570 nm was gradually enhanced, which suggests that Mg^{2+} ion formed a chelate complex with compound **4**. On the other hand, the fluorescent intensity of **4** decreased gradually when increasing the concentration of Cu^{2+} from 0 to 1×10^{-5} mol. dm^{-3} (Fig. 5). Notably, a pronounced change in the fluorescence signal was observed even when the Mg^{2+} and Cu^{2+} ions concentration was as low as 0.3×10^{-5} mol. dm^{-3}. And the detection limits of the present approach were found to be at least as low as 206 nM (Mg^{2+}) and 309 nM (Cu^{2+}), respectively. These fluorescent intensity changes

indicate that such a dual-functional probe could detect Mg^{2+} and Cu^{2+} ions with high sensitivity.

The selectivity of probe **4** for Mg^{2+} and Cu^{2+} ions were also observed by fluorescence measurements. Probe **4** (1×10^{-5} mol dm^{-3}) was treated with different metal ions (1×10^{-5} mol. dm^{-3}). As displayed in Fig. 6, a dramatic enhancement of the fluorescent intensity was observed in the presence of Mg^{2+} ion, which was probably due to the fact that the carbonyl adjacent to the carboxyl group formed a chelate complex with Mg^{2+} ion.[12] This binding result in an enhanced ICT (intramolecular charge transfer) process from the electron-releasing group to Mg^{2+} ion. In contrast, Cu^{2+} ion quenches the fluorescent intensity of compound **4** efficiently, showing the unique response of **4** to Cu^{2+} ion. The ESIPT process of HPBI moiety was disrupted because the complex of **4** - Cu^{2+} formed and a fluorescence quenching was discovered.[11] While the addition of other metal ions, such as Ca^{2+}, Cd^{2+}, Hg^{2+}, Co^{2+}, K^{+}, Al^{3+}, Mn^{2+}, Na^{+}, Fe^{3+}, Sn^{4+}, Zn^{2+} and Pb^{2+}, had no distinct influence on fluorescent intensity. These results indicated that compound **4** exhibits high selectivity toward Mg^{2+} and Cu^{2+} ions.

The competition experiment of **4** towards Mg^{2+} and Cu^{2+} ions was evaluated by adding the mixed solution of $MgCl_2$ and $CuCl_2$. As shown in Fig. 7, the fluorescence intensity of **4** increased with increasing the ratio of Mg^{2+} ion from 0 to 100%. Moreover, the sensing behavior of **4** to Mg^{2+} ion almost experienced no interference by the presence of Cu^{2+} ion. Compound **4** maintained particular fluorescence responses toward Mg^{2+} ion when Mg^{2+} and Cu^{2+} ions co-exist in solution, probably

because the ICT process can bring more significant changes than ESIPT process.

Colorimetric monitoring of this process was also feasible with the excitation at 365 nm using UV lamp. The free probe exhibited a light brown emission color, and a significant color change from light brown to bright yellow was observed upon addition of Mg^{2+} to a solution of **4** (1×10^{-5} mol dm^{-3}). On the contrast, the introduction of Cu^{2+} induced a dramatic quenching in emission color. While the significant color changes are not observed after other metal ions were added, as shown in Fig. 8. These results indicate that compound **4** specifically bind to Mg^{2+} and Cu^{2+} ions and give different response signals.

2.3 Cell imaging

Then we studied the bioimaging application of **4** for detecting Mg^{2+} and Cu^{2+} ions in living cells. Cultured Hela cells were incubated with **4** (10.0 μM) for 30 min at 37 □, and a weak fluorescence of **4** was detected in the cells' interior (Fig. 9 a_0). However, when the cells were incubated with Mg^{2+} ion (20.0 μM) in the culture medium for 30 min at 37 □, a much brighter fluorescence from the cytoplasmic area was observed (Fig. 9 a_1). Bright field microscopic image (Fig. 9, b_0) indicated that the cells remain viable with **4**. In a separate experiment, the cells were incubated with Cu^{2+} ion instead of Mg^{2+} ion. The weak fluorescence of **4** was quenched out efficiently (Fig. 9, a_2). These results indicated that **4** could be used for monitoring intracellular Mg^{2+} and Cu^{2+} ions in living cells.

2.4 Proposed binding mechanism for 4 with Mg^{2+} and Cu^{2+} ions

Thus, according to the obtained results, it is very likely due to the Mg^{2+} ion

induced ICT process. The fluorescence enhancement response of compound **4** toward Mg^{2+} ion is presumably due to Mg^{2+} ion binding to carbonyl and carboxyl group of NA moiety.[12] This selective binding to Mg^{2+} ion results in an enhanced ICT process from NA to Mg^{2+} ion. Different from Mg^{2+} ion, the Cu^{2+} ion inhibits ESIPT process of HPBI moiety and brings a fluorescence quenching. As shown in Scheme 2, compound **4** exhibits weak fluorescence in solution because the HPBI moiety can undergo ESIPT process by the structural transformation from enol to keto tautomers on excitation at 385 nm. After Cu^{2+} ion were added to the solution, the ESIPT process was efficiently inhibited and fluorescence quenching was discovered.[11] These findings suggested that probe **4** can be used to detect Mg^{2+} and Cu^{2+} ions undergo two different mechanisms respectively.

3. Conclusions

In conclusion, an ethenyl-linked HPBI-based chemosensor was synthesized and characterized for recognition of Mg^{2+} and Cu^{2+} ions by different binding sites. Selective binding of compound **4** to metal ions caused immediate and remarkable fluorescence enhancement for Mg^{2+} and fluorescence quenching for Cu^{2+}, which proved that **4** could serve as a sensitive and selective chemosensor of Mg^{2+} and Cu^{2+} ions. The detectability of **4** followed the order of $Mg^{2+} > Cu^{2+} >>$ other metal ions. Furthermore, we have demonstrated that probe **4** is applicable for Mg^{2+} and Cu^{2+} ions imaging in living cells. We expect that the present findings could be applied to detect Mg^{2+} and Cu^{2+} ions in biological and environmental systems.

4. Experimental section

4.1 Materials and general methods

All chemicals and reagents were purchased from Tianjin Jiangtian Chemical Technology Co., Ltd. and used without further purification. 2-hydroxy-5-(hydroxymethyl)benzaldehyde was synthesized by this group according to the method of Nahid Nishat et al..[13] Nalidixic acid (NA) were purchased from Alfa Aesar China (Tianjin) Co., Ltd. Hela cells were purchased from Bogoo (shanghai), cells were incubated in Thermo Scientific Forma Series II Water Jacketed CO_2 Incubator (Thermo).

The ^1H nuclear magnetic resonance (NMR) was recorded on a Bruker DRX-400 AVANCE spectrometer. DMSO-d_6 was used as the solvent. Steady-state fluorescence spectra were recorded on a Hitachi F-4500 spectrophotometer. UV-vis absorption spectra were measured on a Shimadzu UV-3390 spectrophotometer. Elemental analysis was obtained on a Vario MAX CHN apparatus. Fluorescence images in living cells were recorded in Nikon A1 laser scanning confocal microscope.

4.2 Synthesis of HBIN

The synthetic route of HBIN is shown in Scheme 1.

2-(2′-hydroxy-5′-hydroxymethylphenyl)-5-methylbenzimidazole (2)

2-hydroxy-5-(hydroxymethyl)benzaldehyde 0.89 g（5.9 mmol） and $NaHSO_3$ 0.61 g（0.0059 mol）were dissolved in 30 mL ethanol and stirred at room temperature for 4 h. Then 4-methylbenzene-1, 2-diamine 0.72 g (5.9 mmol) was dissolved in 20 mL N,N-Dimethylformamide（DMF）and added slowly to the solution. The solution was stirred at 80℃ for 2 h, then cooled to room temperature and poured into ice

water (500 mL) and stay for 30 min. Then the precipitate was formed, and it was filtered and dried in vacuum at 50℃. The residue was recrystallized with ethanol to obtain a white solid (1.28 g) at 85.4% yield.

^{1}H NMR (400MHz, DMSO-d_6) : δ (ppm) = 13.11（brs，2H，Ph-*OH*, -*NH*），8.00（s，1H，Ar-*H*），7.48（m，2H，Ar-*H*），7.30（d，*J*=8.4Hz，1H，Ar-*H*），7.09（m，1H，Ar-*H*），6.83（d，*J*=8.4Hz，1H，Ar-*H*），5.2（s，1H，CH_2O-*H*），4.51（d， *J*=5.2Hz，2H，-C*H*$_2$-），2.43（s，3H，-*CH*$_3$）. Analytically calculated for $C_{15}H_{14}N_2O_2$: C, 70.85; H, 5.55; N, 11.02%. Found: C, 70.91; H, 5.52; N, 11.04%.

2-(5′-formyl-2′-hydroxyphenyl) -5-methylbenzimidazole (3)

Activated MnO_2 0.8 g（9.3 mmol）was added to the solution of 2-(2′-hydroxy-5′-hydroxymethylphenyl)-5-methylbenzimidazole (**2**) 0.8 g （3.1 mmol）in 20 mL ethanol. After heating to reflux for 25 min, the reaction was quenched. Filtered and washed with 5% HCl, The combined organic layers concentrated in vacuo. Purification by flash column chromatography (Petroleum ether: ethyl acetate = 4:1, v/v) and got the compound **3** as white power 0.39 g at 54.9% yield.

^{1}H NMR (400MHz, DMSO-d_6) : δ (ppm) = 13.92（brs，2H，Ph-O*H*, -*NH*）, 9.92（s，1H，-C*H*O）, 8.66（s，1H，Ar-*H*）, 7.94（d，*J*=8.4Hz，1H，Ar-*H*）, 7.58（d，*J*=8.4Hz，1H，Ar-*H*）, 7.48（s，1H，Ar-*H*）, 7.22（d，*J*=8.4Hz，1H，Ar-*H*）, 7.14（d，*J*=8Hz，1H，Ar-H）, 2.46（s，3H，-*CH*$_3$）. Analytically calculated for $C_{15}H_{12}N_2O_2$: C, 71.42; H, 4.79; N, 11.10%. Found: C, 71.51; H, 4.81; N, 11.19%.

7-[5′-hydroxy-4′- (5′′-methyl-*1H*-benzo[*d*]imidazole-2-yl)styryl]nalidixic acid (4)

A mixture of nalidixic acid (NA) 0.25g (1mmol) and 2-(5′-formyl-2′-hydroxyphenyl)-5-methylbenzimidazole (**2**) 0.23 g (1mmol) in 30% H_2SO_4 (50 mL) was stirred under at 130 □ in a flask for 1 h. The mixture was cooled to room temperature and precipitate was produced. The precipitate was filtered off, washed with ethanol and then dried in vacuum to obtain yellow powder 0.35g at 75.1% yield.

^{1}H NMR (400MHz, DMSO-d_6) : δ (ppm) = 14.41（brs，3H，Ph-*OH*, -*NH,* -CO-*OH*）, 8.63（m，2H，Ar-*H*）, 7.95（m，2H，-*CH*-）, 7.74（d，*J*=8Hz，1H，-*CH*=C-）, 7.62（m，3H，Ar-*H*）, 7.40（d，*J*=8.4Hz，2H，-*CH*=C-）, 7.26（d，*J*=8.8Hz，1H，Ar-*H*）, 3.43（m，2H，-CH_2-）, 2.72（s，3H，-CH_3）, 1.51（t，*J*=7.2*Hz*，3H，-CH_3）.Analytically calculated for $C_{27}H_{22}N_4O_4$: C, 69.52; H, 4.75; N, 12.01%. Found: C, 69.48; H, 4.71; N, 12.24%.

Acknowledgments

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Captions:

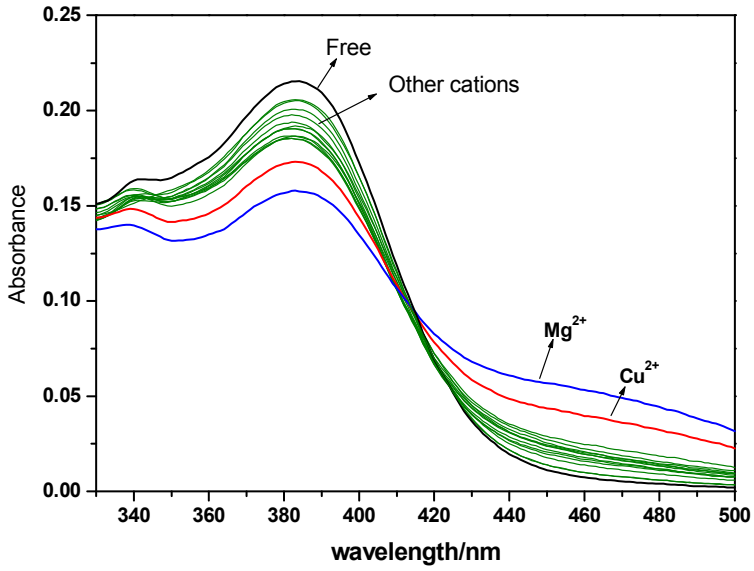
1. Figure 1. UV-vis spectrum of ligand **4** (1×10^{-5} M) in the presence of various metal ions (1×10^{-5} M as their chloride salts).
2. Figure 2.Variation in the UV-vis spectra of **4** (1×10^{-5} M) in MeOH-water (9 : 1, v/v) with increasing concentrations of Mg^{2+} ion (0-1×10^{-5} M) as indicated.
3. Figure 3. Variation in the UV-vis spectra of **4** (1×10^{-5} M) in MeOH-water (9 : 1, v/v) with increasing concentrations of Cu^{2+} ion (0-1×10^{-5} M) as indicated.
4. Figure 4. Fluorescence emission spectra of **4** (1×10^{-5} M) in MeOH-water (9 : 1, v/v) with increasing concentrations of Mg^{2+} ion excited at 385 nm.
5. Figure 5. Fluorescence emission spectra of **4** (1×10^{-5} M) in MeOH-water (9 : 1, v/v) with increasing concentrations of Cu^{2+} ion excited at 385 nm.
6. Figure 6. Fluorescence emission of **4** (1×10^{-5} M) in the presence of various metal ions. Concentration of **4** in MeOH-water (9 : 1, v/v) is 1×10^{-5} M. Concentration of added metal ions are 1×10^{-5} M. All of the measurements were carried out at the equilibrium states.
7. Figure 7. Fluorescence emission plotted vs. concentrations of Mg^{2+} ion. Total concentration of Mg^{2+} and Cu^{2+} ions is always 1×10^{-5} M in mixed ion solution.
8. Figure 8. Top) The selectivity of probe **4** toward various metal ions. Bottom) Color change of probe **4** toward various metal ions. Concentration of **4** in MeOH-water (9 : 1, v/v) is 1×10^{-5} M. Concentration of metal ions are 1×10^{-5} M.
9. Figure 9. Confocal fluorescence images in HeLa cells: Top, a_0-c_0) Cells incubated with HBIN (10 μM) for 1 h. Middle, a_1-c_1) Cells incubated with $MgCl_2$ (20 μM)

and HBIN (10 μM) for 1 h. Bottom, a_2-c_2) Cells incubated with $CuCl_2$ (20 μM) and HBIN (10 μM) for 1 h. Emission was collected at 570 nm upon excitation at 488 nm. Bar = 20 μm.

10. Scheme 1. Synthetic method of compound **4**. Reagents and conditions: (i) 4-methylbenzene-1,2-diamine, $NaHSO_3$, ethanol, room temperature 4 h; DMF, 80 ℃, 2 h; (ii) activated MnO_2, ethanol, reflux, 25 min; (iii) NA, 30% H_2SO_4, 130 ℃, 1 h.

11. Scheme 2. Proposed complexation mechanism of **4** upon addition of $MgCl_2$ and $CuCl_2$.

D. P. Zhai et al Figure 1.



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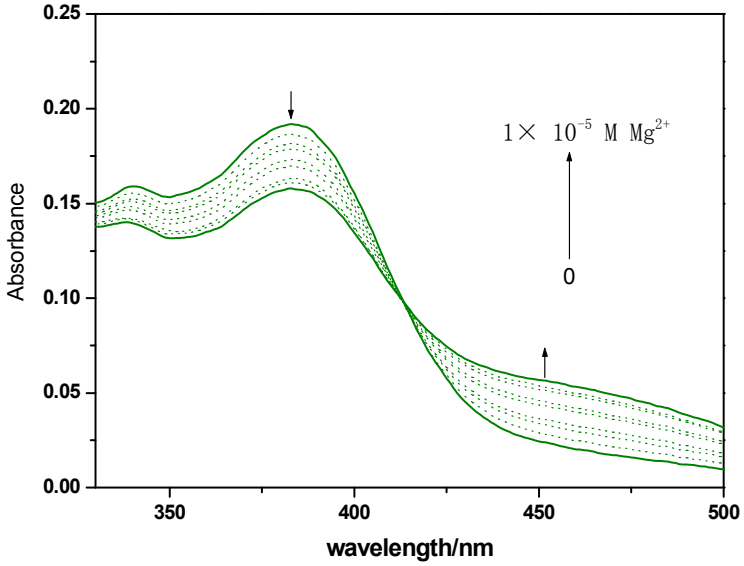

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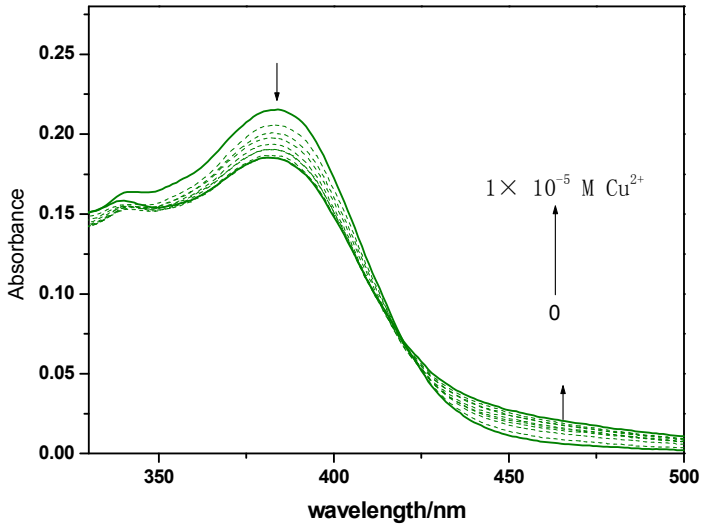

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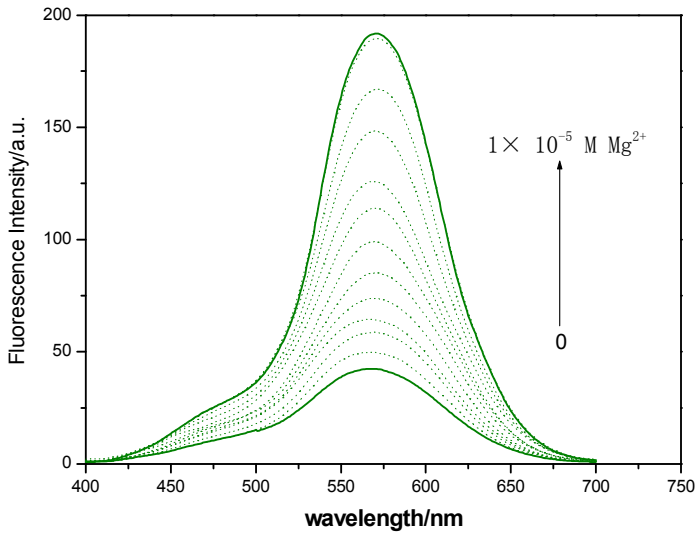

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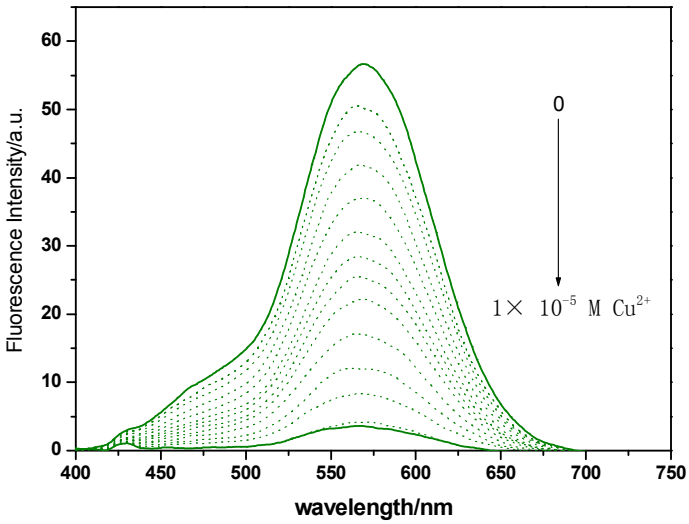

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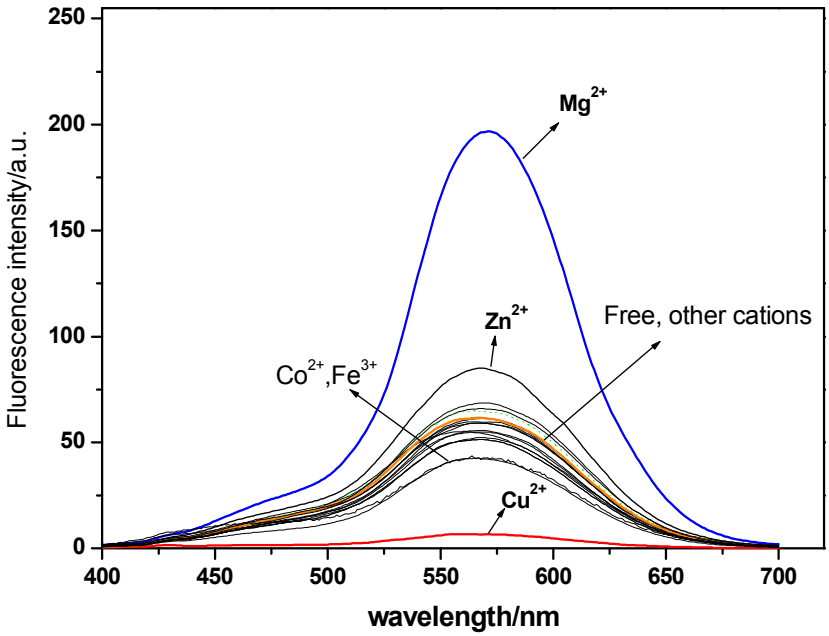

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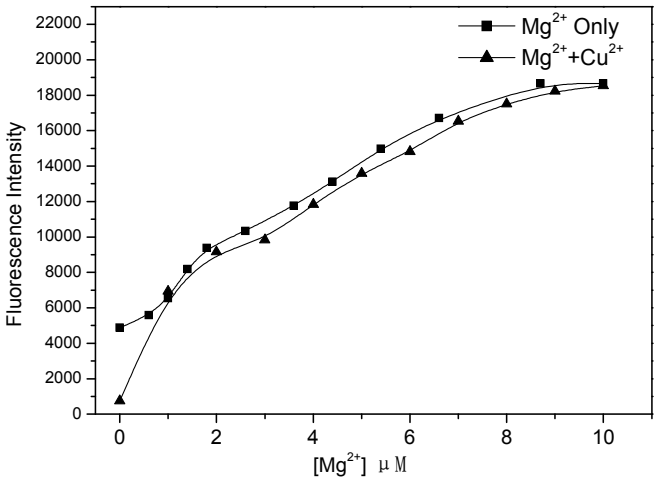

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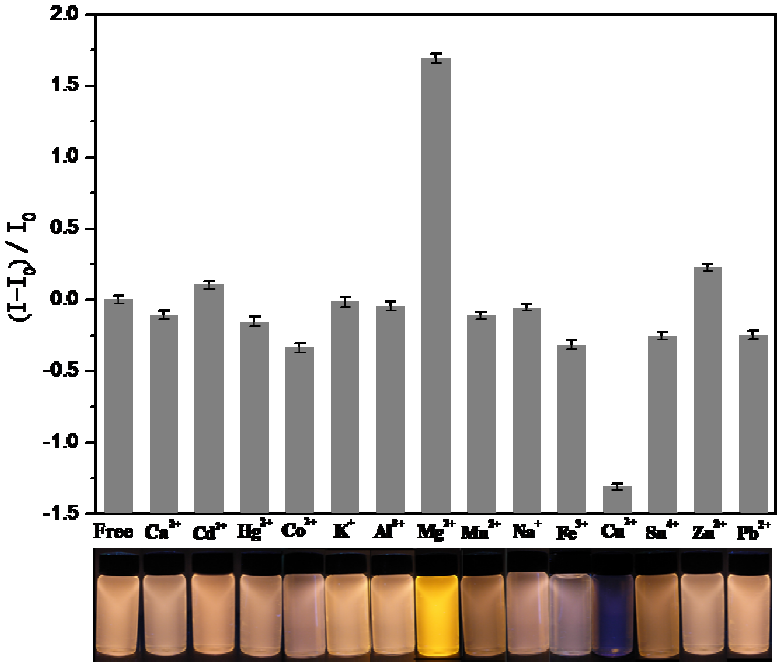

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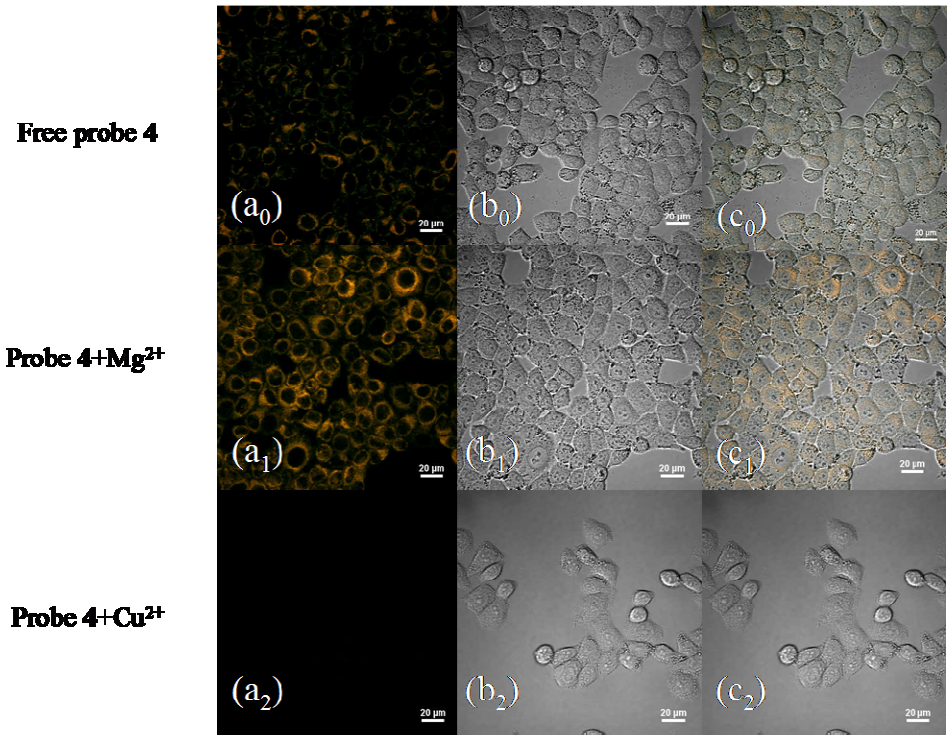
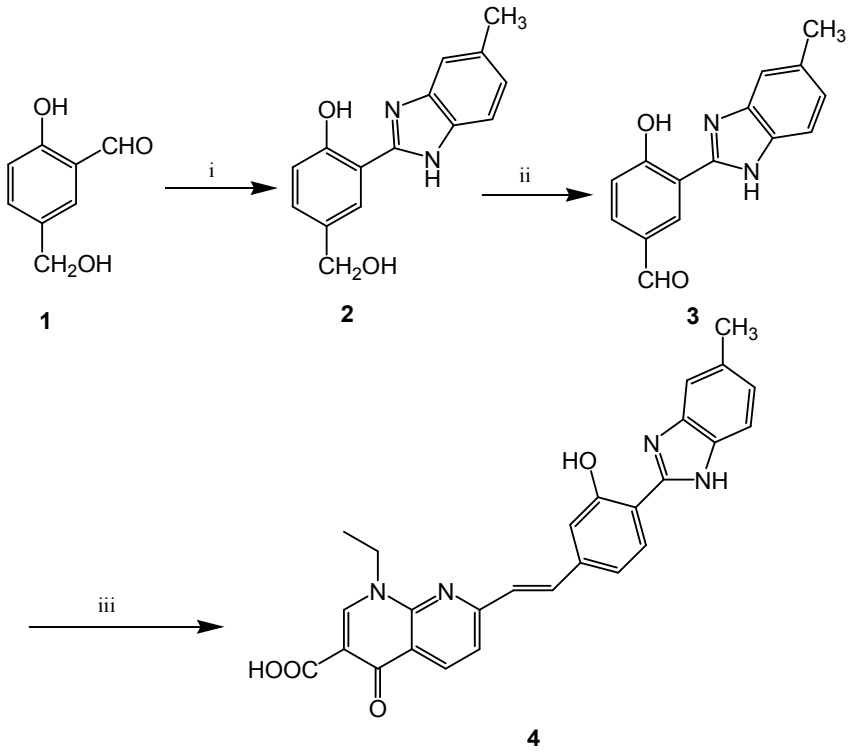


D. P. Zhai et al Scheme 1.



D. P. Zhai et al Scheme 2.