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Tunable Photochromism of Spirobenzopyran via Selective Metal Ion Coordination: An Efficient Visual and Ratioing Fluorescent Probe for Divalent Copper Ion

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In the present paper, a new spirobenzopyran derivative was synthesized and applied in simultaneously colorimetric and fluorescence ratiometric detections of Cu^{2+}. In contrast to the virtually photochromic character of the common spirobenzopyrans in most organic solvents, this spirobenzopyran is colorless in organic aqueous solution even irradiating by ultraviolet light. The formation of red merocyanine in an ethanol-aqueous solution is only induced by Cu^{2+} coordination. Furthermore, the closed form of the spirobenzopyran is highly fluorescent. Upon complexation with Cu^{2+}, it displays not only decreasing in the initial fluorescence emission band but also appearing in a new emission at long wavelength. Thus, the Cu^{2+} quantitative measure can be achieved by fluorescence ratiometry. With the optimum conditions described, the Cu^{2+} concentration can be determined from 5.13×10^{-7} M to 3.81×10^{-4} with a detection limit of 1.06×10^{-7} M. Both the color and the fluorescence changes of the spirobenzopyran are extremely specific for Cu^{2+} over biologically relevant substrates, which meet the selectivity requirements for biomedical application. Serum divalent copper values were determined using this spirobenzopyran, which fell into the normal range of the content reported in the literature and were in good agreement with those obtained by atomic absorption spectroscopy. The combined data from fluorescence titrations and ^1H NMR measurements indicate that the new emission of the spirobenzopyran generated by Cu^{2+} is the result of the metal-induced ring opening and conformation restriction by Cu^{2+} liganding with the opened merocyanine form and the subsequent reduction of the intramolecular charge transfer of the merocyanine.

In the recent years, there has been considerable interest in the design of artificial molecular probes for recognition and quantification of metal ions.[1] Spirobenzopyrans are attractive platforms in such constructions due to their unique photochromic property and molecular recognition ability.[2] Under external physical and chemical stimuli, a spirobenzopyran undergoes isomerization from its spiropyran (spiro-) form to the corresponding *trans*-merocyanine (mero-) form, which has been shown to exhibit extremely sensitive absorption and color changes in the visible range, and the position of this equilibrium usually depends on the solvent polarity and the nature of the substituents.[3] In organic solution, this conversion can be induced by the substrate complexation. Based on this coordination-induced photochromism characteristic, spirobenzopyrans have been employed not only in materials chemistry for molecular switches[4] but also in bioorganic chemistry and analytical chemistry for molecular interaction studies[5,6] and for applications such as molecular transporters[7] and molecular probes for metal ions and organic molecules.[8,9]

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It was previously demonstrated that, for a suitably substituted spirobenzopyran, the conversion of the spiro-form into the mero-form could be achieved by complexation with certain *d*- and *f*-elements[10] or alkali metal ions[11,12] via cooperative ligation of other chelating functionality attached at the 8-position. This metal coordination interaction provides novel and potential approaches for metal sensing. In fact, several spirobenzopyrans have been employed to recognize and detect metal ions.[13,14] Unfortunately, the measuring is usually performed in a low-polar organic solvent and without light irradiating. But the eventual application in environmental and biological fields of a sensor needs to function in an aqueous medium without light-black protection. To the best of our knowledge, no spirobenzopyran-based metal ions probe was reported that meets these requirements.[15] Another challenge of spirobenzopyran for metals sensing is the signaling reporter. In terms of sensitivity and selectivity concerns with quantitative measure, fluorescence spectroscopy offers distinct advantages over absorption spectroscopy. However, most of the spirobenzopyrans hitherto reported have been mainly signaled by UV−vis absorption spectroscopy[11,12,14] due to the common susceptibility of low quantum yield of such dyes.[16] Two 8-hydroxylquinoline-derived spirobenzopyrans have been applied in fluorescent detection of metal ions.[13] The fluorescence emission of the mero-form of the spirobenzopyran in an ethanol solution was greatly increased by chelating with Zn^{2+} or certain alkaline earth metals, whereas complete fluorescence quenching was observed by Cu^{2+}, Co^{2+}, and Ni^{2+}, etc.[13b]

Among the environmentally and biologically important metal ions, Cu^{2+} has been one of the targets of interest.[17,18] In order to assess copper accumulation or deficiency in biological and environmental samples, various efficiently and reproducibly analytical methods, such as spectrophotometry,[19] stripping voltammetry,[20] inductively coupled plasma-mass spectroscopy,[21] atomic absorption spectroscopy (AAS),[22] ion-selective electrodes,[23] and optical polymeric film-based sensors,[24,25] have been developed so far. While these technologies are sensitive, selective, and accurate for Cu^{2+} assay, there is still a need for the development of new methods for multipurpose determination of the metal. Recently, much effort has been placed on exploring Cu^{2+} fluorescent probes, of which a Cu^{2+} recognition site was intramolecularly connected to a fluorescence reporter through a linking bridge. Binding of the recognition unit with Cu^{2+} causes a fluorescence quenching to the fluorophore as a result of electron or energy transfer,[26,27] and a few cause fluorescence enhancement.[28,29] More recently, a paucity of ratiometric fluorescent probes for Cu^{2+} has been developed by Canary et al.,[30] Qian et al.,[31] and Huang et al.[32] based

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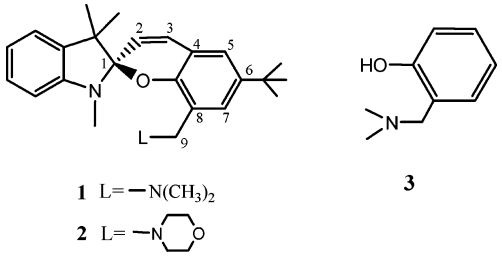
Chart 1. Chemical Structures of the Spirobenzopyrans 1 and 2 and the Reference Compound 3



on the mechanisms of internal charger transfer (ICT), metal binding displacement, and Cu^{2+} complex with the urea groups. Very interestingly, a new probe for simultaneous colorimetric and fluorescent ratiomeric detection of Cu^{2+} was designed by Cui et al. based on the deprotonation of the secondary amines of the receptor which was conjugated to the naphthalimide fluorophore.[33] A colorimetric and fluorescent ratiometric probe combines advantages of the sensitivity of fluorescence with the convenience and aesthetic appeal of a visual assay.[34] However, the approach proposed by Cui et al. displays low sensitivity and a narrow response range for Cu^{2+} detection; moreover, it needs two excitation light sources to perform dual fluorescence emissions.[33]

We have previously reported a fluorescent sensor for Cu^{2+} based on the inner filter effect between a spirobenzopyran derivative (**1**) and a zinc porphyrin.[35] However, the measuring has to be performed in an organic medium since **1** undergoes photoisomerization to its merocyanine form in aqueous solution. Moreover, the Cu^{2+} complexation event was not signaled by **1** itself but by the porphyrin fluorescence quenching, which probably reduces the preciseness and sensitivity of measurement. In our further study on the properties of spirobenzopyran dyes, we found out that not only the photoactivity but also the quantum yield of a spirobenzopyran depends markedly on the substituents present in spiropyran nucleus. In this contribution, we report the design of a new spirobenzopyran (**2**) (Chart 1) and its application in Cu^{2+} sensing. In the molecular backbone of **2**, a morpholine functionality was covalently attached at the 8-position instead of the dimethylamino group of **1**, but **2** displays significantly different photophromic behavior and fluorescence sensing feature for Cu^{2+}. We demonstrate here that **2** could not only function in aqueous solution but also exhibit significant color change and an interesting ratioing fluorescence response for Cu^{2+} due to a dramatic decrease of the original fluorescence emission and a dramatic increase of a new emission at long wavelength upon the Cu^{2+} complex.

Compared to the known ratiometric fluorescent probes for Cu^{2+} reported in the literure,[30−34] **2** possesses some remarkable features: First, the dual fluorescence response was obtained by employing only one fluorophore and excited with one excitation wavelength; this sensing format is well-known to contain many advantages, in particular, the simplicity and cost of one light-emitting diode and laser diode as the excitation source and two narrow band-pass or interference filters to select the emission wavelengths(s). Second, both the excitation and the emission wavelengths of **2** are located in the visible range, and the Stokes shift of the two emission bands is near 180 nm, making it potentially applicable to blood sensing/screening in a physiological environment. Most important, **2** displayed extreme specificity toward Cu^{2+} in an aqueous solution even in the presence of a high concentration of competitive heavy metal ions. In certain biological samples, such as human serum, the concentrations of Zn^{2+} and Fe^{3+}/Fe^{2+} are significantly higher than that of Cu^{2+}, and selective detection of Cu^{2+} in the presence of these metal interfering agents is a critical issue to the application of most common fluorescent probes. But **2** has enabled the direct detection of Cu^{2+} in human serum in the presence of these metals.

EXPERIMENTAL SECTION

General Methods. All reagents and solvents were obtained from Aldrich or Sigma. The stock solutions of 1.0×10^{-3} M **1** and **2** were obtained by dissolving the materials in ethanol. All stock solutions of metal ions were prepared from analytical grade nitrate salts or chloride salts and were dissolved in doubly distilled water. The work solutions of metals were obtained by series diluting the stock solutions with 0.05 M Tris/HCl buffer (pH 7.04, $I = 0.1$ ($NaNO_3$)). Other chemicals were of analytical reagent grade and used without further purification. Proton magnetic resonance spectra were recorded at 400 MHz, and carbon spectra were recorded at 100 MHz on an Invoa-400 (Invoa 400) spectrometer with tetramethylsilane (TMS) as the internal standard. *J* values were given in hertz. Low-resolution mass spectra (MS) were obtained at 50−70 eV by fast atomic bombardment on a Finnigan MAT SSQ-710 mass spectrometer. High-resolution MS were obtained on a Q-Star Pulsar I (Applied Biosystem/PE Sciex). The cold-spray ionization time-of-flight mass spectra (CSI-TOFMS) were acquired using an AccuTOFCS mass spectrometer (JMS-T100CS, Tokyo, Japan). UV−visible absorption spectra were recorded on a Hitachi U-3010 UV/vis spectrophotometer (Kyoto, Japan). Fluorescence emission spectra were recorded on a Hitachi F-4500 fluorescence spectrofluorometer (Kyoto, Japan). Data processing was performed on a Pentium IV computer with Sigmaplot software.

Synthesis. Spirobenzopyran **1** was previously synthesized in our laboratory.[35] Spirobenzopyran **2** was synthesized according to the sequences summarized in Scheme 1. To prepare **2**, hydroxylbenzaldehyde **4** was first allowed to undergo smooth aminomethylation with morpholine and paraformaldehyde affording **5** in 79% yield. Then, in the presence of piperidine, condensation of **5** with *N*-methylated indolenine derivative **6** gave rise to the desired spirobenzopyran. Experimental details and analyses are given below.

Compound ***5*** *[5-tert-Butyl(3-morpholinomethyl)-2-hydroxybenzenaldehyde]*. Under N_2 and at 0 °C, 0.6 mL of morpholine (6.9 mmol) was added into 5.0 mL of glacial acetic acid. The solution was stirred for 15 min. After which, 0.2 g of $(CH_2O)n$ (6.7 mmol) was added at room temperature. 5-*tert*-Butyl-2-hydroxylbenzaldehyde (**4**) (1.19 g, 6.69 mmol) dissolved in glacial acetic acid (3.0 mL) was then injected to the reaction mixture. The solution was

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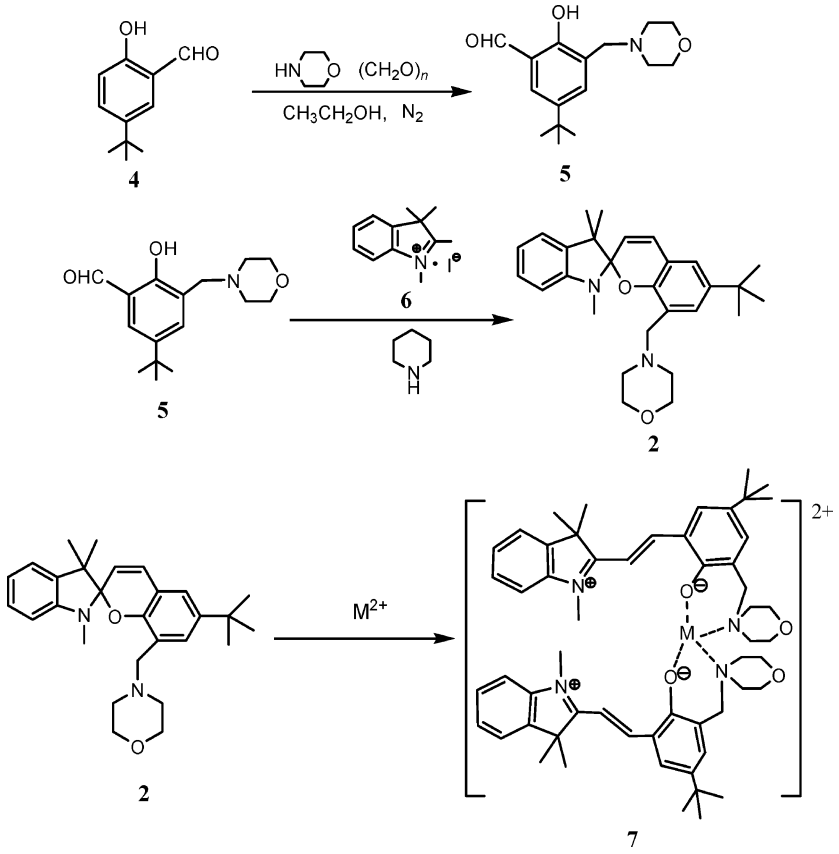
Scheme 1. Scheme for Preparing Spirobenzopyran 2 and Its Metal Complex 7



heated to reflux overnight. The solution was allowed to cool down to room temperature. NaOH (1.0 M, 20.0 mL) was poured into the reaction solution, and the pH was adjusted to slightly basic. The mixture was extracted with CH_2Cl_2 (30.0 mL × 3). The organic layers were combined and dried with Na_2SO_4. The solvent was removed, and the crude product was purified by column chromatography eluted with EA giving **5** as a yellow oil (1.46 g, 79% yield). HRMS: *m/z* calcd. $C_{16}H_{24}NO_3$ $(M + H)^+$: 278.1756, found: 278.1765; ^{1}H NMR (400 MHz, $CDCl_3$): δ1.31 (s, 9H), 2.65 (t, *J*=4.1, 4H), 3.76−3.79 (m, 6H), 7.47 (d, *J*=2.7, 1H), 7.60 (d, *J*=2.7, 1H), 10.02 (s, 1H), 10.18 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 31.32, 34.11, 53.10, 66.74, 121.59, 122.81, 125.60, 133.41, 141.90, 158.55, 192.45.

Spirobenzopyran ***2***. *N*-Methyl-2,3,2-trimethylindolenine **6**[35] (1.87 g, 6.2 mmol) in absolute ethanol (35 mL) was heated to reflux under N_2. Piperidine (0.5 mL, 5.1 mmol) was added. Compound **5** (1.43 g, 5.1 mmol) dissolved in absolute ethanol (10 mL) was then injected to the reaction mixture. The mixture was refluxed for 8 h. The solvent was removed by rotary evaporation. The crude residue was purified by column chromatography by eluting with PE:EA (50:50, v/v) to yield **2** as a pink solid (1.65 g, 73.8% yield). HRMS: *m/z* calcd. $C_{28}H_{37}N_2O_2$ $(M + H)^+$: 433.2855; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.18 (s, 3H), 1.22 (s, 3H), 1.29 (s, 9H), 2.22 (m, 4H), 2.67 (s, 3H), 3.22 (dd, 2H), 3.53−3.56 (m, 4H), 5.69 (d, *J*=8.1, 1H), 6.49 (d, *J*=8.1, 1H), 6.76−6.87 (m, 2H), 6.97 (s, 1H), 7.03−7.15 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.25, 25.72, 29.11, 31.58, 33.99, 51.29, 53.19, 56.55, 67.10, 104.17, 106.62, 117.74, 118.32, 118.80, 121.15, 122.46, 122.79, 127.16, 128.08, 129.98, 136.94, 141.64, 148.14, 150.04.

Spectrophotometric Titrations and Binding Constants. Due to the somewhat solubility of the spirobenzopyrans in water, both the UV and fluorescence titrations were carried out in a 50% ethanol−water (v/v) solution by adding a few μL of a work solution of the metals to 2.0 mL of 1.0×10^{-4} M **1** or **2** with a quartz cell (1.0×1.0 cm^2 cross section). The addition was limited to 100 μL so that the volume change was insignificant. The fluorescence emission spectra were obtained by exciting at the maximal absorption wavelength as determined by UV titrations. The obtained data of the intensity ratio of **2** at 640 and 475 nm were analyzed for apparent association constant, *K*,[36] using the relation that are established with the formation of a 1:2 metal (M^{n+})-to-ligand (L) complex.

$$[M^{n+}] = \frac{1}{2 \cdot K} \cdot \frac{1}{[L]_T} \cdot \frac{1-\alpha}{\alpha^2} \quad (1)$$

$$\frac{1-\alpha}{\alpha} = 2 \cdot \frac{R - R_{min}}{R_{max} - R} \cdot \frac{\Phi_L^{475}}{\Phi_{ML2}^{475}} \quad (2)$$

where $[M^{n+}]$ and [L] denote the free concentration of the metal ion and the ligand **2**, α is the ratio between the free ligand concentration [L] and the initial concentration of the ligand, L_T. *R* is the ratio of the L fluorescence intensity at 650 and 475 nm in the presence of different concentrations of a metal ion, and R_{min}

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and R_{max} are the limiting values of R at zero metal ion concentration and at final (plateau) metal ion concentration, respectively. Φ_L^{475} and Φ_{ML2}^{475} are the quantum yields of the ligand and its metal complex at 475 nm, respectively.

Determination of Fluorescence Quantum Yield (Φ). For measurement of the quantum yields of **1** and **2**, the ethanol solutions of **1** and **2** at pH 7.0 were adjusted to an absorbance of ~0.05. The emission spectra were recorded with the excitation wavelength of 418 nm, and the integrated areas of the fluorescence corrected spectra were measured. The quantum yields were then calculated by comparison with *meso*-tetraphenylporphyrin (TPP) as a reference using the following equation[37]

$$\Phi_F = \frac{I}{I_R}\cdot\frac{A_R}{A}\cdot\left(\frac{n}{n_R}\right)^2\cdot\Phi_R \qquad (3)$$

where Φ_F is the quantum yield of **1** or **2**, I is the integrated area under the fluorescence spectra, A is the absorbance, n is the refractive index of the solvent, and R refers to the reference fluorophore, TPP. $\Phi_R = 0.13$ in cyclohexane was used as the reference quantum yield.[38]

Potentiometric pH Titrations.[39] The potentiometric pH titration experiments were carried out with an ionic strength of 0.1 M $NaNO_3$ at 25 °C. All measurements were performed with an electrode connected to a PHS-3C pH meter (Shanghai, China) at 0.1 mM **2** or 0.1 mM **2** + 0.05 mM Cu^{2+}. The electrode was calibrated using standard buffer solutions, and no corrections were made to pH values determined in the 50% aqueous ethanol (v/v) solution. The FORTRAN program BEST was used to process the potentiometric data and calculate both protonation and stability constants.[40]

Preparation of Human Serum Sample. For Cu^{2+} concentration determination in human serum, the samples were first subjected to the deproteinization process. A 1 + 1 mixture of sulfuric acid and nitric acids was used.[41] Human serum (1.0 mL) was mixed with 1.0 mL of the deproteinizing reagent and heated mildly until the solution vaporized completely, and then the residual solid was dissolved with 1.0 mL of doubly distilled water. After stirring sufficiently, the homogenate was centrifuged (10 min, 100 000 × g). The supernatant was then diluted 5-fold with buffer solution and stored at 4 °C for analytical determination.

The calibration solutions for serum measurement were prepared using a buffer solution, containing the typical coexisting components in human serum. The concentration of each component was chosen to match its normal level reported in human serum,[42] and a small volume of a series of concentration copper ion was added in measure.

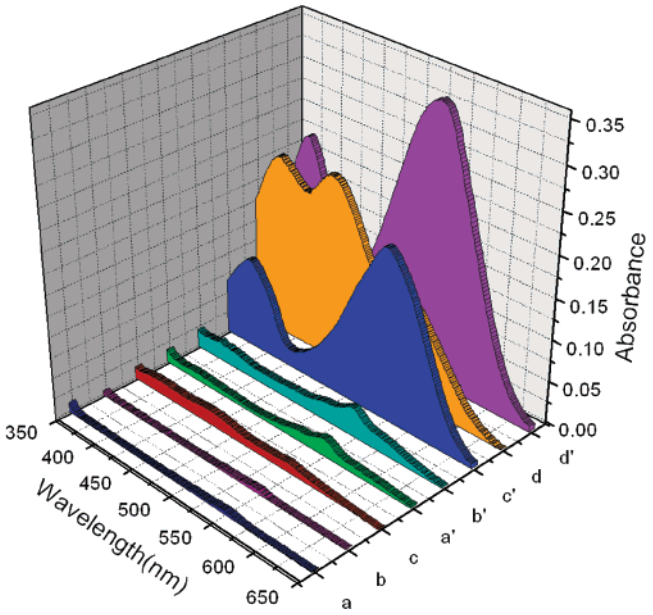

Figure 1. Effects of solvent polarity, ultraviolet light irradiation, and metal complex on the absorption spectra of **1** and **2** (1.0×10^{-4} M, room temperature): (a) absorption of **2** in ethanol, (b) absorption of **2** in ethanol after 30 min of irradiation with ultraviolet light, (c) absorption of **2** in 50% ethanol−water (v/v) solution, (d) c + 1.0×10^{-5} M Cu^{2+}; (a′) absorption of **1** in ethanol, (b′) absorption of **1** in ethanol after 30 min of irradiation with ultraviolet light, (c′) absorption of **1** in 50% ethanol−water (v/v) solution, and (d′) c′ + 1.0×10^{-5} M Cu^{2+}.

RESULTS AND DISCUSSION

Photochromic Behaviors of 1 and 2 in Organic Solvents. The photoreversible property of spirobenzopyran dyes was intensely studied. The closed spiro-form, normally colorless, has an absorption band in the ultraviolet range from 200 to 370 nm. Irradiation in this range initiates photoisomerization, forming the colored merocyanine, which has strong solvatochromic visible absorption bands in the range of 370−600 nm. The position of equilibrium is dependent upon the chemical structure of the spiropyran nucleus and the light exposure conditions as well as the solvent polarity. In general, nonpolar solvents favor the closed colorless form, while polar solvents produce the colored structure. Typical photochromic behaviors of **1** and **2** were studied at room temperature by applying them in the solvents of different polarities. In ethanol, both **1** and **2** (1.0×10^{-4} M) are colorless, and almost no detectable absorption could be observed in the visible region (Figure 1, curves a and a′). Irradiations of the ethanol solutions for 30 min with UV light caused no significant difference of the absorption spectra (curves b and b′), reflecting that both **1** and **2** do not undergo photoisomerization in the solvent. Similar results were obtained by employing them in other organic solvents of different polarities. These observations are distinctly different from the photochromic behaviors of the common 6-nitro substituted spirobenzopyrans, which could be contributed to the electronic and steric effects of the substituent. A strong electron-withdrawing group in the 6-position favors more stable mero-form by dispersing the electronic intensity of the phenolate oxygen, while

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(38) (a) Seybold, P. G.; Gouterman, M. *J. Mol. Spectrosc.* **1969**, *31*, 1−13. (b) Quimby, D. J.; Longo, F. R. *J. Am. Chem. Soc.* **1975**, *97*, 5111−5117.
(39) Georgieva, M.; Velinov, G.; Budevsky, O. *Anal. Chim. Acta* **1977**, *90*, 83−89.
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an electron-donating group such as an alkylated group in the position leads to a more photostationary closed state.[10b,43,44]

Specifically, in ethanol aqueous solution, different photochromic behaviors were observed from **1** and **2**. As the content of water in ethanol was increased, the colorless of **1** changed into red concomitant with two absorption peaks at 540 and 396 nm, respectively. In the 50% ethanol−water solution, **1** has an intense red-violet color with a strong absorption in the visible range (ϵ^{540} nm = 3.11 × 10^3 cm^{-1} M^{-1}), indicative of the formation of the ring-opening component. But **2** remains colorless in the ethanol−water with negligible absorption at the wavelength (ϵ^{540} nm = 235 M^{-1} cm^{-1}), suggesting the nearly complete absence of merocyanine component. We reasoned that the presence of morpholine moiety in **2** is crucial in blocking thermal isomerization to the planar merocyanine structure. The differences of the pK_a between the dimethylamine and morpholine moieties probably make **1** more favorable to form its protonated merocyanine than **2**. This relatively higher photostationary of **2** in water over **1** is advantageous by applying **2** as a metal ion probe in aqueous solution media (via infra).

Metal-Induced Photochromisms of 1 and 2. Another extremely important factor for changing the equilibrium of spiro- and mero-form is metal chelation modulation. The complexation of a metal center with the opened merocyanine is favorable for conversion of spiro-form into mero-form. To evaluate the capability of a metal ion that induces ring opening, the effects of Cu^{2+} on the absorption spectra of **1** and **2** were first studied in a 50% ethanol−water (v/v) solution at pH 7.04. Curves d and d′ in Figure 1 correspond to the absorption spectra of **1** and **2** in the presence of 2.0 × 10^{-5} M Cu^{2+}. Both **1** and **2** exhibit strong absorptions in the visible region (ϵ^{540} nm = 1.64 × 10^4 cm^{-1} M^{-1} for **1** and ϵ^{453} nm = 4.41 × 10^3 cm^{-1} M^{-1} for **2**), respectively, reflecting complete formation of the opened merocyanine forms. However, compared to their free states in the ethanol aqueous solution, the absorption enhancement of **2** by Cu^{2+} is obviously larger than that of **1**. This Cu^{2+} coordination induced photochromic property of **2** constitutes the basis for colorimetric detection of the metal in aqueous solution.

The formation of the photomerocyanine forms of **1** and **2** are significantly dependent on the nature of the metal center. The binding abilities of **2** to various metal ions were studied by visual examination of the metal-induced color and the UV−visible absorption spectral changes in the ethanol−water solution. The nitrate salts of Mg^{2+}, Cd^{2+}, Co^{2+}, Hg^{2+}, Zn^{2+}, Ni^{2+}, Pb^{2+}, and Cu^{2+} ions were used. All titrations studies were conducted using a 1.0 × 10^{-4} M of the ligand. Figure 2 shows the photographs of **2** upon additions of 0.5 equiv of the metal ion, respectively. The ethanol−water solution of free **2** is colorless. No color change was observed upon addition of Cd^{2+}, Co^{2+}, Hg^{2+}, Zn^{2+}, Ni^{2+}, or Pb^{2+}, separately, to the ethanol−water solution. However, upon addition of Cu^{2+}, the solution changed evidently into red color. The photocolorability of the photochromes toward various metal ions can be defined as the changes in long wavelength absorption

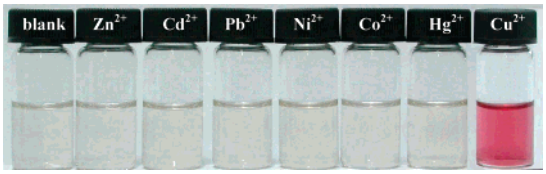

Figure 2. Photographs of solutions of **2** (1.0 × 10^{-4} M) and its metal complexes in 50% ethanol−water. The quantity of all metals used here is 5.0 × 10^{-5} M.

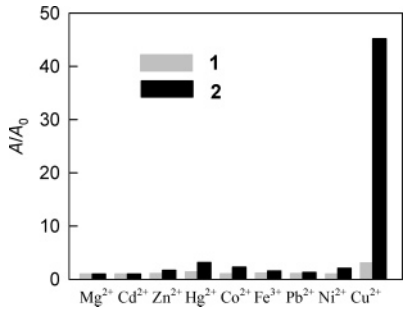

Figure 3. The absorbance change, A/A_0, of **1** (gray, 540 nm) and **2** (black, 453 nm) upon addition of 5.0 × 10^{-5} M metal ions (*x*-axis markers), separately.

($(A-A_0)$ or A/A_0).[45] Here A_0 and A are the absorbance of the photochrome in the absence of and the presence of a cation. Figure 3 shows the dependence of the absorbance ratio, A/A_0, of **2** at 453 nm, on 5.0 × 10^{-5} M metal ions. The absorbance of **2** at 453 nm is increased to 45.6-fold that of the original value by 5.0 × 10^{-5} M Cu^{2+}, but it is only 1.84-fold by Zn^{2+} and 3.21-fold by Hg^{2+}, respectively. The particularly high thermodynamic affinity of Cu^{2+} for the *N*,*O*-chelate ligand and the fast metal-to-ligand binding kinetics result in a remarkably specific complexation of **2** toward Cu^{2+} over other heavy and transition-metal ions, which makes this probe appropriate for selectively colorimetric sensing of Cu^{2+}.

Metal ions bindings of **1** with Cu^{2+} as well as other heavy metal ions were also investigated. In a 50% ethanol−water solution **1** is red-violet, and no significant color change could be observed upon the addition of any metal ions (data not shown). The absorption spectrum of **1** in the ethanol−water solution displays two obvious absorptions at 396 and 540 nm. Although **1** shows a maximal response toward Cu^{2+},[46] the enhancements in absorbance at the two wavelengths are smaller than that of **2** by Cu^{2+} (Figure 3). The absorbance of **1** at 540 nm is increased to 3.07-fold by 5.0 × 10^{-5} M Cu^{2+}, which is significantly lower than the 45.6-fold of A/A_0 observed from **2**. Regarding the response sensitivity (response slope), we could suggest that **2** is a better ligand for Cu^{2+} than **1**.

UV−visible spectroscopy was followed as aliquots of Cu^{2+} were added to the ethanol−water solutions of **2**. Upon a gradual increase in the Cu^{2+} concentration, dramatic increases in the absorbance at both 453 and 384 nm were evident with increasing the concentration of Cu^{2+} up to its concentration approximately to 0.5 equiv relative to the host concentration; further increasing the Cu^{2+} concentration, the two peaks changed into one absorption peak at 418 nm concomitant with the intensity increasing.[46]

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(46) See the Supporting Information.

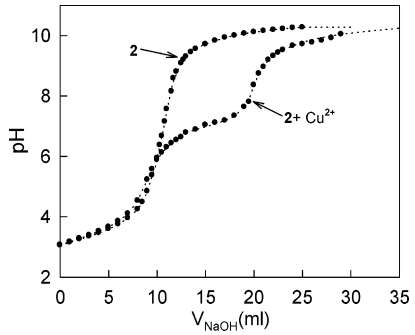
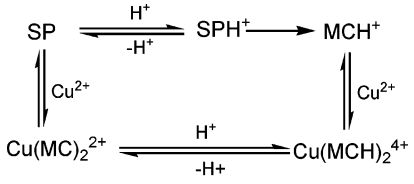


Figure 4. Potentiometric equilibrium curves (----) of 0.1 mM **2** in the absence and presence of 0.05 mM of Cu^{2+} at 25 °C (• is the experimental data).

Scheme 2. Equilibrium Models of the Protonations of 2 and Its Metal Complexes



SP: spiropyran form of **2**
MC: merocyanine form of **2**

Binding analysis using the curve fitting establishes a 1:2 complex between Cu^{2+} and the ligand.[47]

Potentiometric pH Titrations The conversion of spiro-form to merocyanine in the absence and the presence of a metal ion is usually affected by the hydrogen ion present in the solution. The influence of pH on the spectroscopic property of **2** in the absence and the presence of Cu^{2+} was thus determined via potentiometry using commercially available glass electrodes. The accuracy of potentiometry is often superior to spectrophotometry, and, in most systems, it provides the most reliable stability constant.[39] The pH titrations of **2** and **2** in the presence of Cu^{2+} are shown in Figure 4. Typically, a solution containing 0.1 mM of **2** and 1.0 mM of HNO_3 was titrated with 1.0 mM NaOH at constant ionic strength (0.1 M $NaNO_3$) at 25 °C for the determination of the pK_a value. In the second run, the same titration was performed in the presence of 0.5 equiv of Cu^{2+}. The measured data were processed with the program BEST[40] using the equilibrium models as shown in Scheme 2, providing the corresponding protonation and complex formation constants. The protonations of the phenolate oxygen of **2** occurs at a neutral or a slightly acidic condition with a pK_a of 6.57 ± 0.09. However, the initially measured pH indicated no significant protonation of the morpholine nitrogen, the first pK is thus below 2, and it was therefore not considered in the equilibrium model for analysis. As Cu^{2+} begins to coordinate to the ligand, we would expect a shift in the pK_a value. Indeed, this expectation is observed with the titration curve shifting to the right by two proton equivalents, which is reasonable because the ligation of the metal center with **2** would eject a proton into the solution. Based on curve fitting, the corresponding complex formation constant of **2** with Cu^{2+} was 1.52×10^4 M^{-2}. In the present work, a final pH 7.04 was chosen as an ideal experimental condition.

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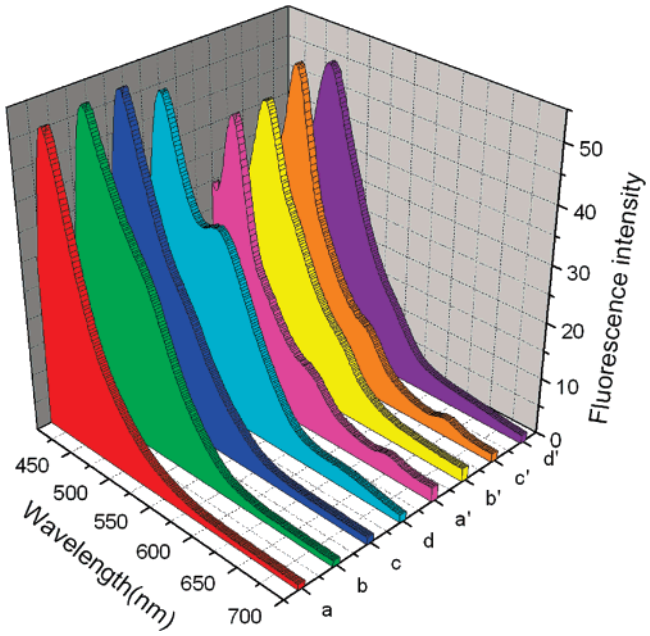

Figure 5. Fluorescence emission spectra of **1** and **2** in different solvents: (a) **1** in toluene (λ_{ex} = 377 nm); (b) **1** in CH_3CN (λ_{ex} = 406 nm); (c) **1** in ethanol (λ_{ex} = 404 nm); (d) **1** in 50% ethanol−water (λ_{ex} = 402 nm); (a′) **2** in toluene (λ_{ex} = 418 nm); (b′) **2** in CH_3CN (λ_{ex} = 418 nm); (c′) **2** in ethanol (λ_{ex} = 418 nm); and (d′), **2** in 50% ethanol−water (λ_{ex} = 418 nm). The concentrations of **1** and **2** were 2.0×10^{-4} M, respectively.

Fluorescence Responses of 1 and 2 toward Cu^{2+}. Neither the spiro- nor the mero-form of the 6-nitro-substituent spirobenzopyran dyes fluoresces appreciably in solution, although the mero-forms possess detectable fluorescence within self-assembling films[16a] or the hydrophobic core of polymer nanoparticles.[16b,c] In our studies, however, we observed that both the spiro-forms of **1** and **2** are fluorescent (Figure 5) in various solvents, which display maximal emission bands in the region of 450−550 nm. The quantum yields of **1** and **2** in ethanol were estimated to be Φ_{F1} = 0.12 and Φ_{F2} = 0.15, respectively. No fluorescence in the red region of 600−700 nm could be observed in the solution, a result that is consistent with the predominance of the spiro-forms in the absence of exogenous metal ion.

1 and **2** show quite different fluorescence response characteristics toward Cu^{2+}. Addition of an increasing concentration of the metal ion to the ethanol−water solution of **1** results in a decrease in the overall emission intensity.[46] Figure 6 shows the fluorescence response curves of **2** toward different concentrations of Cu^{2+}. The responses were recorded at room temperature within 5 min by exciting at 418 nm. Upon addition of Cu^{2+} to the ethanol−water solution of **2**, the fluorescence intensity at 475 nm was decreased with the concomitant formation of a red-shifted, broad emission band center at 640 nm through a well-defined iso-emission point at 578 nm. The fluorescence intensity of the 640-nm band was increased with Cu^{2+} concentration. When Cu^{2+} concentration was ca. 0.05 mM, the emission maximum shifted to 640 nm.

Fluorescence quenching of **1** and **2** by Cu^{2+} is contributed to the proximity of the metal to the unpaired electrons of the ligand which leads to spin−orbit coupling and intersystem crossing.[26,27] We suggest that the new long wavelength emission enhancement

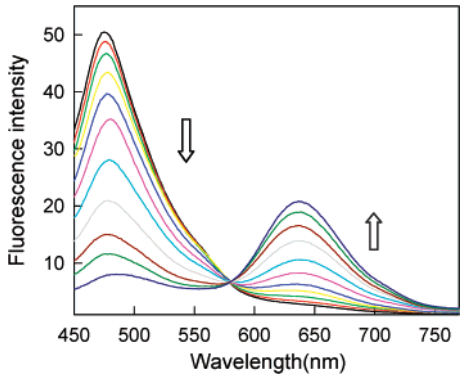

Figure 6. Changes in fluorescence emission spectra (λ_{ex} = 418 nm) of 2.0 × 10^{-4} M **2** upon additions of different concentrations of Cu^{2+}. The arrows indicate the signal changes as increasing in the Cu^{2+}concentrations (0, 0.2, 0.5, 1.0, 2.0, 5.0, 10, 20, 50, 100, and 200 μM).

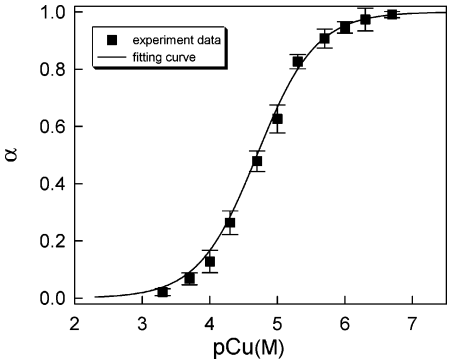

Figure 7. Response parameter value (α) as a function of the negative logarithm of the Cu^{2+} concentration. Fluorescence intensity was recorded at 640 and 475 nm with an excitation wavelength of 418 nm.

of **2** at 640 nm is due not only to an increase in the concentration of the opened form of **2** but also to Cu^{2+} binding to **2** which could induce a conformation restriction and subsequently change the intramolecular charge transfer (ICT) from the phenolate oxygen to the electron-deficient quaternary nitrogen center.[48] The reduction in ICT (effectively unquenching) affords a dramatic enhanced and wavelength shifted fluorescence as a function of the Cu^{2+} concentration. In supporting this assignment, it was found that the spirobenzopyran **1**, which contains a rotationally labile dimethylamino group in the 8-position, showed only fluorescence quenching of the spiro-form but no new emission band at long wavelength when fully complexed with Cu^{2+}.[46]

The dual fluorescence of **2** modulated by Cu^{2+} allows the Cu^{2+} concentration to be determined from the intensity ratio of the two wavelengths. The fluorescence emission ratios of **2** at 640 nm to that 475 nm, I_{640}/I_{475}, increases with Cu^{2+} concentration. A maximal emission ratio was obtained after addition of ~0.2 mM Cu^{2+} ion to the solution. In Figure 7, the relative fluorescence intensity ratio value, α, defined as the ratio between the free **2** concentration and the initial concentration of the ligand, is given as a function of pCu, where pCu is the negative logarithm of Cu^{2+} concentration.[36] The curve fitting for the experimental data points

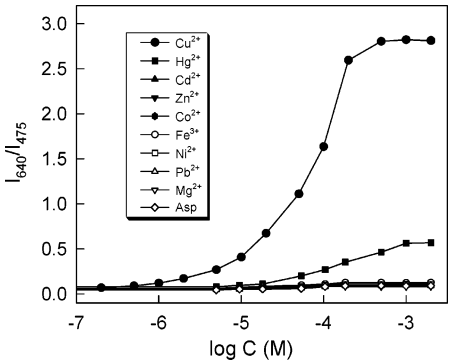

Figure 8. Changes in the fluorescence intensity of **2** at 640 nm to that at 475 nm in the 50% ethanol (v/v) aqueous solution upon addition of different concentrations of metal ions and amino acid, respectively.

was calculated from eqs 1 and 2 with log K = 4.09, which is favorably comparable with that obtained by the pH titration at a fixed Cu^{2+} concentration. The good correlation of the measured data with the theoretical prediction confirms the validity of the proposed method. The dynamic response range covers from 5.13 × 10^{-4} to 3.81 × 10^{-7} M Cu^{2+} ($0.05 \le \alpha \le 0.95$),[49] which is larger than the common Cu^{2+} fluorescent probes based on intensity measuring at one wavelength. The detection limit is 1.06 × 10^{-7} M (α=0.99). Moreover, with a probe concentration of 5.0 × 10^{-5} M, Cu^{2+} can be detected upon to the ppb concentration range.

Selectivity. Probably the most important characteristic feature of a sensor is its response to the species to be measured over that to other relative species present in solution. Since the potential application of the present approach is for the analysis of Cu^{2+} concentration of bioavailability and environment-availability, the selectivity toward other biologically and environmentally relative substrates is particularly important. To define fluorescence responses of **2** toward Cu^{2+} as well as other analytes, complexation experiments with a range of other biologically related substrates were performed at the same conditions. Interaction of **2** with other metal ions, inorganic anions, amino acids, and BSA, separately, each led to no significant changes of the fluorescence intensity ratios at 475 and 640 nm, as shown in Figure 8. Although **2** also responds to Hg^{2+} as well as at a higher concentration of 2.0 × 10^{-5} M, the value of I_{640}/I_{475}, is substantially smaller than that caused by Cu^{2+}. The association constant of **2** with Hg^{2+} was determined to be 2.45 × 10^2 M^{-2}, which is ca. 49-fold smaller than that of **2** with Cu^{2+}. **2** also shows very weak binding toward Co^{2+} (K=107 M^{-2}), whereas the binding constants of other metal ions with **2** could not be determined because the corresponding change in fluorescence emission at the two wavelengths were too small. The metal selectivity factor of Cu^{2+} to Hg^{2+}, which was evaluated by comparing the association constant and the fluorescence signal change at 475 and 640 nm ($K \cdot R$),[50] is ca. 294. Here K is the association constant and R is the fluorescence intensity ratio ($R = I_{640}/I_{475}$) of **2** in the presence of 2.0 × 10^{-4} M of metal ions. The selectivity factors of **2** for the metal ions studied are in the order $Cu^{2+} \gg Hg^{2+} > Co^{2+} > Zn^{2+} \approx Cd^{2+} \approx Pb^{2+} \approx Mn^{2+} \approx Ni^{2+} \approx Mg^{2+}$ (Table 1). To further characterize the binding specificity of **2** for Cu^{2+}, the competitive complexes were also

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Table 1. Fluorescence Signal Changes at 475 and 640 nm, I_{640}/I_{475}, Association Constants, K, and Response Selectivity of 2 to Metal Ions[a]

species	$R(=I_{640}/I_{475})$	K (M^{-2})	response selectivity[b]
2	0.073		
2+ Cu^{2+}	2.638	1.21×10^4	1
2+ Mg^{2+}	0.088	*c*	*c*
2+ Cd^{2+}	0.131	*c*	*c*
2+ Zn^{2+}	0.162	*c*	*c*
2+ Hg^{2+}	0.451	245	3.4×10^{-3}
2+ Co^{2+}	0.230	107	9.1×10^{-4}
2+ Pb^{2+}	0.122	*c*	*c*
2+ Mn^{2+}	0.173	*c*	*c*
2+ Ni^{2+}	0.147	*c*	*c*

[a] The concentration of the metal ion in each case was 2.0×10^{-4} M. [b] Response selectivity = $(K_i \cdot R_i)/(K_{Cu} \cdot R_{Cu})$. [c] The association constant could not be obtained since the fluorescence intensity changes of **2** at 475 and 640 nm are too small in the presence of the metal ion.

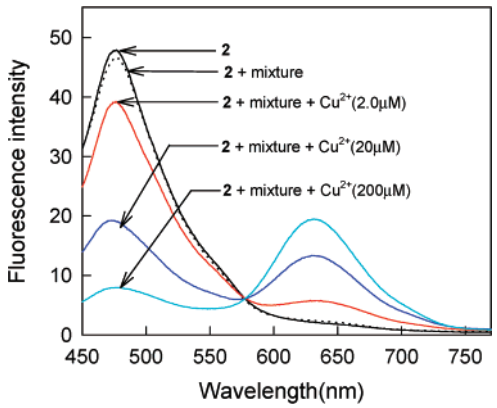

Figure 9. Fluorescence emission spectra of the ethanol−water solutions containing 2.0×10^{-4} M of **2**, **2** + mixture (see text), and **2** + mixture + appropriate concentrations of $CuCl_2$. $\lambda_{ex} = 418$ nm.

conducted in the presence of biologically related substrates. The addition of a mixture of metal ions (Li^+, K^+, Ca^{2+}, and Mg^{2+} (each 0.1 M) and Hg^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, Fe^{3+}, and Pb^{2+} (each 50 μM)), amino acids (glycine, histidine, cysteine, glutamic acid, and aspartic acid (each 50 μM)), and BSA (0.1 mg/mL) did not alter the shape or the intensity ratio of the fluorescence spectra (Figure 9). In contrast, the fluorescence emission spectrum was sensitive to the presence of Cu^{2+} ions, which gives a curve almost superimposable to the one obtained exclusively in the presence of Cu^{2+}. These corroborated mentioned results clearly indicate that **2** not only is insensitive to other targets but also is selective toward Cu^{2+} in their presence, which is important and helpful in validation of the method to meet the selectivity requirements of a Cu^{2+} assay in a physiological field.

Cu^{2+} Binding Reversibility and Response Time. For a practical working sensor to be employed in the detection of specific analytes, the limit of quantification, selectivity, reversibility, and response time are all important aspects. The sensor **2** presented has demonstrated to be sensitive and selective for the detection of Cu^{2+} as the experimental data show. Consequently, it is of significant interest to investigate the reversibility, the response time, and the lifetime of the system. In light of decomposition of the metal complex by visible light, the real-time records of metalation degree of **2** with Cu^{2+} were carried out using

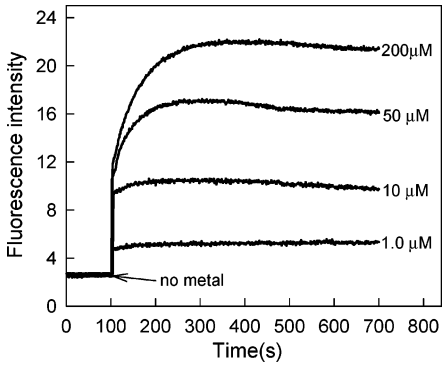

Figure 10. Kinetics of the interaction of **2** (2.0×10^{-4} M) with different concentrations of Cu^{2+}. Fluorescence intensity was recorded at 640 nm in 50% ethanol−water solution. $\lambda_{ex} = 418$ nm.

the 640-nm band fluorescence emission as functions of time (Figure 10). The formation of the metal complex proceeds rapidly at first, followed by a more gradual increase in the emission intensity. On the other hand, the response time depends on the change of the concentration of Cu^{2+}, as the time required to be reached equilibrium increases with the Cu^{2+} concentrations. The stable reading was obtained with 2−6 min. Shaking the cell can shorten the response by ca. 1.5 min. From the fluorescence intensity changes with time as shown in Figure 10, one could also realize that the Cu^{2+}−ligand complex is reasonably stable; irradiation of the metal complex with visible light did not change the emission intensity at 640 nm. Further, we observed that the colored solution of the copper complex was thermally stable at room temperature within four weeks in the presence of the nature light.

We subsequently studied the chemical reversibility behavior of the metal binding of **2** in the ethanol−water solution. Because of the high stability constant of the EDTA−Cu^{2+} complex, it was anticipated that addition of EDTA will sequester Cu^{2+} of the metal complex, liberating the closed **2**. With this intention, 1 equiv of EDTA solution was added to the Cu^{2+} complex of **2**, which visibly turned the intense red color of the solution into the initial colorless. The chemically regenerated **2** can then be recycled for another metal binding event. These results demonstrate that the metal binding of **2** is chemically reversible; it does not establish the facile photochemical reversibility that was characteristic of the 6-nitro substituent spirobenzopyrans. This feature would facilitate the optical detection, for example, the Cu^{2+} measurement could be performed without any light-black protection.

Cu^{2+} Assay in Human Serum. Although a number of Cu^{2+} fluorescent probes have been reported in the literature,[26−34] it is difficult to find a probe that could be applied for effective determination of the Cu^{2+} concentration in a real biological sample[29c] due to fluorescence quenching by other structural related heavy- or transition-metal ions in the sample. The extremely specific fluorescence response of **2** to Cu^{2+} over biologically relevant substrates meets the selectivity requirements for biomedical application. As an example, the quantitative determination of Cu^{2+} concentration in human serum was carried out by using the proposed method. The calibration curve was obtained by using a solution containing different concentrations of Cu^{2+}, along with the typical coexisting components in human serum.[42] The fluorescence responses of the samples were compared to the

Table 2. Results of Determination of Cu^{2+} in Human Serum and Recovery Tests by the Present Method and ICP-AAS

Cu^{2+} added (μM)	method	Cu^{2+} found (μM)	recovery (%)
sample **1**+ 0	present method	23.6 ± 0.48[a]	
	ICP-AAS	25.7 ± 0.69[b]	
sample **2** + 0	present method	22.4 ± 0.56	
	ICP-AAS	22.6 ± 0.58	
sample **3** + 0	present method	26.1 ± 1.26	
	ICP-AAS	24.5 ± 0.73	
sample **1**+ 10	present method	34.8 ± 2.16	112
sample **1**+ 20	present method	46.8 ± 2.71	101
sample **1**+ 50	present method	73.5 ± 8.63	99.7

[a] Average of three determinations. [b] The data were provided by the "Analytical Measuring Center of Peking University".

calibration curve. As shown in Table 2, the determined mean values of Cu^{2+} in the human serum sample correspond very well to the values that are obtained by ICP-AAS and fell in the normal range of the content reported in the literature.[41,51] Further, the recoveries of the method were in the range of 99.7%−112.3%, as given in Table 2, showing that the proposed probe can be satisfactorily applied to the quantitative determination of Cu^{2+} in human serum.

Study on the Interaction Mechanism. The interactions of **2** with metal ions were further studied by ^{1}H NMR spectroscopy in ethanol-d_6 to provide some insights into the interaction mechanism. The ^{1}H NMR spectra of **2** were scarcely affected upon addition of 0.3−1.2 equiv of $ZnCl_2$ to the ethanol-d_6 solution at room temperature, and this ^{1}H NMR spectra combined with the UV−vis absorption and fluorescence emission spectra[46] indicate there is no metal complex formation between Zn^{2+} and **2**. In contrast, addition of $CuCl_2$ to the ethanol-d_6 solution of **2** caused disappearances of proton signals at 3.21 ppm (C_9 protons), 2.20 ppm (methylene protons of morpholine moiety near nitrogen atom), and 3.51 ppm (methylene protons of morpholine moiety near oxygen atom), revealing formation of Cu^{2+} complex. Cu^{2+} is paramagnetic and almost universally causes peaks to disappear in ^{1}H NMR spectra upon complex formation. In addition, the most pronounced downfield shifts of the vinyl protons at 5.72 and 6.48 ppm were observed after the addition of $CuCl_2$. As the protons on vinyl are very sensitive indicators of ring opening,[13a] we surmise that Cu^{2+} interacts with the mero-form of **2**. The substantially downfield shifts of the benzyl protons can also be observed which are ascribed to ring current effects,[52] suggesting an aggregation of the host molecule. The result primarily indicates that the metal interaction occurs through the phenolate hydroxyl group and the nitrogen atom of the morpholine moiety.

To further gain insight into the role of the phenolate oxygen and the nitrogen atom in Cu^{2+} binding, we examined a reference compound, *2*-dimethylaminomethylphenol, **3**, by observing the changes in the UV−visible spectra upon increasing the concentration of Cu^{2+}. Under comparable conditions, **3** exhibits an absorption maximum at 274 nm. Addition of Cu^{2+} to the ethanol−water solution caused an obvious increase in the absorbance value at the wavelength as those obtained by **2**.[46] The result clearly indicates that phenolate hydroxyl group and the morpholine nitrogen are reasonable in the complex formation. Based on the corroborative mentioned results, it is concluded that the fluorescence response of **2** toward Cu^{2+} results from the formation of 2:1 complex with a metal ion. The structure broad band observed at 640 nm can be assigned to the emission of the intramolecular charge transfer (ICT) in the self-assembly of **2**, where binding of the metal center occurs through the phenolate hydroxyl group and the morpholine nitrogen as schematically illustrated in Scheme 1.

CONCLUSION

A new colorimetric and fluorescent ratioing probe for Cu^{2+} in an aqueous solution has been developed based on the metal-coordination tunable photochromism of a spirobenzopyran. The recognition of Cu^{2+} gave rise to a major color change that was clearly visible to the naked eye, while the Cu^{2+} quantification could be achieved by fluorescent ratiometry. The metal recognition and transduction mechanism is one based on the metal-induced conformation isomerization of the ligand which leads to different photophysical properties. The design strategy and remarkable photophysical properties of **2** would help to extend the development of highly selective spirobenzopyran-based fluorescent sensing approaches for other analytes of interest. Further work will be aimed at the application of the probe in living cells to monitor the biological targets by fluorescence microscopy, and the results will be reported in due course.

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SUPPORTING INFORMATION AVAILABLE

Absorption and fluorescence response curves of **1** or **2** to metal ions, ^{1}H NMR, ^{13}C NMR and MS of **2**, and the ^{1}H NMR of **2** with the addition of metal ion. This material is available free of charge via the Internet at http://pubs.acs.org.

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