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Copper-Induced Fluorescence Quenching in a Bis[2-(2′-hydroxyphenyl)benzoxazole]pyridinium Derivative for Quantification of Cu²⁺ in Solution

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Abstract: Accurate determination of Cu²⁺ in solution is crucial for preventing several disease conditions. Spectroscopy-based techniques for metal ion detection are promising methods due to their excellent sensitivity and rapid response time. In this work, we are reporting a newly synthesized 2-(2′-Hydroxyphenyl) benzoxazole-based compound, probe 2, by incorporating a vinyl pyridinium segment into the bis(HBO) 4 system. Probe 2 exhibited excellent specificity toward Cu²⁺ in solution. The ratiometric absorbance (λ₄40/λ₃70) and the quenching of fluorescence at λₑm ≈ 585 nm exhibited an excellent linear correlation. The formation of the 2-Cu complex can be utilized as a highly sensitive spectroscopic method for the detection of Cu²⁺ in solution with a detection limit of 0.15 μM. In addition, Cu²⁺-induced fluorescence quenching in probe 2 occurs mainly via a static quenching mechanism by forming a 2-Cu complex, and the stability constant for the 2-Cu complex was calculated based on spectroscopic measurements.

Keywords: fluorescent dyes; 2-(2′-hydroxyphenyl)benzoxazole (HBO); excited-state intramolecular proton transfer (ESIPT); fluorescence quenching; copper ion

1. Introduction

Small-molecule organic fluorescent dyes have been widely used for various chemical sensing applications including metal ions, anions, pH, temperature, viscosity, and biomolecules [1–11]. An ideal fluorescent chemical sensor should exhibit high sensitivity and selectivity toward the analyte of interest while producing a discernible optical response upon binding of it. The optical response of fluorescent chemical sensors to an analyte binding event will be either an enhancement, quenching, or a shift in its fluorescence emission. 2-(2′-Hydroxyphenyl)benzoxazole (HBO) 1 is a unique molecular skeleton for developing fluorescent chemical sensors due to its ability to undergo excited-state intramolecular proton transfer (ESIPT), which enables HBO derivatives to produce emissions at dual wavelengths and produce a large Stokes’ shift due to enol and keto tautomerization (Scheme 1) [12,13]. In addition, the large Stokes’ shift (i.e., Δλ > 100 nm) observed in HBO structure can reduce the self-absorption of emitted fluorescence and thereby improve probe’s sensitivity. The rational designing of an efficient ESIPT-based chemical sensor is achieved by controlling the dual emissive keto/enol tautomer ratio in solution. Binding of metal ions to simple HBO models such as 1, would produce the metal complex (i.e., 1-M in Scheme 1) that will give only one emission (λₘₐₓ ≈ 376 nm, λₑₘ ≈ 443 nm) as ESIPT is no longer possible [1]. In order to enable ESIPT emission in the HBO-metal complexes, we have introduced several bis(HBO) compounds that have two HBO fragments (i.e., 4) [14–17]. In this molecular design, one HBO subunit is used for metal ion binding, while the other HBO subunit is preserved for ESIPT to give keto emission. Inspired by this principle, we have
developed an interesting bis(HBO)-Cyanine hybrid system 5 (Scheme 1) by incorporating a vinyl pyridinium segment to the bis(HBO) 4 skeleton to extend the conjugation [18]. Probe 5 exhibited an excellent selectivity to fluoride anion (F⁻) and exhibited a large spectral shift (i.e., λ_em ≈ 600 nm to 720 nm) upon F⁻ binding [18].

Scheme 1. The designing and Synthesis of HBO-based metal ion sensors.

Based on efficient anion sensing ability of probe 5, we hypothesized that this new bis(HBO)-Cyanine hybrid model will also be useful for cation sensing via metal ion binding similar to 1-M (Scheme 1). In this work we have synthesized a novel HBO-Cyanine hybrid 2 by introducing a phenyl substituent into the alkyl chain (R = Ph in Scheme 1) of the vinyl pyridinium group to evaluate its cation sensing ability (Scheme 1). Interestingly, probe 2 exhibited an excellent specificity toward Cu²⁺ ions in solution by quenching probe’s fluorescence emission at 585 nm upon introduction of Cu²⁺ ions to the medium: The accurate detection of Cu²⁺ content in an aqueous environment has biological importance as several disease conditions are triggered by high Cu²⁺ content such as Wilson’s disease, Hemolytic anemia, and genetic defects [19]. Therefore, the development of spectroscopy-based detection methods will be advantageous due to their high sensitivity and rapid response time. To date, many fluorescence-based detection methods have been developed for Cu²⁺ detection with calculated detection limits (LOD) ranging from 0.02 to 10 µM range, which clearly indicates the reliability of the fluorescence-based methods for quantification purposes [20–30]. In this study, we have developed spectroscopy-based methods (i.e., absorbance and emission) to analyze Cu²⁺ content in aqueous environments by analyzing quenching of fluorescence (λ_em ≈ 585 nm) as well as the ratiometric absorbance (λ_440/λ_370) response. Due to exhibited high specificity toward Cu²⁺, probe 2 will be a promising candidate toward accurate detection of Cu²⁺ in solution. In this article, we discuss in detail on the design, spectroscopic properties and the application of probe 2 toward the sensitive detection of Cu²⁺ in solution via graphical methods.

2. Materials and Methods

All chemicals for synthesis and spectroscopic studies were purchased from VWR International (Radnor, PA, USA) and Sigma-Aldrich (St. Louis, MO, USA) and used as received. NMR characterization data were acquired by a Bruker 500 MHz NMR spec-
trometer (Billerica, MA, USA). High-resolution mass spectrometry data were acquired by an ESI-TOF MS system (Waters, Milford, MA, USA). UV-vis studies were carried out in a GENESYS 10S UV-Visible spectrophotometer (Thermo Scientific, Waltham, MA, USA) at 25 °C. Fluorescence studies were carried out in a HITACHI F7000 fluorescence spectrophotometer. All spectroscopic work was conducted in spectroscopic-grade chemicals. Compounds 3 and 4 were synthesized according to the previously reported methodology without further modifications \cite{14,15,18}.

2.1. Synthesis of (E)-4-(3,5-Bis(benzo[d]oxazol-2-yl)-2,6-dihydroxystyryl)-1-benzylpyridin-1-i um Iodide (2)

In a 10 mL round bottom flask, 25 mg (0.067 mmol) of the aldehyde 2 was dissolved in 2 mL of ethanol, and 17 mg (0.064 mmol) of the 1-benzyl-4-methylpyridin-1-i um bromide salt was added at room temperature, and the solution was stirred for 5 min. Following the addition of pyridine (0.25 mL), the resulting yellow color solution mixture was heated to 70 °C for 12 h with vigorous stirring. The progress of the reaction was monitored using TLC by monitoring the disappearance of the aldehyde 2. Upon completion, the bright yellow color reaction mixture was cooled down to room temperature, and ethyl acetate (15 mL) was added. Upon the addition of ethyl acetate, a yellow-colored solid product started to precipitate out from the solution. This solution mixture was stirred vigorously at room temperature was 10 min and allowed to settle for another 10 min. Then the solution mixture was filtered by vacuum filtration, and the resulting dark yellow solid product was further washed with hot ethyl acetate (50 °C) (3 × 50 mL). The final product 2 (25 mg; 0.04 mmol) was collected on the Buchner funnel (60% yield).

\[ \text{CH}_3\text{NMR (500 MHz, DMSO-d}_6\text{) } \delta 13.00 (s, 2H), 8.90 (d, J = 6.0 Hz, 2H), 8.34 (s, 1H), 7.93 (d, J = 6.2 Hz, 2H), 7.80 (d, J = 16.3 Hz, 1H), 7.72–7.66 (m, 3H), 7.63 (d, J = 7.6 Hz, 2H), 7.54 (p, J = 7.5, 7.1 Hz, 4H), 7.29 (d, J = 8.8 Hz, 1H), 7.18 (dt, J = 21.6, 7.5 Hz, 4H), 5.74 (s, 2H). \]

\[ \text{C}_3\text{H}_2\text{N}_3\text{O}_4 \text{+}. \] HRMS calculated found (m/z) for [M+] 538.1761 \([C_{34}H_{24}N_3O_4]^+.\) The melting point was found at 338.6 °C.

2.2. Fluorescence Quantum Yield Calculation

The relative fluorescence quantum yields (\(\varphi_{fl}\)) for probe 2 were calculated by using Quinine Sulphate as the standard, where the fluorescence quantum yield of Quinine Sulphate is 0.54 in 0.1 M H_2SO_4. The following equation was used for fluorescence quantum yield determination of probe 2 at 370 nm.

\[ (\varphi_{fl})_{\text{sample}} = \varphi_{\text{ref}} \times \left( \frac{A_{\text{ref}}}{A_{\text{sample}}} \right) \times \left[ \frac{I_{\text{sample}}}{I_{\text{ref}}} \right] \times \left( \frac{n_{\text{sample}}}{n_{\text{ref}}} \right)^2 \]

where A is the absorbance of the sample, I is the integrated fluorescence intensity, and \(n\) is the refractive index of the solvent.

2.3. Spectroscopic Titrations and Calculations

Stock solutions of probe 2 were prepared in spectroscopic-grade DMSO in 10 mM concentration. All cation and anion solutions were prepared with 10 mM concentration in double distilled water. For all spectroscopic studies, the working concentration of probe 2 was 1 × 10^{-5} M (10 μM). For emission studies in different solvents, probe 2 was excited at 370 and 440 nm wavelengths, and the emissions were collected from 460 nm to 720 nm. All spectroscopic analysis experiments were conducted in acetonitrile. For the initial screening of the cation and anion sensitivity, 10 equivalents of the analyte species were introduced to probe 2 (10 μM) in acetonitrile. To study the emission of probe 2 with respect to the addition of each analyte, probe 2 was excited at 370 and 440 nm wavelengths, and the emissions spectra were collected from 460 nm to 720 nm. Since all fluorescence-based analysis studies were conducted in diluted solutions (≈1 × 10^{-5} M), the acquired spectra were not corrected for the inner filter effects, assuming it was negligible.
The linearity plots for the ratiometric absorbance data were obtained by calculating the absorbance ratio at 440 nm and 370 nm wavelengths \((\lambda_{440}/\lambda_{370})\) for each spectroscopic titration plot. The ratiometric emission plots were obtained by dividing the emission of the 2-Cu complex (I\textsubscript{585}) by the emission of probe 2 in the absence of quencher (I\textsubscript{0585}). The Stern–Volmer plot for the quenching of fluorescence emission was calculated by plotting the \(F_0/F\) as a function of Cu\textsuperscript{2+} concentration in the solution, where \(F_0\) and \(F\) are the emission of probe 2 and 2-Cu complex, respectively. The stability constant (K) for the 2-Cu complex was calculated by following the modified Stern–Volmer-type equation.

\[
\log\left(\frac{F_0}{F_0 - F}\right) = \log K + n \log[M^{m+}]
\]

where \(F_0\) is the emission of probe 2 in the absence of the quencher and \(F\) is the emission of the 2-Cu complex at a given titration interval. K is the stability constant for the 2-Cu complex, and \(n\) is the stoichiometric coefficient of the complex.

3. Results and Discussion

3.1. Optical Properties of Probe 2

Spectroscopic properties of probe 2 were studied in different solvents and summarized in Table 1 and Figure 1. The absorption maxima (\(\lambda_{\text{max}}\)) of the probe 2 appeared in 365–390 nm range over a wide range of solvents. The absorption spectra of the probe 2 did not exhibit a noticeable solvatochromic effect while changing from non-polar to polar solvents (Table 1 and Figure 1a). However, in DMSO, probe 2 exhibited a strong absorption peak at \(\lambda_{\text{abs}} \approx 530\) nm (Figure 1a), which is attributed to the deprotonation of the phenolic group (Ph-OH \(\rightarrow\) Ph-O\textsuperscript{−}) [18]. The sharp absorption peak at \(\lambda_{\text{abs}} \approx 530\) nm indicates the increased acidity of the phenolic group in probe 2 structure (in comparison to 4) due to the presence of strong electron-withdrawing vinyl pyridinium substituent. Similarly, the probe also exhibits moderate to weak deprotonation in polar protic solvents such as ethanol and methanol (Figure 1a). In all solvents except DMSO, the probe exhibited a consistent emission at \(\lambda_{\text{em}} \approx 565–590\) nm (Figure 1b,c), which is attributed to the emission resulting from the Excited-state Intramolecular Proton Transfer (ESIPT) process coupled with the intramolecular charge transfer (ICT) as explained in Scheme 2 (i.e., 2 \(\rightarrow\) 2a \(\rightarrow\) 2b). As a result, probe 2 exhibits a large Stokes’ shift (\(\Delta\lambda > 180\) nm), providing strong evidence of efficient ESIPT coupling with ICT (Table 1). The weak emission observed at \(\lambda_{\text{em}} \approx 530\) upon excitation at 440 nm wavelength (Figure 1c) is attributed to the emission produced by the anionic form of 2 produced as a result of solvent-dependent deprotonation (Ph-OH \(\rightarrow\) Ph-O\textsuperscript{−}) [18]. The observed significant hypsochromic shift in the emission resulted in DMSO (\(\lambda_{\text{em}} \approx 515\)) can be attributed to the ICT process occurring in intermediate 2c, which is generated by the deprotonation of probe 2 (Scheme 2). The observed Stokes’ shift of probe 2 (\(\Delta\lambda \approx 130\) nm) was significantly reduced in DMSO (Table 1) due to the hampered ESIPT. The impact of strong ESIPT coupling to ICT was further verified by conducting low-temperature fluorescence studies for probe at \(-188^\circ\)C where the restricted ESIPT/ICT process in the frozen solvent matrix generated a significant hypsochromic shift (\(\lambda_{\text{em}} \approx 535\)) in the emission spectra (Figure 1d).

| Solvent | Hexane | DCM | CHCl\textsubscript{3} | ACN | DMSO | EtOH | MeOH | Water |
|---------|--------|-----|-----------------|-----|------|------|------|-------|
| \(\lambda_{\text{abs}}\) (nm) | 390 | 386 | 383 | 369 | 382 | 372 | 370 | 366 |
| \(\lambda_{\text{em}}\) (nm) | 560 | 582 | 568 | 585 | 515 | 586 | 590 | 592 |
| \(\Delta\lambda\) (nm) | 170 | 196 | 185 | 216 | 133 | 213 | 220 | 226 |
| \(\varphi_{fl}\) | <0.001 | 0.050 | 0.057 | 0.072 | 0.016 | 0.064 | 0.052 | <0.002 |
| \(\varepsilon\) (M\textsuperscript{−1} cm\textsuperscript{−1}) | 3385 | 35,600 | 28,003 | 30,773 | 35,461 | 29,795 | 27,757 | 3908 |

Table 1. Spectroscopic properties of probe 2.
Figure 1. Absorbance (a) and emission (b,c) of probe 2 \((1 \times 10^{-5} \text{ M})\) in different solvents at room temperature and the comparison of room-temperature and low-temperature \((-188 ^\circ \text{C})\) fluorescence of probe 2 \((1 \times 10^{-6} \text{ M})\) in ethanol (d).

Scheme 2. ESIPT-coupled ICT in probe 2.
3.2. Evaluating Cation Sensing Ability of Probe 2 by Spectroscopy

In order to investigate the cation sensing ability of probe 2 in solution, various aqueous cations were tested against probe 2, and the resulting optical spectra were analyzed, as shown in Figure 2. To a 10 µM solution of probe 2 in acetonitrile, 10 equivalence of different cationic species were introduced, and the optical responses (absorbance and emission) were recorded (Figure 2). Probe 2 did not exhibit a noticeable change in the absorbance or emission spectra toward many cationic species (Figure 2). Interestingly, in the presence of Cu$^{2+}$, the absorption spectra of probe 2 exhibited a sharp response at $\lambda_{\text{abs}} \approx 440$ nm, indicating a strong interaction of the probe with Cu$^{2+}$ ions (Figure 2a). Probe 2 also indicated a weak response at $\lambda_{\text{abs}} 440$ nm upon the addition of Ni$^{2+}$ (Figure 2a). These results clearly indicated strong evidence of the potential ability of probe 2 toward Cu$^{2+}$ binding in solution. The fluorescence emission spectra acquired for probe 2 in the presence of metal ions (10 equivalence) exhibited a bright red emission at $\lambda_{\text{em}} \approx 585$ nm except for Cu$^{2+}$, whereas the emission of probe 2 was completely quenched by Cu$^{2+}$ (Figure 2b). Although Ni$^{2+}$ generated a weak response in the absorption of probe 2 at $\lambda_{\text{abs}} \approx 440$ nm (Figure 2a), the emission response of probe 2 remained unchanged upon the addition of Ni$^{2+}$ (Figure 2b). These results suggested an outstanding specificity of probe 2 toward Cu$^{2+}$ and indicated the probe’s ability to detect Cu$^{2+}$ in solution by spectroscopic methods. In order to further validate the consistency and reproducibility of the observed Cu$^{2+}$-induced changes in optical spectra of probe 2 and to rule out the effect of the counter ion during the analysis, different copper salts (i.e., CuCl$_2$, CuSO$_4$, and Cu(NO$_3$)$_2$) were tested against probe 2 (Figure S2). Probe 2 exhibited a steady response toward all Cu$^{2+}$ species independent from the identity of the counter ion, indicating its remarkable specificity toward the copper(II) ions.

Figure 2. Absorbance (a) and emission (b) spectra recorded for probe 2 (1 $\times$ 10$^{-5}$ M) in acetonitrile upon addition of 10 equivalence of aqueous metal ions at room temperature.

3.3. Study of Cu$^{2+}$ Binding Properties of Probe 2 by Spectroscopic Titrations

The observed Cu$^{2+}$-induced strong optical response of probe 2 encouraged us to further investigate the Cu$^{2+}$ sensing mechanism and the binding stoichiometry of probe 2. Therefore, spectroscopic titrations were performed for probe 2 in the presence of Cu$^{2+}$ in acetonitrile (Figure 3). The gradual addition of Cu$^{2+}$ (i.e., 0.1 equiv. portions) into probe 2 (in acetonitrile) led to the appearance of an absorption peak at $\lambda_{\text{abs}} \approx 440$ nm, which continued to increase with further additions of Cu$^{2+}$ (Figure 3a). The absorption peak appeared at $\lambda_{\text{abs}} \approx 440$ nm, reached a maximum with 1 equivalence of Cu$^{2+}$, and further addition of Cu$^{2+}$ exhibited a slight decrease in the peak at $\lambda_{\text{abs}} \approx 440$ nm (Figure 3a). The calculated Job’s plot for copper binding to probe 2 exhibited a 1:1 binding stoichiometry (top inset in Figure 3a). In addition to the gradual increase in the peak at $\lambda_{\text{abs}} \approx 440$ nm,
the addition of Cu$^{2+}$ into probe 2 also exhibited a gradual decrease in the absorbance peak at $\lambda_{\text{abs}} \approx 370$ nm, indicating possible structural modifications to the probe 2 upon binding to Cu$^{2+}$ (bottom insect in Figure 3a). The ratiometric absorbance for $\lambda_{440}/\lambda_{370}$ was plotted as a function of Cu$^{2+}$ concentration, which exhibited an excellent linear relationship ($R^2 = 0.997$), demonstrating the capacity of probe 2 to determine Cu$^{2+}$ concentration in solutions based on the ratiometric absorbance response (Figure 3b). The calculated limit of detection (LOD) for this method was found to be 0.15 $\mu$M. It is important to note that the Cu$^{2+}$-induced absorbance response that appeared at $\lambda_{\text{abs}} \approx 440$ nm is significantly different from the absorption peak observed for probe 2 in DMSO ($\lambda_{\text{abs}} \approx 530$ nm) due to the formation of the phenoxide anion by deprotonation of the phenolic group in probe 2. These data clearly indicate that when probe 2 interacts with Cu$^{2+}$ ions, it does not lead to phenoxide anion generation in the solution. Rather, it generates a coordinated metal complex 2-Cu as proposed in Scheme 1. The observed 1:1 binding stoichiometry by Job’s plot and the single isobestic point observed at $\lambda_{\text{abs}} \approx 400$ nm also provides evidence of the formation of the 2-Cu complex.

Figure 3. (a)—Absorbance spectra recorded for probe 2 ($1 \times 10^{-5}$ M) in acetonitrile at room temperature upon addition of a 10 mM Cu$^{2+}$ solution (in water). The red scatter plot insect shows the absorbance measured at 440 nm during the spectroscopic titration of probe 2 with Cu$^{2+}$ and the green scatter plot insect shows the absorbance measured at 370 nm during the spectroscopic titration. The
green dashed vertical line highlights the absorbance reading at 1:1 (probe 2: Cu\(^{2+}\)) stoichiometry. 
(b)—The plot of ratiometric absorbance (\(\lambda_{440}/\lambda_{379}\)) recorded as a function of Cu\(^{2+}\) concentration. 

(e)—Emission spectra recorded for probe 2 (1 \(\times\) 10\(^{-5}\) M) in acetonitrile at room temperature upon addition of a 10 mM Cu\(^{2+}\) solution (in water). 
(d)—The plot of fluorescence quenching at 585 nm (I\(_{585}/I_{0585}\)) recorded as a function of Cu\(^{2+}\) concentration.

The emission of probe 2 at \(\lambda_{em} \approx 585\) nm decreased gradually with increasing concentration of Cu\(^{2+}\) added into the solution, and probe 2 was nearly non-fluorescent upon the addition of 1 equivalence of Cu\(^{2+}\) (Figure 3c). The relative fluorescence quenching (I\(_{585}/I_{0585}\)) of probe 2 induced by Cu\(^{2+}\) exhibited an excellent linear relationship (R\(^2\) = 0.991) with respect to the Cu\(^{2+}\) concentration in the solution (Figure 3d). However, the linearity of the response gradually diminished and plateaued with increasing Cu\(^{2+}\) concentration. It is also important to note that the addition of 0.1 to 1 equivalence of Cu\(^{2+}\) into probe 2 exhibited a strong fluorescence quenching at \(\lambda_{em} \approx 585\) and further increase in the Cu\(^{2+}\) equivalence in the solution (i.e., 2 equiv.) did not impact significantly (Figure 3c,d). The calculated limit of detection (LOD) for Cu\(^{2+}\)-induced relative fluorescence quenching of probe 2 was found to be 0.38 \(\mu\)M.

The Stern–Volmer plot was generated to analyze the fluorescence quenching event based on the fluorometric titrations of probe 2 with Cu\(^{2+}\) (Figure 4a). In the generated Stern–Volmer plot, the relative quenching efficiency (F\(_{0}\)/F) did not exhibit the conventional linear relationship to the quencher (Cu\(^{2+}\)) concentration (Figure 4a). Although the quenching of fluorescence exhibits a linear relationship with a lower concentration of Cu\(^{2+}\), the quenching efficiency tends to increase exponentially at higher quencher concentrations (Figure 4a). At lower concentrations of the Cu\(^{2+}\), the quenching of fluorescence likely occurs due to the static quenching process by forming the 2-Cu complex, which enables the metal-to-ligand electron transfer (MCLT) process [31–35]. The calculated fluorescence lifetime for probe 2 was found to be \(\approx 2.25\) ns (Figure S6). Based on these data, the bimolecular quenching rate constant (k\(_q\)) calculated for the linear quenching range (i.e., 1–6 \(\mu\)M of Cu\(^{2+}\)) of the graph 4a was 9.11 \(\times\) 10\(^{13}\) M\(^{-1}\) s\(^{-1}\), which was found to be significantly higher than the diffusion-limited dynamic quenching value (k\(_{diff}\) \(\approx 2 \times 10^{10}\) M\(^{-1}\)s\(^{-1}\)). Therefore, the possibility of dynamic quenching can be ruled out for the 2-Cu complex (Figures 4a and S6). However, at higher Cu\(^{2+}\) concentrations, the fluorescence quenching may likely be governed by a combination of static and dynamic quenching processes, which can increase the quenching efficiency exponentially (Figure 4a) [35–37]. The modified logarithmic scale Stern–Volmer plot was generated (Figure 4b) to calculate the stability constant of the 2-Cu complex according to the previously described methodology [9,38,39]. The calculated stability constant for the 2-Cu complex was found to be 6.45 \(\times\) 10\(^4\) M\(^{-1}\), and the stoichiometric coefficient for the complexation was found to be n = 0.9 (\(\approx 1\)), which indicates 1:1 binding stoichiometry (Figure 4b).

Based on the spectroscopic data, we hypothesized that the quenching of probe 2 fluorescence occurs due to the formation of the 2-Cu complex, as described in Scheme 1. Therefore, the removal of the Cu\(^{2+}\) ions from the 2-Cu complex should turn on the fluorescence in 2. In order to validate our hypothesis, spectroscopic back titrations were performed with a 10 mM solution of EDTA to remove Cu\(^{2+}\) from the 2-Cu complex (Figure 5a,b). The addition of EDTA into the 2-Cu complex in acetonitrile gradually suppressed the new absorption peak that appeared at \(\lambda_{abs} \approx 440\) nm, indicating strong spectroscopic evidence for the dissociation of the 2-Cu complex (Figure 5a). Interestingly, the addition of EDTA (0 \(\rightarrow\) 1 eq.) also restored the emission in probe 2 (Figure 5b), which validates our initial hypothesis regarding the mechanism of fluorescence quenching by the formation of the 2-Cu complex. Due to the excellent stability of the Cu-EDTA complex (K\(_{conditional}\) \(\approx 6.3 \times 10^{14}\)), Cu\(^{2+}\) in the 2-Cu complex can be replaced by strong chelators such as EDTA (2-Cu + EDTA \(\rightarrow\) 2 + Cu-EDTA), which releases emissive probe 2 back to the solution. The reversibility of the 2-Cu complex can be useful for developing sensing applications where probe 2 can be successfully utilized as a colorimetric indicator to remove Cu\(^{2+}\) selectively from the solution. Probe 2 was tested against several anionic species (F\(^-\), Cl\(^-\), Br\(^-\), I\(^-\), OH\(^-\), PO\(_4^{3-}\),...
and SO$_4^{2-}$) to investigate its optical response to anions by adding 10 equivalences from each species in solution (Figure 5c,d). As we reported in previous work, probe 5 exhibited a sharp response to F$^-$ by generating a new absorbance band at $\lambda_{abs} \approx 530$ nm, which can be attributed to the phenoxide ion formation by subsequent deprotonation of the probe 2 (Ph-OH + F$^-$ $\rightarrow$ Ph-O$^-$ + HF) as shown in Figure 5c [18]. Similarly, the addition of NaOH (10 equivalence) to probe 2 also exhibited this sharp absorption band at $\lambda_{abs} \approx 530$ nm, confirming the formation of the phenoxide anion (Figure 5c). The emission of probe 2 ($\lambda_{em} = 370$ nm) remained unchanged except for F$^-$ and OH$^-$, where the emission was quenched due to the formation of phenoxide anion via photo-induced electron transfer (PET). However, the distinct differences in absorption spectra (i.e., Cu$^{2+}$: $\lambda_{abs} \approx 440$ nm vs. phenoxide formation: $\lambda_{abs} \approx 530$ nm) can be used as a reliable strategy to distinguish 2-Cu complex formation from the deprotonation event.

Figure 4. (a) Stern–Volmer plot generated for fluorescence quenching in probe 2 by plotting $(F_0/F)$ at 585 nm as a function of Cu$^{2+}$ concentration. (b) Modified logarithmic-type Stern–Volmer plot for determination of the stability and the binding stoichiometry of the 2-Cu complex.

The solution stability of the 2-Cu complex (in acetonitrile) was studied for up to 4 h by analyzing the absorption and emission profiles of the 2-Cu complex (Figures 6a and S3). Interestingly, the 2-Cu complex demonstrated exceptional stability up to 4 h with the absorbance band generated due to the 2-Cu complex formation (i.e., $\lambda_{abs} \approx 440$ nm) remaining unchanged (Figure 6a). Similarly, the emission of the 2-Cu complex remained quenched, confirming high solution stability to the metal–ligand complex. To evaluate the stability of the 2-Cu complex under strong acidic and basic environments, large volumes (i.e., 10 equivalence) of strong base (i.e., NaOH) and strong acid (i.e., HCl) were introduced, and the resulting optical spectra were analyzed (Figures 6b, S4 and S5). Upon addition of a strong base, a noticeable bathochromic shift in the absorption spectra was observed from $\lambda_{abs} \approx 440$ nm to $\lambda_{abs} \approx 530$ nm, which can be attributed to the dissociation of the 2-Cu complex to generate deprotonated probe 2 in the solution (Figure 6b). The emission of probe 2 and 2-Cu remained quenched in the strong base (Figure S4). However, the addition of a strong acid (i.e., HCl) did not alter the 2-Cu absorption band at $\lambda_{abs} \approx 440$ nm, and a significant increase in the molar absorptivity was observed. Based on these results, it is assumed that the 2-Cu complex remained stable to a strong acid (Figure S5). Surprisingly, the 2-Cu complex exhibited a strong fluorescence turn-on at $\lambda_{em} \approx 585$ nm in the strong acid (Figure S5). Although the 2-Cu complex remained unharmed in strong acid, it may likely interfere with the fluorescence quenching process by hampering the metal-to-ligand charge transfer (MLCT) process through subsequent protonation of the ligand. Therefore,
these results suggest that the 2-Cu complex can be utilized as a useful candidate to sense sudden pH fluctuations in aqueous environments. The formation of the 2-Cu complex was verified by mass spectrometric (ESI-TOF) analysis of the 2-Cu complex prepared in situ (in acetonitrile) by treating probe 2 (1 × 10⁻⁵ M) with 1 equivalence of CuCl₂ (Figure 6c,d)). In sharp contrast to the (m/z) = 583 observed for probe 2 only, an intense peak was observed at (m/z) = 634, which was attributed to the 2-Cu complex, as shown in Figure 6d.

**Figure 5.** Absorption (a) and emission (b) spectra recorded for 2-Cu complex (in acetonitrile) upon addition of EDTA (10 mM in water at room temperature). 2-Cu complex was prepared by the addition of 1 equivalence of CuCl₂ (10 mM in water) into probe 2 (1 × 10⁻⁵ M) in acetonitrile. (c,d) represent the absorption (c) and emission (d) spectra recorded for probe 2 (1 × 10⁻⁵ M) in acetonitrile during the addition of 10 equivalence of various anions (10 mM in water) at room temperature.
Figure 6. (a)—The evaluation of the stability of the 2-Cu complex (10 μM in acetonitrile) at room temperature. (b)—The analysis of the 2-Cu complex (10 μM in acetonitrile) upon addition of 50 equivalence of aqueous NaOH at room temperature. Figures (c,d) represent the ESI (TOF) mass spectroscopic analysis of the probe 2 (c) and 2-Cu complex (d).

4. Conclusions

In summary, (E)-4-(3,5-bis(benzo[d]oxazol-2-yl)-2,6-dihydroxystyryl)-1-benzylpyridin-1-ium iodide (2) was synthesized in suitable yields. Probe 2 exhibited excellent selectivity toward Cu²⁺ ions in solution due to the formation of the 2-Cu complex in 1:1 stoichiometry. The formation of the 2-Cu complex revealed a new absorption band at λ_{abs} ≈ 440 nm, and the ratiometric absorbance at (A_{440}/A_{370}) was successfully used for linear detection of Cu²⁺ in solution. The calculated LOD for detection of ratiometric absorbance was found to be 0.15 μM. The formation of the 2-Cu complex resulted in the quenching of fluorescence at 585 nm, and the relative fluorescence quenching (I_{o585}/I_{585}) also exhibited a linear relationship to the Cu²⁺ concentration in the solution. LOD for fluorescence quenching (I_{o585}/I_{585})-based method was found to be 0.38 μM. The formation of the 2-Cu complex was confirmed by spectroscopic and mass spectroscopic analysis. Based on the spectroscopic data, it was concluded that the fluorescence quenching process in the 2-Cu complex occurs via both static and dynamic quenching mechanisms. Therefore, probe 2 will be a reliable and highly sensitive colorimetric and fluorometric sensor for detecting Cu²⁺ in solution efficiently.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/chemosensors10100382/s1, Figure S1: Characterization data; Table S1: Detection limit calculation; Figures S2–S5: Spectroscopic analysis data; Figure S6: Fluorescence lifetime determination.

Author Contributions: C.S.A. designed, synthesized, and characterized the probes. C.S.A., K.J.W. and Y.P. planned, designed, and supervised all experimental setups. B.U.R. conducted all spectroscopic analysis experiments and data collection. Y.L. contributed significantly toward acquiring low-temperature spectroscopic data and fluorescence quantum yield calculations. B.C. conducted fluorescence lifetime measurement experiments. C.S.A., K.J.W. and B.U.R. wrote the manuscript.
with feedback from all authors. All authors have read and agreed to the published version of the manuscript.

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