Solvent-controlled Novel Cu^{+} and $Cu^{+/2+}$ Fluorescent "Turn-ON" Probing

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A novel Schiff base probe, carbamoyl salicylimine benzothiazole hydrazine, was prepared and measured for its probing ability. High sensitivity was shown for Cu^{+} and Cu^{2+}. When solvent polarity was regulated through a combination of H_2O and acetonitrile, selective sensing of Cu^{+} or the total amount of Cu^{+} and Cu^{2+} was possible. In addition, a linear hypsochromic fluorescent shift of about 30 nm was shown. A binding stoichiometry of 1:1 exists at low concentration. Time-dependent emission measurement showed an exponential decay curve ($\tau_1 = 2.99 \times 10^3$ s, 10 equiv of Cu^{+}) and a linear decay line (slope = –0.0216, 5 equiv of Cu^{+}). Interference experiments in 50% acetonitrile in H_2O showed that the emission produced by Cu^{+} and Cu^{2+} was not disturbed by other metal ions or by acidity or basicity. Peroxynitrite changed the emission trends; Cu^{+} emission decreased (78%) and Cu^{2+} fluorescence increased (28-*fold*). Biothiols, L-cysteine, DL-homocysteine, reduced glutathione, and *N*-acetyl-L-cysteine affected the complete reversibility of Cu^{+}-induced emission in 50% (v/v) acetonitrile in H_2O, relative to partial reversibility of Cu^{2+} emission. Thus, by regulating the ratio between acetonitrile and H_2O, Cu^{+} and $Cu^{+/2+}$ can be probed selectively.

Keywords: Solvent polarity, Schiff base, Peroxynitrite, Biothiol, Carbamoyl, Reversibility

Introduction

The determination of $Cu^{+/2+}$ concentrations in biological and environmental systems has become more and more an important diagnostic issue.[1–4] Copper ion is one of the most important metal ions in enzyme-active sites because of its redox properties.[3] Copper, with its two main oxidation states, can be interchanged via the copper version of "Fenton chemistry." This can induce a single electron transfer to biological substrates and mediate important physiological processes. However, uncontrolled free metals in biological systems can give rise to reactive radical species, which can deform many kinds of important molecules and may induce ailments[5] such as certain neurodegenerative diseases.[1,5–8]

It is necessary to develop detection methods for distinguishing between Cu^{+} and Cu^{2+} for the diagnosis of diseases.[2] Commonly, inductively coupled plasma-atomic emission spectroscopy (ICP-AES), ICP-mass spectrometry (ICP-MS), or atomic absorption (AA) are used for the analysis of Cu ions.[9] However, it is very difficult to differentiate between the different oxidation states of the same metal without specific pretreatment. For separate detection of each oxidation state, chemical sensing methods are very useful. There are mainly two strategies for the sensing of copper ions: fluorescence and colorimetric assays. In particular, fluorescence methods have excellent potential to allow researchers to go deeper with *in vivo* studies to effect biological metal sensing with low detection limits.

In particular, sensing of Cu^{+} is challenging because Cu^{+} can easily undergo conversion to Cu^{2+} under ambient conditions via disproportionation.[2] Certain compounds have been known as reliable Cu^{+} sensors, which are usually based on chelation sites involving oxygen, nitrogen, and sulfur[2,10–13] or genetically engineered proteins.[14]

These probing systems use mainly the ether functional group combined with sulfur or nitrogen atoms in a closed ring or open chain-like form.

Here, we report the design of a novel fluorescence "turn-on" molecule for Cu^{+} and Cu^{2+} detection with a carbamoyl salicylimine benzothiazole-based molecule in which salicylaldehyde is a fluorophore (Scheme 1). When *N*-dimethyl carbamoyl chloride was functionalized with the hydroxide of the salicylaldehyde and then the Schiff base was formed (Table S1), the ligand did not show fluorescence because of the existence of a proposed photoinduced electron transfer (PET) mechanism; such latency is important in developing efficient "turn-on" species.[15,16] Fluorescence "turn-on" is then expected to take place in the presence of specific metal ions by blocking PET. Additionally, solvent polarity is important for

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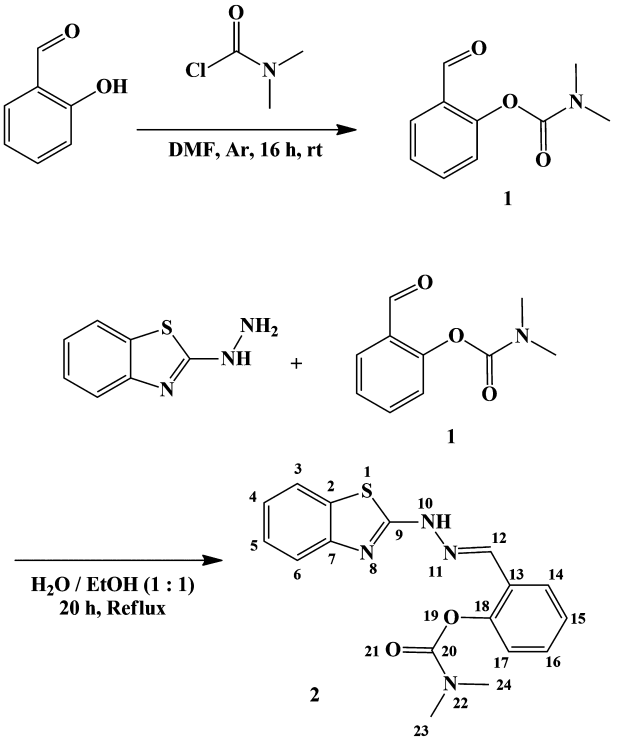

Scheme 1. Synthesis of probe **2**.

the regulation of molecular fluorescence as well as PET or related photomechanisms[17–19]; solvent polarity is related to the stabilization of ground or excited states of the molecules.[20]

Experimental

General Remarks. All reagents of analytical grade were used as received from Sigma Aldrich (Munich, Germany), TCI (Tokyo, Japan), and Junsei (Junsei Chemical Co., Ltd., Tokyo). For characterization of the probe, one-dimensional ^{1}H-NMR, ^{13}C-NMR, two-dimensional correlation spectroscopy (COSY), nuclear Overhauser effect spectroscopy (NOESY), heteronuclear single-quantum correlation spectroscopy (HSQC), and heteronuclear multiple-bond correlation spectroscopy (HMBC) were performed via a Bruker Avance 400 MHz spectrometer (Billerica, MA, USA). Fluorescence analysis was performed with a Shimadzu spectrophotometer (RF–5301pc) (Columbia, MD, USA). UV–vis absorbance was carried out by a Jasco spectroscope (V–530 model) (Jasco, Tokyo, Japan).

Synthesis of the Probe. Compound **1** was synthesized following a previously reported procedure.[21] Salicylaldehyde (2.00 g, 16.4 mmol) was mixed with *N*-dimethylcarbamoyl chloride (3.52 g, 32.8 mmol) in the presence of 1,4-diazabicyclo[2.2.2]octane (DABCO, 3.52 g, 32.8 mmol) in DMF (15 mL) under argon at room temperature. After 16 h, distilled water (150 mL) was added and the reaction mixture was stored at 4 °C for 2 h. The solution was extracted with ethyl acetate (three times). The solvent was evaporated and dried under vacuum for 16 h. A yellow oil was obtained (yield 70%).

For Compound **2**, Compound **1** (1.00 g, 5.18 mmol) was dissolved in ethanol (10 mL) and added to benzothiazole hydrazide that was dissolved in H_2O (10 mL), and refluxed at 90 °C for 24 h. A gray precipitate was obtained, which was filtered, washed with a mixture of deionized water and ethanol (1:1, v/v), dried at room temperature for 24 h, and placed under vacuum for 24 h. A pale grayish powder was obtained (yield, 76%). ^{1}H-NMR (400 MHz, DMSO), δ 12.29 (br, H_{10}), 8.20 (s, H_{12}), 7.88 (dd, $^3J_{H–H}$ = 7.8 Hz, $^4J_{H–H}$ = 1.7 Hz, H_{14}), 7.77 (d, $^3J_{H–H}$ = 7.9 Hz, H_6), 7.46 (d, $^3J_{H–H}$ = 8.0 Hz, H_3), 7.41 (ddd, $^3J_{H–H}$ = 8.1 Hz, $^4J_{H–H}$ = 7.3 Hz, $^5J_{H–H}$ = 1.7 Hz, H_{16}), 7.33–7.28 (m, H_{15}, H_4), 7.18 (dd, $^3J_{H–H}$ = 8.1, $^4J_{H–H}$ = 1.2 Hz, H_{17}), 7.11 (td, $^3J_{H–H}$ = 7.6, $^4J_{H–H}$ = 1.2 Hz, H_5), 3.12 (s, H_{23}), 2.94 (s, H_{24}), ^{1}H-decoupled ^{13}C-NMR (100 MHz, DMSO) δ 166.92 (C_9), 153.70 (C_{20}), 149.43 (C_{18}), 138.50 (C_{12}), 130.22 (C_{16}), 129.40 (C_2), 126.93 (C_{13}), 125.95 (C_7), 125.77 (C_4, C_{15}), 125.52 (C_{14}), 123.56 (C_{17}), 121.71 (C_5), 121.50 (C_6), 118.06 (C_3), 36.49 (C_{23}), 36.25 (C_{24}). ^{1}H-coupled ^{13}C-NMR (100 MHz, DMSO), 167.13 (s, C_9), 153.86–153.74 (m, C_{20}), 150.35–149.47 (m, C_{18}), 138.61 (d, $J_{C–H}$ = 170 Hz, C_{12}), 131.02 (dd, $^1J_{C–H}$ = 140 Hz, $^2J_{C–H}$ = 8.6 Hz, C_{16}), 129.43 (d, $^1J_{C–H}$ = 11 Hz, C_2), 127.18–120.66 (m, C_{13}, C_7, C_4, C_{15}, C_{14}, C_{17}, C_6, C_5), 118.13 (d, $^1J_{C–H}$ = 140 Hz, C_3), 36.50 (quartet, $^1J_{C–H}$ = 140 Hz, C_{23}), 36.24 (quartet, $^1J_{C–H}$ = 140 Hz, C_{24}), HR-LC/MS: calcd for $C_{17}H_{16}N_4O_2S$ + Na: 363.0892, found: *m/z*, 363.0838 $(M + Na)^+$ (Figure S1, Supporting Information).

UV–Visible Absorbance or Fluorescence Measurements. UV–visible absorbance was measured with the Jasco V–530 instrument. After baseline correction, each sample was analyzed through a range of wavelengths (200–700 nm). Fluorescence was checked with a Shimadzu fluorescence spectrometer (RF–5301 PC model). A quartz cuvette was used for both UV-visible and fluorescence analysis (path length 10 mm).

HR-LC/MS. The mass of the probe was analyzed through high-resolution liquid chromatography/mass spectrometry (HR-LC/MS, Bruker Daltonik, (Billerica, MA, USA), micro-TOF-QIImodel, Germany). The following were the settings: ion source type, electrospray ionization (ESI); ion polarity, positive mode; nebulizer, 0.4 bar; capillary, 4500 V; dry heater, 180 °C; scan begin, 50 *m/z*; end plate offset, –500 V; dry gas, 4.0 L/min; collision cell RF, 250.0 Vpp; solvent, methanol.

Screening of Metal Ions. Solutions (0.1 M) of various metal ions (Ag^+, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, K^+, Mg^{2+}, Mn^{2+}, Na^+, Pb^{2+}, and Zn^{2+}, as partnered with perchlorate anions; Cu^+ and Na^+ as acetates; and Al^{3+} as chloride) were prepared in deionized water and added into the probe solution to make 100 100 μM mu;M as the final concentration. In particular, Cu^+, Co^{2+}, Fe^{2+}, and Mn^{2+} solutions were prepared immediately before the reaction and used directly (in less than 1 min) to minimize its transformation into other oxidation states. A 10-min incubation was performed at room temperature after mixing of compound **2** with each metal ion, and

fluorescence and UV-visible absorbance measurements were carried out.

Interference Experiments. Compound **2** (10 μM, 1 equiv) underwent reaction with Cu^{+} (50 μM, 5 equiv) (or Cu^{2+}) followed by 10 min incubation at room temperature. Then, each potentially interfering metal (prepared by the same method described in the section "Screening of metal ions") was treated with compound **2** solutions containing Cu^{+} (or Cu^{2+}). After 10 min incubation, the fluorescence intensity was measured.

Job Plotting. A series of 10 solutions whose total concentrations of compound **2** and Cu^{+} were 22 μM and 50 μM were prepared from 0.0 to 1.0, in increments of 0.1. After 20 min, UV-visible absorbance and fluorescence measurements were obtained.

Reversibility by Biothiols. Compound **2** (10 μM, 1 equiv) and Cu^{+} (50 μM, 5 equiv) (or Cu^{2+}) were mixed and incubated for 10 min at room temperature. Then, solutions of biothiols (L-cysteine, DL-homocysteine, glutathione (reduced), and *N*-acetyl-L-cysteine of concentration 50 μM, 5 equiv) were prepared by dissolving them in distilled water, and adding them into the probe solution containing Cu^{+} (or Cu^{2+}). After 10 min of incubation, the fluorescence was measured.

DFT Calculations. Molecular structures and HOMO (highest occupied molecular orbital)–LUMO (lowest unoccupied molecular orbital) level energies were acquired using density functional theory (DFT) calculations (using Gausian 09. B3LYP method with 6–31g* basis set and 6–311g* basis set for Cu only). All calculations were performed in the gas phase (Table S2).

Effect of ROS/RNS. Stock solutions of reactive oxygen species (ROS) (0.1 M, KO_2, H_2O_2, ^{t}BuOOH, $FeSO_4$ + H_2O_2, $FeSO_4$ + ^{t}BuOOH, NaOCl) were prepared by careful measurement and dissolving these in distilled water, and reacting with the probe solution (Compound **2**, 10 μM) to help prepare a final concentration as 50 μM (5 equiv) for 10 min, followed by fluorescence and UV–visible absorbance measurements (Figure S7). In addition, peroxynitrite, also a member of the reactive nitrogen species (RNS), was synthesized following a previously reported procedure.[22] The mixture of $NaNO_2$ (0.6 M, 1 mL) and H_2O_2 (0.7 M, 1 mL) underwent reaction with HCl solution (0.6 M, 1 mL), followed by addition of NaOH solution (1.5 M, 2 mL) within 5 s at 4 °C. The concentration of peroxynitrite was determined by UV–visible absorbance using the extinction coefficient value of 1670 M/cm (Figure S8).

Results and Discussion

Screening of Solutions of 16 Different Metal Ions with UV–Visible and Fluorescence Spectroscopy in Combination with Acetonitrile and H_2O. Sixteen metal ions were screened for measuring UV–visible absorbance (Figure S2) and fluorescence changes with **2** (Figure 1). For fluorescence screening, three solvent systems were used: 100% acetonitrile, 50% acetonitrile (v/v) in H_2O, and 100% H_2O. Compound **2** was dissolved with acetonitrile and diluted to a final concentration of 10 μM with these three solvent systems. After 20 min, UV–visible and fluorescence spectra were acquired and analyzed.

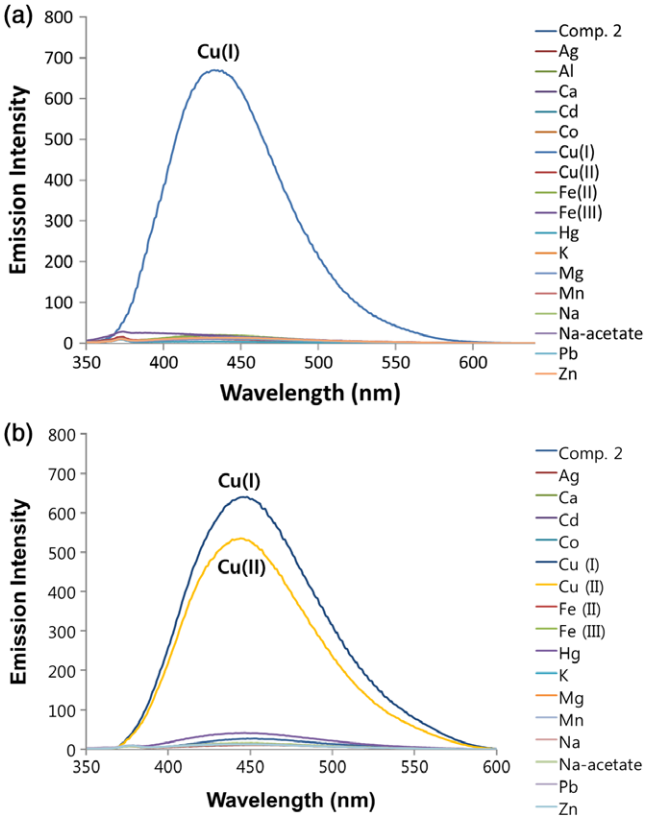

Figure 1. Emission intensity of compound **2** (10 μM, 1 equiv) with 16 metal ions (10 equiv) in (a) 100% acetonitrile, (b) 50% (v/v) acetonitrile in H_2O. Also see Figures S13-S15.

As shown in Figure 1, Cu^{+} showed a large and good selective "turn-on" emission spectrum, 51-fold relative to probe emission. In previously reported literature, for sensing of Cu^{+}, chelating cyclic or acyclic thiaza-ligands involving nitrogen or sulfur atoms were required.[2,13] This new benzothiazole Schiff base ligand showed good "turn-on" selectivity without ether-type functional groups, as was done earlier.

Intriguingly, in the mixed 50:50 (v/v) solvent system, Cu^{2+} as well as Cu^{+} showed a strong fluorescence turn-on signal, 20-and 24-fold, respectively, to the probe fluorescence intensity at the same wavelength (445 nm). In previous literature, there were rare cases for fluorescence turn-on for both Cu^{+} and Cu^{2+}, attributed to the difficulty of designing a chelating ligand that satisfies the probe design requirements for both oxidation states of copper. From our results, it is possible to detect the total amount, or distribution of both oxidation states, of copper in the liquid sample *in vitro* by using the 50:50 solvent system. However, in 100% H_2O solvent, no fluorescence was shown (Figure S3).

Measurement of fluorescence by regulation of solvent volume ratio between acetonitrile and H_2O: In a 50:50 (v/v) solvent system, both turn-on fluorescence for Cu^{+} and Cu^{2+} were as shown in Figure 1(b). To achieve further results, we

changed the volume ratio of the solvent (= acetonitrile/(acetonitrile + H_2O)), in which solvent polarity regulation can be expected. Fluorescence wavelength and emission intensity of the probe with Cu^{+} or Cu^{2+} were measured. A linear blue shift of ~32 and 29 nm were shown for Cu^{+} and Cu^{2+}, respectively (Figure 2(a)). This data is consistent with a fluorescent transition energy gap between the excited state and the ground state, which gradually increases with increasing concentration of acetonitrile. In addition, when the *X*-coordinate is exchanged for "volume/volume ratio," linearity was shown (Figure 2(a)).

In Figure 2(b), the maximum emission intensity of Cu^{+} and Cu^{2+} showed very different trends except at a low acetonitrile ratio (0–0.4). After the ratio of 0.5, Cu^{2+} showed roughly a linear intensity decrease, whereas Cu^{+} displayed a discontinuous trend. Increasing the ratio of acetonitrile loosened the π–π interaction and increased the fluorescence intensity. From 0.5, Cu^{+} showed a relatively high intensity compared to Cu^{2+}.

Importantly, only acetonitrile could discriminate Cu^{+} from Cu^{2+} with a good separation ratio (31-fold) at 436 nm.

Measurement of Time-Dependent Emission Intensity. When continuous fluorescence at different equiv of Cu^{+} was measured, a higher equiv of Cu^{+} showed a more rapid response than lower equiv values (Figure 3).

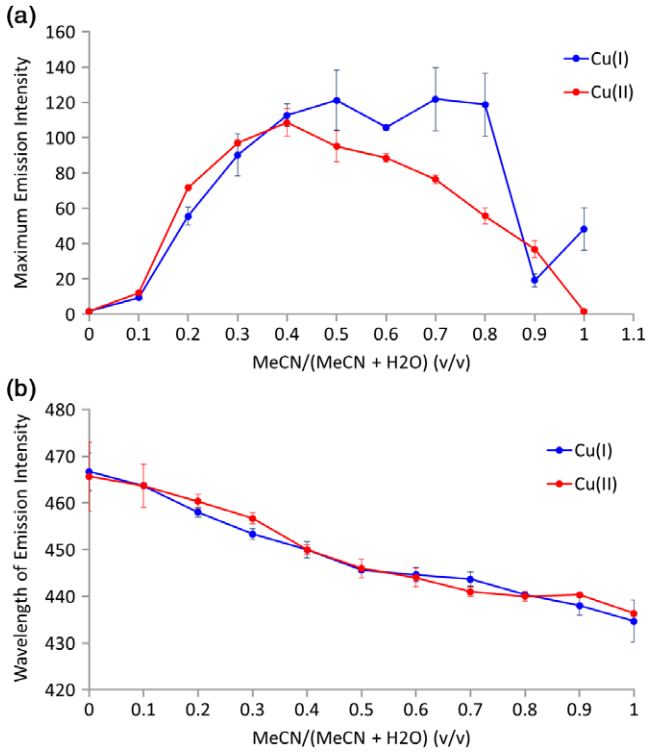

Figure 2. (a) Shift of wavelength at maximum emission and (b) maximum emission intensity of the compound **2** (10 μM, 1 equiv) with Cu^{+} (5 equiv) or Cu^{2+} (5 equiv) in various solvent mixtures (v/v) between acetonitrile and H_2O. Blue line (compound **2** + Cu^{+}), red line (compound **2** + Cu^{2+}), λ_{ex} = 332 nm, Slit width (EX: 3 nm, EM: 3 nm), MeCN: acetonitrile. Error bars indicate standard deviation, three repetitions (Figure S4, Supporting Information).

Interestingly, in less than 400 s, the emission signal at 10 equiv of added Cu^{+} reached the maximum intensity and exhibited an exponential decay curve relative to the linear decay found for 5 equiv loading. The lifetime (τ_1) calculated from the time of the highest peak was 2.99×10^3s ($R^2 = 0.999$) at 10 equiv of Cu^{+} (Figure S5). Some studies have mentioned the disproportion property of Cu^{+} in the absence, or presence, of ligand in polymer chemistry.[23,24] They also evaluated the equilibrium constants for disproportionation of Cu^{+} in water (K_{disp} = ~ 10^6 to 10^7 M^{-1}) and acetonitrile (K_{disp} = 6.3 × 10^{-21}) without ligands. However, the disproportionation of Cu^{+} in acetonitrile can proceed efficiently with halide ligands or with certain types of ligand systems: (Me6TREN, tetradentate tertiary amine-based ligand (K = ~1.4 × 10^3). This result supports the exponential decay at 10 equiv. As shown in the interference result of Figure 4(a), Cu^{2+} showed a quenching effect on Cu^{+}-induced emission. Cu^{2+}, generated by disproportionation of excess Cu^{+} in the presence of compound **2**, can quench the emission enhanced by Cu^{+}, competitively.

The signal at 5 equiv of Cu^{+} showed the most stable probing, adequate for collecting fluorescence information in this probing system. However, 5 equiv of Cu^{+} showed linearity, not an exponential signal, over the range from 1039 to 3600 s, after a maximum was achieved. Linear fitting gave the following relation equation: Emission intensity = –0.0216 × (time) + 181.1 (R^2 = 0.997)" (Figure S5), which means the rate of fluorescence decrease was constant, and is seen arising from competitive equilibria that are proposed to exist between states reflected by the emission created by Cu^{+} and the quenching by Cu^{2+} concentration. At 1 equiv concentration of Cu^{+}, there was no decay during the measuring time (0–3600 s) (Figure S5). From this result, compound **2** at 5 equiv shows potential for a molecular time sensor for use as a determinant for the expected disproportionation of Cu^{+}.

Interference Experiments. In the presence of Cu^{+}, the fluorescence for compound **2** was measured with other metal ions to obtain information about interference (Figure 4; Figure S6).

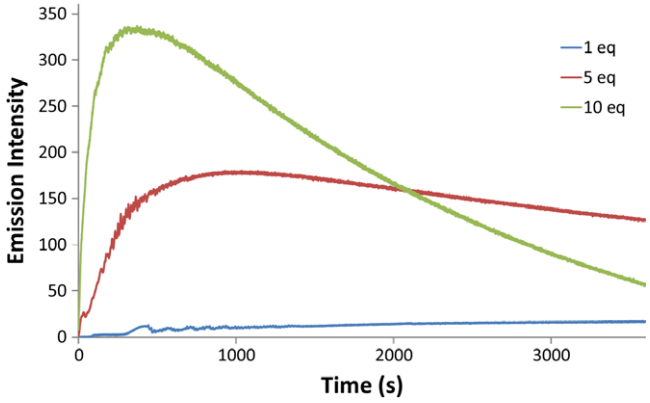

Figure 3. Time-dependent emission intensity of compound **2** (10 μM, 1 equiv) at various equiv of Cu^{+} in acetonitrile solvent. λ_{ex} = 332 nm, λ_{em} = 436 nm, Slit width (EX: 5 nm, EM: 5 nm).

In 100% acetonitrile solvent, Cu^{2+}, Fe^{3+}, and Hg^{2+} showed a strong quenching effect (over 96%) on Cu^{+} emission. Pb^{2+}, Ag^{+}, and Zn^{2+} decreased the emission intensity slightly to 21%, 28%, and 43%, respectively. Interestingly, signals arising from Fe^{2+} and Fe^{3+} were very different. Fe^{2+} showed a 2-fold increase over the control and 50-fold increase over that for Fe^{3+}, which means Fe^{2+} can be differentiated from Fe^{3+} by compound **2** in the presence of Cu^{+}.

In 50% acetonitrile in H_2O, almost all metal ions did not alter Cu^{+} or Cu^{2+} emission intensity (Figure 4 ((b) and (c)). Cu^{2+} and Cu^{+} slightly quenched Cu^{+} and Cu^{2+} emission values by 48% and 32%, respectively, whereas 50% acetonitrile solvent provided relatively stable probing conditions.

When the effect of Brönsted–Lowry acidity and basicity was investigated on Cu^{+} and Cu^{2+} emission in, *e.g.*, the *in vitro* 50:50 (v/v) solvent system, little interference was seen in the fluorescence signal (Figure S7). Limited interference by other metal ions, by acidity, or basicity supports that compound **2** can probe the total amount for both oxidation states $Cu^{+/2+}$ *in vitro* when a 50:50 solvent system is present, although 48% fluorescence can be generated by interference with addition of $Cu^{2+/+}$.

In addition, the possible interference effects of Cu^{+}-based emission by ROS and RNS was investigated. First, although ROS did not turn-on the emission intensity of compound **2**, partial interference patterns were present (Figure S8 ((b) and (c)). This result means that the Cu^{+} probing potential is reasonable in this system despite the presence of ROS. For the absorbance spectrum data, a new selective peak by compound **2** + KO_2 was shown at 430 nm, while there were no new characteristic signals with the presence of compound **2** + Cu^{+} + other ROS. This result suggests that there is a specific and strong interaction/reaction between the probe and O_2^{-}. Second, peroxynitrite, a member of RNS, was tested for interference of emission induced by Cu^{+}. $ONOO^{-}$ showed a 72% decrease on Cu^{+}-induced emission intensity, thought to be mainly arising by the basicity imparted by $ONOO^{-}$ in the solvent (Figure S9). Interestingly, Cu^{2+} showed a 28-fold emission increase in the presence of $ONOO^{-}$. Peroxynitrite thus functioned as a toggle for turn-on/off behavior in emission by Cu^{+} and Cu^{2+} in this probing system (Figure 5).

Determination of Binding Stoichiometry Using Titration and Job Analysis. When the titration of Cu^{+} was performed in 100% acetonitrile using UV–visible absorbance, at low concentration (below 3 equiv) an isosbestic point was observed;

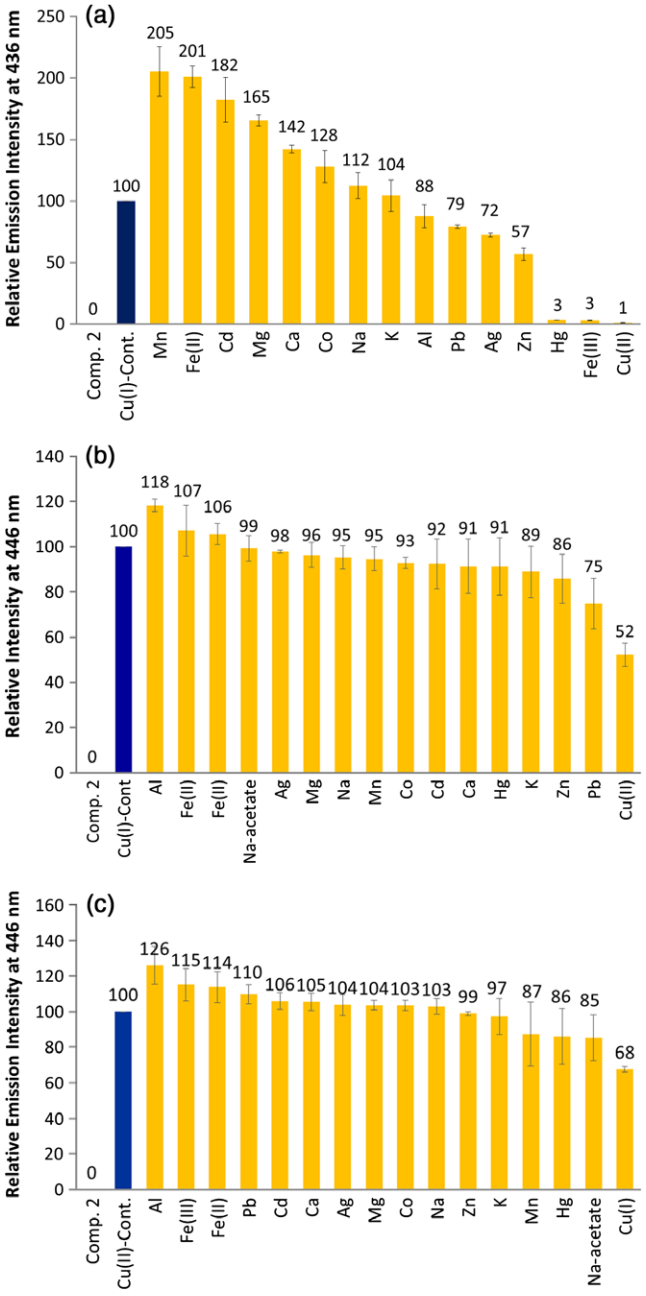

Figure 4. Interference experiments with compound **2** (1 equiv, 10 μM) with 15 kinds of metal ions (5 equiv) in the presence of (a) Cu^{+} (5 equiv) in 100% acetonitrile, (b) Cu^{+} (5 equiv) in 50% acetonitrile in H_2O and (c) Cu^{2+} (5 equiv) in 50% acetonitrile in H_2O. (a) λ_{ex} = 332 nm, slit width (EX: 5 nm, EM: 5 nm), ((b) and (c)) λ_{ex} = 332 nm, slit width (EX: 3 nm, EM: 3 nm). Measurements were acquired three times, and error bars indicate standard deviations.

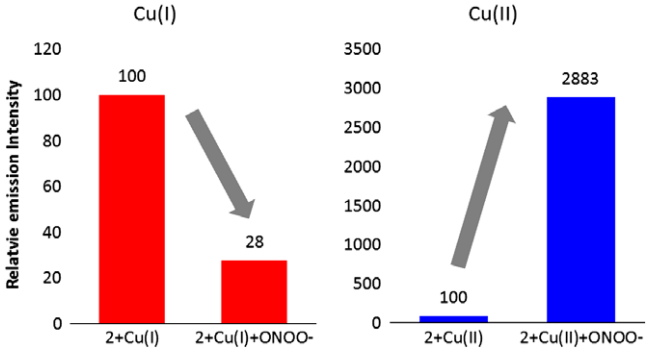

Figure 5. Profound effect of peroxynitrite on the emission intensity by presence of Cu^{+} and Cu^{2+} in acetonitrile solvent. λ_{ex} = 332 nm, slit width (EX: 5 nm, EM:5 nm) (Figure S9).

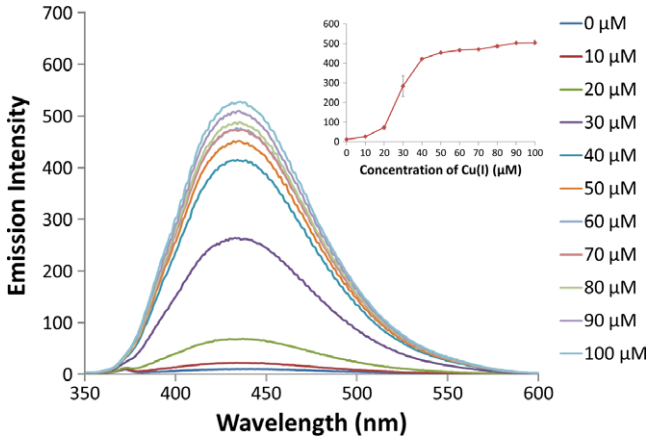

Figure 6. Titration of compound **2** (10 μM, 1 equiv) with copper(I) acetate. Emission spectra are shown in the range of 350 to 600 nm; (inset) emission intensity at 436 nm. λ_{ex} = 332 nm, slit width (EX: 5 nm, EM:5 nm), Solvent: acetonitrile. Repeated three times. Error bar means standard error.

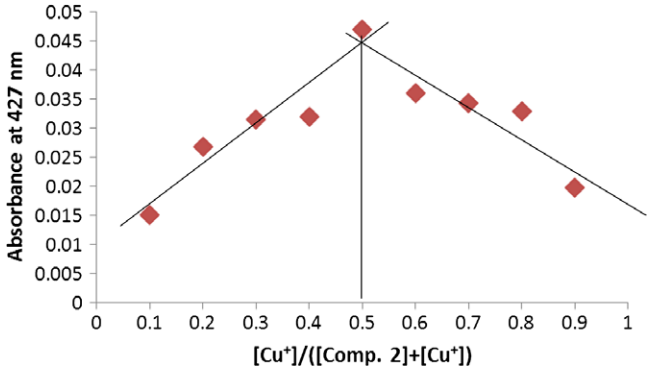

Figure 7. Job plot of compound **2** with Cu^{+}. λ_{ex} = 332 nm, Slit width (EX: 5 nm, EM: 5 nm), Solvent: acetonitrile. Total concentration: 22 μM.

however, no clear isosbestic point was observed over a value of 4 equiv (Figure S10). As shown in Figure 6, the fluorescence intensity increased in a sigmoidal, not linear, fashion. Interestingly, a large variation was shown at 3 equiv of Cu^{+}, indicating a potential breaking point where the structure of the probe may be adapted for sensing of Cu^{+} (Figure 6). Job analysis using fluorescence showed that multiple binding is to be expected, *e.g.*, 1:2–1:4 (Figure S11). However, the binding ratio at low concentration near 20 μM showed a 1:1 value as a total concentration using UV–visible absorbance (Figure 7).

Although there is one main binding pocket observed in the structure of compound **2** shown in Scheme 1, an additional nitrogen, oxygen, and sulfur atom may bind to Cu^{+} allowed by way of a C_{Ar}—C_{Ar} bond rotation, or direct binding, which provides multiple binding possibilities between the probe and metal ions (Figure S16). In particular, the nitrogen atoms in an imine group or in secondary or tertiary amines are important sites commonly involved with PET, which is important to fluorescence turn–OFF behavior".[2,25,26] For these reasons,

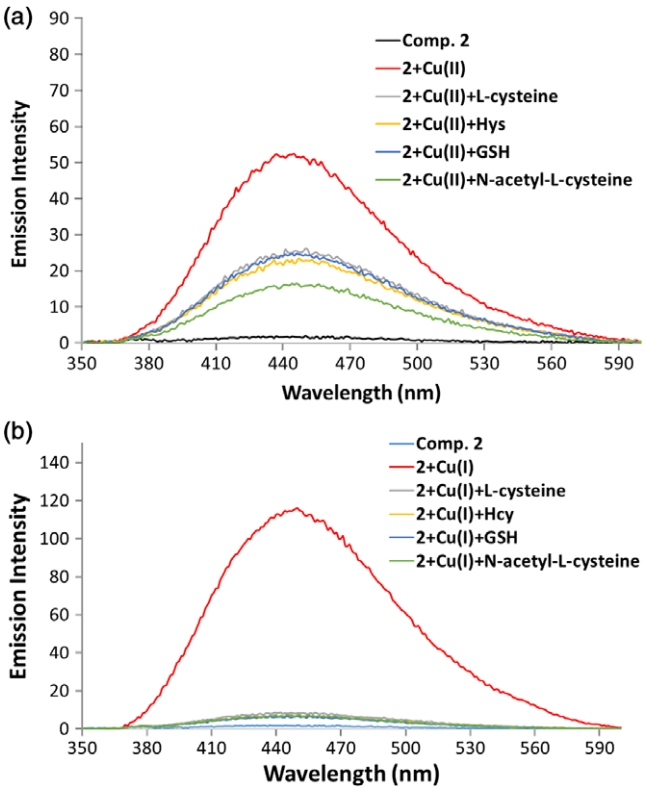

Figure 8. Reversibility of compound **2** (10 μM, 1 equiv) with Cu^{+} (5 equiv) (a) and Cu^{2+} (5 equiv) (b) by biothiols (5 equiv) in 50% (v/v) acetonitrile in H_2O. λ_{ex} = 332 nm, slit width (EX: 3 nm, EM: 3 nm). Hcy: DL-homocysteine, GSH: glutathione (reduced).

1:1 binding stoichiometry was predicted at low concentration because of the design involving one main binding pocket. However, a multi-binding mode, mainly 1:2 after 60 min of incubation, can be shown at high concentration, which implies that an additional binding site is affected by excess Cu^{+} binding, which in turn we propose as related to the PET mechanism.[15,16,25,27]

Reversibility of Cu^{+} and Cu^{2+} by Biothiols, Signal in 50% Acetonitrile in H_2O. In a probing system, reversibility is important for the recovery of the probe and for continuous measurement. For reversibility testing, we used the biothiols L-cysteine, DL-homocysteine (Hcy), reduced glutathione (GSH), and *N*-acetyl-L-cysteine.

Some previous studies reported the affinity between metal ions and R-SH.[28–30] In acetonitrile, there was no reversibility (Figure S12). However, in acetonitrile: H_2O (50:50, v/v), almost complete reversibility was shown for the Cu^{+}-induced fluorescence (Figure 8(a)) by four different biothiol species. This reflects, first, the robustness of the ligand, and second, the likely role that coordinated acetonitrile solvent molecules play, which is important for reversibility; acetonitrile coordination seems more inert than H_2O binding in this probing system. In addition, Cu^{2+}-induced emission exhibited partial reversibility: a near 50% emission decrease could be observed but still the emission was retained (Figure 8(b)). This may be

due to the softness of Cu^{+}, which induces better affinity to biothiols than Cu^{2+}. However, here, we cannot rule out ligand oxidation as a possible route upon Cu^{2+} binding.

Conclusion

Owing to the paramagnetic effect of Cu^{2+} and the disproportionation capacity of Cu^{+}, probe design for discerning Cu^{+} and Cu^{2+} is challenging. From previously reported studies, there are very few examples of fluorescence "turn-on" and differentiation probes for Cu^{+} and Cu^{2+} involving the same chemical system. Here, we synthesized a simple yet interesting benzothiazole Schiff base compound from a chemosensing standpoint. Solutions of 16 different metal ions were screened; exhibition of strong fluorescence sensitivity for Cu^{+} (51-fold) at 436 nm in 100% acetonitrile was detected. In 50% (v/v) acetonitrile in H_2O, both fluorescence for [Cu^{+}] and [Cu^{2+}] were shown at 446 nm. In 100%, a very weak nonselective fluorescence was observed. When the volume ratio of acetonitrile/H_2O was increased from 0.0 to 1.0, a 30 nm solvatochromic blue shift was seen. In addition, whereas Cu^{2+} showed linearity, Cu^{+} exhibited a discontinuous emission trend assigned to metal solvent coordination. Time-dependent emission measurements showed an exponential decay curve (τ_1 = 2.99 × 10^3 s, 10 equiv of Cu^{+}, R^2 = 0.999) and a linear, stable decay line (slope = –0.0216, 5 equiv Cu^{+}, R^2 = 0.996). Interference experiments in 50% (v/v) acetonitrile in H_2O showed that the emissions by Cu^{+} and Cu^{2+} were not disturbed by other metal ions, acidity, or basicity, which means that the total amount of Cu^{+} and Cu^{2+} can be measured by this probing system. While ROS generally did not interfere with the emission by Cu^{+} in acetonitrile solvent, peroxynitrite did decrease the emission arising from [Cu^{+}] (78%); thus, an increase in Cu^{2+}-based fluorescence (28-fold) forms a toggle. Titration and job analysis suggest that multi-site binding modes may exist between the probe and Cu^{+} arising from the probe structure containing many nitrogen and oxygen atoms (Supporting Information). Biothiols, when added, showed almost complete reversibility of the Cu^{+}-induced emission in acetonitrile:H_2O (50%:50%, v/v), relative to the partial reversibility of Cu^{2+}-generated emission. Thus, by regulating the ratio between acetonitrile and H_2O, Cu^{+} could be probed selectively. Both oxidation states of copper could be measured with one small synthetic system.

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Supporting Information. Additional supporting information is available in the online version of this article.

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