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A Highly Selective Reaction-Based Two-Photon Probe for Copper(I) in Aqueous Media

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Dedicated to Professor K. N. Ganesh on the occasion of his 60th birthday

The soft transition metal copper is the third most abundant essential nutrient in the human body. Cu^{+} is the dominant oxidation state rather than Cu^{2+} in a cytosolic reducing environment.[1] It plays a crucial role in a variety of fundamental physiological processes including enzyme functions and transcriptional events.[2] However, alterations in copper cellular homeostasis are connected to severe diseases, such as Menkes and Wilson diseases, Alzheimer's disease, prion disorders, and amyotrophic lateral sclerosis.[3] Therefore, it is crucial to develop novel methods for the detection of this redox-active cation in biological and environmental samples. In general the development of cation-responsive fluorescent probes has been an active research area in recent times owing to a wide range of applications in analytical, environmental, materials, and biological sciences. Fluorescence-based detection methods are more appealing because of their high sensitivity and real-time monitoring of exchangeable metal ions in living cells.[4] Although several efficient fluorescent probes have been reported for Cu^{2+}, probes for monitoring intracellular free copper, that is, Cu^{+}, are rare.[5] The major hurdle in developing fluorescent probes is the redox-active copper-ion-assisted fluorescence quenching process. This problem has been partially addressed by incorporating a suitable spacer between the cation binding site and the fluorophore.[6] These probes show cation-dependent fluorescence response based on a photoinduced electron transfer (PET) mechanism. However, most of the Cu^{+}-selective PET probes display incomplete recovery of fluorescence quantum yield relative to the corresponding isolated fluorophores. Thus, it is necessary to develop an ideal fluorescent probe for the detection of copper in bioavailable form under physiologically reducing conditions.

We and others have reported a reaction-based approach to overcome this fluorescence recovery problem.[7] Taki et al.[7a] and Chang et al.[7b] have developed reaction-based probes for Cu^{+} and Co^{2+} detection by linking tetradentate ligands termed N_4 (tripicolylamine)and N_3O with fluorescein-type dye, respectively. The design strategy comprised selective cation-mediated bond-cleaving reaction to transform a nonfluorescent probe into a highly fluorescent dye and detached paramagnetic metal-ion-bound ionophore unit. We sought to broaden the scope of reaction-based molecular probe design for paramagnetic metal ions by using a different fluorescent reporter dye in the probe. Our design approach also incorporates more than one metal-binding unit to enhance the sensitivity of the reaction-based probes towards the metal ion of interest.

Furthermore, the short ultraviolet excitation wavelength used in commonly employed one-photon fluorescence spectroscopy would adversely stimulate interfering absorbance, scattering, and background autofluorescence generated from endogenous biomolecules. However, two-photon fluorescence spectroscopy (TPS) and microscopy techniques are particularly useful to overcome these drawbacks.[8] Typically, one-photon fluorescence spectroscopy uses a single photon to excite a fluorophore into its excited state, whereas TPS uses two photons of much lower energy to generate a fluorophore excited state.[9] Recently, TPS has gained much attention because it offers a number of advantages, such as less photodamage of samples, low background absorption and scattering, improved spatial resolution and sensitivity, and the ability to image thicker specimens.[10] Thus, the design of fluorescent probes with optional two-photon excitation capability is an added advantage.

Herein, we report a reaction-based two-photon excitable Cu^{+} probe (**XanCu**, see Scheme 1) that combines two fluorescent reporter units with pendant tetradentate ligands through cleavable benzyl ether linkages. We selected blue fluorescent dye xanthone as a reporter and tripicolylamine (N_4) ligands as metal-binding units in our reaction-based nonfluorescent molecular probe. The N_4 ligand is known to be selective for the Cu^{+}-mediated benzylic ether bond-cleavage reaction to generate free fluorescent reporter dye from a nonfluorescent probe under aerobic conditions. The N_4 ligand was obtained in good yield by following the synthetic procedure reported in the literature.[11] The **XanCu** probe was synthesized by the O-alkylation of two hydroxyl functionalities on 3,6-dihydroxyxanthone (Xan) with two equivalents of tripicolylamine chloride (N_4-Cl) under basic conditions in excellent yield (Scheme 1).

We studied the fluorescence properties of **XanCu** in aqueous buffer solution (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.2) in the presence of 2 mM glutathione (GSH) for mimicking intracellular environments. The fluorometric behavior of 1.0 μM **XanCu** was investigated with the addition of several metal ions, such as Li^{+}, Na^{+}, K^{+}, Ba^{2+},

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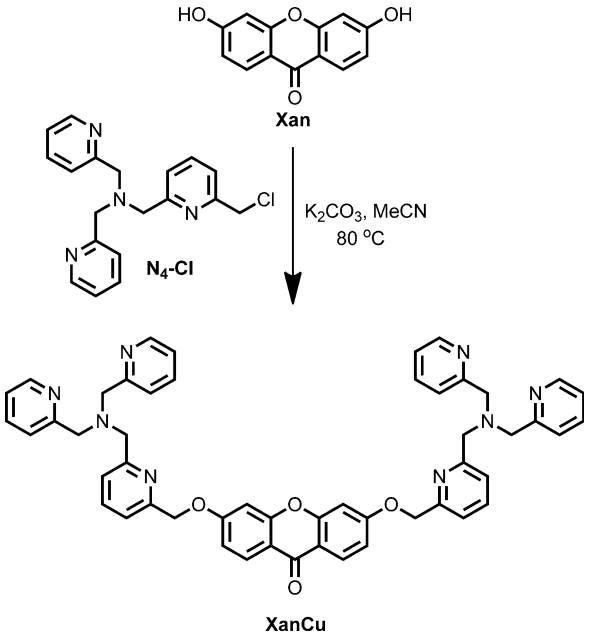


Scheme 1. Synthesis of **XanCu**.

Mg^{2+}, Ca^{2+}, Al^{3+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Ag^{+}, Hg^{2+}, and Pb^{2+}, after 2 h of mixing (Figure 1). Upon excitation at 350 nm **XanCu** was found to be nonfluorescent (quantum yield $\Phi = 0.0088$). The examined millimolar concentrations of alkali and alkaline earth metals had no effect on

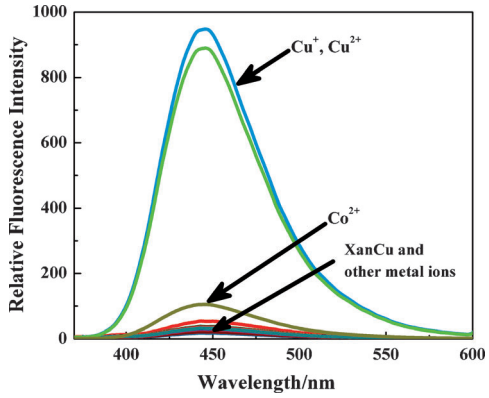


Figure 1. Fluorescence responses of **XanCu** (1.0 μM) upon addition of 1 mM Li^{+}, Na^{+}, K^{+}, Ba^{2+}, Mg^{2+}, Ca^{2+}, and Al^{3+} and 20.0 μM Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Ag^{+}, Hg^{2+}, and Pb^{2+} after 2 h in aqueous solution (50 mM HEPES, pH 7.2, 2 mM GSH; $\lambda_{ex} = 350$ nm).

the fluorescence behavior of **XanCu**. On testing with copper ions, it showed a remarkable approximately 30-fold enhancement in the blue fluorescence emission positioned around 445 nm (quantum yield $\Phi = 0.265$). This significant "switch-on" fluorescence phenomenon is identical for both Cu^{+} and Cu^{2+} ions as GSH (2 mM) rapidly reduces Cu^{2+} to Cu^{+}. The switch-on emission around 445 nm indicates that Cu^{+} reacts with **XanCu** and cleaves the benzyl ether (C–O) linkages in the presence of O_2 releasing blue-emitting phenolic xanthone dye

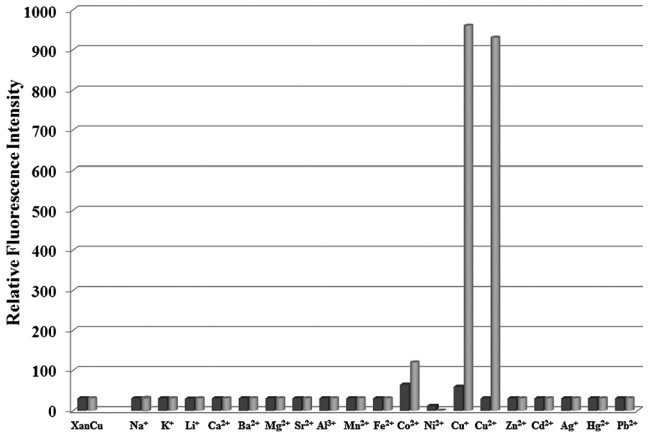


Figure 2. Fluorescence responses of **XanCu** (1.0 μM) in 50 mM HEPES buffer (pH 7.2). The bars represent the fluorescence intensity at 445 nm after 2 h of reaction of 1.0 μM **XanCu** with different metal ions (20.0 μM) in the absence (dark gray bars) or presence (light gray bars) of 2 mM GSH ($\lambda_{ex} = 350$ nm).

(Xan). Cu^{2+} did not show a switch-on fluorescence response in the absence of GSH (Figure 2), whereas Cu^{+} exhibited minimal response as it undergoes spontaneous oxidation to Cu^{2+} in the absence of GSH. Other transition-metal ions except Co^{2+} caused no significant change in the baseline fluorescence intensity (Figure 2); Co^{2+} showed a minimal response in the emission intensity both in the presence and absence of GSH. However, Cu^{+} detection was unaffected by the presence of Co^{2+} as confirmed by a competitive study (Figure S1 in the Supporting Information). We treated 1 μM **XanCu** probe solution containing 2 mM GSH with 20 μM Cu^{+} in the presence and absence of Co^{2+}. The fluorescence signal intensity was unaltered, which indicated the superior selectivity of the probe towards Cu^{+}. Time-dependent study showed that the benzyl ether bond-cleavage reaction in **XanCu** and subsequent detection of Cu^{+} is more rapid than that in previous reports (Figure 3).[7a,c] After addition of Cu^{+} the blue emission reaches a maximum within 30 min. Concentration-dependent study showed that submicromolar levels of Cu^{+} could be detected efficiently as this amount is sufficient to release phenolic xanthone dye (Xan) quantitatively from **XanCu** (1.0 μM) through

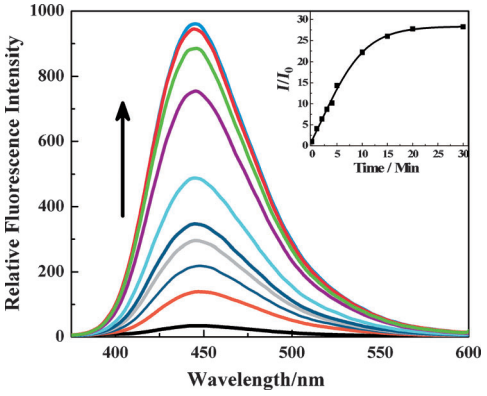
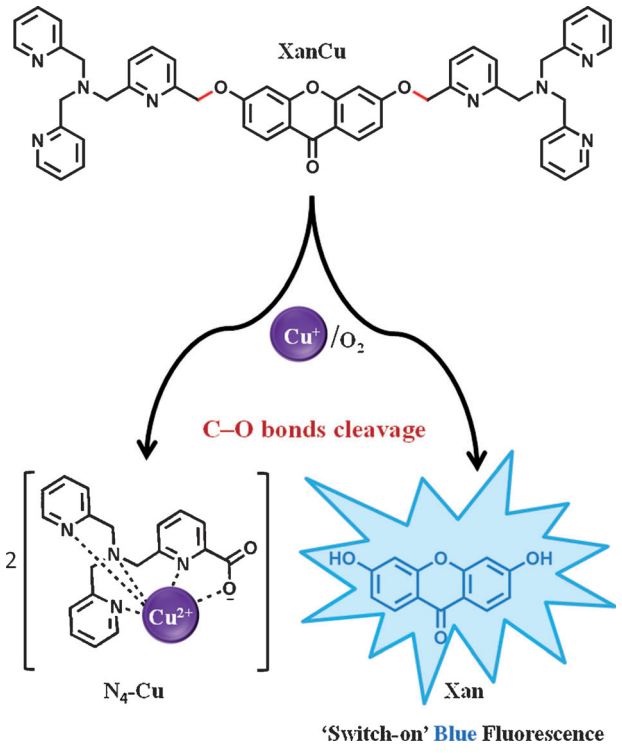


Figure 3. Time-dependent fluorescence responses of **XanCu** (1.0 μM) upon addition of 20.0 μM Cu^{+} in aqueous solution (50 mM HEPES, pH 7.2, 2 mM GSH; $\lambda_{ex} = 350$ nm). Inset: relative emission intensity I/I_0 versus time.

benzyl ether bond-cleavage reaction (Figure S2). Notably, each molecule of the **XanCu** probe contains two tripicolylamino units and therefore can react with two Cu^+ ions.

The effect of pH on the Cu^+-mediated benzyl ether (C–O) bond cleavage was studied to understand the utility of the probe in various pH ranges (Figure S3). **XanCu** reacted efficiently with Cu^+ in the biologically relevant pH range of 6.5–8.5 to release blue-emitting xanthone fluorophore (Xan). Thus, **XanCu** can be used conveniently as a switch-on probe for the detection of Cu^+ without interference from pH-related effects in physiological media.

The ESI–MS data confirmed the benzyl ether bond-cleavage reaction in **XanCu** to release Xan reporter and the copper complex of oxidized N_4 ligand (N_4-Cu; Figure S4). Mass peaks found at *m/z* 397.2 and *m/z* 229.2 correspond respectively to carboxylated N_4-Cu complex (calcd *m/z* 396.06 for $C_{19}H_{17}CuN_4O_2$) and Xan fluorophore (calcd *m/z* 228.04 for $C_{13}H_8O_4$) released from **XanCu**. The Cu^+-catalyzed oxidative benzyl ether bond-cleavage reaction in **XanCu** to release free Xan reporter dye and metal-ion-bound carboxylated N_4 ligands (N_4-Cu) is shown in Scheme 2. This cleavage occurs probably through a proposed mechanism based on the metal-ion-mediated C–N/C–O bond-cleavage reactions reported in the literature.[7b,c,12] The benzylic carbon atoms of the N_4 ligands were oxidized to benzylic radicals (**I**) in the presence of Cu^+ and activated oxygen. The biradical intermediate (**I**) transformed to bis-oxonium ion (**II**), which hydrolyzed to phenolic xanthone dye (Xan) and two equivalents of tripicolylamino-aldehyde. The tripicolylamino-aldehyde intermediate was subsequently oxidized to tripicolylamino-carboxylate by the hydroperoxide of the metal complex through a Baeyer–Villiger-type reaction to form two equivalents of N_4-Cu complex (Figure S9).[13]



Scheme 2. Cu^+/O_2-mediated benzylic ether bond (C–O) cleavage in **XanCu** to release blue fluorescent hydroxyxanthone (Xan) dye and two equivalents of N_4-Cu complex.

After establishing the utility of **XanCu** as a switch-on fluorescent probe by one-photon spectroscopy, we explored the use of **XanCu** for probing Cu^+ by TPS. The two-photon action spectrum of **XanCu** in the presence of Cu^+ showed the maximum two-photon action cross section value of 2.23 GM at 700 nm in 20 mM HEPES buffer (pH 7.2) containing 2 mM GSH (Figure 4). At two-photon excitation the probe showed sufficient fluorescence enhancement in the presence of Cu^+ and can potentially be used as a two-photon excitable sensor for

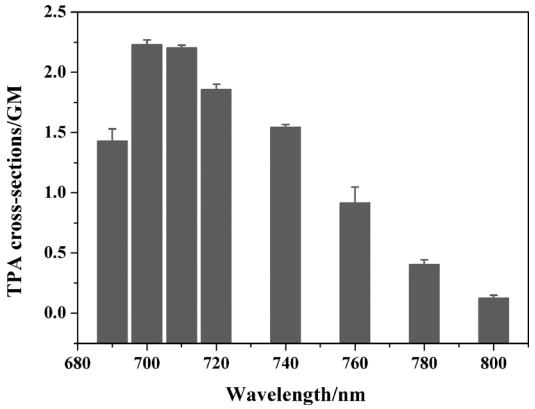


Figure 4. Two-photon action (TPA) cross sections obtained from a solution of 20 μM **XanCu** in the presence of 400 μM Cu^+ with fluorescein at pH 13 as a calibrant. The values correspond to the mean ± standard error of the mean from three independent measurements.

Cu^+. Two-photon excited fluorescence is expected to show a quadratic dependence on the excitation power. To confirm this, fluorescence was recorded for **XanCu** in the presence of Cu^+ at 690 nm on varying the power from 40 to 200 mW at the back aperture. Figure S10 shows the log–log plot of fluorescence obtained as a function of excitation power. The data can be fitted well by a straight line with a slope of 1.93 ± 0.04 (from the first five points), which is close to the value of 2 expected for two-photon excitation. The deviation from linearity at higher power shows saturation and the saturation power is estimated to be approximately 125 mW.

In conclusion, we have successfully developed a **XanCu** fluorescent probe for selective detection of Cu^+ under physiologically reducing conditions. Cu^+-catalyzed oxidative benzylic ether (C–O) bond cleavage releases hydroxyxanthone (Xan) reporter dye from the probe with an approximately 30-fold enhancement in the fluorescence intensity. Thus, **XanCu** can effectively serve as a "switch-on" fluorometric probe for the detection of redox-active paramagnetic copper ions. Furthermore, we also demonstrated the utility of **XanCu** as a two-photon fluorescent probe for the detection of Cu^+. Thus, the **XanCu** probe can be used to track intracellular copper ions and could inspire the development of reaction-based probes for other paramagnetic metal ions.

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