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# Development of Highly Sensitive Fluorescent Probes for Detection of Intracellular Copper(I) in Living Systems

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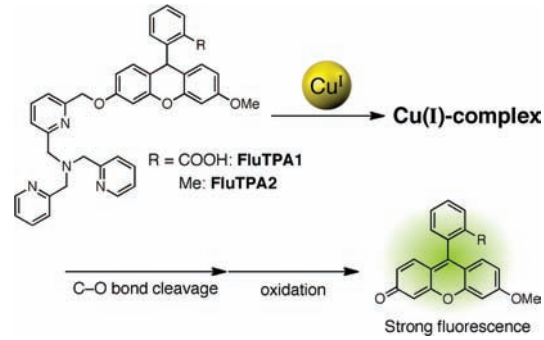
Copper is the third most abundant transition metal in the human body; because of its redox-active nature ($Cu^{I}/Cu^{II}$), it serves as an essential cofactor for a variety of enzymes, including tyrosinase, cytochrome *c* oxidase (C*c*O), and Cu/Zn superoxide dismutase (SOD1), in all living organisms.[1] However, when copper is present in excess, its redox properties turn it into a biological hazard because of its ability to generate reactive oxygen species (ROS), which interfere with cellular metabolism.[1] Sophisticated regulatory mechanisms are available for maintaining a critical balance between the necessity of copper and its toxicity at both the organ and cellular levels;[2] therefore, disruption of copper homeostasis in cells results in severe disorders such as Menkes syndrome,[3] Wilson's disease,[4] amyotrophic lateral sclerosis,[5] and Alzheimer's disease.[6] However, many questions about the trafficking mechanisms, subcellular localization, and quantification of copper in living cells remain unanswered.

Optical imaging with fluorescent probes has been an efficient approach for answering questions about how copper ions function in biological systems.[7] Although a number of satisfactory fluorescent probes for transition metals such as zinc,[8] cadmium,[9] and mercury[10] have been developed over the last few decades, relatively few copper-selective probes have been reported.[11,12] Among these probes, membrane-permeable chemosensors that allow monitoring of intracellular copper with a microscope are rare. Because $Cu^{I}$ rather than $Cu^{II}$ is the dominant oxidation state in a cytosolic reducing environment,[11a] $Cu^{I}$-selective probes, ideally with a turn-on response in fluorescence intensity, are superior for cell imaging.[11a,b] However, most of the reported chemosensors for $Cu^{I}$ have limitations such as a turn-off response, high background fluorescence, and the requirement that two molecules have appropriate concentrations for fluorescence enhancement. Herein, we report the new copper-selective fluorescent probes FluTPA1 and FluTPA2 (Scheme 1) in which the tetradentate ligand tris[(2-pyridyl)-methyl]amine (TPA) is connected to a reduced form of a fluorescein platform through a benzyl ether linkage. We have found that the C−O bond of the benzyl ether of FluTPA1 is selectively cleaved by the reaction with $Cu^{I}$ under physiological reducing conditions and also observed that the fluorescence emission is enhanced significantly. We have further demonstrated that the membrane-permeable probe FluTPA2 reacts with intracellular $Cu^{I}$ and exhibits bright fluorescence in living systems.

All of the spectroscopic measurements on FluTPA1 were performed in an aqueous buffer solution (50 mM HEPES, pH 7.20) containing 2 mM glutathione (GSH), which is the most abundant cellular thiol compound (0.5−10 mM in cells),[13] to simulate intracellular environments. FluTPA1 does not exhibit absorption

***Scheme 1***




in the visible region and has negligible fluorescence; these behaviors remained unchanged for several days under aerobic conditions, indicating that the reduced structure of FluTPA1 can exist stably under physiological conditions. When 20 $\mu$M $Cu^{I}$ was added to the solution of FluTPA1 at room temperature, a significant increase (more than 100-fold) in the emission intensity was observed (Figure 1a). HPLC and electrospray ionization mass spectrometry (ESI-MS) of the reaction mixture revealed that 3′-*O*-methylfluorescein (OMF, $\Phi = 0.37$)[14] was the only fluorescent product, and its yield was determined to be ∼70% after reaction for 2 h. This indicates that the benzyl ether linkage was cleaved and the resulting reduced form of "deprotected" fluorescein was easily air-oxidized to afford OMF, even in the presence of submillimolar GSH. Interestingly, the ESI-MS spectrum of the reaction mixture revealed a set of peaks at *m*/*z* 395.8 (Figure S4 in the Supporting Information) with an isotope distribution pattern calculated for the copper(II) complex having an oxidized ligand of TPA, $[Cu^{II}(TPA-COO^{-})]^{+}$. Thus, it can be suggested that the benzyl radical of the TPA ligand is generated in this C−O bond cleavage reaction.[15] We are currently in the process of elucidating the reaction mechanism involving copper ions. One of the possibilities we are considering is that the copper-active oxygen species is generated as the reactive intermediate.[16] Indeed, when the monodentate probe FluPy, in which the TPA ligand of FluTPA1 was replaced with a 2-pyridylmethyl group, was employed as the control compound, no emission increase for $Cu^{I}$ was observed. In addition, external oxidants such as $H_2O_2$, hypochlorite, and hydroxyl radical did not cause fluorescence from this probe (Figure S5). These results support the idea that C−O bond cleavage of the probe proceeded in an intramolecular manner through the formation of a $Cu^{I}$−FluTPA1 complex with the tetradentate TPA moiety.

The significant fluorescence enhancement of FluTPA1 is specific for copper ions (Figure 1b). Because $Cu^{II}$ is rapidly reduced by GSH to provide the $Cu^{I}$ oxidation state, the fluorescence intensity for $Cu^{II}$ is as high as that observed for $Cu^{I}$. In fact, in the absence

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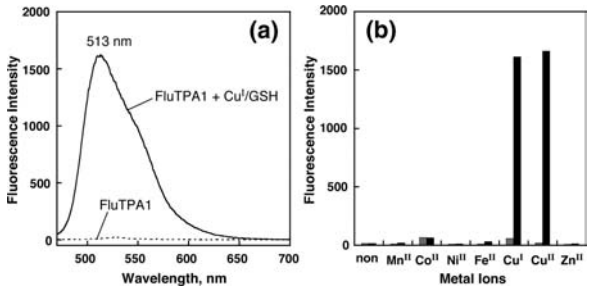


***Figure 1.*** (a) Fluorescence response of 1 $\mu$M FluTPA1 before (dotted line) and after (solid line) reaction with 20 $\mu$M $[Cu^{I}(CH_3CN)_4]PF_6$. The solid spectrum was recorded after 2 h of reaction of FluTPA1 with $Cu^{I}$ in 50 mM HEPES (pH 7.20) containing 2 mM glutathione (GSH). The excitation wavelength was 470 nm. (b) Metal ion selectivity of FluTPA1 in 50 mM HEPES buffer (pH 7.20). The bars represent the fluorescence intensity at 513 nm after 2 h of reaction of 1 $\mu$M FluTPA1 with each type of metal ion (20 $\mu$M) in the absence (gray bars) or presence (black bars) of 2 mM GSH.

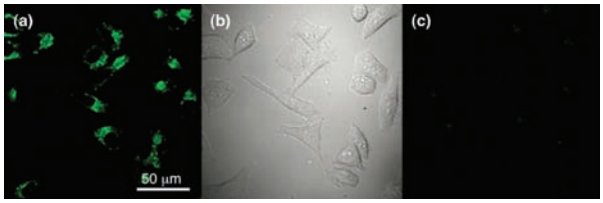


***Figure 2.*** (a) Confocal fluorescence image of HeLa cells supplemented with 100 $\mu$M $CuCl_2$ for 8 h and then further incubated with 5 $\mu$M FluTPA2 for 3 h at 37 °C. (b) Bright-field transmission image of the cells shown in (a). (c) Confocal fluorescence image of FluTPA2-loaded cells grown in a basal medium.

of GSH, $Cu^{II}$ did not show the fluorescence response. Other heavy metal ions apart from $Co^{II}$ caused no discernible change in the emission intensity. Only $Co^{II}$ caused small enhancements in fluorescence intensity both in the presence and in the absence of GSH; however, these enhanced intensities were still much lower than those for copper ions.

The spectroscopic features as well as high selectivity of FluTPA1 for $Cu^{I}$ are satisfactory for practical in vivo applications. However, this probe was membrane-impermeable because of the hydrophilicity of the carboxyl group.[17] Therefore, for application to living cells, we synthesized FluTPA2, which has a hydrophobic character; this probe is based on the TokyoGreen scaffold developed by Urano, Nagano, and co-workers.[18] To examine the performance of FluTPA2, experiments were performed as follows. HeLa cells were incubated with a various concentrations of $CuCl_2$ (20−200 $\mu$M) in the growth medium for 8 h at 37 °C, washed with phosphate-buffered saline containing 200 mM EDTA to remove extracellular $Cu^{II}$, and further incubated with 5 $\mu$M FluTPA2 for 3 h. A strong fluorescence signal with a significantly high signal-to-noise ratio was observed in the $Cu^{II}$-supplemented cells (Figure 2a and Figure S8), whereas no detectable increase in fluorescence was observed in cells grown in a basal medium (Figure 2c). These experiments clearly demonstrate that FluTPA2 is membrane-permeable and can react with intracellular $Cu^{I}$ to afford the highly fluorescent xanthene dye, which means that the C−O bond cleavage reaction with $Cu^{I}$ and the subsequent air-oxidation process as described for FluTPA1 also occur for FluTPA2 inside living cells.

In conclusion, we have developed the new copper-selective fluorescent probes FluTPA1 and FluTPA2 and applied the latter one for visualizing $Cu^{I}$ present in living cells. From the results of this study, it is obvious that only $Cu^{I}$ is the key metal ion that induces the debenzylation reaction of FluTPA probes. Further studies of the reaction mechanism of the fluorogenic process are in progress.

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**Supporting Information Available:** Synthesis and characterization of organic compounds, ESI-MS data, confocal images, and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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