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A fluorescein derivative for nanomolar aqueous copper and monitoring copper ion uptake by transferrin and amyloid precursor protein

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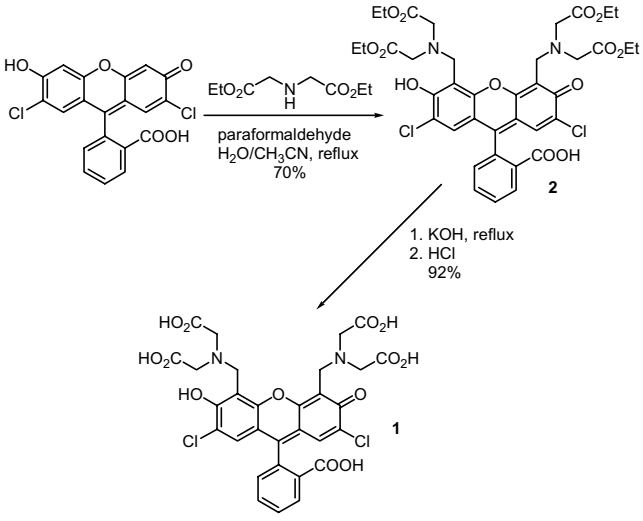
Abstract—A new fluorescent chemosensor based on the fluorescein derivative effectively recognizes Cu^{2+} in nanomolar range at pH 7.4. The Cu^{2+} ion uptake by transferrin and amyloid precursor protein was monitored using the title fluorescent chemosensor.

Fluorescent chemosensors for the detection and measurement of metal ions, especially for cations with biological interest such as Na^{+}, Ca^{2+}, Cu^{2+}, and Zn^{2+}, are actively investigated.[1] In particular, Cu^{2+} is the third in abundance among the essential heavy metal ions in the human body and plays an important role in various biological processes.

Especially, copper is known to play a crucial role in a number of neurodegenerative diseases, such as Alzheimer disease and Wilson's disease. In this regard, copper binding studies in β-amyloid peptide,[2] human- and bovine serum albumins[4] as well as transferrin (Tf)[3d,4] have been actively reported. The serum iron transport protein, Tf, which is known to bind Cu^{2+} and the Cu^{2+}–Tf complex characterized by a $\log K$ value of 12.3.[4b] Also, it is reported that the residues 135–155 of the cystein-rich domain of APP (amyloid precursor protein), a protein highly implicated in Alzheimer's disease, are participating as Cu^{2+} binding site.[2a] Accordingly, the design and synthesis of fluorescent chemosensors for copper ions have become a very active area of research.[1d,5] Calcein especially has been widely used for the various metal ion binding studies including Cu^{2+}.[6]

We report herein a new fluorescein derivative, which can effectively recognize Cu^{2+} in 100% aqueous solution. Also, the Cu^{2+} ion uptake by transferrin and amyloid precursor protein was monitored using the title fluorescent chemosensor.

A diethyl iminodiacetate fluorescein (**2**) was synthesized through a Mannich reaction between 2′,7′-dichlorofluorescein and the iminium ion condensation of product of formaldehyde and diethyl iminodiacetate. The tetra acid **1** was produced in 92% yield by hydrolysis with potassium hydroxide in water (Scheme 1). The product was



Scheme 1. Synthesis of fluorescent chemosensor **1**.

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fully characterized by ^{1}H NMR, ^{13}C NMR, and high resolution FAB mass spectroscopy. The experimental details and characterization data are explained in the supplementary data.

Ca^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Ni^{2+}, and Zn^{2+} ions were used to evaluate the metal ion binding properties of **1**. All fluorescent studies were conducted at pH 7.4 (20 mM HEPES) and using a 1 μM concentration of compound. The fluorescence emission changes of **1** upon the addition of various metal ions (100 equiv) are illustrated in Figure 1. The fluorescence spectra were obtained by excitation into the fluorescein fluorophore at 505 nm, and both the excitation and emission slits were 1.5 nm. As shown in Figure 1, compound **1** displayed large chelation-enhanced quenching (CHEQ) effects with Cu^{2+} and Ni^{2+}. A small red-shift (~6 nm) with Hg^{2+} and a small blue-shift (~7 nm) with Zn^{2+} were observed. Similar red-shift and blue-shift were also observed for Hg^{2+} and Zn^{2+} in their UV spectra (S-Fig. 4). From the fluorescence titration experiments, the dissociation constants of complex **1** with Cu^{2+} (Fig. 2) and Ni^{2+} (S-Fig. 5) were observed to be 26 nM and 700 nM, respectively (errors <10%).[7] Figure 3 explains the titration curves of **1** with Cu^{2+} and Ni^{2+} using fluorescent changes. The selectivity for Cu^{2+} over

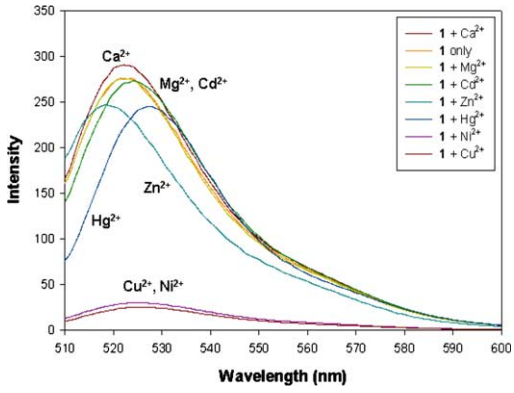

Figure 1. Fluorescence spectra of **1** (1 μM) upon the addition of various metal ions (100 equiv) at pH 7.4 (20 mM HEPES) (excitation at 505 nm).

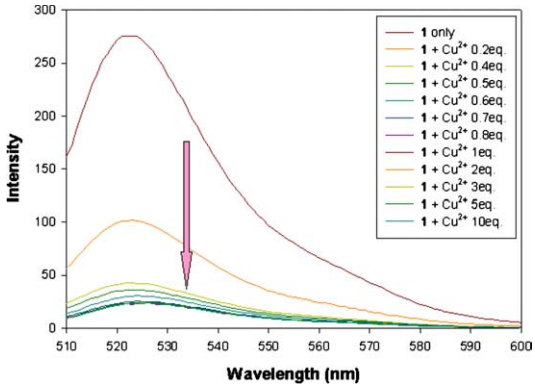

Figure 2. Fluorescent titrations of compound **1** (1 μM) with $Cu(ClO_4)_2$ at pH 7.4 (20 mM HEPES) (excitation at 505 nm).

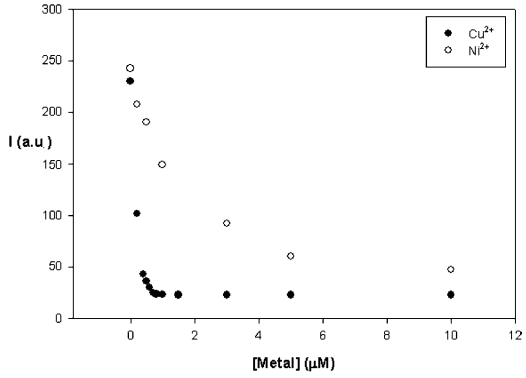

Figure 3. Fluorescent titrations of compound **1** (1 μM) with $Ni(ClO_4)_2$ at pH 7.4 (20 mM HEPES) (excitation at 505 nm and emission at 522 nm).

Ni^{2+} was more than 25 times. As shown in Figure 2, an overall emission change of 10-fold was observed for Cu^{2+}. It is well documented[1d] that when Cu^{2+} binds tightly to the host compound, intracomplex quenching takes place (via energy or electron transfer[8]). To test the further selectivity of the fluoroionophore for Cu^{2+} binding, the fluorescence responses of compound **1** to other metal ions were measured and compared to that of Cu^{2+} ions. Figure 4 explains that emission intensities of **1** with 1 μM Cu^{2+} in the presence of 100 μM of Ca^{2+}, Cd^{2+}, Hg^{2+}, and Zn^{2+} ions were almost the same as that using 1 μM Cu^{2+} alone (S-Fig. 6). Even in the presence of 1 mM of these metal ions, there was not a significant difference in the fluorescence intensities (S-Fig. 6). Furthermore, there was no significant change in the association constant for Cu^{2+} when excess Ca^{2+} (0.1 mM) was present (S-Fig. 7). The job plots using the fluorescence changes indicated 1:1 and 1:2 mixed binding for **1** with Cu^{2+} (S-Fig. 8).

We next examined the feasibility of the biological applications of **1** by using Tf, a model protein for Cu^{2+} uptake.[5a] Figure 5 shows that the fluorescence intensity of **1** is correlated to the concentration of Tf. This tendency arose due to the regeneration of fluorescence of **1** by the Cu^{2+} uptake to Tf instead of **1**, resulting

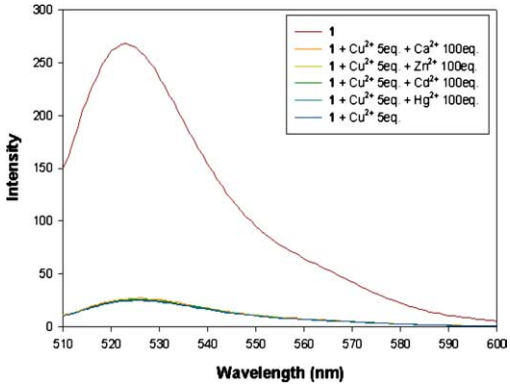

Figure 4. Fluorescence emission spectra of compound **1** (1 μM) with $Cu(ClO_4)_2$ (5 equiv) in the presence of $Ca(ClO_4)_2$, $Cd(ClO_4)_2$, $Hg(ClO_4)_2$, or $Zn(ClO_4)_2$ (100 equiv) at pH 7.4 (20 mM HEPES) (excitation at 505 nm).

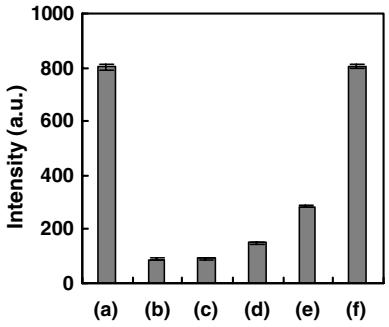

Figure 5. Changes in the fluorescent intensity of **1** upon the addition of Cu^{2+} and Tf (transferrin) at pH 7.4 (20 mM HEPES): (a) **1** (4 μM) only, (b) **1** (4 μM)+Cu^{2+} (4 μM), (c) **1** (4 μM)+Cu^{2+} (4 μM)+Tf (0.1 μM), (d) **1** (4 μM)+Cu^{2+} (4 μM)+Tf (0.5 μM), (e) **1** (4 μM)+Cu^{2+} (4 μM)+Tf (1 μM), (f) **1** (4 μM)+Cu^{2+} (4 μM)+Tf (5 μM) (excitation at 505 nm and emission at 522 nm).

in a positive calibration curve. At 5 μM Tf, **1** fully regenerated the fluorescence quenched by Cu^{2+} ion to the level of intensity, equivalent to the intensity obtained in the absence of Cu^{2+}.

Similar experiments were performed with $APP_{135–155}$ (copper-binding fragment of the human wild-type amyloid precursor protein) as shown in Figure 6. At 50 μM $APP_{135–155}$, **1** fully regenerated the fluorescence quenched by Cu^{2+} ion to the level of intensity, equivalent to the intensity obtained in the absence of Cu^{2+}. The K_d value of $APP_{135–155}$ is reported as 10 μM,[3a] while the Cu^{2+}–Tf complex is characterized by a log K value of 12.3.[5b] Our fluorescent changes upon the addition of Tf or $APP_{135–155}$ are quite consistent with the known affinity of Tf and $APP_{135–155}$.

Fe^{3+} ion can interfere with the Cu^{2+}-selective sensing behavior obtained with Transferrin. In our experiments, in which Cu^{2+} and Fe^{3+} ion at the same concentrations were added together to the mixture of Transferrin and chemosensor **1**, chemosensor **1** was not fully quenched. This seemed to be caused by the competition between Cu^{2+} and Fe^{3+} ions for the metal binding sites of Transferrin. The number of the binding sites on Transferrin previously available for Cu^{2+} became less available for Cu^{2+} by the binding of Fe^{3+} to Transferrin. This explanation can be further supported by the fact that Transferrin can transport both Cu^{2+} and Fe^{3+} ions in the blood.[9]

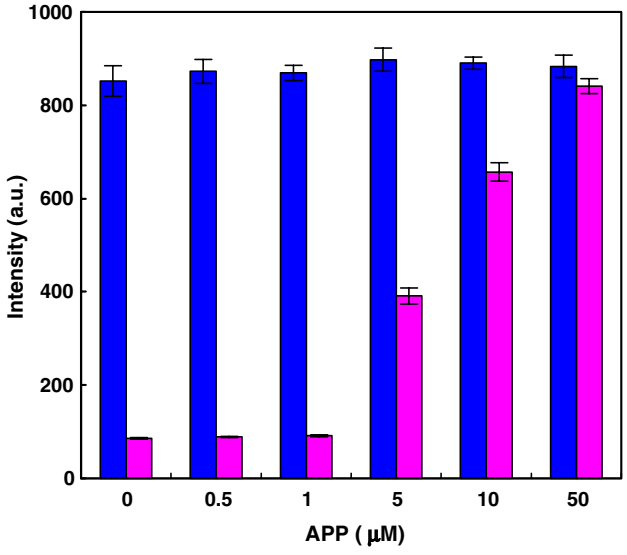

Figure 6. Changes in the fluorescent intensity of **1** upon the addition of Cu^{2+} and $APP_{135–155}$ (amyloid precursor protein) at pH 7.4 (20 mM HEPES); ■: **1** (4 μM)+APP, ■: **1** (4 μM)+APP+Cu^{2+} (4 μM).

In conclusion, we synthesized compound **1** as a fluorescent chemosensor for copper ions, which can be obtained via relatively simple two-step reactions in a moderate yield. It shows a high specificity for nanomolar copper ions at the physiological pH. Furthermore, the usefulness of the title fluorescent chemosensor **1** as a sensor molecule was demonstrated by monitoring Cu^{2+} ion uptake by copper binding proteins such as TF and $APP_{135–155}$, respectively. This highly sensitive Cu^{2+}-selective chemosensor can be suitable for many other biological applications possibly including in vivo experiments.

Acknowledgments

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Supplementary data

Supplementary data (experimental sections including characterizations of **1**, fluorescent spectra, and UV spectra) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2005.12.045.

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