Coumarin-coupled Receptor as a Membrane-permeable, Cu^{2+}-selective Fluorescent Chemosensor for Imaging Copper(II) in HEPG-2 Cell

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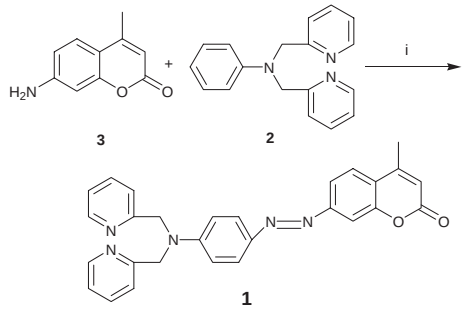
A novel fluorescent chemosensor **1** for imaging labile Cu^{2+} in living biological samples was designed and synthesized; it exhibits very strong fluorescence responses to Cu^{2+}, and its LSM images strongly support the existence of Cu^{2+} in HEGP-2 cell

Among essential heavy metal ions in human body copper is third in abundance.[1] Cells require copper for use in a variety of respiratory and metabolic activites, but alterations in cellular homeostasis are connected to serious neuro-degenerative diseases, including Menkes and Wilson diseases,[2–4] familial amyotrophic lateral sclerosis,[5,6] Alzheimer's disease,[7] and prion diseases.[8] Free copper ions are dangerous to cells owing to their oxidizing potential. The thermodynamically estimated level of free copper in the cytosol of bacterial model systems is less than one ion per cell.[8] So cells exert strict control over intracellular copper distributions.[8–12] Although there is compelling evidence that the intracellular milieu does not contain any free copper ions, the rapid kinetics of copper uptake and release suggests the presence of a labile intracellular copper pool. Yang and co-workers[13] recently provided the first evidence that the labile copper pool appears to be localized in mitochondria and the Golgi region. MicroXANES experiments have confirmed the predominance of low-coordinate, monovalent copper throughout the cell but did not exclude the presence of Cu^{2+}.

Fluorescent chemosensors that can permeate the plasma membrane have proven to be powerful and nondestructive tools for the study of intracellular metal ion distributions of calcium, magnesium, or zinc, yet rigorous analytical techniques for sensitive in vivo measurements of intracellular copper levels are lacking. Some of the currently available copper chemosensors are fairly complex molecules.[14]

Here, to elucidate the presence of Cu^{2+} in the cytosol of the cell, we report the development, characterization, and evaluation of a membrane-permeable copper-selective fluorescent chemosensor for imaging of kinetically labile Cu^{2+}. A novel chemosensor **1** for Cu^{2+} based on photoinduced electron transfer (PET), in which *N*,*N*-bis(pyridin-2-ylmethyl)benzenamine as a receptor group is connected to a coumarin group via a diazo spacer. The coumarin group is chosen as fluorophore, since it has a strong absorption band in the visible region, emits at longer wavelength with high quantum yield and exhibits excellent bioactivity. By attaching an appropriate chelator group to the diazotizated coumarin, we have obtained a novel high-quality Cu^{2+} chemosensor.

Compound **1** originated from *N*,*N*-bis(pyridine-2-ylmethyl)-benzenamine (**2**) (Scheme 1). Compound **2** was coupled at the 4-position (yield = 40%) with diazotized 7-amido-4-methylcoumarin (**3**). Thus, sensor **1** was successfully synthesized via a very short route from inexpensive starting materials. Chemosensor **1**



Scheme 1. Reagents and conditions: $NaNO_2$, HCl, H_2O/DMF, rt.

has advantages over previously available Cu^{2+} chemosensors requiring much more cumbersome synthesis from expensive starting materials. The final compound was characterized by ^1H NMR, ^{13}C NMR, and mass spectrometry (See Supporting Information).[18]

The maximum absorption wavelength of chemosensor **1** is 435 nm, and the maximum emission wavelength is 547 nm. Fluorescence quantum yield of free **1** is 0.003[15] under physiological conditions (pH 7.4, 0.1 M HEPES, 0.1 M KNO_3). Fluorescence is quenched by PET reaction between the receptor and the fluorophore.

Upon addition of Cu^{2+}, the fluorescence intensity of **1** increased by about 7.5-fold, and the corresponding quantum yield increased to 0.026 (see Supporting Information).[18] It is important to point out that the 7.5-fold fluorescence enhancement of **1** is significant, for most previously reported Cu^{2+} chemosensors, the addition of Cu^{2+} caused fluorescence quenching of the fluorophore.[16] Furthermore, maximum fluorescence can be obtained when the ratio of chemosensor and Cu^{2+} is about 1:1

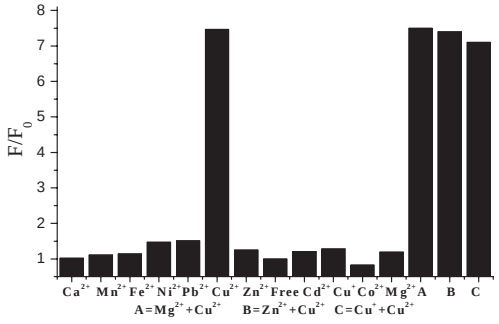


Figure 1. The fluorescence responses of **1** to different metal cations (experimental conditions: 42 μM sensor in DMSO/H_2O = 2:8(V:V), 42 μM metal cation, 100 mM HEPES buffter, 100-mM KNO_3, pH 7.4). F_0 is fluorescent intensity of free **1**, F is fluorescent intensity of **1** and cations.

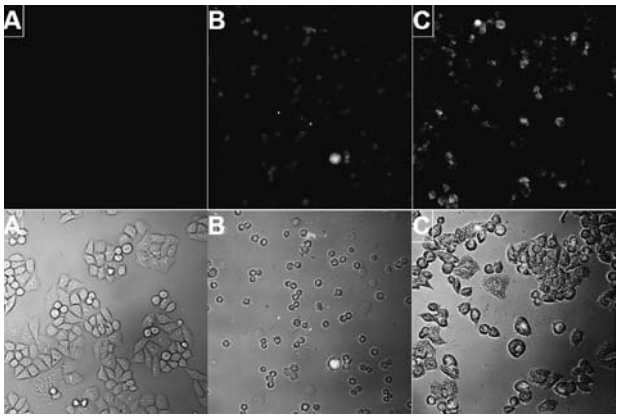


Figure 2. Confocal fluorescence images of HEPG-2 cells. A: Cells incubated in DMEM for 24 h; B: Cells supplemented with 100 μM chemosensor **1** in the growth media at 37 °C for 1 h; C: Cells supplemented with 100 μM $CuCl_2$ in the growth media at 37 °C for 24 h and stained with 100 μM chemosensor **1** for 1 h at 37 °C. Upper: black-field images of live HEPG-2 cells; Lower: black-field and bright-field images of live HEPG-2 were added together.

(see Supporting Information).[18] The result indicates that compound **1** should form a 1:1 complex with Cu^{2+}. Dissociation constant, K_d, between the new chemosensor and Cu^{2+} was determined to be 6.6×10^{-6} M (see Supporting Information).[18]

Our next goal was to check the selectivity of the new chemosensor, we studied the fluorescence response of **1** to other metal cations including Cd^{2+}, Mg^{2+}, Co^{2+}, Ca^{2+}, Fe^{2+}, Cu^{+}, and Mn^{2+}. As shown in Figure 1, Cu^{2+} is the only cation among the tested transition elements that induces fluorescence enhancement.

Emission intensities did not appear to change in the presence of other ions (including Ca^{2+}, Mn^{2+}, Mg^{2+}, Cd^{2+}, Fe^{2+}, Cu^{+}, and Zn^{2+}), except for a little enhancement on addition of Pb^{2+} and Ni^{2+}. This is very nice because under many conditions (e.g., physiological conditions) Fe^{2+} and Zn^{2+} may coexist at relatively high concentrations compared to Cu^{2+}. Thus, our new chemosensor can selectively detect Cu^{2+} under physiological conditions.

We sought to evaluate the ability of **1** to operate within living cells. HEPG-2 cells were incubated in DMEM for 20 min at 25 °C (Figure 2). Supplementing cells with 100 μM $CuCl_2$ growth medium for 24 h at 37 °C and then staining with **1** under the same loading conditions results in a marked increase in observed intracellular fluorescence, as determined from scanning confocal microscopy (LSM) on live samples. To confirm that this fluorescence increase was due to an increase of intracellular Cu^{2+} concentration, we further added TPEN, an intracellular Cu^{2+} chelator (see Supporting Information).[18] This treatment reduced the fluorescence intensity to the initial level. These experiments show that **1** is cell-permeable and can respond to changes in intracellular Cu^{2+} within living cells.

In conclusion, we have synthesized and characterized a membrane-permeable, Cu^{2+}-selective fluorescent chemosensor. Upon addition of Cu^{2+}, the chemosensor exhibits a 7.5-fold emission enhancement and excellent selectivity toward Cu^{2+}. Although the reducing environment of the cytosol is expected to stabilize monovalent copper,[17] the presence of Cu^{2+} in the cytosol of living cells has been suggested. The images of HEPG-2 provide a coherent picture with strong evidence of the existence of the hypothesized labile Cu^{2+}. Because the new chemosensor is based on fluoresence enhancement of fluorophore, compared with the Cu^{2+} fluorescence quenching effect of reported sensors, the new chemosensor offers more practical application in celluar Cu^{2+} imaging and other Cu^{2+} detection fields.

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18 Supporting Information is available electronically on the CSJ-Journal web site, http://www.csj.jp/journals/chem-lett/.

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