Communication

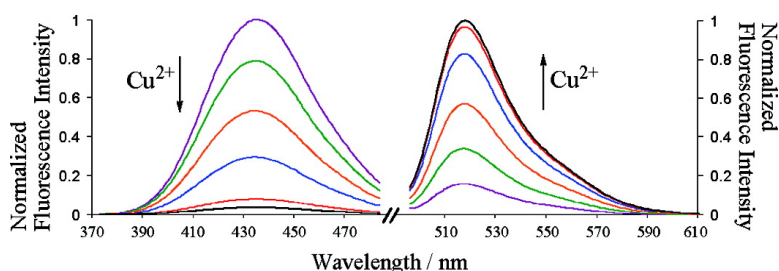# Ratiometric Displacement Approach to Cu(II) Sensing by Fluorescence

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Ratiometric Displacement Approach to Cu(II) Sensing by Fluorescence

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In recent years the development of selective and sensitive fluorescent imaging tools capable of reporting transition metal ions has attracted considerable attention, leading to significant progress in the design of fluorescent probes for Zn(II),[1] Hg(II)[2], and Cd-(II).[3] The design of a Cu(II) sensor has also received consideration given the biological and environmental implications of Cu(II) in a number of neurodegenerative diseases as well as ecological pollution, respectively.[4] However, Cu(II) presents an inherent problem for fluorescent sensing because of the likely quenching of fluorescence by mechanisms inherent to paramagnetic species.[5] Herein we describe a displacement strategy for designing ratiometric fluorescent chemosensor systems and provide an example for determination of a Cu(II) ion.

A practical "off−on" imaging agent must produce a strong fluorescent response upon binding the analyte. Several research groups have addressed Cu(II) fluorescence quenching by designing supramolecular assemblies that suppress the interaction between the metal and the fluorophores,[6] with some success in a limited number of organic solvents. However, these systems suffer from low quantum yields and an inability to function in aqueous media, where biological and environmental Cu(II) is of interest.

To prevent fluorescence quenching by Cu(II) and to achieve a ratiometric response to metal binding, we devised a sensor system that separates the sensing and the signaling events. Two different fluorogenic ligands capable of metal chelation are employed whereby one of the ligands binds Cu(II), while the other produces a fluorescent signal to report the binding event. An equimolar quantity of Cd^{2+} is used, which is displaced from one ligand to the other upon presentation of Cu^{2+}.

The sensor system functions as described in Figure 1. In the absence of Cu^{2+}, ligand **1** binds Cd^{2+}, which causes an enhancement of fluorescence ("on" state). At the same time, ligand **2** is in the uncomplexed form and is thus relatively poorly fluorescent ("off" state). In the presence of Cu^{2+}, Cd^{2+} is displaced from **1**. Formation of [Cu(**1**)] quenches fluorescence, therefore switching **1** "off". Meanwhile, fluorescence of **2** is turned "on" upon binding the liberated Cd^{2+}.

To function as described, the sensor system must meet the following criteria: (a) Both **1** and **2** must show marked change in fluorescence upon complexation of Cd^{2+}, (b) ligands **1** and **2** must form 1:1 complexes with each of the metal ions, (c) the binding affinity of **1** toward Cd^{2+} and Cu^{2+} has to be at least 2 orders of magnitude superior to that of **2**, and (d) in an analogous manner, **1** should bind Cu^{2+} better than Cd^{2+} for the system to achieve a reliable discrimination between "on" and "off" states.

The last two criteria were derived from the graphical analysis, shown in Figure 2, which illustrates how the ratio of the binding constants of **1** and **2** would impact their competitive binding to Cd^{2+}.[7] The magnitude of the metal binding for each ligand corresponds directly to its relative fluorescence intensity. Furthermore, the difference between relative fluorescence intensities of
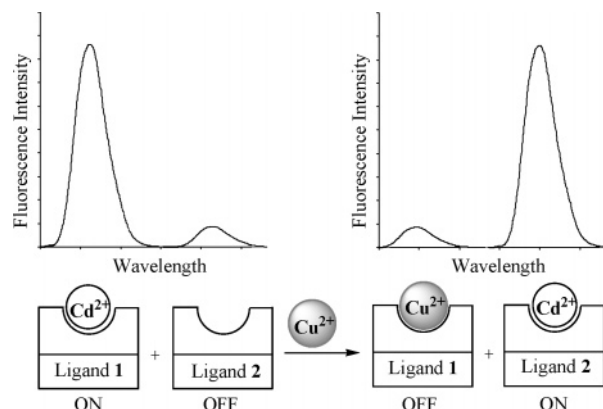

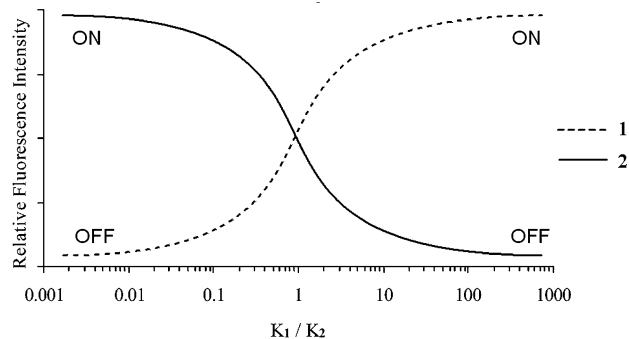**Figure 1.** Schematic representation of a ratiometric Cu^{2+} sensor system.

Figure 2. Simulation of fluorescence-monitored competition between **1** and **2** for Cd^{2+}, as a function of the ratio of the ligand/metal 1:1 association constants ($K = [ML]/[M][L]$). The actual y-scale depends on the spectroscopic characteristics of the system, such as the extent of the chelation enhanced fluorescence.[8]

the two ligands corresponds to the extent of discrimination between their "on" and "off" states.

Several kinetic and spectroscopic criteria also have to be met. Because of the dynamic nature of our sensor system, one has to be concerned with the kinetics of the metal exchange processes. To be able to utilize fluorescence as a means for data readout, the metal exchange rates must be lower than the fluorescence lifetimes of the fluorophores involved. To achieve a ratiometric response, Cd^{2+} should enhance fluorescence of both ligands upon complexation. Different emission wavelengths for the two ligands would facilitate data interpretation. Finally, to prevent resonance energy transfer, it would be preferable for the excitation wavelength of one ligand not to overlap with the emission wavelength of the other.

Given the apparent versatility of the criteria described above, the choice of ligands could be tailored to fit a wide range of studies. Seeking to design a sensor system that could be utilized for biological and environmental Cu(II) sensing, we chose two commercially available ligands that have adequate water solubility and
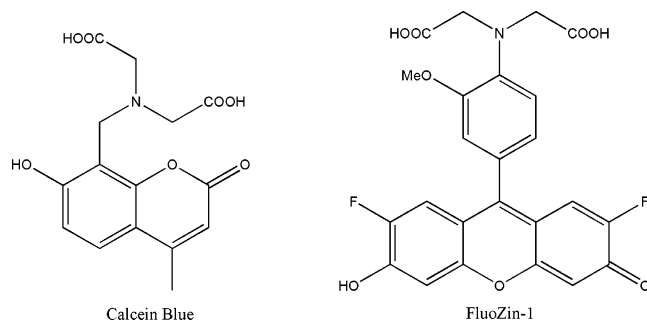
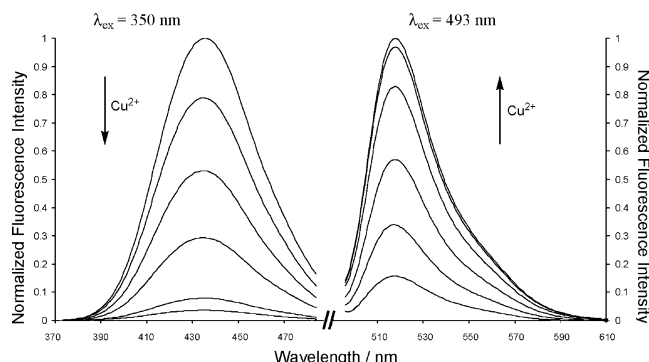**Figure 3.** Fluorescent indicators used in our study.

Figure 4. Normalized fluorescence response for the titration of aqueous solution (pH 7.2) containing 10 μM Calcein Blue, 10 μM FluoZin-1, 10 μM Cd(ClO$_4$)$_2$, 50 mM HEPES, and 100 mM KNO$_3$ with Cu(ClO$_4$)$_2$. Shown spectra contain 0, 2, 4, 6, 8, and 10 μM Cu(ClO$_4$)$_2$.

strong fluorescence. Calcein Blue and FluoZin-1, shown in Figure 3, were chosen as ligands **1** and **2**, respectively.

Calcein Blue and FluoZin-1 fit the criteria discussed above. According to previously reported studies, both ligands form 1:1 complexes with Cu^{2+} and Cd^{2+}.[9] Both free ligands are virtually nonfluorescent because of photoinduced electron transfer (PET) involving the lone pair of the tertiary nitrogen.[5] Upon forming complexes with Cd^{2+}, the lone pair of the nitrogen is coordinated to the metal, thereby eliminating PET and enhancing fluorescence. Calcein Blue binds Cd^{2+} almost 7 orders of magnitude stronger than FluoZin-1 ($K_{dCd} = 1.59 \times 10^{12}$ for Calcein Blue; $K_{dCd} = 1.63 \times 10^{5}$ for FluoZin-1). The stability constant of [Cu(Calcein Blue)] is slightly more than 2 orders of magnitude larger than [Cd(Calcein Blue)] and more than 8 orders of magnitude larger than [Cu-(FluoZin-1)] ($K_{dCu} = 1.88 \times 10^{14}$ for Calcein Blue;[10] $K_{dCu} = 1.02 \times 10^{6}$ for FluoZin-1[11]). The two ligands also meet the spectroscopic requirements, having different excitation and emission wavelengths ($\lambda_{ex} = 350$, $\lambda_{em} = 435$ for Calcein Blue; $\lambda_{ex} = 493$, $\lambda_{em} = 518$ for FluoZin-1).

Ratiometric copper sensing is shown in Figure 4. In the absence of Cu^{2+}, Cd^{2+} is predominantly bound to Calcein Blue. [Cd(Calcein Blue)] is strongly fluorescent ($\Phi = 22.4\%$). Meanwhile, FluoZin-1 is in the apo form, thus showing weak fluorescence at 518 nm ($\Phi = 0.5\%$). Addition of Cu(ClO$_4$)$_2$ causes a decrease of fluorescence at 435 nm due to the formation of [Cu(Calcein Blue)]. The released Cd^{2+} is then consumed by FluoZin-1, enhancing fluorescence at 518 nm ($\Phi = 7.76\%$ for [Cd(FluoZin-1)]). The overall effect upon addition of 1 equiv of Cu^{2+} is a 20-fold quenching of fluorescence at 435 nm and a 5-fold enhancement at 518 nm. Addition of more than 1 equiv of Cu^{2+} results in quenching of fluorescence at 435 nm because of formation of [Cu(FluoZin-1)]. For both 0 and 1 equiv of added Cu(II), the fluorescence spectra of each dye were unaffected by the presence of the other dye.

Finally, we checked whether our sensor system can function in the presence of competing metal ions. Figure 5 shows fluorescence
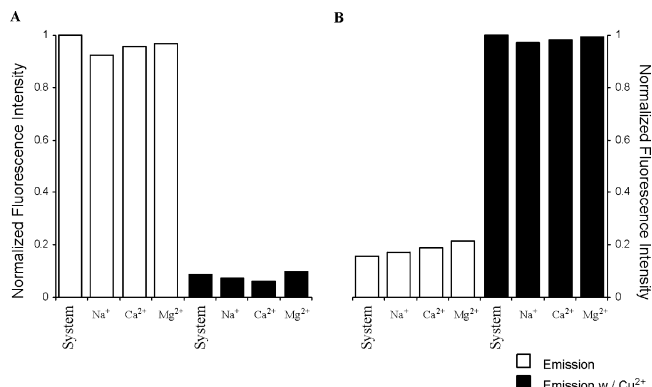

Figure 5. Normalized fluorescence response of the copper sensor system to biologically abundant metal ions. These metal ions were added to aqueous solutions (pH 7.2) containing 10 μM Calcein Blue, 10 μM FluoZin-1, 10 μM Cd(ClO$_4$)$_2$, 50 mM HEPES, and 100 mM KNO$_3$ without and in the presence of 10 μM Cu(ClO$_4$)$_2$. (A) $\lambda_{ex} = 350$; (B) $\lambda_{ex} = 493$.

emission of our Cu(II) sensor system in the absence (System) and in the presence of biologically abundant Na$^+$ Ca^{2+}, Mg^{2+} (K$^+$ was not analyzed since it is already present in excess in the buffer solution). It is evident that these metal ions did not have a significant impact on the ability of our system to sense Cu^{2+}. These competition studies indicated that our system is fit for Cu(II) sensing.

In conclusion, our methodology allows for quantitative measurement of Cu(II) in aqueous solution, under physiologically relevant conditions. The micromolar sensitivity of the system is biologically relevant for certain disease conditions.[12] In principle, the technique offers a general approach to fluorescence detection of metal ions whose complexes are inherently poorly fluorescent.

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