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Toward the Detection of Cellular Copper(II) by a Light-Activated Fluorescence Increase

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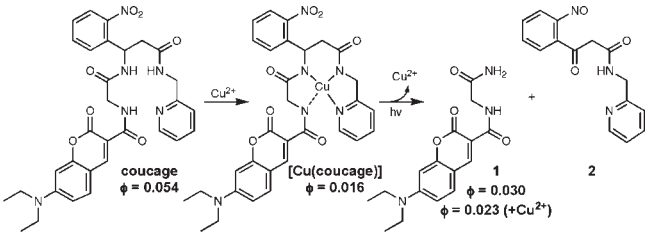
Copper, the third most abundant transition metal in the human body, plays a critical role in many fundamental physiological processes; however, it also catalyzes the production of highly reactive oxygen species that damage biomolecules.[1] Because of copper's dual nature, cells have developed strict regulatory processes to control its cellular distribution.[1] Alterations in copper homeostasis are linked to neurodegenerative diseases such as Menkes and Wilson diseases, Alzheimer's, familial amyotrophic lateral sclerosis, and prion diseases.[2] Being able to visualize the cellular distribution of copper in both of its physiological oxidation states, Cu^{+} and Cu^{2+}, would offer insight into how cells acquire, maintain, and utilize copper while suppressing its toxicity. Whereas reliable fluorescence sensors exist for Cu^{+}, there are fewer options for detecting Cu^{2+} in living cells.[3]

A common strategy in the design of fluorescent probes for metal ions is to link a ligand to a fluorophore such that metal binding causes an increase in fluorescence only in response to the target ion. Cell-permeable fluorescent sensors have proven useful for investigating intracellular metal ion distribution, particularly for Ca^{2+},[4] Zn^{2+},[5] and Cu^{+}.[6] The development of this type of "turn-on" sensor for Cu^{2+}, however, is hampered by the fluorescence quenching effect of this paramagnetic metal ion. As a consequence, many Cu^{2+} sensors have a "turn-off" mechanism,[7] which is generally less sensitive, gives false-positive results, and offers limited spatial resolution. Several examples of turn-on sensors have appeared recently,[3,8] but limitations include sensing mechanisms that operate only in an organic solvent or at nonphysiological pH,[8a−d] low quantum yields in aqueous solution,[8e] or potential off-target responses.[8f−i] Therefore, there is a need to develop new strategies that provide a fluorescent turn-on response in order to investigate intracellular Cu^{2+}. We present here coucage, a new type of fluorescent sensor that uses UV light to uncage a Cu^{2+}-dependent fluorescence response.

Scheme 1. Synthesis and Photolysis of [Cu(coucage)]



Coucage is based on our previously reported copper-caging ligand H_2cage[9] but adapted with coumarin as a fluorescence reporter that is quenched upon Cu^{2+} coordination. The nitrophenyl group incorporated into the backbone of the fluorescent tetradentate chelator is the caging element that blocks activity until activated with light.[10] Exposure to UV light induces bond cleavage, as shown in Scheme 1, which

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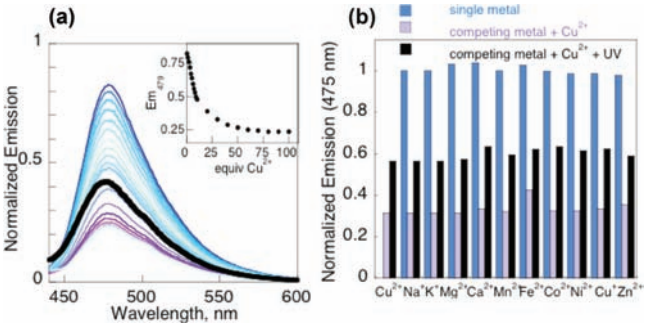

Figure 1. (a) Fluorescence decrease of 1 μM coucage with 0−100 equiv of Cu^{2+}, along with the subsequent increase following UV exposure (thick black trace). Inset: Emission at 479 nm versus added Cu^{2+}. (b) Blue bars: unchanged fluorescence of 1 μM coucage in the presence of 1 mM Na^+, K^+, Mg^{2+}, Ca^{2+}, and Zn^{2+} or 50 μM for others. Purple bars: quenched emission upon the addition of 50 μM Cu^{2+}. Black bars: restored fluorescence after 4 min of UV exposure. All samples were prepared in a 10 mM Hepes buffer at pH 7.4 with 10% dimethyl sulfoxide and excited at 430 nm.

triggers 2-fold activity: release of copper by decreasing the ligand denticity and restoration of fluorescence by disengaging the copper-induced quenching.

Coucage displays an absorbance band at 432 nm that gives a corresponding fluorescence emission maximum at 479 nm with a quantum yield of 0.054. Figure 1a shows that its fluorescence at pH 7.4 is quenched by 75% when saturated with Cu^{2+}, giving a quantum yield of 0.016 and a conditional dissociation constant, K_d, of 7.3 ± 0.9 μM. The 1:1 coucage/Cu^{2+} ratio for complex formation was confirmed by the method of continuous variation (Supporting Information).

The depressed fluorescence of solutions containing coucage and Cu^{2+} can be restored to nearly half the original intensity by irradiation at 350 nm, as shown by the thick spectral trace in Figure 1a. The emission maximum of photolyzed samples shifts slightly to 475 nm, with a quantum yield of 0.023. The fluorescence of the photolyzed products does not return to its initial levels for at least two reasons, the first being that the quantum yield of the independently synthesized photoproduct **1** (0.030) is inherently lower than that of coucage. The second is that Cu^{2+} retains some quenching effect on the photoproducts, although to a much lesser extent than on intact coucage (Figure S5 in the Supporting Information).

Unlike the response observed with Cu^{2+}, no significant fluorescence changes are observed for coucage in the presence of other metal cations, as shown in Figure 1b for Na^+, K^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^+, and Zn^{2+}. When Cu^{2+} is added back to these solutions, the fluorescence decreases by 70% (Figure 1b, purple bars), confirming coucage's high selectivity for Cu^{2+} over other biologically important metal ions. Fluorescence can again be partially restored upon irradiation, as shown by the black bars in Figure 1b.

The increase in fluorescence upon irradiation of [Cu(coucage)] is apparent immediately, and cleavage of the ligand backbone is complete in approximately 3 min. The quantum yields of photolysis for coucage and coucage in the presence of Cu^{2+} are 0.51 and 0.68, respectively, indicating that coordination by Cu^{2+} does not decrease the photolysis efficiency, as was previously observed for [Cu(cage)].[9] Analysis of the reaction mixture by liquid chromatography−mass spectrometry revealed **1** and **2** as photoproducts (Figure S1 in the Supporting Information).

In order for coucage to bind tightly to Cu^{2+}, all three amide protons must be deprotonated. The fact that only 75% fluorescence quenching is achieved at pH 7.4 suggests that the amide proton closest to coumarin is not fully deprotonated at this pH, setting up a H^+/Cu^{2+} competition that precludes maximum fluorescence quenching. Indeed, increasing the pH of coucage/Cu^{2+} solutions above 8 dramatically decreases fluorescence, leaving only a residual 10% signal by pH 9 (Figure S4 in the Supporting Information). Although the greatest fluorescence quenching is observed at high pH, coucage remains biologically applicable because a Cu^{2+} turn-off response is observed at pH 7.4.

To test coucage in living cells, we treated human breast carcinoma MCF-7 cells with coucage and Cu^{2+} and observed the intracellular fluorescence of irradiated versus nonirradiated cells using scanning confocal microscopy. MCF-7 cells incubated with coucage alone initially show a high fluorescence response, as shown in Figure 2b (see also the Supporting Information). After the addition of excess Cu^{2+} to the cell culture medium and incubation for 20 min, the intracellular fluorescence signal decreases by 70%, indicating that Cu^{2+} has coordinated to coucage inside the cells (Figure 2c). Cu^{2+}-treated cells exposed to UV light from a Rayonet photoreactor for 4 min exhibit bulk fluorescence restoration of up to 67% of the original intensity, as seen in Figure 2d. Control experiments in the absence of fluorophore show no background fluorescence, and photobleaching of coucage results in less than 2% intensity loss during the 3 s excitation times used to collect images (see the Supporting Information). Bright-field images after coucage, Cu^{2+}, and UV exposure show that cells remain viable throughout the imaging experiment. In these experiments, cells receive only 0.28 kJ/m^2 of UVA irradiation, which is significantly lower than the 50−300 kJ/m^2 doses known to induce DNA damage and cell death.[11] Cells were also irradiated directly on the microscope (Figure S14 in the Supporting Information). Although this method provides a less distinct fluorescence increase, it demonstrates the possibility of observing the same cells before and after photolysis.

In conclusion, we have presented a new strategy for achieving a fluorescence turn-on response to detect Cu^{2+} in living cells. The sensor relies on a coumarin-tagged ligand that selectively binds Cu^{2+} over other biometals to induce fluorescence quenching, which is subsequently relieved upon UV irradiation to provide the turn-on response. In essence, the strategy reports on the memory of where Cu^{2+} had been available for chelation by the 7 μM binder. Because the probe is destroyed during the readout, this strategy inherently cannot provide real-time monitoring of cellular Cu^{2+} fluctuations. Experiments in live MCF-7 cells demonstrate that coucage is cell-permeable and can detect an increase of intracellular Cu^{2+} under conditions of excess (between 25 and 125 μM) exogenous copper. Copper is imported in its reduced Cu^+ oxidation state and intracellularly is believed mostly to remain in its reduced form. However, subcellular microenvironments may support Cu^{2+}, and the coucage strategy introduced here might find utility in providing snapshots of such Cu^{2+}, provided that improvements can be made to the ligand to make it more sensitive. Future investigations are therefore aimed at improving the quenching efficiency of the copper

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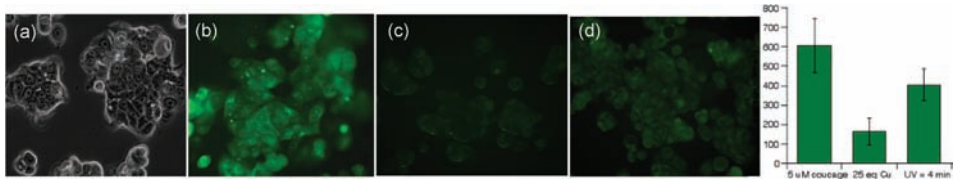

Figure 2. Confocal fluorescence images of coucage and Cu^{2+} in MCF-7 cells. Each panel shows an independent view from the same well: (a) bright-field transmission image; (b) cells incubated with 5 μM coucage for 20 min; (c) image taken 20 min after the addition of 25 equiv of Cu^{2+} to a coumarin-incubated sample; (d) image taken after 4 min of UV light exposure to a coumarin/Cu^{2+}-treated sample. The bar graph represents the average, background-corrected intensity from 10 randomly selected fields of view collected for each condition.

complex at physiological pH and increasing the binding affinity in order to create a more sensitive probe, as well as applying photoactive fluorescent ligands to other biologically interesting metal ions.

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Supporting Information Available: Full experimental details, including the synthesis of coucage and additional fluorescence and microscopy data. This material is available free of charge via the Internet at http://pubs.acs.org.