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$^{2+}$, Zn$^{2+}$, and Cu$^{2+}$.4 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, which is generally less sensitive, gives false-positive results, and offers limited spatial resolution. Several examples of turn-on sensors have appeared recently,3,6 but limitations include sensing mechanisms that operate only in an organic solvent or at nonphysiological pH,6a–d low quantum yields in aqueous solution,6e or potential off-target responses.6f–h 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 couacage, a new type of fluorescent sensor that uses UV light to uncage a Cu$^{2+}$-dependent fluorescence response. Couacage is based on our previously reported copper-caging ligand H$_2$cage9 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 tetradenate 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

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

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μ unchanged fluorescence of 1+K+

that coordination by Cu2+
of other metal cations, as shown in Figure 1b for Na+

Hepes buffer at pH 7.4 with 10% dimethyl sulfoxide and excited at 430 nm.

Figure 1. (a) Fluorescence decrease of 1 μM coucage with 0–100 equiv of Cu2+, along with the subsequent increase following UV exposure (thick black trace). Inset: Emission at 479 nm versus added Cu2+. (b) Blue bars: unchanged fluorescence of 1 μM coucage in the presence of 1 mM Na+, K+, Mg2+, Ca2+, and Zn2+ or 50 μM for others. Purple bars: quenched emission upon the addition of 50 μM Cu2+. 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.

Figure 1, along with the subsequent increase following UV exposure (thick black trace). Inset: Emission at 479 nm versus added Cu2+. 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 photoprodu-duct 1 (0.030) is inherently lower than that of coucage. The second is that Cu2+ 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 Cu2+, no significant fluorescence changes are observed for coucage in the presence of other metal cations, as shown in Figure 1b for Na+, K+, Mg2+, Ca2+, Mn2+, Fe2+, Co2+, Ni2+, Cu2+, and Zn2+. When Cu2+ is added back to these solutions, the fluorescence decreases by 70% (Figure 1b, purple bars), confirming coucage’s high selectivity for Cu2+ 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 Cu2+ are 0.51 and 0.68, respectively, indicating that coordination by Cu2+ does not decrease the photolysis efficiency, as was previously observed for [Cu(cage)].

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 Cu2+, 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+/Cu2+ competition that precludes maximum fluorescence quenching. Indeed, increasing the pH of coucage/Cu2+ 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 Cu2+ 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 Cu2+ 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 Cu2+ to the cell culture medium and incubation for 20 min, the intracellular fluorescence signal decreases by 70%, indicating that Cu2+ has coordinated to coucage inside the cells (Figure 2c). Cu2+-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, Cu2+, and UV exposure show that cells remain viable throughout the imaging experiment. In these experiments, cells receive only 0.28 kJ/m2 of UVA irradiation, which is significantly lower than the 50–300 kJ/m2 doses known to induce DNA damage and cell death. 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 Cu2+ in living cells. The sensor relies on a coumarin-tagged ligand that selectively binds Cu2+ 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 Cu2+ 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 Cu2+ fluctuations. Experiments in live MCF-7 cells demonstrate that coucage is cell-permeable and can detect an increase of intracellular Cu2+ 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 Cu2+, and the coucage strategy introduced here might find utility in providing snapshots of such Cu2+, 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

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.