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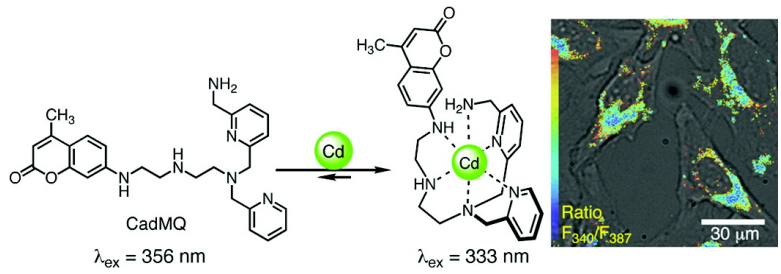

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Fluorescence Imaging of Intracellular Cadmium Using a Dual-Excitation Ratiometric Chemosensor

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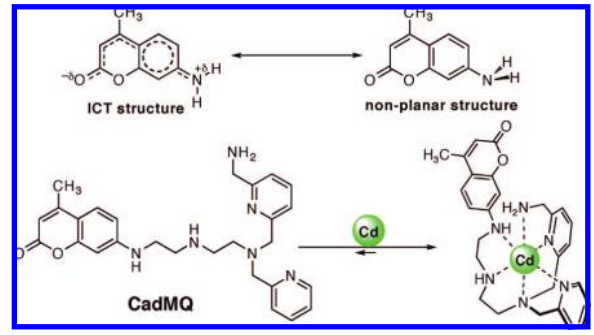
Cadmium (Cd), an inessential element for life, has been recognized as a highly toxic heavy metal and is listed by the U.S. Environmental Protection Agency as one of 126 priority pollutants. Humans are exposed to Cd^{2+} through the ingestion of contaminated food or water and inhalation of cigarette smoke. Cd^{2+} causes a number of lesions in many organs and tissues such as the kidney, liver, gastrointestinal tract, brain, and bone.[1] In addition, chronic exposure to Cd^{2+} has been implicated as a cause of cancers of the lung, prostate, pancreas, and kidney;[2] therefore, the International Agency for Research on Cancer (IARC) has classified cadmium and cadmium compounds as category I carcinogens.[3] Although many experimental results have been obtained on the ability of Cd^{2+} to adversely affect cellular functions, the molecular mechanisms of Cd^{2+}-uptake by cells as well as of carcinogenesis due to Cd^{2+} in humans and other mammals remains unclear.[1]

Fluorescent sensors that can aid in the visualization of specific metal ions of interest in living cells have been indispensable tools for understanding of biological phenomena.[4,5] Many synthetic sensors that exhibit a fluorescence response to Cd^{2+} have been demonstrated; however, most of these sensors can respond to Zn^{2+} as well because the two metal cations often exhibit similar coordination properties.[6] Hence, it has been a significant challenge to develop a Cd^{2+}-selective fluorescent sensor that can discriminate Cd^{2+} from Zn^{2+} under physiological conditions.[7] Recently, the research groups of Peng and of Wang have reported confocal images of Cd^{2+} in living cells using BODIPY- and fluorescein-based sensors, respectively; however, they have not shown the reversible binding of Cd^{2+} in these cells.[7a,b] Furthermore, sensor molecules with higher affinity for Cd^{2+} than either BODIPY- or fluorescein-based sensors are required because cell growth and DNA synthesis would be significantly stimulated by Cd^{2+} concentrations as low as 100 pM,[8] a level that lies beyond the detection limits of either of these sensors. Herein, we report a new Cd^{2+}-selective fluorescent sensor, CadMQ. The excitation wavelength of this molecule exhibits a significant blue shift under physiological conditions when bound to Cd^{2+}, thereby enabling dual-excitation ratiometric imaging of Cd^{2+}, which can, in principle, provide accurate and quantitative measurements of metal ion concentrations in cells.

CadMQ consists of coumarin-120 (C120: 7-amino-4-methyl-1,2-benzopyrone), and a derivative of *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) as the chelator. It has been proposed that C120 exists in an intramolecular charge transfer (ICT) structure in higher polarity solvents, where the bond between the 7-amino group and the 1,2-benzopyrone moiety attains a substantial double bond character.[9] In a nonpolar solvent, however, NH_2 nitrogen is bound to the benzopyrone moiety by a single bond, and the absorption energy has a higher value than it does in a polar solvent.[9] We speculate that if the chelator, which can allow metal-ion binding with the 7-amino group on the coumarin ring, is incorporated into C120, the ICT structure may change into a nonplanar structure and therefore a blue shift of the excitation spectrum will be observed.[10]

Scheme 1



Spectroscopic measurements of CadMQ were performed under physiological conditions (50 mM HEPES, pH 7.20, 0.1 M KNO_3). CadMQ exhibits an intense absorption band at 356 nm ($\epsilon = 1.77 \times 10^5\ M^{-1}\ cm^{-1}$), suggesting the formation of the ICT structure in the excited state. Upon addition of Cd^{2+}, a decrease in the absorbance of this band and a concomitant increase in that of a new band at 333 nm ($\epsilon = 1.42 \times 10^5\ M^{-1}\ cm^{-1}$) were observed with a distinct isosbestic point at 340 nm. A significant hypsochromic shift of 23 nm of the absorption wavelength indicates coordination of the 7-amino group to Cd^{2+} to form the nonplanar structure (Scheme 1). The absorption bands at 356 and 333 nm linearly decreased and increased, respectively, up to a 1:1 $[Cd^{2+}]$/[CadMQ] ratio, which is consistent with a 1:1 complex stoichiometry. The binding of Cd^{2+} resulted in a bathochromic shift of the emission maximum of only 4 nm; this wavelength shift is much less than that observed in the absorption titration. The quantum yields of the free ligand and Cd^{2+}-bound forms were determined to be 0.59 and 0.70, respectively. Therefore, it can be concluded that CadMQ enables the calculation of $[Cd^{2+}]_{free}$ using the ratio of the fluorescence intensities at the different two wavelengths in the excitation spectrum. Figure 1a shows a set of excitation spectra for CadMQ in calibration buffers with various $[Cd^{2+}]_{free}$. The apparent dissociation constant K_d for Cd^{2+} was determined to be 0.16 nM at pH 7.20 by plotting the fluorescence intensities at 328 or 368 nm against $\log[Cd^{2+}]_{free}$ (Figure 1b). This nonlinear fitting analysis reveals that CadMQ is suitable to determine $[Cd^{2+}]_{free}$ between 40 and 660 pM. The fluorescence intensity of CadMQ changed with pH and reached a maximum at pH 6.3 (Supporting Information, Figure S2). However, a significant shift of the fluorescence wavelength was not observed; accordingly, the

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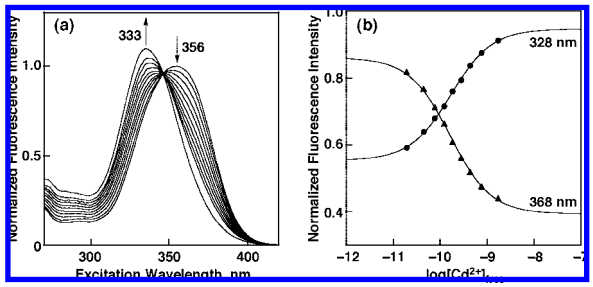

Figure 1. (a) Excitation spectra of CadMQ (2.5 μM) monitored at 440 nm in Cd^{2+}/Mg^{2+}/EDTA buffered system (50 mM HEPES, pH 7.20, 0.1 M KNO_3; 1 mM EDTA, 10 mM $MgSO_4$, 0−0.9 mM $CdCl_2$) and in 50 mM HEPES buffer (pH 7.20) containing ~20 μM $CdCl_2$. (b) Plots of fluorescence intensities at 328 (circle) and 368 (triangle) nm with best-fit curves for the dissociation constant 1.62×10^{-10} M.

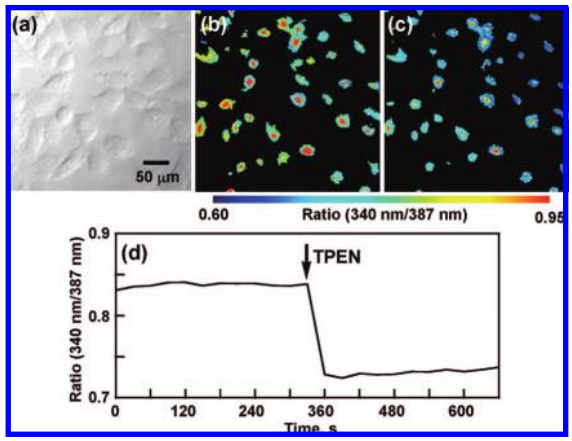

Figure 2. Ratio images of Cd^{2+} in HeLa cells exposed to 5 μM of Cd^{2+} for 3 h at 37 °C, then further incubated with 5 μM CadMQ for 10 min at 37 °C. Images were taken every 30 s: (a) bright-field transmission image; (b) ratio image (340 nm/387 nm) of CadMQ-stained cells prior to TPEN treatment; (c) ratio image of the same cells after treatment with 200 μM TPEN. The image is of the system at 360 s. (d) Average ratio of cells (340 nm/387 nm, $N > 10$) at the corresponding time.

fluorescence ratios between 333 and 356 nm changed only slightly over the wide range of pH.

A large hypsochromic shift in the excitation spectrum observed with Cd^{2+} is unaffected by the presence of high concentrations of Na^+, K^+, Mg^{2+}, and Ca^{2+} (>5 mM), indicating that this probe will be useful in a wide range of biological and microscopic applications. Some of the transition metal ions including Cu^{2+}, Zn^{2+}, and Hg^{2+} interfered with Cd^{2+} binding (Figure S4), which suggests competing binding of these cations.[11] It should be noted that the addition of Zn^{2+} resulted in a slight hypsochromic shift of CadMQ in the excitation spectrum, and little affected the fluorescence ratio. Such the difference in the fluorescence response of CadMQ toward Zn^{2+} and Cd^{2+} can be supported by ^1H NMR spectra in D_2O (pD 7.6, Figure S6). Complexation with Cd^{2+} induced downfield shifts of the coumarin moiety whereas slight upfield shifts were observed for Zn^{2+} complex. This observation strongly demonstrates that the 7-amino group of the coumarin interacts with Cd^{2+}, but not with Zn^{2+}.

Incubation of HeLa cells with 5 μM CadMQ for 30 min at 37 °C revealed a bright punctate staining pattern, indicating that this molecule is membrane permeable. To determine the intracellular distribution of CadMQ, cells were coincubated with LysoTracker Red. A complete overlap of the images between CadMQ and LysoTracker Red revealed that our sensor molecule was located within the acidic compartments of the cells (Figure S7). Subsequent experiments of Cd^{2+}-imaging in living cells were performed with a ratio imaging system. Cells were exposed to 5 μM Cd^{2+} for 3 h at 37 °C, washed with PBS containing 200 μM EDTA to remove extracellular Cd^{2+}, and further incubated with 5 μM CadMQ for 10 min. The fluorescence ratio between the intensities of the 340 and 387 nm bands remained unchanged for 330 s; however, this immediately changed (within 30 s) upon the addition of the heavy metal chelator TPEN (200 μM) to the medium (Figure 2). A decrease and increase in the fluorescence intensities at 340 and 387 nm, respectively, induced by TPEN treatment indicate that CadMQ can probe the change in the intracellular Cd^{2+} levels (Figure S9). In the control experiment, by contrast, initial fluorescence intensity ratio for cells grown without Cd^{2+} was lower than that for Cd^{2+}-exposed cells (Figure S10). In fact, a negligible change in the ratio was observed upon the addition of TPEN.

In conclusion, we have developed a ratiometric fluorescent sensor for Cd^{2+}, CadMQ. This probe exhibits excellent Cd^{2+}-selectivity over other transition metal ions in aqueous media, including Zn^{2+}. It also has high quantum yields in both the apo and Cd^{2+}-bound forms, a strong affinity for Cd^{2+}, and membrane permeability. The ratio-imaging experiments demonstrate that CadMQ will be a useful tool for detecting changes in Cd^{2+} concentrations in living mammalian cells.

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Supporting Information Available: Synthesis and characterization of CadMQ, UV−vis titration for Cd^{2+}, pH-titration, confocal images, and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) K_d for Zn^{2+} was determined to be 44.6 pM. See Supporting Information.

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