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A Novel Two-Fluorophore Approach to Ratiometric Sensing of Zn^{2+}

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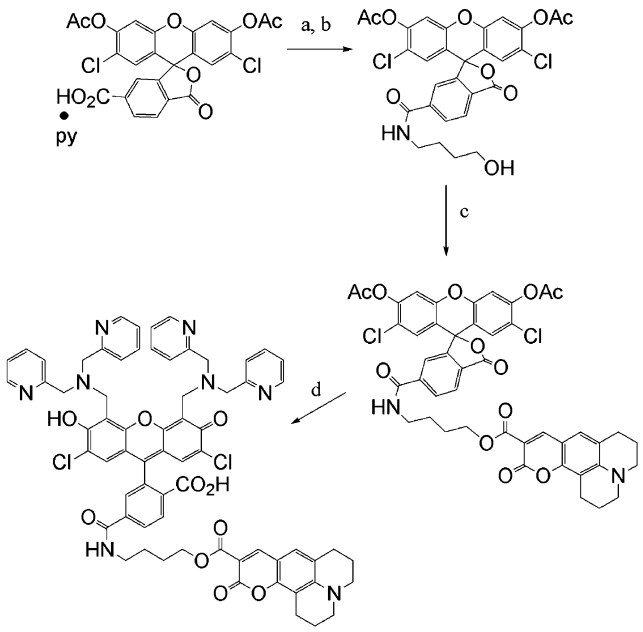
The role of zinc in neurobiology has received significant attention.[1,2] Recent studies indicate that cellular Zn^{2+} levels are tightly regulated.[3] Understanding the biological functions of low millimolar concentrations of loosely bound Zn^{2+} stored in vesicles of hippocampal CA3 neurons[4] is a subject of great interest. The ability to track zinc release at the synapse in a time- and position-sensitive manner requires a fast and quantitative detector such as that provided by intensity-based fluorescent sensors.[5,6] For zinc(II) ions, it can be difficult to obtain a quantitative readout.[7] To address this problem, small-molecule ratiometric Zn^{2+} sensors have recently been reported.[8,9] These probes, however, are based on the Fura-2 and Indo-1 family of Ca^{2+} sensors and require UV excitation, which can be damaging to cells and present problems with autofluorescence when applied in vitro. Available Zn^{2+} sensors with excitation in the visible range are not ratiometric.[10−13] A two-fluorophore nanoparticle system has been described,[14] but use of these particles in cells requires microinjection. We report here an esterase-mediated, small molecule, ratiometric, two-photon compatible system comprising two fluorophores, both excited by visible light, that should be of value in sensing Zn^{2+} in biological environments.

Coumazin-1, the synthesis of which is presented in Scheme 1, is a prosensor containing a Zn^{2+}-insensitive fluorescent coumarin derivative covalently appended to a Zn^{2+}-sensitive fluorescein moiety. The latter is based on the recently reported Zn^{2+} sensor Zinpyr-1 (hereafter, ZP1).[11] The pyridinium salt of 2′,7′-dichlorofluorescein-3′,6′-diacetate-6-carboxylate was prepared in a manner analogous to that of the 2′,7′-difluoro derivative.[15] Activation with oxalyl chloride followed by treatment with 4-aminobutanol afforded the fluoresceinamido alcohol as depicted, which was coupled to coumarin 343 under Mitsunobu conditions. Diacetate-protected dichlorofluoresceins are viable substrates for Mannich reaction with dipicolylamine and para-formaldehyde, obviating an intermediate deprotection step. Coumazin-1 is membrane-permeable and essentially nonfluorescent ($\Phi \leq 0.04$). Treatment of the probe with porcine liver esterase in vitro effects hydrolysis of the ester linkage, yielding coumarin 343 and a PET-based Zn^{2+} sensor analogue of ZP1[11] (Scheme 2).

Fluorescence spectra of esterase-treated micromolar Coumazin-1 solutions with coumarin excitation at 445 nm and ZP excitation at 505 nm before and after the addition of Zn^{2+} are shown in Figure 1. The ratio of ZP to coumarin emission intensity $\lambda_{534}/\lambda_{488}$ varies from 0.5 in the absence of Zn^{2+} to 4.0 in the presence of a 2-fold excess of Zn^{2+}.

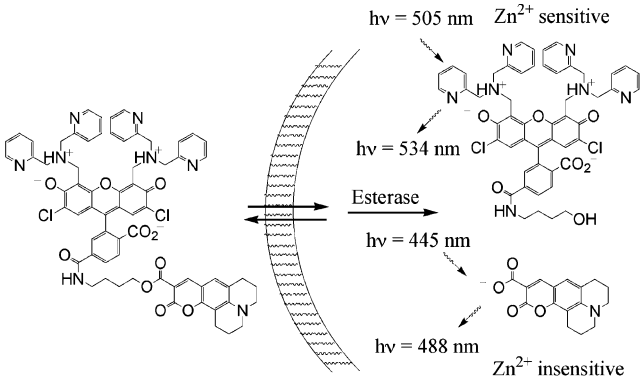
The kinetics of ester hydrolysis were measured by monitoring the increase in coumarin fluorescence over time. A fit to the Michaelis−Menten model gave $k_{cat} = 0.017(4)$ min^{-1} and $k_{cat}/K_m = 0.027(3)$ μmol^{-1} min^{-1} at 25 °C. Intracellular esterase hydrolysis of esterified sensors is a common strategy used to trap the resulting negatively charged carboxylate within the cell.[16] Coumazin-1 is thus transformed into a ratiometric sensing system by esterase processing within the cell.

Scheme 1. Synthesis of Coumazin-1[a]



[a] (a) Oxalyl chloride, DMF, −78 °C; (b) 4-aminobutanol; (c) PPh_3, DIAD, coumarin 343; (d) dipicolylamine, $(CH_2O)_n$, MeCN/H_2O.

Scheme 2. Proposed Activation of Coumazin-1



As indicated in Scheme 2, ester hydrolysis is presumed to generate an amide-substituted ZP sensor. To assess its properties, we prepared and characterized ZPA1 (Figure 2), which contains a hydroxyethyl amide in the 6-position of the fluorescein. ZPA1 has fluorescence and Zn^{2+}-response properties similar to those of the parent sensor ZP-1[11] (Table 1). After esterase processing, excitation of the resulting coumarin product at 445 nm and measurement of emission at 488 nm provides information about the concentration of cleaved sensor. Excitation of the ZP fragment at 505 nm and measurement of emission at 534 nm affords information about the amount of Zn^{2+} present.

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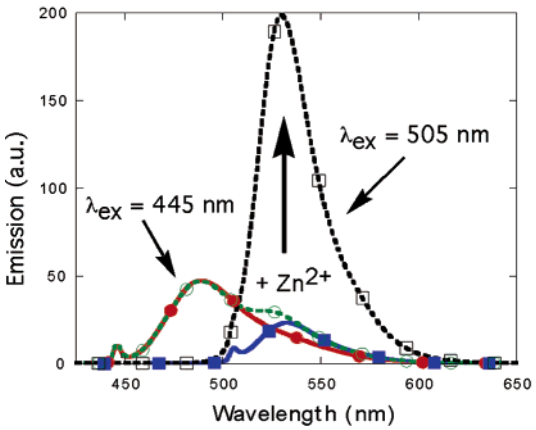

Figure 1. Fluorescence spectra of an esterase-treated solution of Coumazin-1 (2 μM) excited at 445 nm (circles) and 505 nm (squares) before (solid) and after (open) addition of excess $ZnCl_2$ (20 μM).

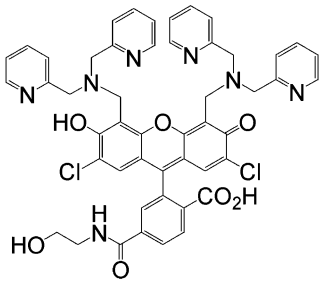

Figure 2. Structure of ZPA1.

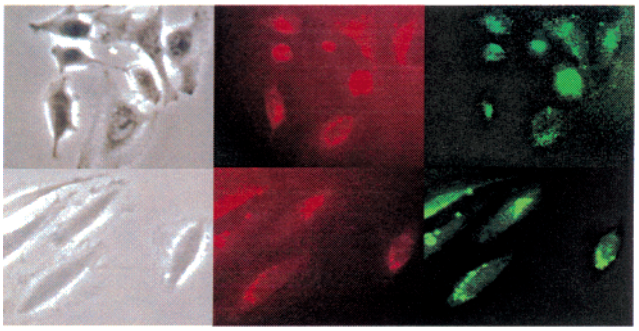

Figure 3. Phase contrast (left) and fluorescence (middle, right) microscopy images of HeLa cells incubated for 6 h with 5 μM Coumazin-1, without (top) and with (bottom) the addition of 5 μM $ZnCl_2$ and 45 μM sodium pyrithione. Fluorescence images were acquired with excitation at 400−440 nm, band-pass of 475 nm (middle) or with excitation at 460−500 nm, band-pass of 510−560 nm (right).

To test these expectations and to determine the extent of colocalizaton of the two fluorophores, we incubated HeLa cells with 5 μM Coumazin-1. Subsequent imaging with a dual-filter fluorescence microscope afforded the images shown in Figure 3. Loading the cells with Zn^{2+} by addition of a $ZnCl_2$-sodium pyrithione solution enhanced the ZP without affecting the coumarin fluorescence. Qualitative comparison of the images indicates good intracellular overlap of the two fluorophores, an essential condition for the effectiveness of this sensing strategy.

In conclusion, we describe a versatile synthetic route to amide-containing ZP1 sensors. We applied this methodology to produce a small-molecule ratiometric sensing system for Zn^{2+} with visible excitation, based upon the esterase-mediated cleavage of a member of the Zinpyr family of Zn^{2+} sensors functionalized with a Zn^{2+}-insensitive reporter fluorophore. This strategy is generally applicable as a modification to existing sensors for all biological analytes.

Table 1. Comparison of Photophysical Properties of Zinpyr and Coumazin Sensors

compound	Φ_{free}	$\Phi_{Zn^{2+}}$	K_d (nM)[c]	pK_a[a]
ZP1	0.38	0.87	0.7 ± 0.1	8.37
ZPA1	0.21	0.64	0.20 ± 0.02	8.43
Coumazin-1	0.01[b,c]	0.02[b], 0.04[c]	0.25 ± 0.03[d]	

[a] Protonation constant for benzylic amine responsible for quenching fluorescence in the unbound state. [b] Excited in the coumarin absorption region, ~470 nm. [c] Excited in the fluorescein absorption region, ~500 nm. [d] Determined after treatment with and in the presence of the esterase.

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Supporting Information Available: Syntheses and experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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