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Detection and Imaging of Zinc Secretion from Pancreatic β-Cells Using a New Fluorescent Zinc Indicator

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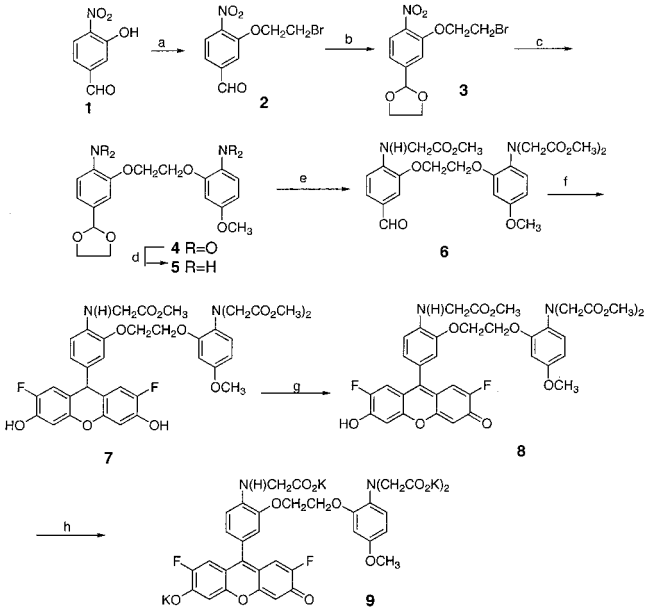
Zinc is an element critical to life. Some of its roles have been long known, such as in gene transcription and metalloenzyme function.[1,2] Other functions are just being discovered and investigated, such as zinc's role in synaptic neurotransmission[3] and in mediating neuronal excitotoxicity.[4,5] Understanding the role of free zinc in living cells has been hindered by a lack of suitable detection and imaging reagents. Fluorescent sulfonamides of 8-aminoquinolines have been used with a moderate degree of success.[6−9] However, these probes suffer from relatively low signal levels and require potentially damaging UV excitation. More recently, visible wavelength fluorescent probes of [Zn^{2+}] have been reported.[10−12] These newer probes have higher affinity for zinc and are brighter than the first generation UV-based probes, but they suffer from a different drawback: their chelator moieties all include aliphatic tertiary amines, which are significantly protonated at physiological pH. This protonation limits the pH range in which the probes are useful measures of [Zn^{2+}], and also results in intracellular localization to acidic compartments.[11] This property is in contrast to the anionic character of fluorescent calcium indicators, which have been of enormous benefit for measuring cytosolic [Ca^{2+}] in all areas of biology.[13] Herein we report a novel visible wavelength, zinc-specific fluorescent probe (**9**, FluoZin-3) that is tetraanionic and its application in the imaging of zinc secretion from pancreatic β-cells.

The structure of **9** is related to the fluorescent [Ca^{2+}] probes fluo-3[14] and fluo-4.[15] Fluo-3/4 bind Zn^{2+} with low nM affinity, and have Ca^{2+} $K_d \approx 300$ nM.[13] We sought to reduce the ion affinity of the chelator portion of the fluo-3/4 molecule, so as to retain physiologically relevant Zn^{2+} affinity while lowering the Ca^{2+} affinity to a physiologically irrelevant concentration value. This was accomplished by simply removing one of the *N*-acetic acid moieties. Synthesis of the bis-anilino dioxolane **5** proceeds from appropriately substituted 2-nitrophenols. Subjection of **5** to alkylation conditions (hot DMF, excess methyl bromoacetate, diisopropylethylamine) gave the benzaldehyde **6**. The acetal group in **5** cleaved back to the carboxaldehyde moiety during the reaction, which inhibits bis-alkylation at the *p*-anilino nitrogen atom, giving the desired trialkylated product (**6**). Cleavage of dioxolanes to their corresponding carbonyl compounds in the presence of electrophiles is well established,[16−18] and this cleavage is promoted by the electron-donating *p*-anilino substituent that stabilizes the incipient benzylic cation.[19] Prolonged reaction times did in fact produce low yields of the *N*,*N*,*N*′,*N*′-tetraalkylated product from **5**. Acid-mediated condensation of **6** with 4-fluororesorcinol,[20] followed by dehydrogenative oxidation, gave the tetraester **8**, which was converted into the fluoroionophore **9** by saponification (Scheme 1). The methoxy group serves to increase the affinity of the chelator toward zinc by donation of electron density to the *p*-nitrogen atom. In the absence of zinc, that is, the presence of the zinc chelator *N*,*N*,*N*′,*N*′-tetra-

Scheme 1 [a]



[a] Reagents: a) 1,2-dibromoethane, K_2CO_3 (77% yield); b) ethylene glycol, *p*-TsOH (97%); c) 5-methoxy-2-nitrophenol, K_2CO_3 (88%); d) H_2, Pt/C (49%); e) DMF, $BrCH_2CO_2CH_3$, DIEA (40%); f) 4-fluororesorcinol, $MeSO_3H$ (98%); g) *p*-chloranil (60%); h) KOH (50%).

(2-picolyl)ethylenediamine (TPEN), the fluorescence quantum yield is negligible (<0.005). Titration with increasing concentrations of buffered Zn^{2+} solutions gives an apparent K_d of 15 ± 2 nM, with a Hill coefficient of 1, consistent with a 1:1 binding of Zn^{2+}:**9** (Figure 1). The fluorescence reached a maximum near 100 nM Zn^{2+} (Figure 1). The fluorescence increases several hundred-fold, and a fluorescence quantum yield of 0.43 ± 0.04 was measured in the presence of 5 μM Zn^{2+}.[21] In the presence of saturating Zn^{2+} (5 μM), FluoZin-3 has stable fluorescence from pH 6 to 9. The fluorescence of the complex decreases as the pH is lowered below 6.0 with an apparent pK_a of 4.8, and no fluorescence is observed at pH < 4. This pH dependence correlates exactly with the fluorescence pH dependence of other 2,7-difluoroxanthen-3-ol-6-ones,[20] which lose their fluorescence as the phenolic hydroxyl group (pK_a = 4.8) becomes protonated. This behavior indicates that the pH sensitivity of **9** is due exclusively to protonation of the fluorescent part of the molecule at low pH, and not of the chelator portion. The K_d and titration profile are unchanged in the presence of 1 μM free Ca^{2+}, indicating good selectivity of **9** for zinc over calcium.

A potential biological application of **9** is for monitoring secretion from single cells. Secretion assays, especially at the single cell level, are important in assessing signal transduction of secretion, effects of new drugs on secretion, and intercellular communication. Zn^{2+}

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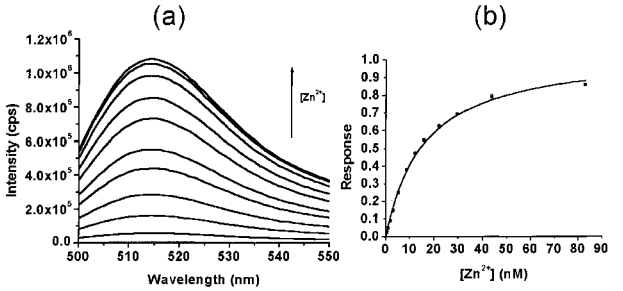

Figure 1. (a). Fluorescence emission spectra (excitation 488 nm) of FluoZin-3 (**9**, 0.5 μM) in buffered Zn^{2+} solutions with free (unbound) Zn^{2+}-concentrations of 0, 1, 3, 5, 9, 12, 20, 30, 45, and 80 nM and 10 μM, respectively. The up-arrow indicates the increase of free [Zn^{2+}] from 0 to 10 μM. The first spectrum almost overlaps with the *x*-axis indicating the very low fluorescence in the absence of Zn^{2+}. The spectra were measured at 22 °C, pH 7.4 in buffered Zn^{2+} solutions comprising 20 mM HEPES, 135 mM NaCl, 1.1 mM total EGTA, and 0−1.1 mM $ZnCl_2$. Free Zn^{2+} concentrations were calculated from the equation K_d = [Zn^{2+}][EGTA]/[Zn^{2+}-EGTA] using K_d = 1.1 nM of EGTA for zinc.[32] Fluorescence intensity is in arbitrary units. (b). Normalized fluorescence response as a function of free [Zn^{2+}]. The fluorescence emission intensities at 515 nm (maximum emission) were normalized to the full scale response obtained at 10 μM free Zn^{2+}. An apparent K_d of 15 nM was observed by analyzing the data with nonlinear least-squares fitting.

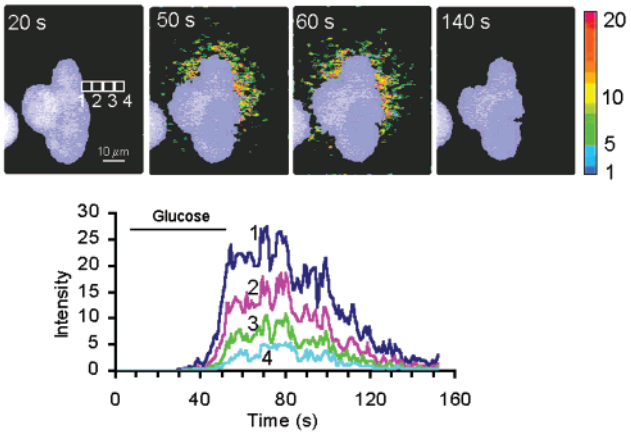

Figure 2. Imaging of Zn^{2+} secretion from pancreatic β-cells. Images are shown in ratios of fluorescence intensities against a reference image collected in the beginning of the sequence. The time at which each image was acquired is indicated as 20, 40, 50, 140 s, respectively. The temporal responses of Zn^{2+} secretion were analyzed using the four regions of interest (ROI) (4 μm^2) as indicated as 1, 2, 3, 4 in the first image. The traces from top to bottom correspond to the ROI 1, 2, 3, 4, respectively. Cells were incubated in Krebs-Ringer buffer containing 2 μM FluoZin-3 (**9**) and stimulated to secrete by the application of 20 mM glucose. The bar on top of the traces indicates the application of stimulation. The details of the imaging experiments and data analysis are as previously described.[29]

is released from neurons, where it appears to act as a neurotransmitter,[8,22−24] and from β-cells of the pancreas where it may serve an autoregulatory role on the β-cell.[25] In addition, Zn^{2+} secretion can serve as an indicator of insulin secretion since insulin and Zn^{2+} are co-stored in secretory vesicles and co-released by exocytosis.[26−29]

To assess **9** for monitoring secretion of Zn^{2+}, β-cells were bathed in a solution of 2 μM of the dye while monitoring fluorescence by laser-scanning confocal microscopy. Cells were stimulated by applying 20 mM glucose using a micropipet.[28] As shown in Figure 2, once glucose is applied large fluorescence signals are observed around the outside of the cell presumably corresponding to detection of Zn^{2+} released from the cell. Zn^{2+} is secreted by exocytosis to the extracellular milieu where it reacts with the dye to form the fluorescent complex. In the presence of 50 μM Zn^{2+} chelator TPEN, no fluorescence enhancements were observed from cells following stimulation confirming that the observed fluorescence enhancements are dependent on the presence of free Zn^{2+} and the formation of the Zn^{2+}:FluoZin-3 complex. The pH independence of the signal suggests that the fluorescence changes are not associated with local pH changes during exocytosis. As shown in Figure 2, the total Zn^{2+} concentration near the cells reached 600 nM at peak response while the total Zn^{2+} concentration decreased moving away from the cell consistent with diffusional dilution of released Zn^{2+}.[30] The spatial resolution of the imaging technique allows observation of heterogeneity in secretion among cells as some cells in the cluster do not release Zn^{2+} while others are active. In addition, within individual cells, some regions of the cell membrane give rise to secretion while others do not suggesting active zones of secretion on the cell surface.

In previous work[29] similar measurements were made using Zinquin,[31] one of the UV-excited Zn^{2+}-specific 8-aminoquinoline sulfonamide fluorophores. The new dye **9** has several advantages for this application with the most significant being the improvement in signal-to-noise (S/N) ratio. The fluorescence enhancement seen during secretion is >100-fold over baseline with FluoZin-3 while typical measurements with Zinquin yielded only a 3−5-fold enhancement over baseline. This improvement can be attributed to greater sensitivity of the dye and lower background autofluorescence from both the cell and surrounding solution due to the longer wavelength for excitation. Furthermore, as a tetraanionic dye FluoZin-3 is less permeable to the cell than the monoanionic Zinquin[7] and creates lower background fluorescence and less fluctuations inside the cell. Besides the obvious advantage of detecting lower levels of Zn^{2+}, the greater S/N allows better characterization of the cloud of Zn^{2+} formed by secretion. In previous work[29] Zn^{2+} was only detected in the immediate vicinity of the cell (~1−2 μm away); however, in the image shown in Figure 2 Zn^{2+} can be detected at least 15 μm away from the cell, giving an enhanced view of the transport of Zn^{2+} away from the cell. The higher sensitivity may also be translated into better temporal resolution. Ongoing investigations with this dye have shown that bursts of Zn^{2+} secretion due to exocytosis can be detected with video-rate imaging. Regarding probe ion selectivity, it should be noted that the cells being imaged with **9** were bathed in Krebs-Ringer buffer containing 2.4 mM Ca^{2+}, 1.2 mM Mg^{2+}, 118 mM Na^+, and 5.4 mM K^+, yet no fluorescence was observed until extracellular Zn^{2+} was added or Zn^{2+} secretion was induced, even though in vitro weak fluorescence could be induced from **9** with 40 μM Ca^{2+} in the absence of Zn^{2+}. In vitro screening of **9** against various other biologically relevant heavy metals showed some sensitivity toward Fe^{2+} and Hg^{2+}, although substantially higher concentrations than for Zn^{2+} were needed to induce fluorescence; **9** was even less sensitive to Cu^{2+}, Cd^{2+}, Ni^{2+}, Co^{2+}, Ba^{2+}, Pb^{2+}, Mn^{2+}, Tb^{3+}, and La^{3+} than to Ca^{2+}.

In conclusion, we have reported on the synthesis and fluorescence properties of an outstanding new visible wavelength Zn^{2+}-selective fluorescent probe (**9**), and its application in imaging and quantification of Zn^{2+} secretion from pancreatic β-cells. Further work is underway to utilize the probe in other cell-secretion assays.

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Supporting Information Available: Detailed experimental procedures and analytical data for the synthesis of compounds **2**−**9**; data from the fluorescence pH sensitivity of the **9**:Zn^{2+} complex; tabulated fluorescence sensitivity of **9** to other metals, relative to Zn^{2+}(PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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