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A New Cell-Permeable Fluorescent Probe for Zn^{2+}

Grant K. Walkup,[†] Shawn C. Burdette,[‡]
Stephen J. Lippard,*[,‡] and Roger Y. Tsien*[,†,§]

Department of Pharmacology and Chemistry and Biochemistry and Howard Hughes Medical Institute University of California at San Diego La Jolla, California 92093-0647
Department of Chemistry Massachusetts Institute of Technology Cambridge, Massachusetts, 02139

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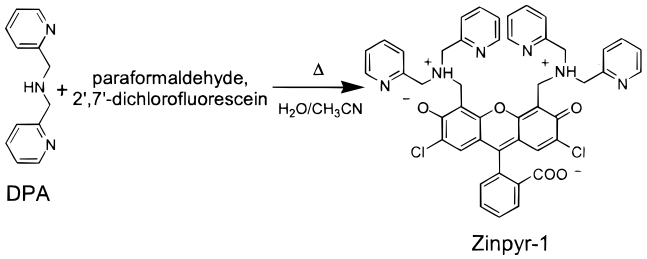
Zinc is an essential cofactor, critical for numerous cellular functions.[1] The combination of its unique chemical properties[2] and its central role in processes including gene expression,[3] apoptosis,[4] enzyme regulation,[5] and neurotransmission[6,7] suggests that Zn^{2+} may be a major regulatory ion in the metabolism of cells.[8,9]

Although Zn^{2+} is abundant in eukaryotes and most is tightly bound, pools of chelatable Zn^{2+} have been imaged in living cells with concentrations ranging from sub-nM in undifferentiated mammalian cells[10] to ∼0.3 mM in hippocampal nerve synaptic vesicles.[11] Currently, the most widely applied probes for cellular zinc are aryl sulfonamides of 8-aminoquinoline such as 6-methoxy-(8-*p*-toluenesulfonamido)quinoline (TSQ),[12] Zinquin[4,13] and TFLZn[11] (TSQ analogues). The distinction between chelatable and free Zn^{2+} is problematic due to the fact that these quinoline-based dyes can form mixed complexes, sensing Zn^{2+} that is already partially coordinated. Recent studies have clarified some of the details regarding the aqueous Zn^{2+}-binding equilibria of such dyes, enhancing their value as quantitative probes.[14] Quinoline probes require ultraviolet excitation (∼350 nm), however, which can be damaging to cells, and have relatively dim fluorescence with quantum yields ≈ 0.1 and extinction coefficients ≈ 10×10^3 M^{-1} cm^{-1}.[15]

Because the availability of better Zn^{2+}-specific probes would provide additional insight into the cell biology of Zn^{2+}, interest in the field remains high. Several fluorescent sensors for Zn^{2+} have been described, including those with polypeptide[16−18] or protein-based[19] scaffolds, as well as fluorophore-appended macrocycles.[20−22] Although each has potential advantages over the quinoline probes, none has been successfully applied to measure Zn^{2+} within living cells, either because of unacceptable binding affinity, optical properties, or the need to perform microinjections for their use. Probes traditionally used to measure Ca^{2+} or Mg^{2+} have also been employed, but the signals from these species are hard to separate from those of Zn^{2+}.[23,24]

To achieve high affinity binding without quinoline sulfonamide or EGTA-based chelating moieties, we incorporated the bis(2-pyridylmethyl)amine (di-2-picolylamine or DPA) moiety into our probe to chelate zinc. The $[Zn(DPA)]^{2+}$ complex has an apparent K_d of 70 nM at pH 7 and essentially no measurable affinity for Ca^{2+} or Mg^{2+}.[25] Furthermore, the DPA ligand is not expected to present a challenge to membrane permeability, owing to its structural similarity to the membrane-permeant heavy metal chelator *N*,*N*,*N*′,*N*′-tetra(2-picolyl)ethylenediamine (TPEN).[26] Finally we selected fluorescein as the reporting group because of its large extinction coefficient, high quantum yield, membrane permeability, and the ready availability of optical filter sets for fluorescence microscopy.

Access to a DPA-derivatized fluorescein was achieved through a Mannich reaction between 2′,7′-dichlorofluorescein (DCF) and the iminium ion condensation of product of formaldehyde and DPA. The chlorines in DCF restrict substitution to the 4′- and



5′-positions of the fluorescein ring, lower the phenolic pK_a so that it is largely deprotonated at physiological pH, and provide a small redshift to the chromophore. The resulting molecule, termed Zinpyr-1, displays a highly Zn^{2+}-selective fluorescence response.[27] Analysis of the crude material (TLC, ^{1}H NMR) suggests that it is obtained in >80% purity after trituration from boiling ethanol. Material of analytical purity used for all of the present studies is obtained in 46% yield after chromatography on alumina or reverse phase silica (30%).

At physiological ionic strength and pH (50 mM PIPES (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), 100 mM KCl, KOH

* To whom correspondence should be addressed.
[†] Department of Pharmacology, University of California, San Diego.
[§] Department of Chemistry and Biochemistry and Howard Hughes Medical Institute, University of California, San Diego.
[‡] Massachusetts Institute of Technology.

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(27) The name Zinpyr-1 was chosen to indicate the structural composition of the ligand, containing four pyridyl groups, as well as its ability to "peer" into the Zn^{2+} concentration of samples. Only Zn^{2+} and Cd^{2+} have been observed to enhance Zinpyr-1 fluorescence, and other transition metal ions can quench fluorescence, see Supporting Information.

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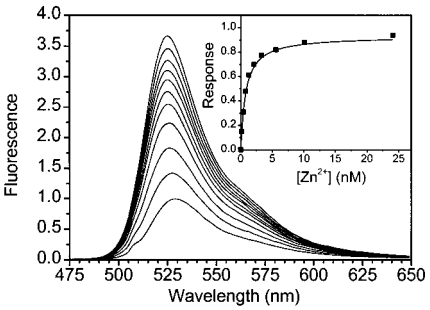

Figure 1. Fluorescence emission response of Zinpyr-1 (0.5 μM) to buffered Zn^{2+} solutions. Spectra were acquired in 100 mM KCl, 50 mM PIPES, pH 7.00 at 25 °C. Excitation was provided at 507 nm with 1 nm slit widths. Emission data were corrected for the response of the detector, using the manufacturer-supplied curve, and the emission data points at 507 nm, which were perturbed by scatter, have been removed for clarity. The first 10 spectra shown correspond to free Zn^{2+} concentrations between 0 and 24 nM. The eleventh spectrum was obtained with ~25 μM free Zn^{2+}. Inset: fluorescence response obtained by integrating the emission spectra between 509 and 650 nm, subtracting the baseline (0 Zn^{2+}) spectrum and normalizing to the full scale response obtained after overwhelming the zinc buffer (25 μM free Zn^{2+}). The observed data (rectangles) were analyzed by nonlinear least-squares fitting (solid line) to determine an apparent dissociation constant of 0.7 nM.

to pH 7.0) Zinpyr-1 has an excitation maximum at 515 nm (ϵ = 79.5 × 10^3 M^{-1} cm^{-1}) and a fluorescence quantum yield of 0.39 in the absence of Zn^{2+}. With saturating Zn^{2+} (25 μM) the excitation maximum shifts to 507 nm (ϵ = 84.0 × 10^3 M^{-1} cm^{-1}), and the quantum yield increases to 0.87.[28,29] The fluorescence of the metal-free ligand also changes with pH, with maximal fluorescence occurring at pH 5.5 and essentially no fluorescence at pH > 12. The response fits to an apparent pK_a of 8.3. Accordingly, the Zn^{2+}-induced fluorescence enhancement is much greater at higher pH, consistent with a mechanism of intramolecular photoinduced electron transfer from a deprotonated benzylic amine resulting in fluorophore quenching and alleviation of such quenching by Zn^{2+} chelate formation.

Preliminary estimates indicated an apparent K_d for the Zinpyr-1·Zn^{2+} complex of ~1 nM. To characterize the Zn^{2+} affinity of Zinpyr-1 more accurately, we prepared a dual-metal single-ligand buffer system[30] comprising 1 mM total EDTA, 2 mM total Ca^{2+} (or Mg^{2+} up to 5 mM), and 0−1 mM total Zn^{2+}. Under these conditions, the $[Zn(EDTA)]^{2-}$ complex buffer system has an apparent K_d of 2.11 nM. This system not only facilitates the controlled variation in $[Zn^{2+}]$ necessary to characterize its binding to this new ligand, it also demonstrates the selectivity of the fluorescence response.

The results of a typical titration are shown in Figure 1. The quantum yield of fluorescence increases 2.25-fold, and additional changes in the absorption spectra between the ligand and zinc complex are synergistic, allowing a ~3.1-fold increase in the integrated emission to be achieved with excitation at 507 nm, the Zinpyr-1·Zn^{2+} excitation maximum. This analysis was performed in triplicate using different preparations of Ca^{2+}/EDTA/Zn^{2+} buffers to determine an apparent Zn^{2+} K_d of 0.7 ± 0.1 nM (mean ± esd), with a Hill coefficient of 1, consistent with a 1:1 ligand:Zn^{2+} species responsible for the fluorescence increase.

The ability of Zinpyr-1 to respond to changes in Zn^{2+} within Cos-7 cells was assessed. Labeling of cells with 5 μM Zinpyr-1 at room temperature for 0.5 h produced bright punctate staining (Figure 2) that co-localized with the acidic-compartment probe LysoTracker (Molecular Probes, Eugene OR). These observations

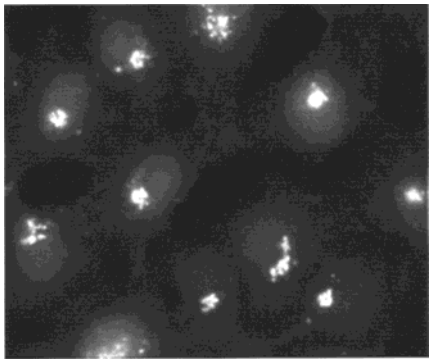

Figure 2. Fluorescence microscopy images of Cos-7 cells labeled with 5 μM Zinpyr-1 for 0.5 h at 37 °C. The bright perinuclear punctate staining is sensitive to the addition of the zinc ionophore Zn^{2+}/pyrithione and reversible by treatment with the high affinity membrane-permeable heavy-metal chelator, TPEN.

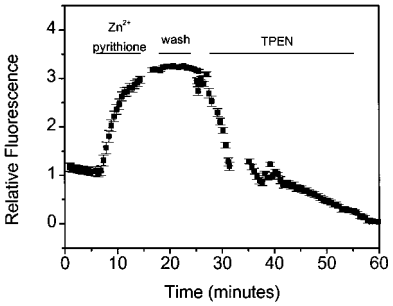

Figure 3. Fluorescence intensity analysis of Cos-7 cells shown in Figure 2. Eight of the bright perinuclear punctate regions were normalized by their initial intensity, and the mean ± std. error plotted at each time point. Cytosolic and nuclear regions selected for analysis did not respond to exogenously added Zn^{2+}/pyrithione or TPEN (not shown).

parallel similar findings made with quinoline-based Zn^{2+} probes in undifferentiated eukaryotic cells.[10,31] On the basis of the morphology of the juxtanuclear Zinpyr-1 staining, we suspected the Golgi to be the labeled organelle. Double-labeling experiments with a galactosyl transferase-enhanced cyan fluorescent protein fusion (GT-ECFP) that co-localizes with the medial/trans-Golgi[32] confirmed that the Zinpyr-1 stains the Golgi or a Golgi-associated vesicle. Furthermore, addition of exogenous Zn^{2+} (50 μM) with the zinc ionophore 2-mercaptopyridine *N*-oxide (pyrithione, 20 μM) resulted in enhanced fluorescence from the puncta only. The increase in fluorescence could be reversed with TPEN, establishing the metal-requirement of the signaling (Figure 3). Whereas Zinpyr-1 preferentially stains the Golgi and acidic cellular compartments, this property will be addressed in future designs.

In conclusion, we have prepared a new, high affinity, selective fluorescent sensor for Zn^{2+} that is membrane permeable. The fluorescein core of Zinpyr-1 exhibits bright fluorescence, can be excited at visible wavelengths, and overlaps well with the 488 nm Ar/ion laser line, which will facilitate confocal microscopy with this probe. Zinpyr-1 represents the first Zn^{2+} indicator to be developed in an ongoing program to tune the binding and optical properties of sensors for the neurosciences.

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Supporting Information Available: Synthesis and characterization of Zinpyr-1, and experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(28) Reported quantum yields are based on fluorescein having a quantum yield of 0.95 in 0.1 N NaOH. We estimate these values to be accurate within 5%.

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