

A Highly Selective Turn-On Colorimetric, Red Fluorescent Sensor for Detecting Mobile Zinc in Living Cells

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We describe ZRL1, a turn-on colorimetric and red fluorescent zinc ion sensor. The Zn²⁺-promoted ring opening of the rhodamine spirolactam ring in ZRL1 evokes a 220-fold fluorescence turn-on response. In aqueous media, ZRL1 turn-on luminescence is highly selective for Zn²⁺ ions, with no significant response to other competitive cations, including Na⁺, K⁺, Ca²⁺, Mg²⁺, Mn²⁺, Fe²⁺, Co²⁺, Ni²⁺, Cu²⁺, Cd²⁺, or Hg²⁺. In addition to these characteristics, preliminary results indicate that ZRL1 can be delivered to living cells and can be used to monitor changes in intracellular Zn²⁺ levels.

Mobile zinc plays an important role in life processes, and disruption of zinc homeostasis has been implicated in health disorders including Alzheimer's disease^{1,2} and diabetes.³ Methods for sensing Zn²⁺ uptake, accumulation, trafficking, and efflux have therefore attracted much recent attention.^{4,5} Although several Zn²⁺-selective fluorescent sensors are now available,^{6–9} novel constructs remain in high demand to provide one or more of the following features: (1) selectivity for Zn²⁺; (2) pH-independent response under physiological conditions; (3) large dynamic range; (4) red or near-infrared emission to avoid autofluorescence by biomolecules; (5) improved photostability; and (6) ratiometric detection.

Rhodamine B and its derivatives, because of their high quantum yields and good photostability, are of particular interest for cation sensing. The mechanism of fluorescent turn-on for these compounds typically involves opening of a spirolactam ring by external reagents, including Cr³⁺, Fe³⁺,

Cu²⁺, Hg²⁺, Pb²⁺, Au³⁺, and Ag⁺.^{10–20} The spirocyclic forms of these dyes, which are colorless and nonfluorescent, become colorful and strongly fluorescent when cations react with the sensors. To our knowledge, there are only two examples of zinc sensing based on such a rhodamine dye spirocyclic ring-opening mechanism.^{21,22} One, termed Rhoda-P, is selective for zinc, but its properties were investigated only in organic solvents and there was no information about its reversibility or applicability for live cell imaging.²¹ Moreover, we find that Rhoda-P evokes no appreciable turn-on response to zinc ions in buffered aqueous solution (Figure S1, Supporting Information, SI). The other example is a ratiometric zinc sensor based on a hybrid fluorescein–rhodamine lactam platform.²² Its photophysical properties were investigated in 50% aqueous acetonitrile solution, and there was fluorescence interference by [Hg²⁺] > 20 μM and protons in the pH range 6.0–7.0.

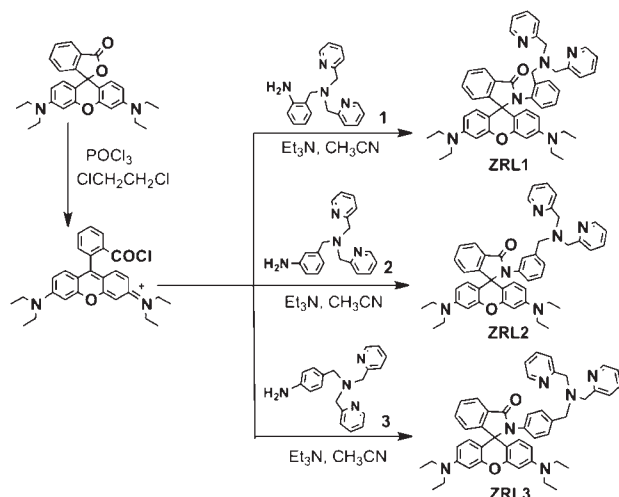
In the present Communication, we describe the synthesis and properties of a water-soluble, turn-on red fluorescent sensor, ZinRhodaLactam-1 (ZRL1), which exhibits extremely low background fluorescence and a large turn-on response when bound to zinc in aqueous media. ZRL1 has

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Scheme 1. Synthesis of ZRL1, ZRL2, and ZRL3



excellent selectivity, is pH-independent in the range 5.4–11.0, and exhibits a large dynamic range for mobile Zn^{2+} . Fluorescence microscopy experiments further establish that ZRL1 is membrane-permeable and can be used to monitor intracellular Zn^{2+} in live cells.

The design of ZRL1 combines a rhodamine B nonfluorescent lactam and a zinc-binding ligand, 2-[bis(2-pyridylmethyl)aminomethyl]aniline (**1**; Scheme 1). ZRL1 was synthesized by treating rhodamine B base with POCl_3 , followed by dropwise addition to a solution of **1**, prepared by our published method.²³ After column chromatography (alumina, eluent 50:1 (v/v) $\text{CH}_2\text{Cl}_2/\text{MeOH}$), ZRL1 was obtained in 17% yield and was quite stable over 6 months, even when kept at room temperature. The analogues ZRL2 and ZRL3 were prepared by the same route, except that 3-[bis(2-pyridylmethyl)aminomethyl]aniline (**2**) and 4-[bis(2-pyridylmethyl)aminomethyl]aniline (**3**) were used in place of **1**. The synthetic procedures for compounds **2** and **3** are similar to those for **1**, as fully described in the SI. ZRL1, ZRL2, and ZRL3 were characterized by ^1H and ^{13}C NMR spectroscopic and HRMS methods, and the structure of ZRL1 was determined by X-ray crystallography (Figure 1 and Tables S1 and S2, SI).

ZRL1 forms colorless and nonfluorescent solutions in either aqueous media or organic solvents, including DMSO, acetone, MeOH, or CH_3CN . Addition of zinc ions to ZRL1 in aqueous solution or organic solvents leads to the development of a pink color and red fluorescence, indicating that ZRL1 can detect zinc ions via opening of the spirolactam ring. Quantitative photophysical studies were conducted under simulated physiological conditions (50 mM PIPES with 100 mM KCl, pH 7.0). The absorption and fluorescence spectral titrations by zinc ions are shown in Figure 2. The metal-free sensor initially displayed no appreciable absorption or emission band above 500 nm ($\Phi < 0.001$), indicating the existence of only the lactam form in aqueous media. Addition of Zn^{2+} ions triggered formation of a visible absorption band centered at 569 nm and an emission band with a maximum at 595 nm. The solution changed from colorless to pink. These results indicate that Zn^{2+} ions promote the transformation of ZRL1 from the lactam to

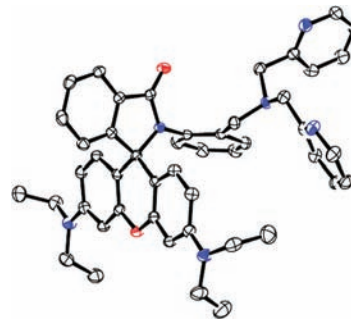


Figure 1. ORTEP diagram of ZRL1 showing 50% probability thermal ellipsoids on all non-hydrogen atoms. Hydrogen atoms are omitted for clarity. Oxygen atoms are red, carbons are white, and nitrogens are blue.

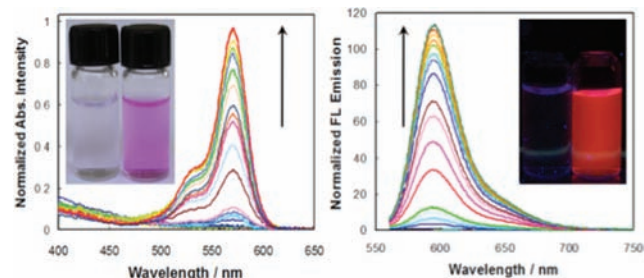


Figure 2. Absorption (left) and fluorescence (right) spectra of ZRL1 ($10 \mu\text{M}$) in a buffered solution upon addition of different amounts of ZnCl_2 . Excitation was performed at 550 nm. The inset pictures show, following addition of 100 equiv of zinc ions to ZRL1 ($10 \mu\text{M}$) in a buffered solution, visible color formation by the naked eye (left) and fluorescence (right) under illumination by a hand-held UV lamp. All spectra were acquired 2 h after mixing ZRL1 with zinc in 50 mM PIPES at pH 7.0 containing 100 mM KCl.

ring-opened form. Incremental addition of Zn^{2+} [$(0-1.8) \times 10^{-3} \text{ M}$] provided a steady fluorescence turn-on with no shift in either the absorption or emission maximum. When the concentration of Zn^{2+} was greater than $1.0 \times 10^{-3} \text{ M}$, the optical features reached a plateau, with $\epsilon_{569} \sim 20800 \text{ M}^{-1} \text{ cm}^{-1}$ and the emission quantum yield $\Phi_{\text{FL}} = 0.22$. The ~ 220 -fold increase in the fluorescence quantum yield provides an excellent turn-on ratio for zinc sensing in aqueous media. Moreover, the response of ZRL1 to zinc ions can be reversed. Titration of the ZRL1- Zn^{2+} complex with a metal ion chelator, either TPEN or EDTA, restored the fluorescence to baseline levels (Figures S2 and S3, SI). The effect of pH on the fluorescence spectrum of ZRL1 was also investigated in aqueous media (Figure S4, SI). In the pH range from 5.4 to 11.0, the zinc-induced ZRL1 fluorescence is pH-independent and the free ligand has no appreciable emission in the visible region of the spectrum. This property is valuable for applications to living cells, where pH changes are caused by biological stimuli.

For comparison, the photophysical properties of ZRL2 and ZRL3 were also investigated in buffer (50 mM PIPES containing 100 mM KCl, pH 7.0). However, both compounds failed to exhibit any appreciable turn-on response to Zn^{2+} ions (Figures S5 and S6, SI), indicating the importance of close proximity between the carbonyl group on the lactam ring and the adjacent dipicolylamine zinc-chelating moiety for ring opening of the lactam.

The ZRL1 zinc binding affinity was analyzed by fluorescence spectroscopy. The fit to a plot of normalized intensity versus $[\text{Zn}^{2+}]_{\text{total}}$ (Figure S7, SI) revealed a turn-on constant

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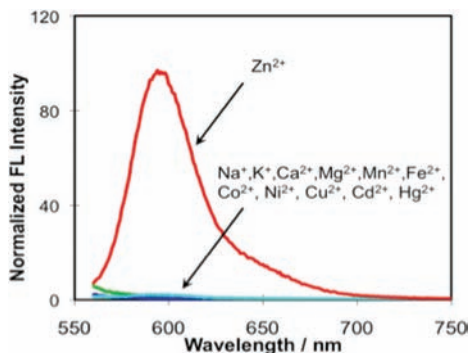


Figure 3. Fluorescence spectroscopic response of ZRL1 to various metal ions. All spectra were acquired after 2 h upon mixing ZRL1 with zinc in 50 mM PIPES at pH 7.0 containing 100 mM KCl. Normalized fluorescence intensities represent the addition of an excess of the appropriate metal ion (1 mM for Na^+ , K^+ , Ca^{2+} , and Mg^{2+} ; all others are 200 μM) to a 10 μM solution of ZRL1. Excitation was provided at 550 nm.

K_t of $73 \pm 2 \mu\text{M}$, where K_t represents both zinc dissociation and lactam formation, for ZRL1 binding to Zn^{2+} . The K_t value of ZRL1 is at the high end compared to K_d values of many related sensors with dipicolylamine Zn^{2+} -binding groups⁸ but in the functional range for sensing of the ion in many neurochemical applications.^{2,5}

The fluorescence turn-on of ZRL1 is highly selective for Zn^{2+} over many other monovalent and divalent metal ions (Figures 3 and S8, SI). Even large excesses of Na^+ , K^+ , Ca^{2+} , and Mg^{2+} (1 mM), the most biologically relevant, potentially competing metal ions, barely perturb the fluorescence of the zinc-free and zinc-bound forms of ZRL1. Moreover, addition of 200 μM concentrations of any of the following divalent transition-metal ions into ZRL1, Mn^{2+} , Fe^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Cd^{2+} , or Hg^{2+} , does not induce an appreciable fluorescence turn-on, although Co^{2+} , Ni^{2+} , and Cu^{2+} show an obvious absorption response (Figure S9, SI). The paramagnetism of these last three ions most likely quenches the fluorescence of the resulting ZRL1 complexes. Thus, only Zn^{2+} evokes a fluorescent turn-on response over all other competing metal ions tested. Many reported fluorescent Zn^{2+} sensors, such as those in the Zinpyr family,⁸ lanthanide chemosensors,²⁴ and some ZnAF family members,²⁵ cannot distinguish Zn^{2+} from Cd^{2+} and/or Hg^{2+} . At present, we do not have a good explanation for this behavior.

The ability of ZRL1 to track zinc ions in living cells was also examined (Figure 4). HeLa cells incubated with 10 μM

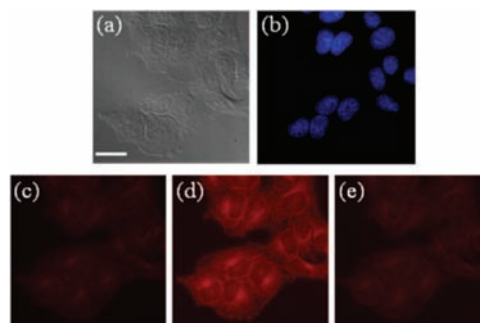


Figure 4. Live-cell imaging of HeLa cells after incubation with ZRL1 (10 μM) and Hoechst 33258 nuclear stain (blue): (a) Bright-field transmission image. (b) Nuclear image stained by Hoechst 33258. (c) Fluorescence image without addition of Zn^{2+} . (d) Fluorescence image of cells incubated with ZRL1 for 15 min, washed twice, and then treated with 50 μM Zn^{2+} pyrrhione for 20 min. (e) ZRL1-loaded, 50 μM Zn^{2+} -supplemented cells treated with 80 μM TPEN after 10 min. Scale bar = 25 μm .

ZRL1 for 15 min at 37 °C exhibit a weak intracellular red fluorescence (Figure 4c). After addition of 50 μM Zn^{2+} carried into the live cells by the ionophore pyrrhione, the red fluorescence was much stronger and clearly observable (Figure 4d). The red fluorescence substantially decreased upon introduction of 80 μM TPEN (Figure 4e). Figure 4b shows cells treated with the nuclear stain Hoescht 33258. These results indicate that ZRL1 is cell-permeable and can be used to image Zn^{2+} within living cells.

In conclusion, ZRL1 based on rhodamine B in its lactam form undergoes a large, turn-on red fluorescence specifically in the presence of zinc ions in aqueous media and in live cells. The increase in quantum yield on binding zinc is ~ 220 -fold, and the fluorescence can be reversed upon addition of an appropriate zinc chelator. These properties will make ZRL1 of value in studies of the neurochemical functions of mobile zinc that are currently in progress.

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Supporting Information Available: Full synthetic procedures, characterization data, crystallographic details in CIF format of ZRL1, pH titration of ZRL1, and data fitting method for fluorescence turn-on constant. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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