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Development of ratiometric fluorescent probe for zinc ion based on indole fluorophore

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ABSTRACT

A water-soluble ratiometric fluorescent probe ZID-1 has been developed on the basis of an internal charge transfer (ICT) mechanism. Upon complexation with Zn^{2+} under physiological conditions, ZID-1 exhibits a significant blue shift of 77 nm in the emission spectrum. The fluorescent behavior of ZID-1 suggests that the pyridyl group incorporated into the fluorophore coordinates the metal ion as the fourth ligand and affords an appropriate binding affinity (K_d = 17.1 nM) for the intracellular imaging of Zn^{2+}.

Fluorescent chemosensors are used for the detection of a specific metal ion in living cells and are excellent tools for elucidating the functions of metal ions in biological systems.[1] These chemosensors can be used to visualize pools of labile metal ions with high optical sensitivity by means of fluorescence microscopy.[2] In particular, ratiometric fluorescent probes that exhibit a shift in either excitation or emission maxima upon the formation of a complex can be used to perform quantitative measurement of a change in metal ion concentration.[3] Determination of the ratio of fluorescence intensities measured at two suitably selected wavelengths results in the cancellation of artifactual factors, such as illumination intensity, photobleaching of the probe, cell thickness, and dye concentration within cells, in the fluorescent signals. An internal charge transfer (ICT) mechanism has been widely used as the basis for the design of ratiometric fluorescent sensors.[1] Fura-2 and Indo-1 are typical examples as the ICT probe in which Ca^{2+} binding alters the electron-donating properties of the electron-rich chelating group in the excitation state and causes a significant blue shift in its fluorescence spectrum.[3]

Among a series of biologically important metal ions, zinc (Zn^{2+}) has attracted considerable attention because of its structural significance and catalytic functions in metalloproteins.[4] Although these Zn^{2+} ions are often tightly bound, chelatable (weakly bound) Zn^{2+} ions also exist in several tissues, including brain tissue,[5] pancreatic tissue,[6] and seminal plasma.[7] To understand the roles of Zn^{2+} in these tissues, a variety of Zn^{2+}-selective fluorescent probes that are based on quinoline,[8] fluorescein,[9] benzazole,[10] coumarin,[11] other fluorophores,[12] or proteins[13] have been developed. However, the functions of Zn^{2+} in these tissues or even within single-celled organisms are not fully investigated, because of the lack of suitable ratiometric probes comparable with Fura-2 and Indo-1 for Ca^{2+},[3] using which valuable information about its intracellular behavior has been obtained. Therefore, the development of efficient ratiometric probes for Zn^{2+}, matching for Fura-2 and Indo-1, is indispensable for the investigation of the biological roles of Zn^{2+}. Although several kinds of ratiometric fluorescent probes have recently been developed for the detection of Zn^{2+},[14,15] most of these suffer from some weak points such as small shift in the fluorescence wavelength, large fluorescence increase or decrease in the Zn^{2+}-bound form, low water solubility, or low binding affinity to Zn^{2+}. Furthermore, an emission ratiometric probe is required for imaging using two-photon excitation (TPE) fluorescence microscopy, which provides significant advantages over standard laser confocal approaches.[16] Therefore, there is still a requirement for ratiometric probes that can provide a large peak shift in emission spectra upon their binding with Zn^{2+}.

Herein, we report the development of a new Zn^{2+}-selective fluorescent probe ZID-1, which utilizes a 2-(4-aminophenyl)indole derivative as the fluorophore. Such a derivative is also utilized in Indo-1,[3] Mag-indo,[17] and IndoZin,[15a] which are sensor molecules for Ca^{2+}, Mg^{2+}, and Zn^{2+}, respectively. In order to achieve high selectivity and affinity for Zn^{2+} detection, we introduced a pyridylmethyl arm into the fluorophore of ZID-1 through an oxygen atom at the *o*-position of the amino group. The fluorescence properties, metal ion selectivity, and dissociation constant for Zn^{2+}, determined under

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physiological conditions, strongly suggest that ZID-1 can be used as an effective ratiometric fluorescent probe for intracellular imaging of Zn^{2+}.

The synthesis of ZID-1 started from 2-aminophenol and performed in 6 steps, as outlined in Scheme 1. The amino group was alkylated by the reaction with 2 equiv of *tert*-butyl bromoacetate according to the reported procedure.[18] The resulting phenol **1** was then treated with NaH and 2-picolyl chloride to yield compound **2**, which composes the Zn^{2+} binding moiety of the probe. Successive formylation under the Vilsmeier conditions, the Wittig reaction, and reductive cyclization using triethyl phosphite gives indole-triester **5** as a white powder. Finally, the ester was successively hydrolyzed by boron trifluoride diethyl ether complex ($BF_3{\cdot}Et_2O$) and KOH to yield ZID-1, which was purified by size-exclusion chromatography and reverse-phase chromatography.

Under physiological conditions (50 mM HEPES, pH 7.2, 0.1 M KNO_3), ZID-1 exhibited an absorption maximum at 351 nm ($\varepsilon = 3.84 \times 10^4\ M^{-1}\ cm^{-1}$), which is in good agreement with those reported for the apo forms of Indo-1[3] and its derivatives.[15a,17] Upon addition of Zn^{2+}, a decrease in the absorbance of this band and a concomitant increase in that of a new band at 329 nm ($\varepsilon = 3.26 \times 10^4\ M^{-1}\ cm^{-1}$) were observed with a distinct isosbestic point at 335 nm (Fig. 1). The absorption bands at 351 nm and 329 nm linearly decreased and increased, respectively, up to a 1:1 $[Zn^{2+}]/[ZID\text{-}1]$ ratio (Fig. 1 inset), indicating the formation of a 1:1 complex with a strong binding affinity.

Figure 2a shows emission spectra of ZID-1 excited at 335 nm, which is the isosbestic point of the UV–vis traces, at various free Zn^{2+} concentrations. The apo form exhibits a characteristic band at 470 nm ($\Phi = 0.097$) that shifts to 393 nm ($\Phi = 0.11$) with an isoemissive point upon binding with Zn^{2+}. The fluorescence response of ZID-1 fits a Hill coefficient of ~1 (Fig. 2a inset); it is again confirmed that ZID-1 forms a 1:1 complex with Zn^{2+} even at the diluted concentration of ZID-1 such as 1 μM. A significant hypsochromic shift (77 nm) of the emission wavelength indicates that the ICT excited state of ZID-1 (apo form) is strongly affected by coordination with Zn^{2+}; this in turn indicates that ZID-1 would be more useful than reported emission ratiometric fluorescent Zn^{2+} probes, the fluorescence shifts of which are much smaller than the present value. From the plot of the fluorescence intensities at 395 nm or 470 nm against $\log[Zn^{2+}]_{free}$ (Fig. 2b), the apparent dissociation constant K_d for Zn^{2+} was determined to be 17.1 ± 0.2 nM at pH 7.20. The K_d value of ZID-1 is comparable to those of reported tetradentate ligands (0.1–20 nM) but considerably smaller than

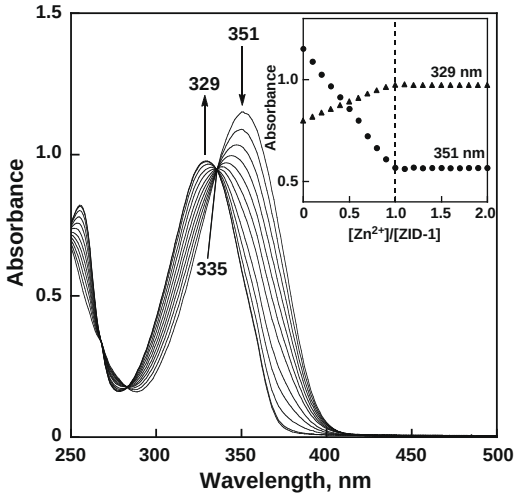
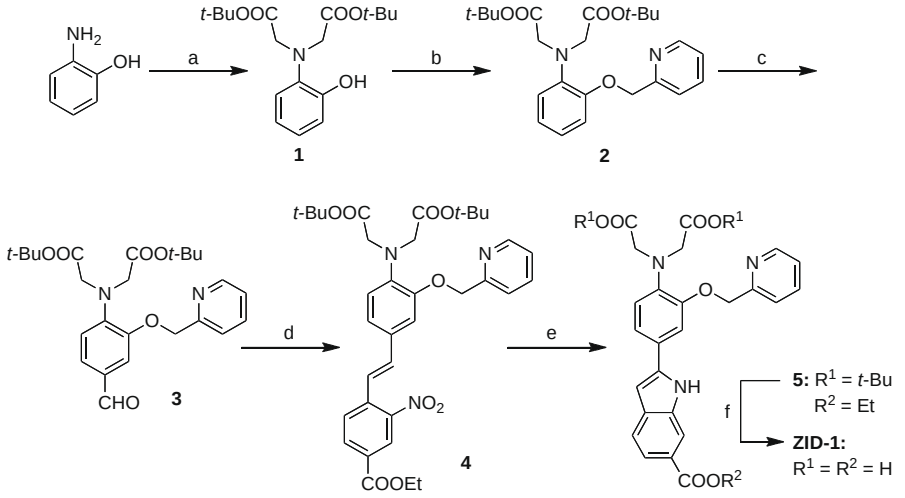


Figure 1. UV–vis spectral change by addition of Zn^{2+} to 30 μM of ZID-1 in 50 mM HEPES buffer (pH 7.20, 0.1 M KNO_3). Inset: mol ratio plots of absorbance at 329 nm and 351 nm.

that of IndoZin (3 μM),[19] in which a methyl group replaces pyridylmethyl group. This result reveals that the incorporated pyridine moiety coordinates the metal ion as the fourth ligand to give a stable chelate. Furthermore, this nonlinear fitting analysis reveals that ZID-1 is suitable for detecting $[Zn^{2+}]_{free}$ between 4.1 and 68 nM.[20]

We then examined the fluorescence responses of 1 μM ZID-1 to various biologically relevant metal ions, shown in Figure 3. Na^+, K^+, Mg^{2+}, and Ca^{2+}, which exist in high concentrations in cells, had a very small effect on the fluorescence spectrum, even at metal concentrations as high as 5 mM; furthermore, they did not interfere with Zn^{2+} binding, indicating that this probe can be employed for a wide range of biological applications using microscopic techniques. Other transition metal ions such as Fe^{3+}, Co^{2+}, Ni^{2+}, and Cu^{2+} formed complexes and quenched the fluorescence of ZID-1. Cd^{2+} induced the emission shift of ZID-1 as observed for Zn^{2+}. However, these free cations would have little influence on intracellular Zn^{2+} imaging, because of their presence in very low concentrations.[21]

In summary, we have developed a new ratiometric fluorescent probe for Zn^{2+}, ZID-1, on the basis of an ICT mechanism. Upon complexation with Zn^{2+}, this probe exhibits a significantly large blue shift (77 nm) in the emission spectrum. It also has a binding affinity such that it is suitable for biological applications; such an affin-



Scheme 1. Synthetic procedure of ZID-1. Reagents and conditions: (a) *t*-butyl bromoacetate, proton sponge, KI, CH_3CN, reflux, overnight, 80%; (b) NaH, KI, 2-picolyl chloride·HCl, THF, room temperature, overnight, 22%; (c) $POCl_3$, DMF, room temperature, overnight, 77%; (d) Wittig reagent, K_2CO_3, DMF, 90 °C, overnight, 78%; (e) $P(OEt)_3$, 125 °C, 4 h, 46%; (f) $BF_3{\cdot}Et_2O$, KOH, then 0.1 M HCl; 100%.

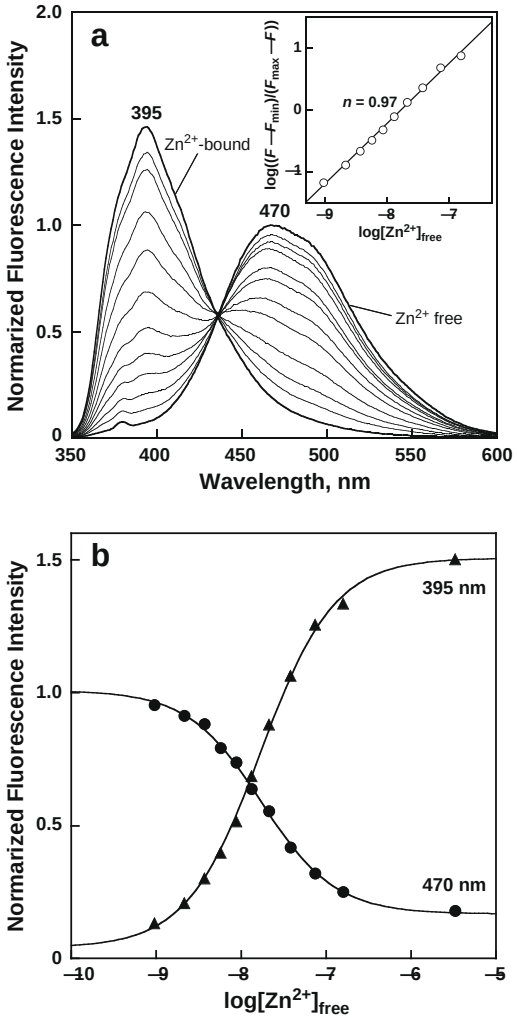


Figure 2. (a) Emission spectra of ZID-1 (1 μM) excited at 335 nm in Zn^{2+}/NTA buffered system (50 mM HEPES, pH 7.20, 0.1 M KNO_3; 10 mM NTA, 0–9.5 mM $ZnSO_4$) and in 50 mM HEPES buffer (pH 7.20) containing 4 μM $ZnSO_4$. Inset: Hill plot at 470 nm. The Hill coefficient (n) was determined from the slope. (b) Plots of fluorescence intensities at 395 nm (triangles) and 470 nm (circles) with best-fit curves for the dissociation constant of 17.1×10^{-9} M.

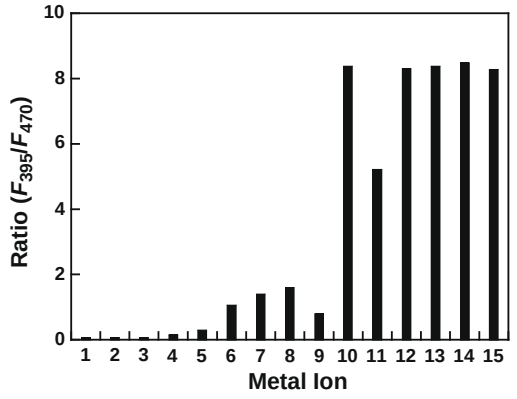


Figure 3. Fluorescence ratio of ZID-1 (1 μM) between 395 nm and 470 nm as a function of various added metal cations (5 mM for Na^+, K^+, Ca^{2+}, and Mg^{2+}, 10 μM for all other cations) in 50 mM HEPES (pH 7.20, 0.1 M KNO_3). 1: no metal; 2: Na^+; 3: K^+; 4: Mg^{2+}; 5: Ca^{2+}; 6: Fe^{2+}; 7: Co^{2+}; 8: Ni^{2+}; 9: Cu^{2+}; 10: Zn^{2+}; 11: Cd^{2+}; 12: Zn^{2+} + Na^+; 13: Zn^{2+} + K^+; 14: Zn^{2+} + Mg^{2+}; 15: Zn^{2+} + Ca^{2+}.

ity is successfully achieved by incorporating a pyridylmethyl group (as the binding ligand) into the fluorophore. Intracellular imaging of zinc ions using this probe is currently in progress for evaluating the suitability of ZID-1 for biological applications.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.01.026.

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