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Abstract: It is still a significant challenge to develop a Zn$^{2+}$-selective fluorescent sensor with the ability to exclude the interference of some heavy and transition metal (HTM) ions such as Fe$^{3+}$, Co$^{3+}$, Ni$^{2+}$, Cu$^{2+}$, Cd$^{2+}$, and Hg$^{2+}$. Herein, we report a novel amide-containing receptor for Zn$^{2+}$, combined with a naphthalimide fluorophore, termed ZTRS. The fluorescence, absorption detection, NMR, and IR studies indicated that ZTRS bound Zn$^{2+}$ in an imidic acid tautomeric form of the amide/di-2-picolylamine receptor in aqueous solution, while most other HTM ions were bound to the sensor in an amide tautomeric form. Due to this differential binding mode, ZTRS showed excellent selectivity for Zn$^{2+}$ over most competitive HTM ions with an enhanced fluorescence (22-fold) as well as a red-shift in emission from 483 to 514 nm. Interestingly, the ZTRS/Cd$^{2+}$ complex showed an enhanced (21-fold) blue-shift in emission from 483 to 446 nm. Therefore, ZTRS discriminated in vitro and in vivo Zn$^{2+}$ and Cd$^{2+}$ with green and blue fluorescence, respectively. Due to the stronger affinity, Zn$^{2+}$ could be ratiometrically detected in vitro and in vivo with a large emission wavelength shift from 446 to 514 nm via a Cd$^{2+}$ displacement approach. ZTRS was also successfully used to image intracellular Zn$^{2+}$ ions in the presence of iron ions. Finally, we applied ZTRS to detect zinc ions during the development of living zebrafish embryos.

Introduction

Fluorescent sensors are powerful tools to monitor in vitro and/or in vivo biologically relevant species such as metal ions because of the simplicity and high sensitivity of fluorescence.¹ A typical fluorescent sensor contains a receptor (the recognition site) linked to a fluorophore (the signal source) which translates the recognition event into the fluorescence signal.² Therefore, an ideal fluorescent sensor must meet two basic requirements. First, the receptor must have the strongest affinity with species of interest (binding selectivity), which is the central processing unit of a sensor. Second, the fluorescence signal should not be perturbed by the environment (signal selectivity). Most reported fluorescent sensors display an increase or decrease in the emission intensity upon binding to species of interest. However, ratiometric responses are more attractive because the ratio between the two emission intensities can be used to measure the analyte concentration and provide a built-in correction for environmental effects, such as photobleaching, sensor molecule concentration, the environment around the sensor molecule (pH, polarity, temperature, and so forth), and stability under illumination.³

Optical imaging with fluorescent sensors for Zn$^{2+}$ has attracted great attention, owing to the biological significance of zinc.⁴ Zinc is the second most abundant transition metal ion in the human body after iron, and is an essential cofactor in many biological processes such as brain function and pathology, gene transcription, immune function, and mammalian repro-

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Zn$^{2+}$-Triggered Amide Tautomerization Produces a Highly Zn$^{2+}$-Selective, Cell-Permeable, and Ratiometric Fluorescent Sensor

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duction.5,6 This ion is also involved in pathological processes, such as Alzheimer’s disease, epilepsy, ischemic stroke, and infantile diarrhea.7–9 Although most biological zinc ions are tightly bound to proteins (playing structural and catalytic roles), loosely bound or chelatable zinc, which are the main target of fluorescent sensors, are present in various human tissues, including the brain,10 intestine,11 pancreas,12 and retina.13 Up to now, a variety of fluorescent sensors for Zn2+ have been developed with some successful applications to image Zn2+ in living cells or hippocampal slices,14–16 perhaps most notably by Lippard15 and Nagano.16 Recently, Guo et al. reported a fluorescent sensor to trace intact Zn2+ in zebrafish embryos.17 However, only a few ratiometric fluorescent sensors for Zn2+ have been reported.18 Additionally, to our best knowledge, all these reported sensors have a shortcoming in that they suffer from interference of some heavy and transition metal (HTM) ions such as Fe2+, Co2+, Ni2+, Cu2+, and Hg2+. Even though the reported sensors show a selective turn-on fluorescence signal for Zn2+, they often display poor binding selectivity for Zn2+ over other HTM ions.19 Their low selectivity for Zn2+ may result from the use of di-2-picolylamine (DPA),15,16,19 acrylic and cyclic polyamines,20 iminodiacetic acid,21,22 bipyridine,21,22 quinalone,23 and Schiff bases24 as Zn2+-chelators, which have similar affinities to other HTM ions. In addition, some available Zn2+ sensors have difficulty in distinguishing Zn2+ and Cd2+, since Cd2+ is in the same group of the periodic table and has similar properties with Zn2+. Therefore, similar fluorescence changes including the change of intensity and the shift of wavelengths are usually observed when Zn2+ and Cd2+ are coordinated with fluorescent sensors. In recent years, Cui et al.25 and Jiang et al.25b,c reported sensors which can discriminate Zn2+ and Cd2+ with different emission wavelengths; however, these sensors have a stronger affinity for Cd2+.

After the first attachment to fluorescein in 1996,26 DPA has been used as the most popular receptor for Zn2+ sensors. Some DPA-derivatives, such as N,N-di-(2-picolyl)ethylenediamine (DPEN),27 tris(2-pyridylmethyl)amine (TPA)28c,29 and N,N,N′-tris(pyridin-2-ylmethyl)ethylenediamine (TRPEN),30,31 were subsequently devised as Zn2+-chelators, because DPA-related chelators confer selectivity for Zn2+ over cations that occur in much higher concentrations in biological samples, such as Ca2+, Mg2+, K+, and Na+. Since these chelators can also bind other HTM ions strongly, new strategies should be exploited to improve the Zn2+ selectivity of receptors. One possible approach is to impose the conformational restraint to the chelator of ions.
in sensors.\(^{30}\) Dai et al. developed TPA-based sensors with a trigonal bipyramidal coordination geometry to improve the Zn\(^{2+}\)/Cu\(^{2+}\) selectivity.\(^{30}\) In this contribution, an amide group is introduced into a DPA-type receptor to increase the Zn\(^{2+}\) selectivity. The amide linkage is a key facet in the structure of proteins, peptides, and other biologically important molecules.\(^{31}\) The hindered C–N bond rotation of amides (the peptide bond) is due to amide resonance (Scheme 1a) and provides proteins with the ability to form secondary and tertiary structures fundamental to biological activity. In our scaffold, an amide group was inserted into a sensor (ZTRS) to link two moieties of the 1,8-naphthalimide fluorophore and a DPA chelator. 4-Aminonaphthalimide is a cell-permeable fluorophore possessing a visible emission wavelength, high photostability, and facile synthesis of various derivatives.\(^{32}\) The amide oxygen and nitrogen atoms are well-known chelating sites.\(^{33}\) The binding of Zn\(^{2+}\) to the amide-DPA receptor of ZTRS induced an enhanced shift in emission wavelength due to binary effects of photoinduced electron transfer (PET) and intermolecular charge transfer (ICT) mechanisms, and therefore displays a turn-on signal. More importantly, high binding selectivity for Zn\(^{2+}\) was achievable by complexation of various metal ions in alternative amide tautomeric forms (Scheme 1b).

**Results and Discussion**

**Synthesis.** The route used to synthesize ZTRS is initiated by the coupling of 4-amino-N-butyl-1,8-naphthalimide (1) and 2-chloroacetyl chloride to produce 2 in 86% yield (Scheme 2). Reaction of 2 with DPA under basic conditions gives ZTRS in 84% yield. As a reference compound, ZTF without a fluorophore was prepared by the condensation of 2-chloro-N-phenylacetamide with DPA in 92% yield.

**Effect of pH on the Fluorescence of ZTRS.** The influence of pH on the fluorescence of ZTRS was initially examined by fluorescence titration in acetonitrile/water (50:50) solution (Figure 1). The fluorescence spectrum of ZTRS exhibits an emission band with a maximum at 483 nm (ε = 83300 M\(^{-1}\) cm\(^{-1}\), Φ = 0.016). Since the carbonyl group in ZTRS decreases the electron-donating ability of the amide nitrogen, ∼40 nm blue-shift was observed in emission compared to that of 4-amino-1,8-naphthalimide (520–530 nm). The fluorescence of ZTRS at 483 nm remained unaffected between pH 12.8 and 6.3 but dramatically increased from pH 6.3 to 5.4 due to the inhibited PET process by protonation of the tertiary amine in DPA; with increasing acidity from pH 4.7 to 2.6, a significant decrease in the 483 nm emission and a blue-shifted emission band centered at 456 nm were observed. This phenomenon may be attributed to the protonation of the amide oxygen, which leads to a decrease in electron-donating ability and a blue-shift in emission. The stable fluorescence of ZTRS at around pH 7.0 is favorable for in vivo applications.

**Zn\(^{2+}\) Selectivity.** The selectivity of the fluorescent response of ZTRS to zinc ions was then examined. Figure 2a shows the
fluorescence response of ZTRS to various metal ions in aqueous solutions (CH3CN/0.5 M HEPES, pH 7.4) 50:50). Selective and large fluorescent enhancements (FE) were observed upon addition of Cd2+ (21 fold) and Zn2+ (22 fold) to the solution of ZTRS. Notably, Cd2+ induced a blue-shift in the emission of ZTRS to 446 nm (blue fluorescence, ε = 84700 M⁻¹ cm⁻¹, Φ = 0.34), while Zn2+ caused a red-shift to 514 nm (green fluorescence, ε = 87500 M⁻¹ cm⁻¹, Φ = 0.36). This difference in response allows ZTRS to easily distinguish between Cd2+ and Zn2+ in aqueous solution, even with the naked eye (Figure 3). The Job plots indicate the ZTRS/Zn2+ and ZTRS/Cd2+ complexes all have 1:1 stoichiometry (Figure 2b and Supporting Information, Figure S1). The apparent dissociation constants (Kd) of ZTRS with Zn2+ and Cd2+ were determined by fluorescence spectroscopy as shown in Figure 4 to be 5.7 nM and 48.5 nM, respectively. In addition, ZTRS responds to metal ions in the same way in DMSO aqueous solutions (DMSO/0.5 M HEPES, pH 7.4 = 10:90) (Supporting Information, Figure S2). Also, it is worth mentioning that even in 100% aqueous solutions ZTRS can selectively sense Zn2+ (8 fold: ε = 68600 M⁻¹ cm⁻¹, Φ = 0.096) and Cd2+ (7 fold: ε = 63500 M⁻¹ cm⁻¹, Φ = 0.084) with less enhanced fluorescence (Supporting Information, Figure S3). The good water solubility of ZTRS demonstrates its potential for biological imaging.

In contrast to the fluorescent response of ZTRS to metal ions in aqueous solutions, in 100% CH3CN Zn2+ and Cd2+ result in blue-shifted emissions with the maximum wavelength change from 481 to 430 and 432 nm, respectively (Supporting Information, Figures S4, S5); however, the addition of Zn2+ and Cd2+ to ZTRS in 100% DMSO cause red-shifted emissions with the maximum wavelength change from 472 to 512 and 532 nm, respectively (Supporting Information, Figures S6, S7).
addition of other HTM ions results in blue-shift in emissions in both CH3CN and DMSO (Supporting Information, Figures S8, S9). However, a small blue-shift of the absorption maximum of ZTRS in CH3CN, DMSO, and aqueous solution upon addition of Zn2+ and Cd2+ (Supporting Information, Figures S10–S15) indicates that the red-shifted emission does not result from the deprotonation of amide NH group, because the deprotonation of the NH group conjugated to 1,8-naphthalimide would cause a red-shift in absorption spectra.18h,25a These spectral data suggest that ZTRS binds Zn2+ and Cd2+ in different tautomeric forms, depending on the solvent and metal ions (Scheme 3). ZTRS complexes both Zn2+ and Cd2+ in the amide tautomer in CH3CN, and the imidic acid tautomer in DMSO predominantly. However, other HTM ions bind to the amide tautomer in both CH3CN and DMSO.

Further evidence for the amide and imidic acid tautomeric binding modes (Scheme 3) is provided by 1H NMR titration experiments of ZTRS with Zn2+ and Cd2+ in CD3CN (Supporting Information, Figures S16, S17) and DMSO-d6 (Supporting Information, Figures S18, S19). 2D NOESY of ZTRS/Zn2+ (1:1 complex) in CD3CN (Figures 3, Supporting Information, Figures S20, S21) and DMSO-d6 (Figures 3, S22–23), and IR spectra of ZTRS/Zn2+ (1:1 complex) in CH3CN (Supporting Information, Figure S24) and DMSO (Supporting Information, Figure S25). As a reference, the binding properties of ZTF with Zn2+ were also examined by means of 1H NMR and IR spectra.

The blue-shifts in emission of ZTRS with HTM ions in acetonitrile are attributed to the coordination of the amide oxygen with metal ions which increases the electron-withdrawing ability of the amide group via ICT mechanism. As expected, the absorption maximum of ZTRS undergoes a blue-shift from 371 to 348 nm upon addition of both Zn2+ and Cd2+ (Supporting Information, Figures S10, S11). 1H NMR analysis provides further evidence to support the M–O bond formation, which results in large upfield shifts of the resonance of the adjacent NH proton.34 For example, addition of 1 equiv of Zn2+ or Cd2+ promotes a large upfield shift (11.72 to 9.73 and 9.49 ppm, respectively) of the resonance of the adjacent NH proton in ZTRS (Supporting Information, Figures S16, S17). In contrast, the same proton in ZTRS in CD3CN with the addition of 1 equiv of Zn2+ and Cd2+ (Supporting Information, Figures S18, S19), undergoes a much smaller upfield shift from 11.51 to 11.26 and 11.37 ppm in DMSO, respectively. 1H NMR analysis of ZTF with Zn2+ also shows a large upfield shift of NH from 10.91 to 9.29 in CD3CN, while there is a clear downfield shift of OH from 10.54 to 10.75 in DMSO (Figure 5). With the electron-withdrawing nature of the carbonyl group, the lone pair of electrons on the amide nitrogen is delocalized by resonance, thus forming a partial double bond with the carbonyl carbon and putting a partial negative charge on the oxygen (amide resonance, Scheme 1A). The complexation of the carbonyl oxygen with Zn2+ in CD3CN blocks the resonance structure B and then shifts the NH resonance upfield. Correspondingly, the binding of the amide nitrogen with Zn2+ in DMSO acts as an electron-withdrawing group to shift the OH resonance downfield. Therefore, the chemical shift of the amide NH can be used to distinguish between Zn2+ (or other metal ions) is bound to carbonyl oxygen or imidic acid nitrogen.

The single crystal structure and data of ZTF-Zn2+ in CH3CN are shown in Figure 6 and Supporting Information, Table S1, respectively. As expected, the amide oxygen (O1) cooperates with the DPA (N2–N4) and one CH3CN molecule (N5) as a receptor to bind Zn2+ (Figure 6). The bond length of Zn(1)–O(1) (2.002 Å) is much shorter than the other four Zn1–N bonds.

2D NOESY studies of ZTRS/Zn2+ (1:1) in CD3CN and DMSO-d6 give the direct evidence for the amide and imidic acid tautomeric binding modes (Figure 7). In NOESY, the nuclear Overhauser effect (NOE) between nuclear spins is used to establish the correlations. Hence the cross-peaks in the resulting two-dimensional spectrum connect resonances from spins that are spatially close. As shown in Figure 7, in the amide tautomeric form, H4 and H6 are spatially close, so that there are only cross peaks between H2–H6 and H3–H6; but in the imidic acid tautomeric form, H4 and H6 are spatially close, so that besides those between H2–H6 and H3–H6, there is also a strong cross peak between H4–H6 which supports the existence of the OH proton.

IR spectra also confirm the imidic acid binding mode. As shown in Supporting Information, Figure S24, the IR spectrum of ZTRS/Zn2+ (1:1) complex in CH3CN displays a typical C=O amide I band (1662 cm−1) and C–N stretching absorption at 1099 cm−1. The typical O–H (3457 cm−1) and C–O (1102 cm−1) stretching absorptions further verify the ZTRS/Zn2+ (1:1) complex in DMSO has the imidic acid binding pattern (Supporting Information, Figure S25). The IR spectrum of ZTF/Zn2+ (1:1) complex in DMSO also exhibits an O–H stretching absorption (Supporting Information, Figure S27).

Significantly therefore, we conclude that in aqueous solutions of Cd2+ the receptor ZTRS adopts an amide tautomer binding mode showing blue-shifted emission, while in aqueous solutions of Zn2+ ZTRS binds the metal ion via an imidic acid tautomer.

(34) A paper that reported the binding of amide oxygen with HTM ions resulting in upfield shifts of the resonance of the adjacent NH proton in 1H NMR spectra; ref 33a.
showing red-shifted emission. The red-shift in emission of ZTRS/Zn²⁺ is likely to be due to the expansion of the fluorophore-conjugated system.

To further check the Zn²⁺-selective tautomeric transformation of ZTRS over other metal ions, competition experiments were conducted in the presence of 300 equiv of Na⁺, K⁺, Mg²⁺, or Ca²⁺ and 3 equiv of Li⁺, Co²⁺, Ni²⁺, Cu²⁺, Cd²⁺, Fe²⁺, Fe³⁺, Cr³⁺, Ag⁺, Hg²⁺, or Pb²⁺, with the subsequent addition of 1 equiv of Zn²⁺. As shown in Figure 8a, the emission profile of the ZTRS/Zn²⁺ complex is unperturbed in the presence of these metal ions, indicating the strongest affinity and selectivity for Zn²⁺. A reasonable explanation would be the displacement of these metal ions by Zn²⁺ and the induced transformation of chelation from an amide to an imidic acid tautomeric form. It is notable that the addition of Zn²⁺ to these solutions induced an immediate ZTRS/Zn²⁺ fluorescence profile except in Cu²⁺ solution. The Cu²⁺ solution with 1 equiv Zn²⁺ displayed an enhanced fluorescence centered at 514 nm after 48 h.

Figure 5. ¹H NMR spectra of ZTF in the presence of Zn²⁺ in (a) CD₃CN and (b) DMSO-d₆.

Figure 6. Crystal structure of ZTF·Zn²⁺. All hydrogen atoms and perchlorate counterions are omitted for clarity. Thermal ellipsoids are shown at the 50% probability level. Selected bond distances (Å) and bond angles (deg): 
- Zn(1)–O(1) = 2.002(1), Zn(1)–N(2) = 2.206(1), Zn(1)–N(3) = 2.046(1), Zn(1)–N(4) = 2.032(2), Zn(1)–N(5) = 2.045(2), O(1)–Zn(1)–N(2) = 80.65(5), O(1)–Zn(1)–N(3) = 114.68(5), O(1)–Zn(1)–N(4) = 119.02(6), O(1)–Zn(1)–N(5) = 119.02(6), Zn(1)–N(5) = 95.44(6), N(2)–Zn(1)–N(3) = 80.65(5), N(2)–Zn(1)–N(4) = 80.72(5), N(2)–Zn(1)–N(5) = 176.04(6), N(3)–Zn(1)–N(5) = 118.54(6), N(3)–Zn(1)–N(5) = 101.29(6), N(4)–Zn(1)–N(5) = 101.20(6).

Figure 7. Partial 500 MHz ¹H–¹H NOESY spectra of ZTRS/Zn²⁺ (1:1) in (a) CD₃CN and (b) DMSO-d₆.
The selectivity of the fluorescence responses of ZTRS to Cd²⁺ was also examined by the addition of various metal ions to the solution of ZTRS−Cd²⁺ complex (1:1). As shown in Figure 8b, the addition of Zn²⁺, Cu²⁺, or Cd²⁺ quenches the fluorescence of ZTRS−Cd²⁺ complex at 446 nm. In contrast, other metal ions promote slight changes in the fluorescence of ZTRS−Cd²⁺ complex. This may mean that ZTRS has a higher affinity with Cd²⁺ than most of the HTM ions except for Zn²⁺, Co²⁺, and Cu²⁺.

Ratiometric Detection of Zn²⁺ Based on the Displacement Approach. The addition of Zn²⁺ to ZTRS induces an enhanced fluorescence with a 31 nm red-shift in emission. For practical applications, ratiometric signal should show a large shift in absorption or emission. Most of the reported ratiometric Zn²⁺ sensors are constructed on the basis of an ICT mechanism. Here, we develop a new approach to detect Zn²⁺ ratiometrically relying on a displacement strategy. In the displacement assay approach pioneered by Anslyn, an indicator is first allowed to bind reversibly to a receptor. Then, a competitive analyte is introduced into the system causing the displacement of the indicator from the host, which in turn modulates an optical signal. Based on this principle, the major requirement for an indicator displacement approach is that the affinity between the indicator and the receptor should be comparable to that between the analyte and the receptor. In our case, the affinity of ZTRS with Zn²⁺ ($K_a = 5.7$ μM) is stronger than that with Cd²⁺ ($K_a = 48.5$ μM). The ZTRS/Cd²⁺ complex displays a broadband with a maximum at 446 nm. When Zn²⁺ was added to the solution of ZTRS/Cd²⁺ complex, Cd²⁺ was displaced by Zn²⁺, resulting in a significant decrease in the 446 nm emission and an increase of a red-shifted emission band centered at 514 nm (attributed to the formation of a ZTRS/Zn²⁺ complex) with a clear isosbestic point at 472 nm (Figure 9). The inset in Figure 9 exhibits the dependence of the intensity ratios of emission at 514 nm to that at 446 nm ($I_{514}/I_{446}$) on Zn²⁺.

Detection of Intracellular Zn²⁺ with ZTRS. In vitro studies demonstrated the ability of ZTRS to detect Zn²⁺ with excellent selectivity. To examine whether this ability is preserved in vivo, A549 cells (lung cancer cells) were used to detect exogenous zinc ions in live cells. The cells treated with 5 μM ZTRS alone exhibited very weak background fluorescence (Figure 10a). However, the cells incubated with 1 μM ZnCl₂ and ZTRS displayed enhanced green fluorescence (Figure 10b). When the cells exposed to ZTRS and Zn²⁺ were further treated with a membrane-permeable zinc chelator (N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine, TPEN) that decreases the intracellular level of zinc, the treated cells showed a very weak fluorescent signal, indicating that green fluorescence is caused by response of ZTRS to intracellular zinc ions (Figure 10c). Interestingly, while blue fluorescence was observed in cells incubated with 5 μM CdCl₂ and ZTRS, the cells initially treated with CdCl₂ and ZTRS and subsequent exposure of the cells to 1 μM ZnCl₂ exhibited green fluorescence (Figure 10d,e). These experiments indicate that ZTRS can discriminate in vivo Zn²⁺ and Cd²⁺ with green and blue fluorescence, respectively. More attractively, Zn²⁺ could be ratiometrically detected in vivo with a large fluorescence color change from blue to green via the Cd²⁺ displacement approach. Furthermore, intracellular zinc ions were detected by use of ZTRS even in the presence of iron ions (Figure 10f,g). The cadmium-displacement method provides an appealing ratiometric change but this assay may have a drawback due to the toxicity of cadmium in biological systems. However, cytotoxicity of cadmium ions was not observed up to 40–50 μM concentrations, and thus this assay could be applied for biological systems. These cell experiments show that ZTRS is cell-permeable and can be used to monitor Zn²⁺ selectively in vivo and to further distinguish between Cd²⁺ and Zn²⁺ in living cells.

Imaging of Intact Zn²⁺ in Zebrash in Zebrafish with ZTRS. We then applied ZTRS to trace the distribution of intact zinc ions in

When 54 h-old zebrafish were exposed to external Zn\textsuperscript{2+} or an endogeneous zinc pool in fish. It was observed that, green-spotted band may result from sequestration of in the disappearance of the green spots (Figure 11e). The of external zinc ions, and (c) after treatment with ZTRS and 1 \mu M \text{ZnCl}_2 and subsequent treatment of the cells with 25 \mu M TPEN. (d) Cells treated with ZTRS and 5 \mu M \text{CdCl}_2 and (e) after treatment with ZTRS and 5 \mu M \text{CdCl}_2 and subsequent treatment of the cells with 1 \mu M \text{ZnCl}_2. (f) Cells treated with ZTRS and 5 \mu M \text{Fe(ClO}_4)_2; and (g) after treatment with ZTRS and 5 \mu M \text{Fe(ClO}_4)_2; and subsequent treatment of the cells with 1 \mu M \text{ZnCl}_2 (bar = 50 \mu m).

Figure 11. Zebrafish incubated with 5 \mu M ZTRS. (a) Images of 19 h-old, (b) 36 h-old, and (c) 48 h-old zebrafish incubated with ZTRS for 1 h. (d) Image of 54 h-old zebrafish incubated with ZTRS for 1 h, (e) image of 54 h-old zebrafish after initial incubation with 100 \mu M TPEN for 1 h, and subsequent treatment of washed zebrafish with ZTRS for 1 h (a, b, c: left, bright field images; right, fluorescence images). Scale bar = 250 \mu m.

living organisms. Zebrafish is a good animal model to monitor ions using sensors due to convenient detection of ions by fluorescence microscopy and permeability of ions and sensors in fish. Therefore, zebrafish recently has been widely used to detect various ions such as Hg\textsuperscript{2+}, Cu\textsuperscript{2+}, and Zn\textsuperscript{2+}. Zebrafish embryos were incubated with 5 \mu M ZTRS at various time points during development. At 19 h post fertilization (hpf) of the embryo, a green-spotted band was observed in the bottom of the venter (Figure 11a). During development, the necklace-like band composed of green spots was brighter and moved to the top of the venter until 48 hpf (Figure 11b,c). After 54 hpf, the green-spotted band was no longer observed, and only scattered bright spots were distributed around the pericardial sac (Figure 11d). The treatment of 54 h-old zebrafish with 100 \mu M TPEN resulted in the disappearance of the green spots (Figure 11e). The green-spotted band may result from sequestration of ZTRS or an endogeneous zinc pool in fish. It was observed that, when 54 h-old zebrafish were exposed to external Zn\textsuperscript{2+} (20 \mu M) followed by treatment with ZTRS, overall green fluorescence in the fish was increased. This suggests that the green-spotted band may result from endogeneous zinc ions in fish and not sequestration of the probe. In a recent study, Guo and co-workers found rather similar green spots in the zebrafish stained with a NBD-based sensor (NBD–TPEA). With the preliminary in vivo Zn\textsuperscript{2+} imaging of intact 4-day-old zebrafish larvae with NBD–TPEA staining, TPEN addition experiment of 5-day-old larvae, and the evidence of ICP-MS data for zinc in the separated zygomorphic luminescent areas, Guo and co-workers believe the green spots in zebrafish with NBD–TPEA staining are correlated to Zn\textsuperscript{2+} storage for the development of zebrafish. No abnormal developmental defects were observed upon treatment with ZTRS, indicating that it is biologically orthogonal. These results demonstrated the usefulness of ZTRS for monitoring biologically relevant ions in living organisms.

Conclusion

We have designed and synthesized a new naphthalimide-based fluorescent probe ZTRS for ratiometric Zn\textsuperscript{2+} sensing which contains an amide-DPA receptor. ZTRS has the strongest affinity with Zn\textsuperscript{2+} among competitive metal ions and displays an excellent fluorescent selectivity for Zn\textsuperscript{2+} with an enhanced red-shift in emission resulting from the Zn\textsuperscript{2+}-triggered amide tautomerization. Although ZTRS can bind to both Zn\textsuperscript{2+} and Cd\textsuperscript{2+}, these metal ions can be differentiated by this sensor; upon binding to Zn\textsuperscript{2+} and Cd\textsuperscript{2+} to the sensor, green and blue fluorescence were observed, respectively. Also, ratiometric detection of Zn\textsuperscript{2+} with a large emission wavelength shift from 446 to 514 nm can be achieved via a Cd\textsuperscript{2+} displacement

approach. Furthermore, this sensor is cell permeable and can be applied to trace zinc ions during the development of a living organism. The connection of the amide-DPA with other fluorophores is in progress.

Experimental Section

Materials and Methods. Unless otherwise noted, materials were obtained from Aldrich and were used without further purification. The synthesis of compound 1 was according to the published procedure. Melting points were measured using a Büchi 530 melting point apparatus. 1H NMR and 13C NMR spectra were recorded using Bruker 250 MHz or Varian 500 MHz. Chemical shifts were given in ppm and coupling constants (J) in Hz. UV absorption spectra were obtained on UVKON 933 double beam UV/vis spectrometer. Fluorescence emission spectra were obtained using RF-5301/PC spectrofluorophotometer (Shimadzu).

Synthesis of Compound 2. A solution of 102 mg (0.9 mmol) of 2-chloroacetyl chloride in 5 mL of dry CH2Cl2 was added dropwise to a solution of 200 mg (0.75 mmol) 4-amino-N-butyl-1,8-naphthalimide (1) and 150 mg (1.23 mmol) 4-dimethylaminepyridine (DMAP) in 30 mL of dry CH2Cl2 stirred in an ice bath. After stirred 2 h at room temperature, the mixture was removed under reduced pressure to obtain a pale-yellow solid, which was purified by silica gel column chromatography using dichloromethane as eluent to afford 4-(2-chloroacetyl)amino-N-butyl-1,8-naphthalimide (2). Yield: 221 mg (86%). Mp: 243–244 °C. 1H NMR (CDCl3, 250 MHz) δ 0.98 (t, J = 7.2 Hz, 3H), 1.39–1.48 (m, J = 7.2 Hz, 2H), 1.57–1.74 (m, J = 7.2 Hz, 2H), 4.16 (t, J = 7.2 Hz, 2H), 4.39 (s, 2H), 7.80 (t, J = 8.4 Hz, 1H), 8.16 (d, J = 8.5 Hz, 1H), 8.45 (d, J = 8.0 Hz, 1H), 8.61 (s, 2H), 9.15 (s, 1H, N–H). 13C NMR (CDCl3, 62.5 MHz) δ 13.86, 20.38, 30.18, 40.31, 43.39, 119.02, 119.65, 123.54, 123.80, 125.66, 127.23, 128.80, 131.34, 132.08, 137.00, 143.75, 154.28, 163.45, 163.95, 164.19. HRMS (ESI) calcd for C18H18ClN2O3 [MH+] 339.1739, found 339.1729.

Synthesis of Compound 3. A solution of Zn(ClO4)2 (100 mg, 0.29 mmol) and di-(2-picolyl)amine (DPA) (70 mg, 0.35 mmol) in 2 mL of dry CH3CN was treated with 5 mL of 0.1 M ZTRIS aqueous solution (CH3CN/0.5 M HEPES (pH 7.4) = 50:50) was prepared. For the metal-bound studies, 15 µL of 10 mM Zn(ClO4)2 or Cd(ClO4)2 was added to 5 mL of 10 µM ZTRIS in aqueous solution (CH3CN/0.5 M HEPES (pH 7.4) = 50:50). The concentration of the reference was adjusted to match the absorbance of the test sample at the wavelength of excitation. Emission for ZTRIS was integrated from 375 to 650 nm with excitation at 360 nm. The quantum yields were calculated with the expression in eq 1.

$$\Phi_{\text{sample}} = \Phi_{\text{standard}} \times \frac{\int \text{emission}_{\text{sample}}}{\int \text{emission}_{\text{standard}}}$$  (1)

X-ray Crystallographic Analysis. Single crystals were cooled to 180 K immediately after removal from the solution, and single crystal X-ray diffraction data were collected at 180 K on a Nonius Kappa CCD diffractometer using MoKa radiation (λ = 0.71073 Å) equipped with an Oxford Cryosystem cryostream. The structure was solved by direct methods using the program SHELXS-97 and refined on F2 against all data using SHELXL-97. All non-hydrogen atoms were refined with anisotropic displacement parameters. The hydrogen atoms were included in the models in calculated positions and were refined as constrained to bonding atoms.

Determination of Apparent Dissociation Constant. Fluorescence spectroscopy was used to determine the apparent dissociation constants (Kd) of ZTRIS (10 µM) with Zn2+ and Cd2+, using the reported method. Free Zn2+ and Cd2+ concentrations were controlled by metal ion buffers (e.g., NTA (nitrilotriacetic acid) in this study, 10 mM). log K (ZnNTA) = 10.66 (20 °C, = 0.1), and log K (CdNTA) = 9.80 (20 °C, = 0.1). The fluorescence intensity data (Figure 4) were fitted to eq 2, and Kd was calculated.

$$F = F_0 + (F_{\text{max}} - F_0) [\text{M}^{2+}]_{\text{free}} - K_d + [\text{M}^{2+}]_{\text{free}}$$  (2)

where F is the fluorescence intensity, Fmax is the maximum fluorescence intensity, F0 is the fluorescence intensity with no addition of Zn2+ and Cd2+, and [M2+free] is the free Zn2+ and Cd2+ concentration.

Imaging of Mammalian Cells Incubated with ZTRIS and CdCl2 or ZnCl2. A549 cells (human lung cancer cells) were seeded in a 24-well plate at a density of 2 × 10^3 cells per well in culture media (RPMI-1640 supplemented with 10% fetal bovine serum (FBS)). After 24 h, 5 µM ZTRIS in the culture media containing 0.1% (v/v) DMSO was added to the cells, and the cells were incubated for 1 h at 37 °C. After washing twice with 400 µL of Dulbecco’s phosphate buffered saline (DBPS, without calcium and magnesium) to remove the remaining sensor, the cells were further treated with 5 µM CdCl2 or 1 µM ZnCl2 in DPBS for 15 min. The treated cells were imaged by fluorescence microscopy (Eclipse TE2000-S, Nikon, Japan).

For a cadmium-displacement experiment, 5 µM ZTRIS in the culture media containing 0.1% (v/v) DMSO was added to the cells, and the cells were incubated for 1 h at 37 °C. After washing twice with 400 µL of DPBS to remove the remaining sensor, the cells

were treated with 5 µM CdCl₂ in DPBS for 15 min. Without washing, the cells were further treated with 1 µM ZnCl₂ for 15 min. The treated cells were imaged by fluorescence microscopy.

For a TPEN experiment, 5 µM ZTRS in the culture media containing 0.1% (v/v) DMSO was added to the cells, and the cells were incubated for 1 h at 37 °C. After washing twice with 400 µL of DPBS to remove the remaining sensor, the cells were treated with 1 µM ZnCl₂ in DPBS for 15 min. Without washing, the cells were further treated with 25 µM TPEN for 15 min. The treated cells were imaged by fluorescence microscopy.

For an iron competition experiment, 5 µM ZTRS in the culture media containing 0.1% (v/v) DMSO was added to the cells, and the cells were incubated for 1 h at 37 °C. After washing twice with 400 µL of DPBS to remove the remaining sensor, the cells were treated with 5 µM Fe(ClO₄)₂ in DPBS for 15 min. Without washing, the cells were further treated with 1 µM ZnCl₂ for 15 min. The treated cells were imaged by fluorescence microscopy.

Fluorescence images were obtained as the following: the excitation wavelength range of the UV-2A filter is from 330 to 380 nm, including 360 nm of the maximum excitation wavelength of the ZTRS. The long-pass emission (barrier) filter employed in the UV-2A combination is designed to collect signals at wavelengths exceeding 420 nm, enabling visualization of red, green, and blue emission from fluorophores excited in the ultraviolet. Under this UV-2A filter, the cells treated with CdCl₂ and ZnCl₂ show blue and greenish-blue, respectively.

**Tracing Distribution of Zinc Ions in Zebrafish.** Zebrafish were kept at 28 °C and maintained at optimal breeding conditions. For mating, male and female zebrafish were maintained in one tank at 28 °C on a 12-h light/12-h dark cycle, and then the spawning of eggs was triggered by giving light stimulation in the morning. Almost all eggs were fertilized immediately. All stages of zebrafish were maintained in E3 embryo media (15 mM NaCl, 0.5 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂, 0.15 mM KH₂PO₄, 0.05 mM Na₂HPO₄, 0.7 mM NaHCO₃, 10⁻⁵% methylene blue; pH 7.5). Zebrafish embryos at 19, 36, 48, and 54 hpf were incubated with 5 µM ZTRS in E3 media containing 0.1% (v/v) DMSO for 1 h at 28 °C.

Alternatively, 54 h-old zebrafish were exposed to 100 µM TPEN in E3 media containing 0.1% (v/v) DMSO for 1 h at 28 °C to remove intact zinc ions in zebrafish. After washing with E3 media to remove the remaining TPEN, the zebrafish were further incubated with 5 µM ZTRS in E3 media for 1 h at 28 °C. The treated zebrafish were imaged by fluorescence microscopy equipped with UV-2A filter.

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**Supporting Information Available:** Fluorescence and UV—vis absorption spectra of ZTRS with metal ions; 1H NMR, 2D NOESY, and IR spectra of ZTRS/Zn²⁺ and ZTRS/Cd²⁺ complex in CD₃CN and DMSO; X-ray crystallographic data (CIF). This material is available free of charge via the Internet at http://pubs.acs.org.