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Near-Infrared Fluorescent Probes with Large Stokes Shifts for Sensing Zn(II) Ions in Living Cells

Shuwei Zhang[†], Rashmi Adhikari[†], Mingxi Fang[†], Nethaniah Dorh[†], Cong Li[†], Meghnath Jaishi[‡], Jingtuo Zhang[†], Ashutosh Tiwari^{*,†}, Ranjit Pati^{*,‡}, Fen-Tair Luo^{*,§}, and Haiying Liu^{*,†}

[†]Department of Chemistry, Michigan Technological University, Houghton, Michigan 49931, United States

[‡]Department of Physics, Michigan Technological University, Houghton, Michigan 49931, United States

[§]Institute of Chemistry, Academia Sinica, Taipei, Taiwan 11529, Republic of China

Abstract

We report two new near-infrared fluorescent probes based on Rhodol counterpart fluorophore platforms functionalized with dipicolylamine Zn(II)-binding groups. The combinations of the pendant amines and fluorophores provide the probes with an effective three-nitrogen-atom and one-oxygen-atom binding motif. The fluorescent probes with large Stokes shifts offer sensitive and selective fluorescent responses to Zn(II) ions over other metal ions, allowing a reversible monitoring of Zn(II) concentration changes in living cells, and detecting intracellular Zn(II) ions released from intracellular metalloproteins.

Graphical abstract

*Corresponding Authors: tiwari@mtu.edu, patir@mtu.edu, luoft@gate.sinica.edu.tw, hylu@mtu.edu.

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Detailed synthetic procedures, ¹H and ¹³C NMR spectra of fluorescent probes A and B, detailed supplemental optical spectra of fluorescent probes A and B in the absence and presence of different metal ions, ESI-MS spectra for probes A and B in the absence and presence of Zn(II) ions, photostability, binding constants, and detection limits of probes A and B, Job's plot, selectivity of probes A and B to Zn(II) ions over hydrogen peroxide, in vitro cell imaging, and intracellular detection of Zn(II) by using probes A and B (PDF)

Notes: The authors declare no competing financial interest.

Keywords

fluorescent probe; near-infrared; Rhodol counterpart; Zn(II) ions; photoinduced electron transfer

Zinc (II), the second most abundant transition metal ion in the human body after iron, is often present in a tightly bound form in proteins. It plays several vital roles in a variety of physiological and pathological processes including cellular metabolism, gene expression, cell apoptosis, regulation of metalloenzymes, neural signal transmission and mammalian reproduction.^{1–3} The disruption of Zn(II) homeostasis may contribute to Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, cerebral ischemia (ischemic stroke), epilepsy, prostate cancer, diabetes, immune dysfunction, and infantile diarrhea.^{1,4–6} Therefore, the development of fluorescent probes to effectively quantify and visualize Zn(II) concentration in biological systems is key to understanding of Zn(II) important roles in biological processes. Furthermore, the fluorescent probes with high sensitivity and spatial resolution of fluorescence would assist in identifying the molecular mechanisms that link zinc homeostasis and human pathophysiology. Several fluorescent probes for Zn(II) sensing have been developed by using different fluorophores such as 2-aryl benzimidazoles,^{7,8} benzoxazoles,^{9,10} indoles,¹¹ benzofurans, quinoline,^{12,13} dansyl,¹⁴ anthracene,¹⁵ coumarin,^{16,17} naphtha-limide,¹⁸ fluorescein,^{19–25} Rhodamine,^{24,26,27} cyanine dye,^{28–31} BODIPY dyes,³² and porphyrins.³³ They can detect Zn(II) concentration in the range of 10^{-10} M in the cytoplasm to 10^{-4} M in some vesicles.^{1,6,34} Most of these probes still suffer from some significant drawbacks such as autofluorescence and high light scattering. Near-infrared fluorescent probes have been developed to overcome these limitations by reducing background signals and photoinduced damages to biological samples. However, the near-infrared (NIR) fluorescent probes for Zn(II) ions are still less common, as only a very few of them could be used to monitor the levels of endogenous Zn(II) ions in living cells and organisms.^{28,33,35} The search for readily accessible NIR fluorescent probes with excellent hydrophilic properties, high specificity, large Stokes shifts and dynamic responsive range is

still a challenging task for imaging of Zn(II) concentration changes inside living cells. Fluorescent probes with large Stokes shifts possess advantages as spectral overlap between absorption and emission spectra can be eliminated. It allows for detection of fluorescence by avoiding measurement errors by excitation and scattered lights.

In this paper, we report a rational design and synthesis of near-infrared fluorescent probes (A and B) with large Stokes shifts to image the changes in Zn(II) concentration in living cells by incorporating the di-2-picolyamine as a Zn-specific receptor into the near-infrared emissive Rhodol counterpart fluorophores (Figure 1). Fluorescent probes A and B display absorption peaks at 576 and 586 nm and weak fluorescence peaks at 701 and 702 nm with large Stokes shifts of 125 and 116 nm in 10 mM HEPES buffer (pH 7.0), respectively. Near-infrared fluorescent probes A and B display sensitive and selective fluorescent responses to Zn(II) over most other metal ions with detection limits of 0.19 and 0.086 μM , allow for reversibly monitoring Zn(II) concentration in living cells, and sensitively detect intracellular levels of free Zn(II) ions which are released from intracellular metalloproteins when the cells were treated with 2,2'-dithiodipyridine. First-principles density functional calculations are carried out to gain electronic structure level understanding of the fluorescence behavior of probes A and B.

Experimental Section

Instrumentation

^1H NMR and ^{13}C NMR spectra were collected via a 400 MHz Varian Unity Inova NMR spectrophotometer. ^1H NMR and ^{13}C NMR spectra were recorded in CDCl_3 solutions. Chemical shifts (δ) were given in ppm relative to solvent residual peaks (^1H , δ 7.26 for CDCl_3 ; ^{13}C , δ 77.3 for CDCl_3) as internal standards. High-resolution mass spectrometry data (HRMS) of the intermediates and fluorescent probes were measured with fast atom bombardment (FAB) ionization mass spectrometer, double focusing magnetic mass spectrometer or matrix assisted laser desorption/ionization time-of-flight mass spectrometer. Absorption spectra were taken on a Perkin Elmer Lambda 35 UV/vis spectrometer. Fluorescence spectra were recorded on a Jobin Yvon Fluoromax-4 spectrofluorometer.

Cell Culture and Fluorescence Imaging

Breast cancer (MDA-MB-231) and normal endothelial (HUVEC-C) cell lines obtained from ATCC were cultured according to the published protocols.³⁶ Cells were plated on 12-well culture plates at a density of 1×10^5 cells/mL for live cell imaging. After 24 h incubation at 37 °C with a 5% CO_2 incubator, the media was removed and cells were rinsed three times with PBS. Fresh serum free media with 2, 5, or 10 μM of probe A or B was added, and then cells were further incubated for 1 h at 37 °C with 5% CO_2 . After the cells were rinsed three times with PBS, fresh serum-free media with either 100 μM Zn(II) or 100 μM Zn(II) plus sodium pyruvate (Pyr) was added. Cells were then incubated for 30 min at 37 °C with 5% CO_2 . At this point, the cells were rinsed three times with PBS, and fresh serum free media was added before acquiring images using an inverted fluorescence microscope (model AMF-4306; EVOS, AMG). Fluorescence images were obtained at either 20 $\times$ or 60 $\times$ magnification and the exposure times were kept constant for each image series. 100 μM of

TPEN (*N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine) was added to the wells containing 100 μM Zn(II) + 100 μM sodium pyrrhione, and then cells were incubated for 10 min at room temperature. Fluorescence images were acquired at 20 $\times$ magnification using the inverted fluorescence microscope as described previously. Similar experiments with TPEN were also conducted by using 5 μM of each probe at either 10 or 30 μM Zn(II) plus sodium pyrrhione. The ability to detect intracellular levels of Zn(II) ions in HUVEC-C cells was evaluated using 2,2'-dithiodipyridine (DTDP) using a modified protocol.³⁷ Cells were serum starved for 3 h at 37 °C with 5% CO₂ and then incubated with 2, 5, or 10 μM of probe A or B for 1 h at 37 °C with 5% CO₂. To corresponding wells, 100 μM DTDP was added and cells were further incubated for 30 min at 37 °C with 5% CO₂. Finally, cells were imaged at 20 $\times$ or 60 $\times$ using the inverted fluorescence microscope (model AMF-4306; EVOS, AMG).

Materials

Unless specifically indicated, all reagents and solvents were obtained from commercial suppliers and used without further purification. 3-((Bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxy-benzaldehyde (**1**),³⁸ 9-(2-carboxyphenyl)-6-(diethylamino)-1,2,3,4-tetrahydroxanthylum perchlorate (**2**),^{39,40} and 4-(2-carboxyphenyl)-7-(diethylamino)-2-methylchromenylium perchlorate (**3**)⁴¹ were prepared according to reported procedures.

Synthesis of Fluorescent Probes A and B

Fluorescent Probe A—3-((Bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxybenzaldehyde (**1**) (0.30 g, 0.90 mmol) and 9-(2-carboxyphenyl)-6-(diethylamino)-1,2,3,4-tetrahydroxanthylum perchlorate (**2**) (0.43 g, 0.90 mmol) were dissolved in acetic acid (10 mL) in a 25 mL round-bottom flask. The reaction mixture was heated at 100 °C and stirred for 3 h. When the solvent was evaporated under reduced pressure, the crude product was purified by silica gel chromatography using CH₂Cl₂/CH₃OH/CH₃COOC₂H₅/CH₃COOH (20/2/10/0.32, v/v/v/v) as eluent to obtain fluorescent probe A as deep blue solid (0.12 g, 16.8%). ¹H NMR (400 MHz, CDCl₃) δ 8.51 (d, *J* = 4.0 Hz, 2H), 8.04 (d, *J* = 8.0 Hz, 1H), 7.62–7.58 (m, 3H), 7.54–7.51 (m, 2H), 7.31–7.26 (m, 3H), 7.23 (s, 1H), 7.15–7.11 (m, 3H), 6.86 (d, *J* = 8.0 Hz, 1H), 6.65–6.62 (m, 2H), 6.50–6.47 (m, 1H), 3.85–3.78 (m, 6H), 3.42–3.37 (m, 4H), 2.78–2.64 (m, 2H), 2.15–2.11 (m, 1H), 1.83–1.79 (m, 1H), 1.63–1.59 (m, 2H), 1.17 (t, *J* = 4.0 Hz, 6H); ¹³C NMR (100 MHz, CDCl₃) δ 169.9, 158.3, 158.1, 154.7, 151.6, 148.8, 137.4, 133.6, 133.3, 131.4, 129.6, 129.4, 127.7, 127.3, 125.3, 123.5, 123.3, 122.6, 116.9, 111.7, 97.0, 59.0, 57.0, 45.2, 27.5, 24.3, 22.3, 12.8. HRMS (ESI): calculated for C₄₄H₄₃N₄O₄⁺ [M-ClO₄]⁺, 691.3279; found, 691.3275.

Fluorescent Probe B—3-((Bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxybenzaldehyde (**1**) (0.12 g, 0.26 mmol) and 4-(2-carboxyphenyl)-7-(diethylamino)-methylchromenylium perchlorate (**3**) (0.09 g, 0.26 mmol) were dissolved in acetic acid (4 mL) in a 10 mL round-bottom flask. The reaction mixture was heated at 100 °C and stirred for 2 h. After the solvent was evaporated under reduced pressure, the crude product was purified by silica gel chromatography using CH₂Cl₂/CH₃OH/CH₃COOC₂H₅/CH₃COOH (30/3/10/0.4, v/v/v/v) as eluent to obtain fluorescent probe B as deep blue solid (0.04 g,

19.3%). ^1H NMR (400 MHz, CDCl_3) δ 8.57 (d, J = 4.0 Hz, 2H), 7.93 (d, J = 4.0 Hz, 1H), 7.65–7.61 (m, 3H), 7.56–7.52 (m, 2H), 7.37–7.30 (m, 3H), 7.26–7.24 (m, 2H), 7.19–7.16 (m, 3H), 6.91 (d, J = 8.0 Hz, 1H), 6.55 (d, J = 8.0 Hz, 1H), 6.47–6.45 (m, 1H), 6.41–6.37 (m, 1H), 3.89 (s, 4H), 3.81 (s, 2H), 3.40–3.34 (m, 4H), 1.18 (t, J = 4.0 Hz, 6H); ^{13}C NMR (100 MHz, CDCl_3) δ 170.2, 158.8, 158.3, 153.3, 152.7, 149.8, 148.9, 137.3, 134.4, 132.3, 129.7, 129.5, 128.8, 128.3, 127.3, 127.2, 124.2, 123.5, 122.6, 118.3, 117.4, 109.5, 105.9, 100.5, 97.9, 94.6, 59.1, 57.2, 44.7, 30.0, 12.8. HRMS (ESI): calculated for $\text{C}_{41}\text{H}_{39}\text{N}_4\text{O}_4^+$ $[\text{M}-\text{ClO}_4]^+$, 651.2966; found, 651.2966.

Results and Discussion

Synthetic Approach to Fluorescent Probes A and B

Dipicolylamine (DPA), a zinc binding ligand with three nitrogen atoms for $\text{Zn}(\text{II})$ chelation, was incorporated into near-infrared emissive Rhodol counterpart fluorophores where an oxygen atom from a hydroxyl group can involve in $\text{Zn}(\text{II})$ chelation to enhance $\text{Zn}(\text{II})$ binding strengths. We conducted a condensation reaction of 3-((bis(pyridin-2-ylmethyl)amino)-methyl)-4-hydroxybenzaldehyde with 9-(2-carboxyphenyl)-6-(diethylamino)-1,2,3,4-tetrahydroxanthylum perchlorate (**2**), and 4-(2-carboxyphenyl)-7-(diethylamino)-methylchromenylium perchlorate (**3**) in acetic acid solution at 100 °C, respectively, affording fluorescent probes for $\text{Zn}(\text{II})$ (A and B) (Scheme 1). We intentionally controlled one carbon chain length between zinc-binding ligand and the fluorophores in order to manipulate the sensitive fluorescence responses of the probes to $\text{Zn}(\text{II})$ ions via effective photoinduced electron transfer effect of a tertiary amine from zinc-binding ligand as an electron donor to the fluorophores. The fluorescent probes were characterized by ^1H NMR, ^{13}C NMR, and high-resolution mass spectrometry.

Optical Properties Of Fluorescent Probes A And B In Different Solvents

All optical data of fluorescent probes were obtained with 10 μM concentration of fluorescent probe A or B in different solvents. Fluorescent probes A and B were very stable in 10 mM HEPES buffer (pH 7.0) containing 1% EtOH solution. These fluorescent probes possess advantageous photophysical properties including a large absorption extinction coefficient, excellent photostability, and large Stokes shifts with near-infrared emission (Table 1 and Figure S7). Probes A and B display large Stokes shifts of 111 and 101 nm with absorption and emission peaks at 581, 594 nm and 692, 695 nm in ethanol solution, respectively. However, the fluorescent probes exhibit smaller Stokes shifts in less polar solvents such as dichloromethane and toluene. The fluorescent probes possess low fluorescence quantum yields in different solvents (Table 1). Probe B shows slightly longer absorption and emission wavelengths in all solvents because of its slightly better π -conjugation than probe A although they have very similar structures (Table 1). The slightly better π -conjugation of the fluorescent probe B is due to less steric hindrance between two bulky groups in a trans configuration in a double bond (Scheme 1).

Optical Responses of Fluorescent Probes A and B to $\text{Zn}(\text{II})$ Ions

The optical responses of the fluorescent probes to $\text{Zn}(\text{II})$ ions were investigated in aqueous HEPES buffer (pH 7.0) containing 1% EtOH solution. Probe A displays a main absorption

peak at 576 nm and a shoulder peak at 430 nm. Upon gradual addition of Zn(II) to 10 μM solution of fluorescent probe A, the main and shoulder absorption peaks of the probe A undergo red shifts by 26 and 20 nm, respectively, and absorbance of the probe A increases as the molar absorptivity of probe A increases from $1.77 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ in the absence of Zn(II) ion and to $2.06 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ in the presence of 10 μM Zn(II) ions (Figure 2). Fluorescent probe B exhibits a main broad absorption peak at 586 nm and a shoulder peak at 435 nm in the same buffer solution. Gradual addition of Zn(II) to 10 μM fluorescent probe B solution triggers red shifts of the main and shoulder absorption peaks by 38 and 21 nm, respectively, and results in enhancement of the absorbance as the molar absorptivity of probe B increases from $1.80 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ in the absence of Zn(II) ion and to $2.10 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ in the presence of 10 μM Zn(II) ions (Figure 2).

Fluorescent probe A displays a very weak fluorescence peak at 701 nm with a fluorescence quantum yield of 0.06% in HEPES buffer (pH 7.0) containing 1% EtOH solution in the absence of Zn(II) ions. This is due to fluorescence quenching effect via photoinduced electron transfer from the tertiary amine of a Zn(II)-binding dipicolylamine ligand as an electron donor to the Rhodol counterpart fluorophore (Table 1). Gradual addition of Zn(II) to a 10 μM solution of fluorescent probe A causes significant fluorescence increases because binding of Zn(II) to the dipicolylamine ligand effectively suppresses the fluorescence quenching effect and recovers the probe fluorescence (Figure 3). Probe A shows a linear fluorescence response to Zn(II) from 0.3 to 4.0 μM with a detection limit of 0.19 μM (Figure S8). The 1:1 stoichiometry was further confirmed by a nonlinear fitting of the titration data at 701 nm by assuming a 1:1 association between fluorescent probe A and Zn(II) (Figure S14). Fluorescent probe B displays a slight higher fluorescence background with fluorescence quantum yield of 0.10% in HEPES buffer than the probe A (Figure 3), and possesses a linear fluorescence response to Zn(II) from 0.3 μM to 4.0 μM with a lower detection limit of 0.086 μM when compared to the probe A (Figure S13). Probe B displays similar fluorescent responses to zinc(II) ions with 1:1 stoichiometry which is similar as probe A. This 1:1 binding stoichiometry between probe B and Zn(II) was verified by Job's plot that is based on nonlinear fitting of the titration curve using 1:1 binding model (Figure S15).

Theoretical Modeling

We performed first-principles quantum mechanical calculations to gain an electronic structure level understanding of the fluorescence behavior of fluorescent probes A and B acting as multidentate chelators for Zn(II) ions. The stable conformations of the aforementioned probes and their corresponding Zn(II)/probe complexes were determined through optimization of their geometries (Figure 4). A posteriori hybrid density functional method (B3LYP) that includes a portion of the exact Hartree–Fock exchange, and a 6-311+G(d,p) Gaussian basis set^{42,43} were used for our calculation. We used an implicit solvent model (taking water as the solvent) to mimic the solvent effect.

The tetra dentate ligands undergoing the Zn(II) chelation involve three nitrogen atoms of the dipicolylamine (DPA) and an oxygen atom from a nearby hydroxyl group. The hydroxyl group is found to be deprotonated upon binding to Zn(II) for both the probes A and B. The

dihedral angle between the two pyridinal planes of DPA in probes A and B are calculated to be 115.12° and 102.12° respectively. Upon the addition of Zn(II) ion, the dihedral angle changes to 132.90° and 129.90° in the so formed Zn(II)/probe A and B complexes, respectively, suggesting a significant conformational change upon binding. The calculated Zn–N and Zn–O bonding distances in Zn(II)/probes A and B complexes are explicitly shown above in Figure 4b and d. Further analysis reveals that the addition of Zn(II) ion in the probes A and B is also accompanied by the redistribution of charge between Zn(II) ions and the associated ligands. The Mulliken charges on three nitrogen atoms an oxygen atom in probes A and B before binding to Zn(II) are found 0.57 e, 0.08 e, –0.07 e, –0.20 e and 0.68 e, –0.07 e, –0.09 e, –0.21 e, respectively. Upon the addition of Zn(II), the charge configuration in these species are found 0.43 e, 0.21 e, 0.03 e, –0.34 and 0.44 e, 0.18 e, 0.04 e, –0.32 e, respectively. On the other hand, the charge on Zn ion in fluorescent complex A and B after binding are found to be 1.21 and 1.12 e, respectively.

Next, to understand the observed fluorescence phenomenon, we performed the excitonic calculations using time-dependent density functional methods (TD-DFT)^{44,45} for all of the above-mentioned stabilized conformations of the probes. The Lorentzian fitted plot of the absorption spectrum with a half width maximum value of 5 nm were shown in the Figure 5. The variation of oscillator strength (*f*) with wavelength is presented (Figure 5). The oscillator strength (*f*) defines the probability of absorption of electromagnetic radiation for transitions between energy levels of the molecule. The strength of maximum absorption is found to be higher in both the Zn(II)/probe A and B complexes than that in fluorescent probes A and B, respectively. The maximum *f*-values for probes A and B are found to be 0.89 and 1.13, while in the case of Zn(II)/probes A and B complexes, it is found to be 0.98 and 1.28, respectively. This clearly explains the observed sensitivity of Zn(II) ions toward fluorescence behavior due to a much higher absorption observed in both the complexes, which match experimental results of absorbance enhancement when Zn(II) ions bind to fluorescent probes (Figure 2).

For the fluorescent probes A and B, the calculated dominant absorption are found at wavelengths 547 and 540 nm, respectively. Upon the addition of Zn(II), the maximum absorption in Zn(II)/probe A complex shifts by 34 nm toward the red end to lie at 581 nm. A similar shift of 28 nm in the maximum absorption is seen in the case Zn(II)/probe B complex which is found to lie at 568 nm. Thus, in both cases of probes A and B, our results agree well with the experimentally reported findings of absorption spectra. Further analysis also reveals that in all of these four cases, the dominant absorption peak, without any ambiguity, does correspond to the HOMO–LUMO excitation. It is important to point out that, the experimentally observed higher values of the emission wavelength (λ_{em}) of 701 and 702 nm for the Zn(II)/probes A and B complexes may be attributed to the transition of electron from an excited state to a higher energy metastable structure with different conformation than the true ground state. It should also be noted that the calculation presented here is carried out for a single molecule in the presence of an implicit solvent unlike that in the experiment. Nevertheless, the red shifts observed upon binding with Zn(II) to probe A or B is in good agreement with our experimental results.

Selectivity of Fluorescent Probes to Zn(II) Ions over Other Metal Ions

Selectivity of fluorescent probes A and B to Zn(II) over other metal ions was investigated in 10 mM HEPES buffer (Figure 6). No significant responses of fluorescent probes A and B were observed to 20 μ M alkali, and alkaline-earth metal ions such as Na^+ , K^+ , Ca^{2+} , Mg^{2+} , and Al^{3+} ions, and some transitional metallic ions such as Mn^{2+} , Fe^{3+} , Ni^{2+} , Co^{2+} , Hg^{2+} , Ag^+ , and Cr^{3+} ions. However, the presence of Cu^{2+} ions causes fluorescence quenching of the fluorescent probe A or B as the fluorescence quenching may be due to metal-to-ligand electron transfer upon excitation for paramagnetic ions such as Cu^{2+} for fluorescence quenching which was encountered in fluorescein-based probes for Zn(II) ions.^{19–24} The fluorescent probes can be used to detect Zn(II) with minimum interference from other competing metal ions except Cd^{2+} ions because Cu^{2+} ions are rarely present as free ions in the living cells.

In Vitro Imaging of Zn(II) in Living Cells

After we established the fluorescence responses of the fluorescent probes to Zn(II) ions, we investigated whether the fluorescent probes A and B are cell permeable and stable, and respond to Zn(II) ions reversibly in living cells. We used mixtures of Zn(II) and pyrithione, a cell-permeable ionophore, to deliver exogenous Zn(II) to the cells, and employed *N,N,N',N'*-tetrakis(2-picolyl)ethylenediamine (TPEN), a cell-permeable metal ion chelator to test fluorescently responsive reversibility of the probes to Zn(II) ions in living cells according to our reported procedures.⁴⁶ Fluorescent probe A at 5 μ M concentration displayed extremely weak fluorescence background signals in HUVEC normal cells and MDA-MB231 breast cancer cells before the addition of exogenous Zn(II) ions (Figures 7 and 8). This is because of the low level of endogenous fluorescence from the cells in near-infrared region, and the low fluorescence quantum yield of the probe. No significant fluorescence change was observed when the cells treated with fluorescent probe A were supplemented by Zn(II) ions alone. However, fluorescence turn-on was observed when sodium pyrithione was added to the cells treated with fluorescent probe A and supplemented by Zn(II) ions (Figures 7 and 8). Increase of fluorescent probe A concentration from 2 to 10 μ M in living cells significantly enhances turn-on fluorescence intensity of the probe (Figures S22–S29). These results demonstrated that the fluorescent probe A is cell-permeable and intact in living cells, and can be effectively applied to image intracellular Zn(II) ions in living cells. In contrast, fluorescent probe B showed weaker fluorescence turn-on in both HUVEC and MDA-MB231 cells (Figures 7, 8, and S22–S29).

We tested whether the fluorescent probes can respond reversibly to Zn(II) ions in living cells by using *N,N,N',N'*-tetrakis(2-picolyl)ethylenediamine (TPEN), a cell-permeable Zn(II) ion chelator.⁴⁶ Upon addition of TPEN to the cells, fluorescence intensity of fluorescent probe-labeled cells decreased dramatically by more than 90% (Figure 9), indicating that the fluorescent probes respond to Zn(II) reversibly since TPEN effectively removes Zn(II) ions from the fluorescent probes (Figures S30 and S31).

We also investigated whether the fluorescent probes could be used to detect intracellular Zn(II) concentration changes in living cells by using 2,2'-dithiodipyridine (DTDP) to promote the release of Zn(II) ions from intracellular metalloproteins.⁴⁷ When DTDP is used

alone, only modest increases (nM) in intracellular Zn(II) ions have been reported.^{48,45} The HUVECC cells treated with fluorescent probe A showed very weak fluorescence without DTDP treatment. However, the cells displayed accumulation of endogenous Zn(II) ions because addition of DTDP to the HUVEC-C cells treated with fluorescent probe A significantly enhances the fluorescence intensity of fluorescent probe A even at 2 μ M concentration in living cells without the addition of external ionophores (Figures 10 and S32). Further, the cells treated with TPEN (zinc chelator) showed the decrease in fluorescence intensity of fluorescent probe-labeled cells (Figure 10). These results unambiguously demonstrated that fluorescent probe A can be effectively used to detect intracellular Zn(II) ion. Fluorescent probe B displays much weaker fluorescence responses to intracellular Zn(II) changes in living cells when compared with fluorescent probe A (Figure S32).

Conclusion

In this study, we report synthesis and characterization of novel near-infrared fluorescent probes for Zn(II) based on Rhodol counterpart fluorophores functionalized with dipicolylamine Zn(II)-binding groups. The fluorescent probes display selective and sensitive responses to Zn(II) ions over most other metal ions. The binding of Zn(II) ions to fluorescent probes effectively triggers fluorescence turn-on of the fluorophores by suppressing fluorescence quenching effect via photoinduced electron transfer from tertiary amine of dipicolylamine residues to Rhodol counterpart fluorophores. The fluorescent probes offer the reversible visualization of exogenously supplemented Zn(II) ions in living normal and cancer cells. In addition, these probes effectively detect intracellular Zn(II) ions which are released from intracellular metalloproteins in the cells treated with 2, 2'-dithiodipyridine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Chemical structures of near-infrared fluorescent probes for Zn(II) ions.

Figure 2. UV/vis absorption spectra of 10 μM probes A (a) and B (b) upon gradual addition of Zn(II) from 1 μM to 20 μM in 10 mM HEPES buffer solutions (pH 7.0).

Figure 3. Fluorescence spectra of 10 μM fluorescent probes A (a) and B (b) upon gradual addition of Zn(II) from 1 to 20 μM in 10 mM HEPES buffer solution (pH 7.0) with excitation at 595 nm. Fluorescence responsive curves of fluorescent probes A (c) and B (d) to Zn(II) ions.

Figure 4. Optimized conformations of fluorescent probes. Fluorescent probe A (a) and Zn(II)/probe A complex (b). Fluorescent probe B (c) and Zn(II)/probe B complex (d), respectively.

Figure 5. Absorption spectra of probe A (a), Zn(II) /probe A complex (b), probe B (c), and Zn(II) /probe B complex (d).

Figure 6.

Fluorescence spectra of 10 μM probes A (a) and B (b) to 20 μM of various metal anions in 10 mM HEPES buffer solutions (pH 7.0).

Figure 7.

Fluorescence images of 5 μM fluorescent probes A and B in HUVEC-C cells. Cells were then supplemented with either 100 μM zinc(II) chloride or 100 μM each of zinc(II) chloride plus sodium pyruvate (Pyr) for 30 min before acquiring images. The images were acquired using an inverted fluorescence microscope at 20 $\times$ magnification.

Figure 8.

Fluorescence images of 5 μM probes A and B in MDA-MB231 cells. Cells were supplemented with 100 μM zinc(II) chloride or 100 μM each of zinc(II) chloride and sodium pyrithione (Pyr) for 30 min before acquiring images.

Figure 9.

Fluorescence images of MDA-MB231 cells incubated with fluorescent probe A. 100 μM each of zinc(II) chloride and sodium pyrithione (Pyr) were supplemented to cells for 30 min before acquiring images. Then 100 μM TPEN (zinc chelator) was added for 10 min before acquiring the second set of images.

Figure 10.

Fluorescence images of HUVEC-C cells in the presence of 5 μM each of probes A and B. Cells were supplemented with 100 μM 2,2'-dithiodipyridine (DTDP) for 30 min before acquiring images. Then 100 μM TPEN (zinc chelator) was added and cells were further incubated for 10 min before acquiring the images.

Scheme 1. Synthetic Route to Fluorescent Probes A and B

Table 1
Optical Properties of Fluorescent Probes A and B

	solvent	λ_{abs} (nm)	λ_{em} (nm)	ϵ_{max} ($10^4 \text{ M}^{-1} \text{ cm}^{-1}$)	Φ_{f} (%)
probe A	buffer	576	701	1.77	0.06
	ethanol	581	692	2.78	0.60
	DCM	615	671	2.68	0.49
	toluene	625	668	0.62	<0.01
probe B	buffer	586	702	1.80	0.10
	ethanol	594	695	2.20	0.64
	DCM	640	682	0.84	1.12
	toluene	649	679	0.53	<0.01