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Original article

Ratiometric and selective two-photon fluorescent probe based on PET-ICT for imaging Zn^{2+} in living cells and tissues

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ABSTRACT

A two-photon fluorescent probe TPZn was developed for specific ratiometric imaging Zn^{2+} in living cells and tissues. Significant ratiometric fluorescence change was based on photoinduced electron transfer and intramolecular charge transfer. The synthetic method of TPZn was simple. It was successfully used to selectively image Zn^{2+} based on the higher binding affinity for Zn^{2+} than for Cd^{2+} . TPZn was easily loaded into the living cell and tissues with high membrane permeability in a complex biological environment. TPZn could clearly visualize endogenous Zn^{2+} by TP ratiometric imaging in hippocampal slices at a depth of 120 μ m. Thus, TPZn is a useful tool to image of Zn^{2+} in living cells and tissues without interference from Cd²⁺.

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1. Introduction

Zinc is of great interest in the field of neurobiology [1,2]. Recent studies indicate that cellular Zn^{2+} levels are tightly regulated [3]. There is a pool of Zn^{2+} that is loosely bound in the vesicles of hippocampal CA3 neurons [2]. It is also known that disorder of Zn^{2+} metabolism is closely associated with many severe neurological diseases, including Alzheimer's disease (AD) [4], cerebral ischemia, and epilepsy [5]. To understand the biological functions of Zn^{2+} , many fluorescent sensors have been reported [6].

Most of the reported Zn^{2+} probes are based on one-photon excitation [7]. In these works, quinolin [7], pyridinehydrazone [8] and so on were used as fluorophores for the detection of Zn^{2+} . They are only used in the photography of cells, as the light penetration is limited and highly localized photography images in deep tissue are difficult to obtain. To visualize the biological activity deep inside living tissue (>80 µm) without the interference of surface preparation artifacts [9], it is crucial to use two-photon microscopy (TPM). The technique, which employs two-photons of lowerenergy for the excitation, has the advantages of an increased penetration depth, a localized excitation, and a prolonged observation time [9]. Recently, Cho *et al.* reported some twophoton Zn^{2+} -selective probes to be used in the imaging of cells and

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tissues, for example, AZn1, AZn2 and Zinbo-5 [10]. These probes have significantly enriched our knowledge about the role Zn^{2+} plays in many biological processes.

Herein, we report a novel two-photon ratiometric probe for Zn^{2+} ions (TPZn, Scheme 1) in living cells and tissues, which is based on photoinduced electron transfer (PET) and intramolecular charge transfer (ICT). TPZn is derived from 1,4-dicyano-2,5-bis(styryl)benzene as the fluorophore based on a donor-acceptor-donor structure possessing a large two-photon cross section and *N*,*N*-bis(pyridine-2-ylmethyl)benzenamine as a Zn^{2+} receptor (ICT donor). We also note that TPZn successfully discriminates Zn^{2+} from Cd²⁺, which typically interferes in the Zn^{2+} detection.

2. Experimental

TPZn was synthesized according to the general route by Witting reaction of 1,4-bis(diethylphosphorylmethyl)-2,5-dicyanobenzene with 4-(bis(pyridine-2-ylmethyl)amino)-benzaldehyde in 35% yield (Scheme 1).

All the solvents were of analytical grade. The solutions of metal ions were prepared by dissolving $Mn(NO_3)_2$, $Pb(NO_3)_2$, $Co(N-O_3)_2.6H_2O$, $Zn(NO_3)_2.6H_2O$, $Ca(NO_3)_2.4H_2O$, $NaNO_3$, $Cu(NO_3)_2.3H_2O$, $3H_2O$, $Ni(NO_3)_2.6H_2O$, KNO_3 , $Cd(NO_3)_2.2H_2O$, $Hg(NO_3)_2.1/2H_2O$, $Cr(NO_3)_3.9H_2O$, $Mg(NO_3)_2.6H_2O$, $FeCl_2.4H_2O$, and $BaCl_2.2H_2O$ in distilled water. ¹H NMR and ¹³C NMR spectra were recorded on a VARIAN INOVA-400 spectrometer with chemical shifts reported as ppm (in CDCl₃, TMS as internal standard). Mass spectrometric data

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Scheme 1. The synthesis of TPZn.

were obtained on an HP1100LC/MSD MS spectrometer and an LC/Q-Tof MS spectrometer. Fluorescence measurements were performed on a PTI-700 Felix and Time-Master system, and the slit width was 3 nm for both excitation and emission. Absorption spectra were measured on Lambda 35 UV/vis spectrophotometer. All pH measurements were made with a Model PHS-3C meter. The fluorescence quantum yield (Φ) of TPZn was measured using standard methods on air-equilibrated samples at room temperature. Quinine bisulfate in 0.05 mol/L H₂SO₄ (Φ = 0.546) was used as a reference. The TPA cross sections (δ_{TPA}) of TPZn were determined under various conditions by the two-photon-induced fluorescence method by using fluorescein (10⁻⁴ mol/L in 0.1 mol/L NaOH) as the reference.

Compound **4** was synthesized according to our previous work [11].

Compound **2**: To a solution of 2-chloromethylprydine (1.524 g, 12 mmol) in H₂O (0.5 mL), aniline (0.558 g, 6 mmol), 5 mol/L NaOH (3 mL), and hexadecytrimethylammonium chloride (20 mg) were added under N₂ protection. The mixture was stirred vigorously for 24 h at room temperature. The mixture was extracted with CH₂Cl₂, and the extract was washed with H₂O and dried with MgSO₄. After evaporation of the solvent, the desired product **2** (1.262 g) was obtained as a beige solid in 76% yield *via* column chromatography (silica, CH₂Cl₂/AcOEt, 4/1, v/v). ¹H NMR (400 MHz, CDCl₃): δ 4.83 (s, 4H), 6.71–6.73 (m, 3H, *J* = 20 Hz), 7.17–7.21 (m, 4H, *J* = 12 Hz), 7.27–7.28 (d, 2H, *J* = 8 Hz), 7.62 (t, 2H, *J* = 16 Hz), 8.60–8.69 (d, 2H, *J* = 4 Hz). ¹³C NMR (100 MHz, CDCl₃): δ 57.37, 112.55, 117.29, 120.86, 122.12, 129.39, 136.93, 148.24, 149.81, 158.91. TOF-MS calcd. for C₁₈H₁₇N₃ [M+H]⁺: 276.1501, found: 276.1506.

Compound **3**: POCl₃ (1 mL, 17 mmol) was added into a solution of DMF (2 mL, 26 mmol) in portions over 0.5 h and cooled in an ice bath. Then, the solution was stirred for 0.5 h. Compound **2** (0.600 g, 2.18 mmol) in DMF (1 mL) was added in portions over 20 min. The mixture was heated for 3 h at 90 °C and stirred into H₂O (5 mL), and then neutralized to pH 6–8 with K₂CO₃. The mixture was extracted with CH₂Cl₂, and dried with MgSO₄. *Via* column chromatography (silica, petroleum/acetone, 5/3, v/v), the desired product (0.263 g) was obtained as a yellow oil in 40% yield. ¹H NMR (400 MHz, CDCl₃): δ 4.92 (s, 4H), 6.80–6.89 (d, 2H, *J* = 8 Hz), 7.21–7.25 (m, 4H, *J* = 16 Hz), 7.66–7.72 (m, 4H, *J* = 24 Hz), 8.61–8.71 (d, 2H, *J* = 4 Hz), 9.71 (s, 1H). ¹³C NMR (100 MHz, CDCl₃): δ 57.08, 112.00, 120.68, 122.46, 126.38, 132.04, 137.00, 149.94, 153.11, 157.20, 190.21. TOF-MS calcd. for C₁₉H₁₇N₃[M+H]⁺: 304.1450, found: 304.1442.

Probe TPZn aldehyde **3** (0.34 g, 1.2 mmol), and NaH (30 mg, 1.5 mmol) were dissolved in 3 mL of THF, and the solution was cooled to 0 °C under N₂. To this solution, Compound **4** (0.428 g, 1.0 mmol) in 9 mL of THF was added dropwise. The reaction mixture was stirred for 1 h at 0 °C, and then for 12 h at room temperature, followed by the removal of THF under reduced pressure. Water was added to the reaction mixture, and the product was extracted with dichloromethane (4× 10 mL). The organic layer was dried with anhydrous Na₂SO₄ followed by evaporation of the solvent. The crude product was separated by column chromatography with a gradient of hexane in dichloromethane (0%–50%). The product was obtained as a red powder in 42% yield. ¹H NMR (400 MHz, CDCl₃): δ 8.63–8.74 (d, 4H, *J* = 4.8 Hz), 7.91 (s, 1H),

7.70–7.81 (t, 4H, J = 7.2 Hz), 7.52 (s, 1H), 7.40–7.49 (d, 2H, J = 8.0 Hz), 7.29–7.34 (d, 4H, J = 4.8 Hz), 7.16–7.21 (d, 4H, J = 16.0 Hz), 7.09–7.14 (d, 4H, J = 16.0 Hz), 6.99–7.09 (t, J = 7.2 Hz, 4H), 6.72–6.79 (d, J = 8.6 Hz, 2H), 4.92 (s, 8H). ¹³C NMR (100 MHz, CDCl₃, Me₄Si): δ 158.24, 149.98, 149.31, 138.78, 137.14, 134.46, 129.21, 129.06, 125.03, 122.45, 120.94, 117.90, 117.14, 114.38, 112.87, 77.51, 77.20, 76.88, 57.39. TOF-MS calcd. for C₄₈H₄₀N₈ [M+2H]⁺: 728.3376, found: 728.3402.

3. Results and discussion

In the TPZn molecule, DPA was used not only as the receptor for Zn^{2+} , but also as the donor in a push–pull electronic system. The absorption spectrum of TPZn in CH₃CN–water solution is characterized by a very intense band centered at 450 nm, which is responsible for the orange-yellow color of the solution. Fluorescence of TPZn was quenched ($\Phi = 0.09$), because PET occurred. When Zn^{2+} was added gradually, the λ_{ab} showed a 50 nm blue shift from 450 nm to 400 nm with an isosbestic point at 425 nm. The PET process was restrained, and there are blue shift in the absorption and fluorescence spectra, because the electron-donating ability of the nitrogen atom at the *N*,*N*-bis(pyridin-2-ylmethyl) aniline conjugated onto the fluorophore was reduced, which is an ICT process.

Free TPZn exhibited λ_{em} at 586 nm with a quantum yield of 0.09. Upon addition of Zn²⁺, the λ_{em} underwent a blue shift from 586 nm to 535 nm with a quantum yield of 0.43, and the fluorescence color changed from orange-red to green. Other metal ions had no such fluorescence response. Cd²⁺, the most common interfering ion, only slightly changed the fluorescence intensity with a smaller wavelength shift (15 nm, Φ_{Cd} = 0.15 Fig. 1a and b). Upon gradual addition of Zn²⁺ to a solution of TPZn, the fluorescence intensity increased linearly with the Zn²⁺ concentration in the range of 0–2.0 mmol/L (Fig. 1a and c).

From the fluorimetric experiments, we realized that TPZn would have higher binding affinity for Zn²⁺ than for Cd²⁺. As expected, the addition of Zn²⁺ into TPZn-Cd solution resulted in quick fluorescence changes (less than 1 min). The λ_{em} of TPZn-Cd underwent a blue shift from 586 nm to 535 nm, indicating that Zn^{2+} can displace Cd^{2+} to form the TPZn-Zn complex (Fig. 1b). Although displacement assays have been used in cation recognition [12], ratiometric displacement approaches with two-photon fluorescent sensor have not been successful. Although there is some quenching in the presence of Co^{2+} , Ni^{2+} and Cu^{2+} , the ratio F_{535}/F_{586} does not change after addition of other metal ions (Fig. 1b and c). The dissociation constants (K_d^{TP}) for TPZn-Zn calculated from TP fluorescence titration curves were $K_{11} = 4.86 \times 10^{-5}$, K_{12} = 2.01 × 10⁻⁴ (F_{535}) and K_{11} = 6.47 × 10⁻⁵, K_{12} = 1.78 × 10⁻⁴ M (F_{535}/F_{586}) , respectively. TPZn shows excellent selectivity for Zn²⁺ compared with various metal ions and is pH-insensitive in the biologically relevant pH range, showing no interference in the pH range 6-8 for TPZn and TPZn-Zn (Fig. 1d).

Physiologically important metal ions which exist in living cells, such as Ca^{2+} , Mg^{2+} , Na^+ , and K^+ , did not give any responses at 50-fold excess concentration. Most heavy and transition metal ions, such as Hg^{2+} , Mn^{2+} , and Pb^{2+} , also had no interference. Ni²⁺, Co^{2+} and Cu^{2+} obviously quenched the fluorescence to some extent, a



Fig. 1. (a) Emission spectra with excitation wavelength of 425 nm (isosbestic wavelength) in Tris–HCl (0.01 mol/L, MeCN/water, 50/50, v/v, pH 7.4) in the presence of increasing concentrations of Zn²⁺ (0–30 eq.). The concentration of TPZn was 10 μmol/L. (b) Fluorescence spectra of TPZn (λ_{ex} 425 nm) (10 μmol/L) in the presence of different metal ions (50 μmol/L) in Tris–HCl (0.01 mol/L, CH₃CN/water, 50/50, v/v, pH 7.4). (c) The saturation titration of TPZn (10 μmol/L) with Zn²⁺, the minimum concentration of the added Zn²⁺ is 10 μmol/L. (d) The interference of pH in 6–8 for TPZn and TPZn-Zn. (e) Fluorescence responses of TPZn (10 μmol/L), λ_{ex} 425 nm) to Zn²⁺ (50 μmol/L) in the presence of selected metal ions (50 μmol/L) in Tris–HCl (0.01 mol/L, MeCN/water, 50/50, v/v, pH 7.4). From left to right: Zn²⁺, K⁺, Na⁺, Ca²⁺, Mg²⁺, Cd²⁺, Mn²⁺, Ba²⁺, Pb²⁺, Fe²⁺, Cu²⁺, Co²⁺, Ni²⁺, and Hg²⁺.

problem faced in the other metal ion sensors [13]. The competition experiments of Zn^{2+} mixed with the metal ions shown no significant variation in the ratio of fluorescence intensity (F_{535}/F_{586}). Fortunately, when the heavy quenchers Ni²⁺, Co²⁺ and Cu²⁺

existed, enhancement of the ratio of fluorescence intensity (F_{535}/F_{586}) was observed (Fig. 1e).

The two-photon action cross sections ($\delta \Phi$) of TPZn and TPZn-Zn were determined by using the two-photon-induced fluorescence



Fig. 2. TP action spectra of TPZn, TPZN-Zn, and TSQ-Zn.

measurement technique [13]. The maximum two-photon action cross sections of TPZn and TPZn-Zn were 77 GM and 185 GM at 830 nm, respectively (see Fig. 2). Coordination of the Zn^{2+} to the ligand that is conjugated to the 1,4-dicyano-2,5-bis(styryl)benzene core suppressed the ICT process, and this process also contributes to the fluorescence intensity enhancement, so the two-photon action cross section of TPZn-Zn is larger than TPZn. The TP action spectra of the Zn^{2+} complexes with TPZn in Tris–HCl (0.01 mol/L, CH₃CN/water, 50/50, v/v, pH 7.4) was 46 times larger than those of TSQ-Zn (Fig. 2). This finding indicates that TPM images for samples stained with TPZn would be much brighter than those stained with commercial sensors.

We next examined the application of TPZn for ratiometric fluorescence imaging of Zn^{2+} in cultured living cells (HEK 293 cells). Two-photon microscopy (TPM) imaging of HEK 293 cells was observed under a LSM 510 Meta two-photon microscope (Zeiss).

The double-channel fluorescence images at 515-545 nm and 570-600 nm are shown in Fig. 3. HEK 293 cells incubated with DMEM containing 5.0 µmol/L TPZn for 0.5 h at 37 °C were stained, indicating that TPZn can permeate into the cells and accumulate in them. When cells stained with compound TPZn were incubated with $Zn(NO_3)_2$ (5.0 μ mol/L) in phosphate-buffered saline (PBS) for 0.5 h and washed, the ratio of fluorescence intensities at 515-545 nm and 570–600 nm increased immediately (Fig. 3) Ratio imaging. The intensity ratio data were obtained using Image I (National Institutes of Health) software (Fig. 3). The cells remained viable and no apparent toxicity and side effects were observed throughout the imaging experiments (about 2-3 h). These experiments indicate that TPZn can provide ratiometric detection for intracellular Zn²⁺ ions. Therefore, it could be a useful molecular probe for studying biological processes involving Zn²⁺ ions within living cells.

We further investigated the utility of TPZn in tissue imaging. TPM images were obtained from a part of an acute rat hippocampal slice from a 2-day postnatal rat incubated with 10 µmol/L TPZn for 30 min at 37 °C. They reveal intense fluorescence in the CA3 regions as well as the hilus of dentate gyrus (Fig. 4a) [14]. The TPM images obtained at a depth of 80–160 μ m revealed the [Zn²⁺] distribution in the mossy fibers of dentate granule neurons near the hilus exclusively in the given plane along the z direction (see Fig. 5). These findings demonstrate that TPZn is capable of detecting intracellular free Zn^{2+} ions at a depth of 80–160 μ mol/L in living tissues by using TPM. Moreover, Fig. 4c-h shows the results of TPM imaging of the mossy fiber axon terminals of pyramidal neurons in the CA3 region at a depth of 120 µm. The double-channel fluorescence images at 515-545 nm and 570-600 nm both have regions of intense fluorescence, which seemed to be derived from localization of the dye. However, this influence was diminished in the ratio image, and higher ratios were clearly observed in the CA3 region rich in vesicular zinc. Treatment of the cells with the membrane-permeable metal ion chelator TPEN



Fig. 3. TPM of live HEK 293 cells. (a and b) Cells incubated with 5.0 μ mol/L TPZn for 0.5 h at 37 °C, emission collected at 570–600 nm (a) and emission collected at 515–545 nm (b). (c and d) Cells supplemented with 5.0 μ mol/L Zn²⁺ in the growth media for 0.5 h at 37 °C emission collected at 570–600 nm (c) and emission collected at 515–545 nm (d). The excitation wavelength was 830 nm. TP ratio fluorescence ($F_{535/}F_{586}$) images of Zn²⁺ in HEK 293 cells. (Up) HEK 293 cells incubated with compound TPZn (5.0 μ mol/L) and then further incubate with 5.0 μ mol/L Zn(NO₃)₂.



Fig. 4. TPM images of a part of an acute rat hippocampal slice. (a) Images of living tissue stained with 10 μ mol/L TPZn in ACSF (pH 7) buffer at a depth of *ca.* 120 μ m with magnification 10×. (b) Magnification at 100× shows sin the hilus (H) of dentate gyrus (DG) regions (purple regions of a) at a depth of about 120 μ m. (c and d) Images with emission collected at 515–545 nm using magnification at 100× in the CA3 regions at a depth of about 120 μ m before (c) (red regions of a) and after (d) addition of 200 μ mol/L TPEN to the imaging solution. (e and f) Images with emission collected at 570–600 nm using magnification at 100× in the CA3 regions at a depth of about 120 μ m before (e) and after (f) addition of 200 μ mol/L TPEN to the imaging solution. (g) Ratiometric images generated from (c) and (e). (h) Ratiometric images generated from (d) and (f). The TPEF images were acquired with excitation at 830 nm with a femtosecond pulse.



Fig. 5. TPM images show the hilus (H) of the dentate gyrus (DG) by magnification at $100 \times$ of a rat hippocampal slice stained with 10μ mol/L TPZn at different depths (a: 80 μ m, b: 100 μ m, c: 120 μ m, d: 140 μ m, e: 160 μ m). The TPEF images were collected at 520–550 nm upon excitation at 790 nm with fs pulses.

reversed the fluorescence ratio enhancements to baseline levels (Fig. 4h), which confirms that the increase in fluorescence ratio results from Zn^{2+} coordination and not from other phenomena, such as proton flux or sensor photoactivation. These ratiometric signals disappeared upon addition of TPEN, indicating that TPZn can detect endogenous Zn^{2+} in the CA3 region. To the best of our knowledge, this is the first example of TP ratiometric fluorescence imaging at a depth of 120 μ m to visualize endogenous Zn^{2+} in hippocampal slices. These experiments indicate that TPZn is an effective ratiometric probe for biological applications and can reversibly monitor changes in intracellular Zn^{2+} in living tissues. We believe TP ratiometric sensors with these advantages will be a vital implement in investigations of the biological significance of Zn^{2+} .

4. Conclusion

In this paper, we have developed a new TP ratiometric sensor TPZn for Zn^{2+} based on ICT-PET mechanisms, and pH insensitivity in a large physiological pH range. TPZn is selective against other biologically competing alkali- and alkaline-earth-metal ions, and

has higher binding affinity for Zn^{2+} than for Cd^{2+} in fluorimetric experiments. The TP action spectra of TPZn-Zn indicated a $\delta \Phi$ value of 185GM at 830 nm, 46 times larger than those of TSQ-Zn. TPZn was confirmed to be directly applicable to monitor changes in intracellular Zn^{2+} in living tissues, owing to its membrane permeability. TP Ratiometric imaging with TPZn at a depth of 120 μ m could clearly visualize endogenous Zn^{2+} in hippocampal slices [9].

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