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Dicyanostilbene-Derived Two-Photon Fluorescence Probe for Free Zinc Ions in Live Cells and Tissues with a Large Two-Photon Action Cross Section

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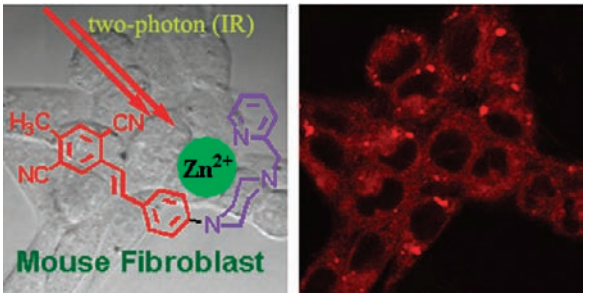
ABSTRACT



A novel two-photon fluorescence probe for Zn^{2+} derived from dicyanostilbene as a two-photon fluorophore and 4-(pyridine-2-ylmethyl)piperazine as a novel Zn^{2+} ligand was developed. The probe shows a 72.5-fold fluorescence enhancement in response to Zn^{2+}, a large two-photon action cross-section (580 GM), a noncytotoxic effect, and pH insensitivity in the biologically relevant range, and its dissociation constant (K_d^{TP}) is 0.52 ± 0.01 μM. The probe can selectively detect free Zn^{2+} ions in live cells for 1500 s or so and in living tissues at a depth of 80−150 μm without interference from other metal ions and the membrane-bound probes.

Two-photon excitation fluorescence microscopy (TPM), which uses two photons of lower energy as the excitation source, has rapidly evolved into a widely used tool in biological and biomedical research and is increasingly popular. Compared to traditional fluorescence microscopy, TPM offers intrinsic three-dimensional (3D) resolution combined with reduced phototoxicity and photobleaching, increased specimen penetration, and negligible background fluorescence.[1]

However, most fluorophores presently used as labels or sensor platforms in TPM belong to one-photon (OP) ones which have small two-photon absorption cross sections (δ) that limit their usage.[2] Therefore, to make TPM a more versatile tool in biology, researchers need a wider variety of two-photon (TP) probes with large δ for specific applications.

Molecular design strategies of organic molecules with large δ are well-established.[3] In general, the magnitude of δ increases with an increasing degree of intramolecular charge transfer (ICT) upon excitation.

We have reported a TP fluorophore, 4-methyl-2,5-dicyano-4′-amino stilbene (**DCS**) with remarkably large δ, which has been successfully employed in the design of

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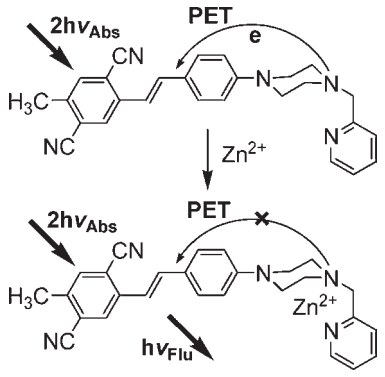
Scheme 1. Structure of **DZn** and Its Two-Photon PET Mechanism



TP fluorescence (TPF) probes for Hg^{2+} and Ag^{+} ions.[4] **DCS** is a push−pull chromophore with the strong electron donor of a *N*,*N*′-disubstitutedamino group at its extremity and the strong electron acceptor of two cyano groups on its single aromatic ring. The interaction between the amino group and cyano groups can significantly improve ICT and increase the excited state dipole moment, which drastically boosts the δ value of the fluorophore.

Zinc is a vital component of enzymes and proteins.[5] In the brain, a few millimoles of intracellular free Zn^{2+} ions are stored in the presynaptic vesicles, which are released with synaptic activation, and seem to modulate excitatory neurotransmission.[5a] To understand the biological roles of Zn^{2+}, a variety of OP fluorescence (OPF) probes derived from quinoline (TSQ, Zinquin and TFLZn) and fluorescein (FluoZin-3, Zinpyr, ZnAF, etc.) have been developed.[6,7] However, most of them require a rather short excitation wavelength or suffer from pH-sensitivity.

To overcome these problems, people began to develop TPF probes for Zn^{2+}, and four such probes have been reported.[8] Although two among them have good achievement in application and one can be used for bioimaging, the values of their δ are comparatively small, and the largest δ is just 193 GM.

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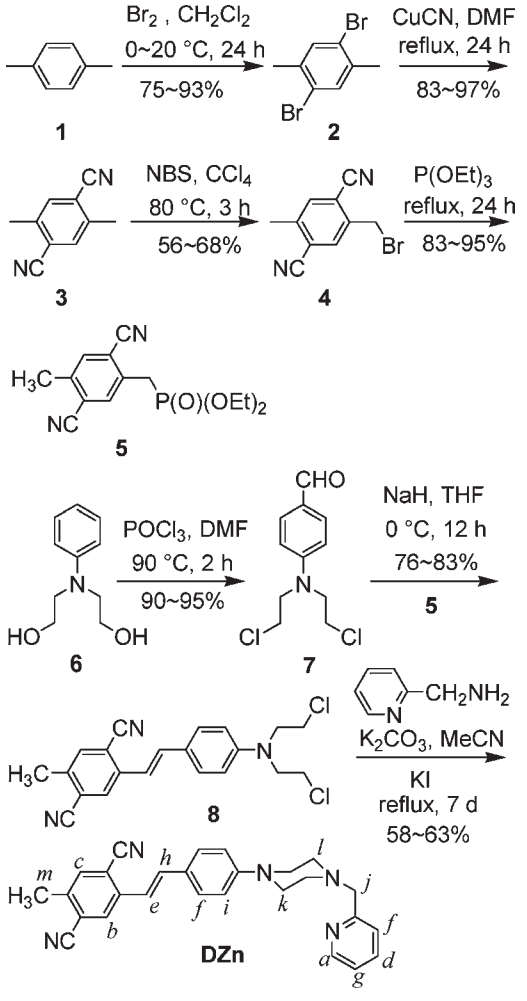
As an efficient bioimaging TPF probe, it should have long-wavelength emission (near the ideal NIR imaging window 650−900 nm), large δ, excellent photostability, proper water solubility, and good cell permeability.

Herein, we extend our earlier work[4] and report a new TPF probe for Zn^{2+} derived from **DCS** as a TP fluorophore and 4-(pyridine-2-ylmethyl)piperazine (**PMP**) as a novel Zn^{2+} ligand. **PMP** is a original receptor and exhibits a good affinity to Zn^{2+}. To the best of our knowledge, no probes for Zn^{2+} using **PMP** as a receptor have been reported. We report that **DZn** (Scheme 1) is capable of imaging the intracellular free Zn^{2+} ions in live cells for a long period of time and in living tissue at a depth of $> 80\,\mu$m without mistargeting and photobleaching problems.

Scheme 2. Synthetic Procedure of **DZn**



The preparation of **DZn** is given in the Supporting Information (SI) and Scheme 2. The solubility of **DZn** in water was 20 μM, which is sufficient to stain the cells (Figure S1, SI). The absorption intensities of **DZn** are almost independent of Zn^{2+} (Figure S2, SI), indicating **DZn** is a PET (photoinduced electron transfer)-based probe. However, **DZn** exhibits an unexpected strong sensitivity to Zn^{2+} in the OP and TP processes (Figures 1a, S3, SI). As expected, the TP action cross section or fluorescence brightness ($\Phi\delta$)

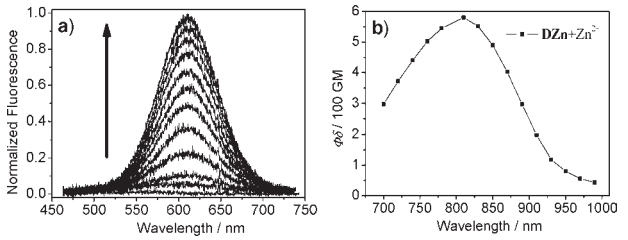


Figure 1. (a) TP emission spectra of 1 μM **DZn** (MOPS buffer) in the presence of free Zn^{2+} (0−6 μM). (b) TP action spectra of 1 μM **DZn** in the presence of 6 μM free Zn^{2+}.

and δ of **DZn** increased 72.5-fold from 8 to 580 GM and 2.3-fold from 400 to 935 GM (Table S1, SI) upon saturation with Zn^{2+} in 30 mM MOPS buffer (100 mM KCl, 10 mM EGTA, pH 7.2; EGTA = ethylene glycol bis(2-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid, and MOPS = 3-(morpholino)-propanesulfonic acid), respectively. This exceeds the brightness of many fluorophores widely used in biology, including fluorescein, BODIPY, or Acedan (2-acetyl-6-(dimethylamino)naphthalene), and the TP action cross section of **DZn**-Zn^{2+} is more than six times that of **AZn2**-Zn^{2+} (Table S1, SI). **AZn2** is a TPF probe for Zn^{2+} derived from acedan and *N*,*N*-di-(2-picolyl) ethylenediamine (DPEN).[8b] TP emission spectra of **DZn** are similar to those of OP (Figures 1a, S3, SI), and its TPF intensities enhanced with increasing Zn^{2+} concentrations. Likewise, its two-photon excitation spectrum in the presence of 6 μM Zn^{2+} is analogous to the OP absorption spectrum, and it had a TP excitation maximum at 810 nm (Figure 1b) (OP absorption and emission maximum at 403 and 610 nm, respectively). **DZn** is pH-insensitive in the biologically relevant pH range (Figure S5, SI) and highly selective toward zinc ions and does not yield a positive fluorescence response to Cd^{2+} (Figure S4, SI), which has been a challenge in the selective detection of zinc ions.

The dissociation constants (K_d^{OP} and K_d^{TP}) for **DZn** calculated from the OP and TP fluorescence titration curves (Figures 1a, S3, S6, S8, SI) are 0.51 ± 0.02 μM and 0.52 ± 0.01 μM (Figures S7, S9, SI), respectively; the detection limit of the probe is in the submicromolar range. A Job plot (Figure S10, SI) indicated that **DZn** coordinated to Zn^{2+} with 1:1 stoichiometry in water solution.

H NMR titration data clearly indicated that, except for H_b, H_c, and H_m, all the peaks of the other 10 types of H_s (H_a, H_d−H_l) experienced distinct downfield shifts and broadened with increasing Zn^{2+} concentration, and the chemical shifts for two sorts of hydrogen atoms (H_k and H_l) of the piperazidine group evinced a great increase.

More surprisingly, upon increasing Zn^{2+} concentration from 1 to 10 equiv, the chemical shifts of H_d and H_g caught up and overtook those of H_c, and H_e and H_f, respectively (Scheme 2, Figure S11 and Table S2, SI). This could be attributed to a deshielding effect, arising from the decrease of the electron density in the **DCS** fluorophore caused by the strong complexation of N−Zn^{2+}.

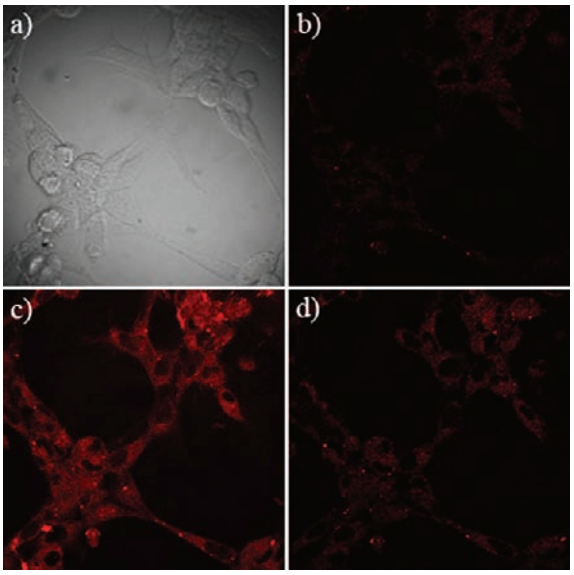


Figure 2. (a) Bright-field image. (b−d) TPM images of 1 μM **DZn**-labeled mouse fibroblast collected at 550−650 nm, before (b) and after (c) addition of 10 mM SNOC to the imaging solution; (d) After addition of 0.1 mM TPEN to (c). The TP excitation fluorescence (TPEF) images were collected upon excitation at 810 nm with a femtosecond pulse. Cells shown are representative images from replicate experiments (n = 5).

The TPM images of mouse fibroblast labeled with **DZn** showed considerably poor TPF at 550−650 nm (Figure 2b), which was due to fluorescence quenching based on the PET mechanism after the addition of 10 mM S-nitrosocysteine (SNOC), an endogenous NO donor that triggers the release of Zn^{2+}, to the above cell culture fluids; the TPF intensity increased gradually with time (Figure 2c) and then decreased abruptly upon the addition of 0.1 mM TPEN (*N*,*N*,*N*′,*N*′-tetrakis(2-pyridyl) ethylenediamine) (Figure 2d), a membrane-permeable Zn^{2+} chelator that can effectively remove Zn^{2+}.[8b] Without interference from the membrane-bound probes (no fluorescence at 360−460 nm) in this visual window,[8b] therefore, **DZn** can detect Zn^{2+} concentrations in live cells and has no cytotoxic effect.

To further investigate the utility of this probe in deep tissue imaging, TPM images were obtained from a part of a mouse brain tissue slice incubated with 10 μM **DZn** for 30 min at 37 °C. Three TPM images were obtained in the same plane at a depth of about 120 μm. Without the addition of SNOC, the TPM image was dimmish (Figure 3a), while, after the addition of 30 mM SNOC, it became extremely bright (Figure 3b). Subsequently, upon the addition of 0.3 mM TPEN, the TPM image became rapidly faint (Figure 3c). The fluorescence of the **DZn**-Zn^{2+} complex lasted 1500 s or so (Figure 3d). Similarly, the fluorescence lasted for a long time in the cell-imaging process. Moreover, the TPM images obtained at a depth of 80−150 μm revealed the [Zn^{2+}] distribution in the brain tissues in the given plane along

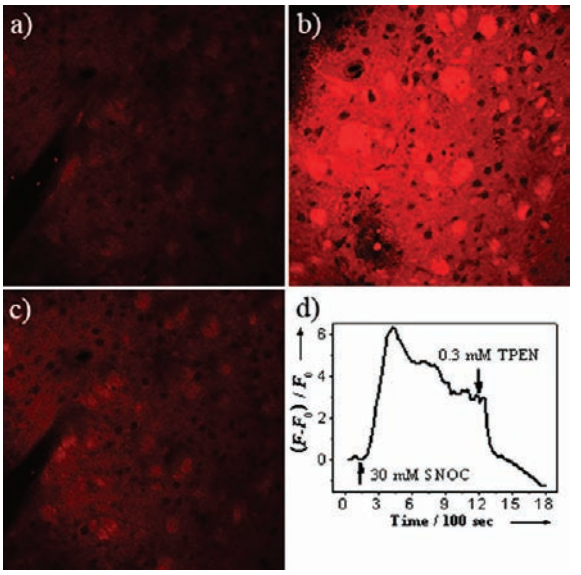


Figure 3. TPM images of a mouse brain tissue slice stained with 10 μ**M DZn**. (a) At a depth of ca. 120 μm with magnification 100×. Before (a) and after (b) addition of 30 m**M** SNOC to the imaging solution; (c) After addition of 0.3 m**M** TPEN to (b). (d) Relative TPEF intensity of a mouse brain tissue slice stained with 10 μ**M DZn** collected at 550−650 nm as a function of time. The TPEF images were collected at 550−650 nm upon excitation at 810 nm with a femtosecond pulse.

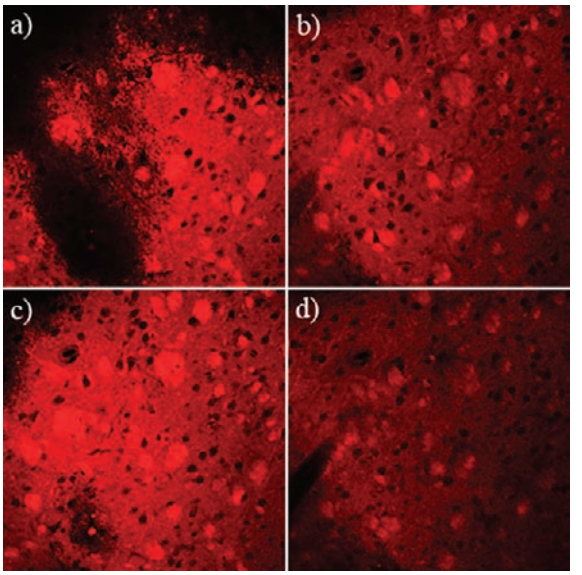


Figure 4. TPM images of a mouse brain tissue slice stained with 10 μ**M DZn** by magnification 100× at different penetration depths of (a) 80, (b) 100, (c) 120, and (d) 150 μm. The TPEF images were collected at 550−650 nm upon excitation at 810 nm with fs pulses.

the z direction (Figure 4). These findings demonstrate that **DZn** is capable of detecting intracellular free Zn^{2+} ions at a depth of 80−150 μm in living tissues by using TPM.

In conclusion, we have developed a TPF probe **DZn** with small molecule size, large $\Phi\delta$ (580 GM), noncytotoxic effect, long-wavelength emission at 610 nm (adjacent to the ideal imaging visual window 650−900 nm), large Stokes shift (207 nm), excellent photostability, moderate water solubility, and good cell permeability. **DZn** shows a 72.5-fold fluorescence enhancement in response to Zn^{2+} and pH insensitivity in the biologically relevant range, and its dissociation constant (K_d^{TP}) is 0.52 ± 0.01 μM. **DZn** can selectively detect intracellular free Zn^{2+} ions in live cells for 1500 s or so and in living tissues at a depth of 80−150 μm without interference from other metal ions and the membrane-bound probes.

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Supporting Information Available. Synthesis, photophysical study, cell culture, and two-photon imaging. This material is available free of charge via the Internet at http://pubs.acs.org.