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Ultra-fast zinc ion detection in living cells and zebrafish by a light-up fluorescent probe

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ABSTRACT

As the second most abundant transition metal after iron in biological systems, Zn^{2+} takes part in various fundamental life processes such as cellular metabolism and apoptosis, neurotransmission. Thus, the development of analytical methods for fast detection of Zn^{2+} in biology and medicine has been attracting much attention but still remains a huge challenge. In this report, we develop a novel Zn^{2+}-specific light-up fluorescent probe based on intramolecular charge transfer combined with chelation enhanced fluorescence induced by structural transformation. Addition of Zn^{2+} in vitro can induce a remarkable color change from colorless to green and a strong fluorescence enhancement with a red shift of 43 nm. Moreover, the probe shows an extremely low detection limit of 13 nM and ultra-fast response time of less than 1 s. The Zn^{2+} sensing mechanism was fully supported by TDDFT calculations as well as HRMS and ^1H NMR titrations. The recognition of Zn^{2+} in living Hela cells as well as the MTT assay demonstrate that the probe can rapidly light-up detect Zn^{2+} in vivo with low cytotoxicity and good cell-permeability. Furthermore, the probe can also be successfully applied to bioimaging Zn^{2+} in living zebrafish.

1. Introduction

As the second most abundant micronutrient transition metal behind iron in biology, it is well known for decades that Zn^{2+} plays multifunctional roles in fundamental biological processes, such as gene transcription and expression, neural signal transmission, cell division and proliferation, immune activity [1,2]. Due to zinc being an important structural cofactor and catalytic center of hundreds of zinc proteins and regulators of enzymes, deficiency of zinc ion might be associated with severe risks of physical growth retardation and neurological disorders such as cerebral ischemia and Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease and hypoxia-ischemia [3]. However, an excess zinc level might contribute superficial skin diseases, diabetes, and brain diseases. Furthermore, trace amount of free Zn^{2+} might significantly perturb zinc homeostasis in biology due to its strong affinity [4,5]. The clinic studies confirmed that zinc may also prevent esophageal cancer cells proliferation through Orai1-mediated intracellular Ca^{2+} oscillated ions [6]. Therefore, there is an increasing demand for effective and portable detection methods to spatiotemporally monitor biological zinc ion fluctuation.

Compared with conventional methods for Zn^{2+} detection including potentiometry, enzymatic assay, surface acoustic wave spectroscopy, mass spectrometry and electrochemistry, fluorescence spectroscopy is a particularly preferable tool for detection of interesting analytes due to its operational simplicity, high sensitivity and selectivity, and ease of observation [7–10]. Thus, numerous Zn^{2+}-specific fluorescent chemosensors have been reported based on various fluorescent dyes as signal groups, such as 3-hydroxychromone [11], peptide [12], diazafluorene [13], coumarin [14], naphthalene [15,16], quinoline [17,18], boron-dipyrromethene [19], fluorescein [20] and others [21,22], which are bearing chelating moieties as Zn^{2+}-sensing groups such as piperidine/pyridine tripods. Binding of Zn^{2+} with those sensor molecules can significantly regulate electron processes including photo-induced electron transfer (PET), intramolecular charge transfer (ICT) [23], excited-state intramolecular proton (ESIPT) [24,25] or chelation enhanced fluorescence (CHEF) leading to dramatic spectra or color changes.

It is to be noted that most probes in literatures demonstrated excellent fluorescence and/or color changes with short-wavelength light excitation and/or emission probably inducing photobleaching, cellular auto fluorescence, and photo-damage to tissues or organelles. To overcome this drawback, several groups reported their contributions with excitation of long wavelength light [26–31]. For example, Kim's group reported a Golgi-localized two-photon (TP) probe for imaging zinc ions based on the TP fluorophore 6-(benzo[*d*]thiazol-20-yl)-2-(*N*,*N*-dimethylamino) naphthalene in EtOH/MOPS buffer (v:v, 1/1) on excitation at 750 nm [32]. Zhao's group developed a three-photon probe for the imaging of exogenous Zn^{2+} in live cells with varying emission under excitation at 1200 nm [30]. Recently, Tian's group demonstrated a two-photon ratiometric fluorescent probe for bioimaging Zn^{2+} in hippocampal tissue and zebrafish in

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EtOH/HEPES buffer (v:v, 1/1) upon excitation at 800 nm, which suffered the interference of Cu^{2+} [33]. Meng's group reported a mitochondria-targeted ratiometric two-photon fluorescent probe for biological Zn^{2+}, in which Cd^{2+} slightly interfere the detection [34]. Unfortunately, these TP and OP fluorescent probes for Zn^{2+} detection more or less showed at least one of drawbacks including long response time, or high limits of detection besides the interference of Cu^{2+}, Cd^{2+} or Fe^{3+} due to their similar properties [35–37].

Our former investigations showed that the cyano electron-withdrawing group can modulate the electron transfer process which finally induce changes of the absorption/emission spectra [38–40]. Therefore, we envision that introduction of a cyano group could extremely lower the HOMO and LUMO level of probe-analyte complex other than the free probe, which probably induced a red shift. Encouraged by these excellent studies and considering those limitations in mind, herein we describe a simple fluorescent probe for ultrafast detection of Zn^{2+} with high selectivity and sensitivity designed by coordination-induced structural transformation. The probe was easily prepared with good yield via one-step condensation of 3′-formyl-4′-hydroxy-[1,1′-biphenyl]-4 carbonitrile and 2-aminobenzohydrazide under mild conditions. As expected, complexation with Zn^{2+} can switch on a large fluorescence enhancement of **P-OH** in 1 s, showing extremely low detection limit and fast response time. The MTT assay and recognition of Zn^{2+} in living Hela cells showed low cytotoxicity and good cell-permeability of the probe. Furthermore, **P-OH** could be successfully applied to bioimaging Zn^{2+} in living zebrafish.

2. Experimental

2.1. Reagents and Chemicals

All chemical reagents were obtained commercially from Aladdin, J&K or Sinopharm Chemical Reagent Co. and used as received without further purification unless otherwise stated. All solvents were purified using standard methods. 3′-formyl-4′-hydroxy-[1,1′-biphenyl]-4 carbonitrile (**1**) and 2-aminobenzohydrazide (**2**) were synthesized according to revising procedures following literatures [38].

2.2. Instruments and Measurements

Ultrapure water was purified from Millipore. ^{1}H NMR and ^{13}C NMR spectra were carried out on a Bruker Advance DRX 400 spectrometer at 400 MHz/100 MHz with TMS as an internal reference. Mass spectra (ESI) were conducted on Bruker Waters-Q-TOF-Premier spectrometer or a Shimadzu LCMS-IT-TOF spectrometer. UV–Vis and Fluorescence spectra were recorded on a TU-1901 spectrophotometer and a Hitachi F-7000 luminescence spectrometer with xenon lamp, respectively. The pH value was measured using a Metrohm 808 Titrando pH meter. Twice-distilled water used throughout all experiments was purified by a Milli-Q system (Milipore, USA). The fluorescence images of cells and zebrafish were taken using a confocal laser scanning microscope (Leica, TCS SP5, Germany) with an objective lens (×40).

2.3. Synthesis of P-OH

Under N_2 atmosphere, 2-aminobenzohydrazide (1 mmol, 0.15 g) and 3′-formyl-4′-hydroxy-[1,1′-biphenyl]-4 carbonitrile (1 mmol,0.20 g) were mixed in 20 mL of ethanol. The mixture was stirred for 6 h under refluxing. After the reaction finished, the solvent was removed under reduced pressure. The crude product was recrystallized from ethanol to give 200 mg of the pure title compound as a yellow solid in 60% yield. ^{1}H NMR (400 MHz, DMSO d_6) δ ppm: 12.05 (s, 1H), 11.83 (s, 1H), 8.65 (s, 1H), 7.95 (d, J = 2.0 Hz, 1H), 7.90 (s, 4H), 7.72 (dd, J = 8.4, 2.2 Hz, 1H), 7.61 (d, J = 7.7 Hz, 1H), 7.26–7.19 (m, 1H), 7.07 (d, J = 8.6 Hz, 1H), 6.77 (d, J = 8.2 Hz, 1H), 6.62–6.56 (m, 1H), 6.51 (s, 2H). ^{13}C NMR (100 MHz, DMSO d_6) δ ppm: 165.6, 158.6, 150.8, 147.5, 144.3, 133.2, 133.1, 130.1, 129.7, 128.8, 128.6, 127.3, 119.8, 119.5, 117.7, 117.0, 115.1, 112.8, 109.7. HRMS (ESI) calc. for $[C_{20}H_{17}N_3O_2\text{-}H]^-$, 355.11950; found, 355.11991.

2.4. Synthesis of *Ref*

The compound was prepared using benzaldehyde following the same procedure as **P-OH** in 73% yield. ^{1}H NMR (400 MHz, DMSO d_6) δ ppm: 11.62 (s, 1H), 8.40 (s, 1H), 7.71 (d, J = 7.1 Hz, 2H), 7.63–7.55 (m, 1H), 7.45 (dt, J = 12.4, 6.8 Hz, 3H), 7.26–7.16 (m, 1H), 6.82–6.73 (m, 1H), 6.64–6.55 (m, 1H), 6.39 (s, 2H).

2.5. General Procedure for Spectra Measurement

A stock solution containing 1.0 mM of **P-OH** was prepared in THF for all fluorescence measurements below. Stock solutions of analytes (Zn^{2+}, Ni^{2+}, Mg^{2+}, Al^{3+}, Ag^{+}, Ba^{2+}, Ca^{2+}, Cr^{3+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, K^{+}, Li^{+}, Mn^{2+}, Na^{+}, Pb^{2+}, Cd^{2+} as their chloride, perchlorate, or nitrate) were prepared in distilled deionized water, respectively. A typical test solution was prepared by mixing 0.01 mL of probe **P-OH** (1.0 mM) and 0.390 mL of THF, appropriate aliquot of each analyte stock solution which was diluted to 1 mL with PBS buffer (20 mM, pH 7.4). The resulting solutions were mixed well for 10 min before recording the fluorescence and UV absorption spectra. The corresponding fluorescence spectrum was performed under excitation at 360 nm.

2.6. Cell Culture and Fluorescence Imaging

HeLa cells were incubated in Dulbeco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (10%), penicillin (100 U/mL), and streptomycin (100 U/mL) at 37 °C in a 95% humidity atmosphere containing 5% CO_2. After washing with Dulbecco's phosphate-buffered saline (DPBS) twice, HeLa cells were incubated for 12 h in a flatbottom 96-well plate in 100 μL of culture medium and incubated in 5% CO_2 at 37 °C. The cytotoxic effect of **P-OH** was determined by MTT assays after the 24 h incubation of the cells with the probe at different concentrations (0.0, 2.0, 5.0, 10.0, 15.0, and 20.0 μM).

2.7. In Vivo Imaging of Zebrafish

2-day old zebrafish were provided by the Eze-Rinka Biotechnology Co., Ltd. For fluorescence, the zebrafish were incubated with the aqueous solution of Zn^{2+} (100 μM) for 10 min, and then imaged after PBS buffer. In the experimental group, the zebrafish pretreated with Zn^{2+} (100 μM) for 10 min were incubated with **P-OH** (5 μM). The images of zebrafish were obtained on a confocal microscope with an excitation at 405 nm and the collection wavelength range from 500 to 550 nm.

3. Results and Discussion

3.1. Design and Synthesis of P-OH

Rational combination of donor and acceptor moieties has been extensively used to construct various fluorescent probes specifically for metal ion detection due to their high binding ability [41]. **P-OH** was successfully synthesized by a one-step condensation of 2-aminobenzohydrazide and 3′-formyl-4′-hydroxy-[1,1′-biphenyl]-4 carbonitrile in good yield (See Scheme 1). All compounds were fully characterized by ^{1}H NMR, ^{13}C NMR and ESI-MS analysis (SI, Figs. S1–S4).

With the probe in hand, we initially investigated whether **P-OH** was suitable for Zn^{2+} detection by UV–visible spectra. As shown in Fig. 1, the spectrum of free **P-OH** (10 μM) exhibited two intense absorption bands at 297 and 357 nm in THF aqueous solution (v/v, 4:6, pH 7.4, HEPES 20 mM). Addition of Zn^{2+} resulted in the 5 nm red shift of the band at 297 nm and the disappearance of the band at 357 nm. Simultaneously, a new small band at around 400 nm formed, which was attributed to