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A Novel Hydrophilic Bis(1,2,3-triazolyl)fluorenyl Probe for In Vitro Zinc Ion Sensing

Dao M. Nguyen¹, Xuhua Wang¹, Hyo-Yang Ahn¹, Luis Rodriguez¹, Mikhailo. V. Bondar³, and Kevin D. Belfield^{1,2,*}

Kevin D. Belfield: belfield@mail.ucf.edu

¹ Department of Chemistry, University of Central Florida, 4000 Central Florida Parkway, Orlando, FL 32816, USA

² CREOL, The College of Optics and Photonics, University of Central Florida, 4000 Central Florida Parkway, Orlando, FL 32816, USA

³ Institute of Physics, Prospect Nauki, 46, Kiev-28, 03094, Ukraine

Abstract

A hydrophilic bis(1,2,3-triazolyl)fluorene derivative was synthesized as a multiphoton absorbing, zinc ion sensing fluorescent probe. The fluorescence response was approximately five-fold greater in presence of Zn^{2+} , resulting in a large binding constant (10^9) for a 1:2 ligand to zinc complex. A four-fold increase in the two-photon absorption cross section was achieved upon binding Zn^{2+} . In vitro two-photon fluorescence microscopy imaging revealed a significant fluorescence increase upon introduction of Zn^{2+} into HeLa cells and reversible Zn^{2+} binding, demonstrating the potential of this probe for zinc ion sensing.

Keywords

1,2,3-triazolyl-fluorene; two-photon microscopy bioimaging; fluorescent probe; zinc ion sensing; metal sensing; binding constant

Zinc is the second most abundant transition metal ion *in vivo* and plays a significant role in the metabolic regulation, ion channels and receptors, neuronal transmission, and certain metalloenzymes.^{1,2} In neurophysiology, zinc ion is thought to participate in the formation of β -amyloid in Alzheimer's disease.³ Zinc ion release also causes hypoglycemia-induced neuronal death.⁴ The zinc ion is relatively inert with a closed shell of $3d^{10}$ electronic configuration, hence fluorescence methods are advantageous for detection of Zn^{2+} .^{5,6}

Designing new fluorescent probes for Zn^{2+} sensing *in vitro* and *in vivo* in biological environments should involve selectivity for Zn^{2+} over other relevant metal ions. The probe should have high affinity, as well as bind rapidly and reversibly. Zinc ion sensing utilizing fluorescein,⁷ porphyrin,⁸ benzoxazole,⁹ and other derivatives¹⁰ have been reported. Since the advent of two-photon absorption (2PA) and techniques that rely on 2PA, a number of advantages have been identified for 2PA, e.g., molecules can be excited at a long

CORRESPONDING AUTHOR FOOTNOTE. Fax: 1-407-823-2252 belfield@mail.ucf.edu.

Supporting Information Available. Synthesis and NMR spectra of **1**, TGA analysis, photophysical characterization, additional UV-vis absorption and emission spectra, binding constant determination of **1** with Zn^{2+} ion, reversibility, viability assay of probe **1** in HeLa cells, and experimental cell incubation and imaging are provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

wavelength, usually in the near infrared (NIR) region, deeper penetration and less scattering, and reduced photodamage and photobleaching.¹¹ Recently, our laboratory developed 2PA fluorescent probes for targeting cellular proteins^{12,13} and other biological applications. The fluorene-based construct is employed for its generally high fluorescence quantum yield, reasonably large 2PA cross section, and high photostability.^{14–19}

The metal sensing probe reported herein is constructed of an acceptor- π -electron bridge-donor (A- π -D) fluorene-based chromophore system, consisting of a 1,2,3-triazole-based recognition moiety for an integrated fluoroionophore.⁵ The bis(1,2,3-triazolyl)fluorene-based probe was synthesized by “click” chemistry,^{20,21} and it exhibited significantly enhanced fluorescence emission upon binding Zn^{2+} ions over other biological relevant heavy metals. The linear and nonlinear photophysical properties of the new probe **1** were investigated in common solvents. The metal to ligand stoichiometry ratio and the binding constant were determined, as was the reversibility of metal complexation. *In vitro* cell imaging revealed a fluorescence enhancement upon introduction of Zn^{2+} ions, and was reversible, as described below.

Bis(1,2,3-triazolyl)fluorene **1** was synthesized from fluorene derivative **A** (Scheme 1), involving a Sonogashira coupling of **A** with 2-(4-ethynylphenyl)benzothiazole **B** to obtain **C**, followed by reduction using hydrazine on Pd/C in a mixture of EtOH and THF at 70 °C to yield **D**. Arylamine **D** underwent N-alkylation with propargyl bromide affording bis-alkyne **E**. Finally, a “click” reaction of **E** and 2-azidoethylacetate, using copper(I) catalyst at room temperature, afforded bis(1,2,3-triazolyl)fluorene **1** in 70% yd. 2-Azidoethylacetate was prepared by a new method and its NMR spectra were consistent with the literature.²² The HRMS of **1** confirmed a molecular ion at 980.4249 which is consistent for the formula $\text{C}_{54}\text{H}_{60}\text{O}_8\text{N}_8\text{S}$.

Bis(1,2,3-triazolyl)fluorene **1** exhibited high thermal stability as the thermogravimetric analysis (TGA) of **1** showed it decomposed above 300 °C (Fig. S3 in the supporting information).

The linear photophysical properties of **1** in ethanol, acetonitrile, aqueous acetonitrile, THF, and cell medium (RPMI) are summarized in Table 1. The UV-vis absorption and fluorescence emission of **1** with metal ions in common organic solvents were quite similar. The fluorescence emission of **1** displayed a large Stokes shift with increasing solvent polarity (Fig. S4 in the supporting information). Several relevant metal ions were evaluated with the new probe **1** by UV-vis absorption and fluorescence emission spectroscopy. The fluorescence response factor (F/F_0) was measured by comparison of the maximum integrated area of the corrected emission spectrum of the metal complex (F) with the probe **1** before addition of metal ion (F_0). The UV-vis absorption of **1** with selected heavy metal ions (Zn^{2+} , Hg^{2+} , Cd^{2+} , Pb^{2+}) showed a hypsochromic shift from 2–20 nm. The fluorescence emission of metal ions, Li^+ , Mg^{2+} , Ca^{2+} , Ba^{2+} , Fe^{2+} , Co^{2+} , Cu^+ , Hg^+ , La^{3+} , and Au^{3+} were relatively unchanged while Cu^{2+} , Fe^{3+} , and Pd^{2+} quenched the fluorescence. However, Hg^{2+} , Cd^{2+} , Ag^+ , Pb^{2+} , and Zn^{2+} enhanced the fluorescence (Fig. 1). The UV-vis absorption and fluorescence emission of **1** towards Zn^{2+} in ethanol are shown blue-shifted that could be due to the increase in energy gap between the HOMO-LUMO as a function of Zn^{2+} concentration with isosbestic points with a single equilibrium complexation (Fig. S5 in the supporting information). Moreover, an approximately five-fold fluorescence increase upon binding to Zn^{2+} (Fig. 2), resulted in a large binding constant of 10^9 M^{-2} for a 1:2 ligand to metal stoichiometry ratio, determined by the linear fitting (Fig. S6 in the supporting information).²⁴ The fluorescence quantum yield and lifetime dramatically increased for Zn^{2+} complexation with **1** in comparison to the free probe **1** (Table 1). The behavior of **1** in cell medium (pH = 7.4) and in acetic acid buffer²⁵ (pH = 5) was unchanged. However, at lower

pH (< 4) the emission spectrum showed an additional absorption at 450 nm due to the protonated form, and at a higher pH (> 9) the compound precipitated.

The complex of **1** with metal ions formed rapidly upon agitation and was stable for several days. Moreover, complexation with **1** was reversible as demonstrated by displacement with EDTA (ethylenediamine tetracarboxylic acid), liberating probe **1** from its zinc complex (Fig. S7 in the supporting information).

Figure 3 illustrates the two-photon absorption (2PA) cross sections of **1** and its zinc complex in ACN. A significant increase in the 2PA cross section in region $\lambda = 740\text{--}760\text{ nm}$ was observed for the complex relative to free **1**. The 2PA process is a third order nonlinear optical property with a strong dependence on the intramolecular charge transfer (ICT) process.²⁶ The observed increase in δ_{2PA} of its zinc complex suggests that metal binding does not occur primarily via the amino functionality, rather it is suggestive of binding to the 1,2,3-triazolyl carboxylate moieties, since binding the the electron-donating moiety would be expected to decrease ILCT and the δ_{2PA} .^{10a, 27,28} The nature of Zn ion binding to this class of molecule and its dependence on 2PA is complex and a subject of future investigation.

The 2PA cross sections of the zinc complex of **1** at 740–760 nm were ca. 100–280 GM, suitable for applications in two-photon fluorescence microscopy (2PFM) zinc ion sensing and imaging.

The cytotoxicity of probe **1** was evaluated by the Alamar Blue cytotoxicity assay to assess its suitability for *in vitro* imaging.²⁹ Relatively low cytotoxicity of **1** in HeLa cells was demonstrated with optimal concentrations for imaging at 5–15 μM (Fig. S8 in the supporting information).

In vitro Zn^{2+} sensing and 2PFM imaging was investigated for probe **1** in live HeLa cells (Fig. 4); cells were maintained on a stage incubator and images were acquired before and after a zinc containing medium was introduced into cells. Next, N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) was introduced as a chelator³⁰ to displace Zn^{2+} in the zinc complex of **1**, resulting in a decrease in fluorescence (Fig. 4B-D) *in vitro*.

The new probe **1** exhibited both good two-photon absorptivity and fluorescence enhancement upon Zn^{2+} binding, had low cytotoxicity, had high Zn^{2+} binding constants, and, importantly, was soluble in aqueous media. Bis(1,2,3-triazolyl)fluorene **1** proved useful for *in vitro* zinc ion sensing by 2PFM, suggesting future potential for *in vivo* Zn^{2+} sensing and imaging, a subject of future investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIGURE 1. Fluorescence response of **1** at 2×10^{-6} M in ethanol towards selected metal ions in concentrations ranging from 10^{-6} to 10^{-4} M.

FIGURE 2. Fluorescence emission spectra of **1** at 2×10^{-6} M in ethanol excited at 383 nm as a function of Zn^{2+} concentration.

FIGURE 3.

UV-vis absorption, fluorescence emission, anisotropy, and 2PA of **1** and its zinc complex at 10^{-4} M in ACN.

FIGURE 4. (A) DIC image of HeLa cells incubated with 15 μ M of probe **1** for 2 h. (B) 2PFM image at $\lambda_{\text{ex}} = 740$ nm and power = 50 mW. (C) 2PFM image after 15 min treatment with zinc sulfate (50 μ M). (D) 2PFM image after further incubation with 50 μ M TPEN for 15 min.

SCHEME 1.
Synthesis of bis(1,2,3-triazolyl)fluorene **1**

TABLE 1
Linear photophysical properties of **1** and select metal complexes in organic solvents

Solvent	Metal ion	Φ_{Fl}	τ (ns)	$\epsilon \cdot 10^{-4}$ ($M^{-1}cm^{-1}$)	λ_{ab} (nm)	λ_{em} (nm)	$\Delta\lambda$
EtOH	1	0.1 ^a	0.3	7	383	613	230
	1 + Zn^{2+}	0.3	1.6		366	513	149
	1 + Hg^{2+}	0.1			362	550	180
	1 + Cd^{2+}	0.2			372	568	196
ACN	1 + Pb^{2+}	0.1			381	595	214
	1	0.1		6	385	643	258
	1 + Zn^{2+}	0.3			368	568	200
	1	0.02			384	663	279
ACN:H ₂ O (95:5)	1	0.04			369	627	258
THF	1	0.9		7.2	390	568	178
RPMI	1	0.37			395	555	160
	1 + Zn^{2+}	0.44			388	514	126

^a photodecomposition quantum yield²³ at 405 nm, $\Phi_D = 2 \times 10^{-6}$