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Synthesis and photophysical properties of a fluorescent TREN-type ligand incorporating the coumarin chromophore and its zinc complex

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Abstract—A new, UV-excited fluorescent Zn^{2+} indicator was synthesized and the spectral profile of its free and Zn^{2+} bound forms were studied. The fluorescent properties of this probe are due to the 7-amino-4-methylcoumarin fluorophore, which is conjugated with the tris(2-aminoethyl)amine (TREN) that functions as the zinc-chelating moiety. The compound exhibits a Zn^{2+} dissociation constant of 18.0 μM. The fluorescence spectra of the probe showed a clear shift in excitation wavelength maxima upon Zn^{2+} binding, indicating its potential use as ratiometric Zn^{2+} indicator.

Zn^{2+} is the second most abundant transition metal, after iron, in biological systems.[1] Since the 1940s, there has been a steady stream of data implicating Zn^{2+} in a number of biological processes. Zinc may serve as a structural element in enzymes and transcription factors, as well as at the catalytic site of a number of enzymes. The interest in Zn^{2+} is compounded by its involvement in a number of neuropathologies such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Parkinson's disease, hypoxia–ischemia, and epilepsy.[2]

Zn^{2+} is often referred to as the 'silent ion' since, unlike other biological transition metal ions (such as Fe^{2+}, Mn^{2+}, or Cu^{2+}), it does not have an intrinsic spectroscopic or magnetic signal because of its $3d^{10}$ $4s^0$ electronic configuration. However, its ability to form tetracoordinated complexes with a variety of organic moieties that can be connected to a fluorophore can be used as the basis for synthesizing fluorescent probes that may be useful in biological systems.[3]

The design and synthesis of fluorescent zinc probes requires the combined expertise of chemistry, biology, and medicine. A number of articles analyzing the reasoning behind the design of such molecules have been published and, as a result, a host of fluorescent zinc probe syntheses have been reported and reviewed in recent articles.[4–8]

In this letter, we describe the synthesis and the fluorescence spectra of a new chemosensor for zinc **1**, where the 7-amino-4-methylcoumarin fluorophore and the tris(2-aminoethyl)amine (TREN) as zinc-chelating moiety have been conjugated. The choice of the fluorescent molecule **1** as a zinc probe was based on the following rationale: depending on the nature of the photo-induced process, fluorescent ion probes with a linked fluorophore and ionophore can be classified into two types: photo-induced electron transfer (PET) and photo-induced charge transfer (PCT) probes.[9] In PET sensors, upon excitation of the ion free form, a donor–acceptor electron transfer causes fluorescence quenching. When the sensor is bound to a target cation, such transfer is prevented and the net result is a fluorescence enhancement. In PCT sensors, the fluorophore contains an electron-donating group (in this case the 7-amino group) conjugated to an electron-withdrawing group (in this case the coumarin carbonyl), which upon excitation, undergoes internal charge transfer (ICT) from the donor to the acceptor.[10] Coordination of the target ion with the electron-donor moiety destabilizes the system resulting in a hypsochromic shift in its excitation spectrum.

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Such a shift is highly desired in fluorescence ion probes, since it offers the possibility of ratiometric measurements of ion concentrations. Given that in **1**, one of the nitrogens is part of both fluorophore and chelator, the probe is expected to belong to the latter type and its excitation spectrum should undergo a blue shift upon zinc ion binding.

The stepwise synthesis of **1** is depicted in Scheme 1. 7-Amino-4-methylcoumarin, protected as tosyl amide **2**, was reacted with tris(2-chloroethyl)amine hydrochloride in the presence of Cs_2CO_3 to yield dichloride **3**. Deprotection of the amine via treatment with H_2SO_4 and conversion of the dichloride **4** to the corresponding diazide **5**, followed by catalytic reduction using 10% Pd/C as catalyst, furnished the desired TREN analog **1** in 18% overall yield.[11]

A stock indicator solution was prepared in nanopure water at 0.02 M concentration. An aliquot of this stock solution was then added to a 10 mM HEPES buffer, containing 140 mM NaCl and 2.5 mM KCl, to make a final indicator concentration of 10 μM, and the pH was adjusted to 7.8. Zn^{2+}-containing solutions were prepared by adding Zn^{2+} (0.02 M $ZnSO_4$ solution prepared in nanopure water) to the final indicator solution, to give Zn^{2+} concentrations ranging from 1 to 500 μM.

The spectral profile of probe **1** was studied in solutions of increasing Zn^{2+} concentration. As shown in Figure 1, the Zn^{2+} free probe exhibits an excitation maximum wavelength at 359 nm, which shifts to 337 nm upon zinc binding. This set of spectra has an isosbestic point at 340 nm. When excited at 359 nm the molecule shows

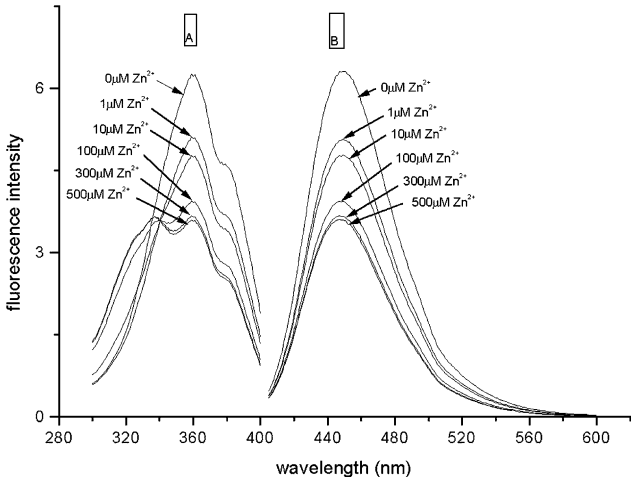
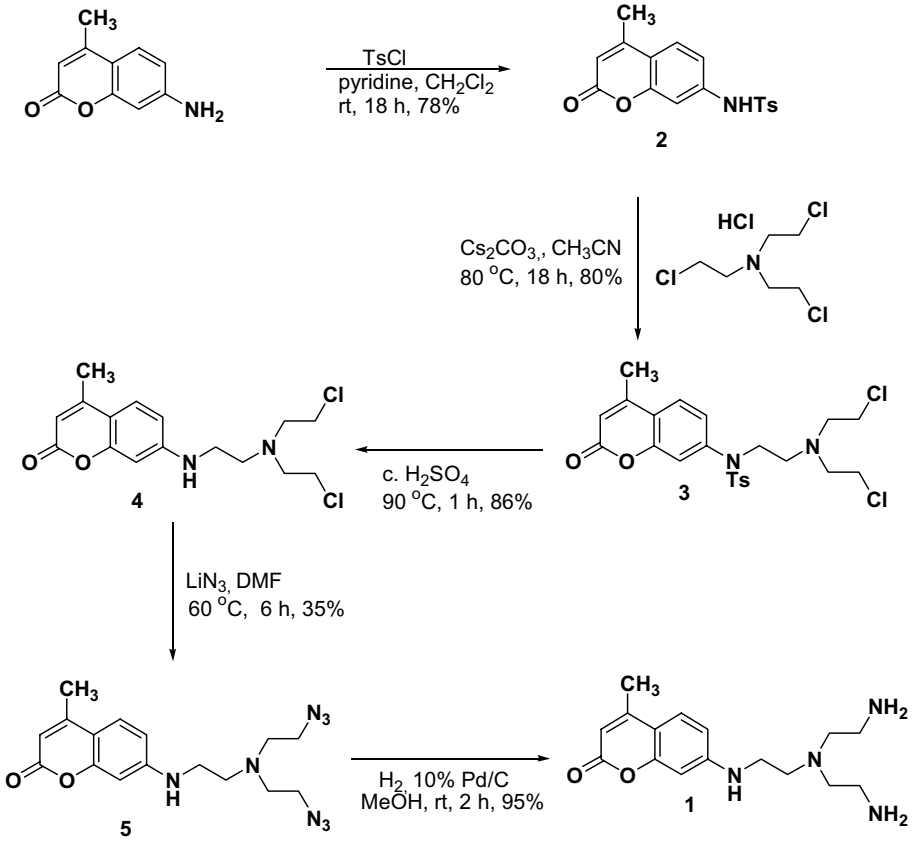


Figure 1. Excitation (A) and emission (B) spectra of 10 μM TREN-type probe **1** as a function of increasing Zn^{2+} concentration in 10 mM HEPES buffer, containing 140 mM NaCl and 2.5 mM KCl, adjusted to pH 7.8. Spectra were obtained at 22 °C in an Aminco Bowman Series 2 fluorimeter with excitation and emission slit widths set at 4 nm. The emission and excitation wavelengths were set at 450 and 359 nm, respectively.



Scheme 1.

an emission maximum at 450 nm, which does not shift even at saturation Zn^{2+} levels. However, a 42% decrease in fluorescence intensity was observed upon addition of 300 μM Zn^{2+}. When excited at 337 nm, the fluorescence spectra of the probe solutions exhibit an emission maximum at 450 nm and a small increase in fluorescence intensity as the concentration of Zn^{2+} increases. From the hypsochromic shift and the change in excitation intensity observed, it appears that compound **1** is indeed a PCT-type probe suitable for ratiometric measurements.

The dissociation constant value, calculated according to Tsien's algorithm for ratiometric dyes,[12] was in the micromolar region (K_d = 18 μM) and comparable to those reported for cyclen-type sensors ZnACF-1, ZnACF-2,[13] RF-2,[14] a di(2-picolyl)amine analog of 6,7-dimethoxy-4-methylcoumarin,[15] and a cyclen-coupled analog of 6,7-dimethoxy-4-methylcoumarin.[16] However, the probe has considerable lower affinity than anthracene- or dansyl-coupled polyazamacrocyclics.[4] Apparently, conjugation of the free electron pair of the 7-amino group with the coumarin system reduces its availability for coordination with Zn^{2+}, thus reducing its affinity to the ion as compared to a calculated K_d value of 1.9×10^{-10} M for TREN.[17] A tridansylated TREN as well as a tris(4-dimethylaminobenzyl)-TREN probe have been reported in the literature, but K_d values were not calculated in these studies.[18,19]

Given that aliphatic polyamine sensors are susceptible to protonation, we examined the spectral profile of the free and zinc-bound forms at pH 6.0–8.0. An 8% reduction of fluorescence was observed when the pH was increased from 6.0 to 7.0 in solutions of the zinc-free sensor. The corresponding decrease in fluorescence, when the pH value was further increased from 7.0 to 8.0, was 4%. No shift in either excitation or emission maxima was observed (data not shown).

In contrast to the profile exhibited by the free probe, the ion-bound form undergoes drastic changes upon the same increase in pH values. A total fluorescence decrease of 41% was observed and, at a pH range 6.0–7.0, the excitation spectrum is identical to that of the free probe (Fig. 2). However, increasing the pH from 7.0 to 8.0 results in a change of the excitation spectrum to that of the bound form. As suggested by the excitation spectra profile, zinc binding is pH dependent since the sensor is protonated in acidic to neutral media and Zn^{2+} is able to coordinate with the TREN system at pH values above 7.5. Similar arguments have been used to explain the fluorescence profile of the other two TREN-type zinc probes in solutions of decreasing acidity.[15,20]

The potential of this compound for use as a ratiometric dye in biological systems is shown in an ion competition study of a range of metals binding to the sensor. Samples were excited at $\lambda_{exc\,free}$ (359 nm) and $\lambda_{exc\,bound}$ (337 nm) and the fluorescence ratio was calculated, integrating the emission between 370 and 650 nm (Fig. 3). In the first set of measurements the increase of fluorescence ratio in the presence of zinc is shown (black bar), as compared to that of the free dye (gray bar). Such an increase in the fluorescence ratio is expected, since addition of zinc increases F_{337} while decreasing F_{359}. The rest of the measurement sets indicate the ratio of fluorescence of the dye in the presence of 1 equiv of metal (gray bars) versus the ratio of fluorescence after addition of 1 equiv of zinc to the sample (black bars). The results

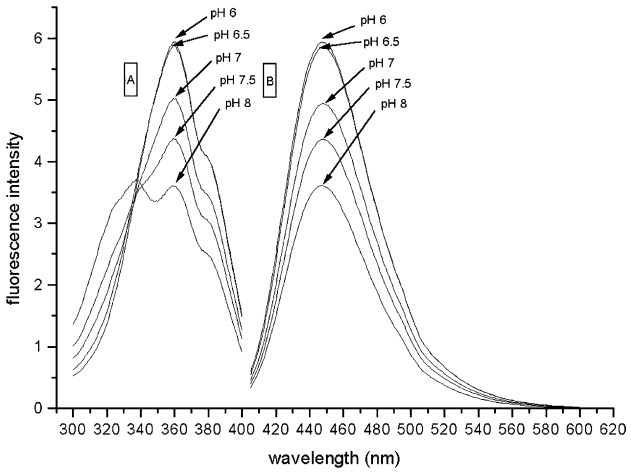


Figure 2. Excitation (A) and emission (B) spectra of 10 μM TREN-type probe **1** as a function of pH in 10 mM HEPES buffer, containing 140 mM NaCl, 2.5 mM KCl, and 300 μM Zn^{2+}. The pH was adjusted by addition of HCl solution. Spectra were obtained at 22 °C in an Aminco Bowman Series 2 fluorimeter with excitation and emission slit widths set at 4 nm. The emission and excitation wavelengths were set at 450 nm and 359, respectively.

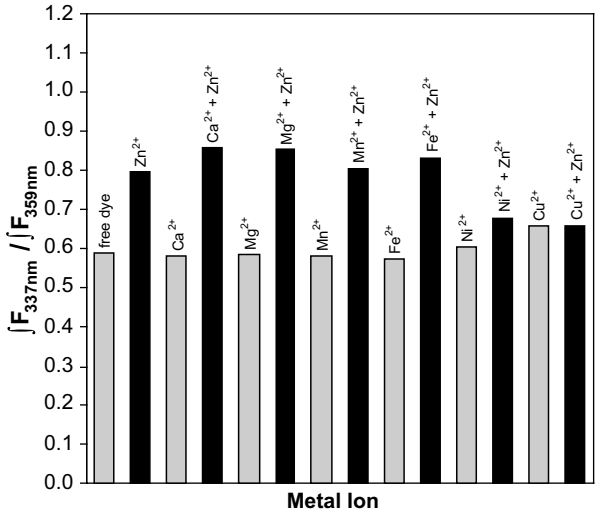


Figure 3. M^{n+}-selectivity profile of TREN-type sensor: Bars represent ratio integrated fluorescence response $\int F_{337\,nm}/\int F_{359\,nm}$, where 337 and 359 nm are the $\lambda_{exc\,max}$ of the zinc-bound and the free dye, respectively. Emission was integrated between 370 and 650 nm. Spectra were acquired in 10 mM HEPES buffer, containing 140 mM NaCl and 2.5 mM KCl, adjusted to pH 7.8 at 25 °C. Gray bars: Aliquots of concentrated stock solutions of each metal ion were added to the 10 μM dye solution to provide 10 μM total metal ion concentration, and the fluorescence response was calculated. Black bars: Zn^{2+} (10 μM) was added subsequently to the solution containing the metal ion, and the response was measured.

of this study are very similar to those published for zinc fluorescent sensor RF2.[14] Binding by these metal ions appears to be weak, since the presence of metals does not reduce the fluorescence ratio of the dye and subsequent addition of Zn^{2+} to these TREN-probe solutions enhances fluorescence. As expected, the latter effect is cancelled in the case of paramagnetic Ni^{2+} and Cu^{2+} ions due to their fluorescence quenching properties.

In conclusion, we have synthesized a new UV-excited fluorescent TREN-type Zn^{2+} indicator incorporating the coumarin chromophore, and studied the spectral profile of its free and Zn^{2+} bound forms. The compound exhibits a Zn^{2+} dissociation constant of 18.0 μM. The fluorescence spectra of the probe showed a clear shift in excitation wavelength maxima upon Zn^{2+} binding, indicating its potential use as a ratiometric zinc indicator.

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11. *Selected data*. Compound **1**: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.27 (d, J = 9 Hz, 1H, Ar), 6.53 (br d, J = 9.0 Hz, 1H, Ar), 6.43 (br s, 1H, Ar), 5.91 (br s, 1H, CH_3–C=C*H*), 3.18 (br t, J = 5.5 Hz, 2H, NHCH_2), 2.79 (t, J = 5.5 Hz, 4H, CH_2NH$_2$), 2.73 (t, J = 5.5 Hz, 4H, NCH_2), 2.58 (t, J = 5.5 Hz, 2H, CH_2N), 2.29 (s, 3H, CH_3). ESI-MS (positive ion mode): *m/z* 305 $[M+H]^+$; ESI-ion trap MS^n (positive ion mode): MS^2 *m/z* 305→288 $[M+H-NH_3]^+$, MS^3 *m/z* 288→271 [Product ion(288)$-NH_3]^+$, MS^4 *m/z* 271→214 [Product ion(271)$-2C_2H_4-H^{\cdot}]^+$, MS^5 *m/z* 214→186 [Product ion(214)$-CO]^+$, MS^6 *m/z* 186→158 [Product ion(186)$-C_2H_4]^+$.
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