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A Sensitive Probe for the Detection of Zn(II) by Time-Resolved Fluorescence

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Fluorescent probes for metal ions have found widespread use in biological studies.[1] For example, the fluorescent dyes TSQ and Zinquin have facilitated the examination of Zn^{2+} homeostasis by fluorescence microscopy.[2] Various new dyes have been developed more recently[3,4] and have allowed the study of important neurological functions of zinc.[5] Ratiometric agents are highly desirable because of the ability to visualize both ligand and metal concentration.[6,7] Wavelength-ratiometric agents display different fluorescence emission maxima of the ligand and its metal complex. The potential advantage of this approach is the ready visualization by color, although quantitation invariably requires digitization and processing of the data. We examine here the development of a novel and sensitive probe for the ratiometric detection of Zn(II) utilizing time-resolved fluorescence (TRF) techniques that provide invaluable information about the system when multiple fluorescence-emitting components contribute to steady-state fluorescence intensities. Using frequency domain techniques, the fluorescence lifetimes of chemosensors for metals,[8] including Zn(II) has been reported.[9] Here, using time domain TRF techniques, we demonstrate that a marked improvement over steady-state fluorescence methods for the ratiometric detection of Zn(II) can be achieved using a novel compound (**1**).

Compound **1** resembles tripodal N_4-ligands that we examined previously as chelation-enhanced fluorescent chemosensors for Zn^{2+}.[10] The ligand is structurally related to tris(2-pyridylmethyl)-amine (TPA), which forms a highly stable 1:1 complex with zinc.[11] One arm of compound **1** contains the elements of 8-hydroxyquinaldine (HQ), a well-known chromophore and an established analytical tool for zinc chelation.[12] HQ forms a 2:1[13] or 3:1[14] complex with most metal ions, but the highly chelating ligand **1** was designed to display 1:1 binding stoichiometry and strong sensitivity for Zn(II) similar to or better than TPA. A strong enhancement in the fluorescence quantum yield was obtained by inclusion of two dimethyl sulfonamide groups.[15] The mechanism of enhancement is complex:[16] HQ compounds are poorly fluorescent ($\Phi = 2\%$) in part due to photoinduced electron transfer (PET) involving the lone pair of the tertiary nitrogen[17] and in part due to excited-state proton transfer (ESPT) caused by the phenolic proton and the nitrogen of HQ.[18] Upon complexation of Zn^{2+}, the lone pair of the tertiary nitrogen is coordinated to the metal, thereby eliminating PET. In addition, metal complexation results in loss of the phenolic proton, eliminating ESPT.

Compound **1** shows excellent Zn^{2+} chelation-enhanced steady-state fluorescence properties. Figure 1 shows a titration of ligand **1** with Zn^{2+} and an X-ray structure of the complex with 1:1 stoichiometry. Fluorescence enhancement was observed upon addition of 1 equiv of zinc perchlorate to the HEPES-buffered aqueous solution of **1** ($\Phi = 24.4\%$ for [Zn(**1**)]). A series of metal solutions indicated subpicomolar sensitivity. The detection limit of **1** was from 10 fM to 1 pM (log $K_1 = 13.29$).[19]

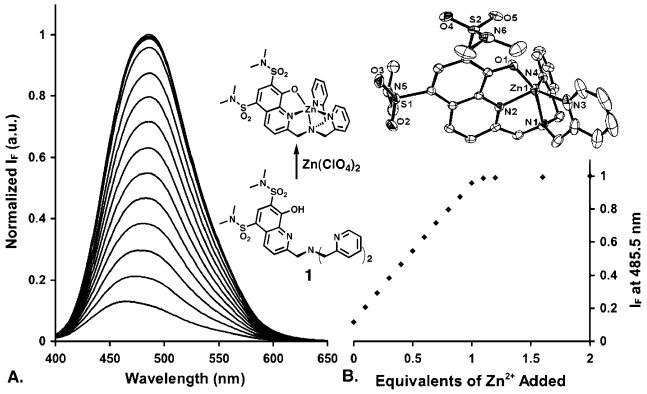

Figure 1. (A) Fluorescence enhancement of **1** as a function of Zn^{2+} concentration. Spectra were acquired in aqueous solutions (0.1 M KNO_3, 50 mM HEPES, pH 7.0, 25 °C). **1** (1 μM) was titrated with 0.1 μM aliquots of $Zn(ClO_4)_2$. (B) Fluorescence as a function of added Zn(II) monitored at 485 nm (molar ratio plot). Inset: ORTEP representation of [Zn(**1**)] showing 50% thermal ellipsoids and selected atom labels. Solvent molecules, hydrogen atoms, and perchlorates were omitted for clarity. Crystallographic data are listed in Table S3 of the Supporting Information.

Other biologically significant metal ions and first row transition metal ions (except Cu^{2+}) had little effect (Supporting Information) on the fluorescence of [Zn(**1**)].

Time-resolved fluorescence spectroscopy has emerged as a valuable tool for imaging cellular components.[20] For example, measurements of TRF decay profiles can differentiate between different populations of fluorescence-emitting fluorophores with different lifetimes, whereas steady-state fluorescence measurements merely reflect the unresolved contributions of each component to the overall fluorescence. Thus, lifetime-based measurements are rich in information and provide unique insights into the systems under investigation.[21] In a simple two-state system, the fluorescent probe has a lifetime τ_f in the free state and τ_{Zn} in a complex with a Zn^{2+} ion, and the fluorescence intensity as a function of time, $I(t)$ (following excitation with a brief light pulse with a width $\Delta t \ll \tau_f, \tau_{Zn}$), is:

$$I(t) = C[A_f \exp(-t/\tau_f) + A_{Zn} \exp(-t/\tau_{Zn})] \qquad (1)$$

where A_f and A_{Zn} represent the relative contributions of each component to $I(t = 0)$ at the observation wavelength. These fractions are proportional to $\epsilon_f c_f$ and $\epsilon_{Zn} c_{Zn}$, where c_f and c_{Zn} are the concentrations of free and Zn-bound molecules, and ϵ_f and ϵ_{Zn} are the respective molar extinction coefficients. Furthermore, $A_f + A_{Zn} = 1.0$, and C is a proportionality constant. If the emission spectra of the two components are similar (Figure 1), and as long as $\epsilon_f \approx \epsilon_{Zn}$, the ratio $R(Zn) = A_{Zn}/(A_f + A_{Zn})$ represents the fraction of probe molecules complexed with Zn^{2+} ions. Fitting eq 1 to the experimentally determined fluorescence decay curve yields values of the parameters A_{Zn}, A_f, τ_f, and τ_{Zn} from which $R(Zn)$ can be estimated. Steady-state fluorescence measurements yield values that

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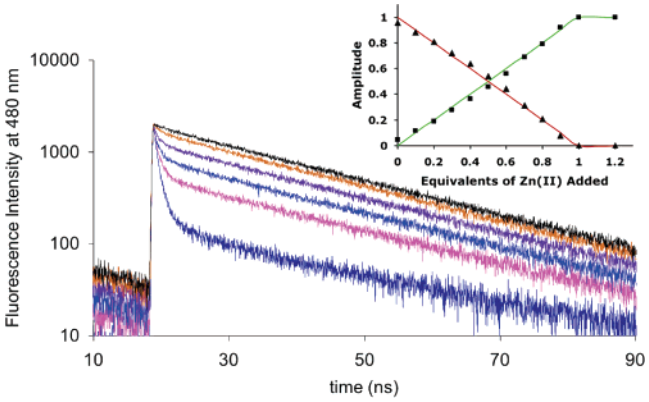

Figure 2. Exponential fluorescence decays as a function of Zn^{2+} concentration measured at 480 nm. Spectra were acquired in aqueous solutions (0.1 M KNO_3, 50 mM HEPES, pH 7.0, 25 °C). A 1 μM solution of **1** was titrated with 0.1 μM aliquots of $Zn(ClO_4)_2$. Spectra shown contain no Zn^{2+} (lowest, blue curve) and increasing amounts (bottom to top) of Zn^{2+} (0.2, 0.4, 0.6, 0.8, and 1 μM). Inset: A_f (▲) and A_{Zn} (■) as a function of Zn^{2+} concentration. The solid lines were calculated with the use of a standard quadratic equation describing the Zn + **1** Ω [Zn(**1**)] equilibrium with a binding constant $K > 10^9$ M^{-1} (Supporting Information).

are proportional to the sum of the mean contributions of the free and Zn-bound probe components to the overall fluorescence, $\langle I(t)\rangle = A_f\tau_f + A_{Zn}\tau_{Zn}$. The values of $\langle I(t)\rangle$ at different Zn^{2+} concentrations depend on the relative values of τ_f and τ_{Zn}, and the steady-state fluorescence measurements cannot ascertain whether the two-state model is applicable. On the other hand, the contributions of each component, $A_f\tau_f$ and $A_{Zn}\tau_{Zn}$, can be determined separately by TRF methods.

Several representative fluorescence decay profiles were obtained during titration of 10 μM of **1** with zinc perchlorate. These profiles were analyzed using the FluoFit software,[22] which enabled the extraction of the two lifetime decay constants. In the absence of Zn^{2+}, the $I(t)$ signal decays rapidly with a dominant component with τ_f (1) = 0.64 ns, A_f (1) = 0.96, and a minor second component with τ_f (2) = 24.9 ns, A_f (2) = 0.044. The contribution of a slower fluorescence component gradually increases as the concentration of Zn^{2+} is increased (Figure 2). Upon addition of a full equivalent of Zn^{2+}, only the slow component with a lifetime of τ_{Zn} = 22.5 ns is observed ($A_{Zn} \approx 1.0$, single-exponential decay).

The Figure 2 inset depicts what is essentially a calibration plot of A_f and A_{Zn} the fractional composition of the two components in the various solutions as a function of Zn^{2+} concentration. Excellent ratiometric behavior was observed, consistent with strong, stoichiometric binding. The lifetime constant of [Zn(**1**)] ($\tau_{Zn} \cong 22$ ns) is ~30 times longer than that of the free probe **1** ($\tau_f \cong 0.70$ ns). We note that the dynamic range of ~30 in Figure 2 is significantly higher than the dynamic range of ~10 in Figure 1 based on steady-state fluorescence spectral measurements at different Zn^{2+} concentrations. The latter, smaller ratio is due to aggregation of the probe in aqueous solutions (the short lifetime component observed in Figure 2 when $[Zn^{2+}] = 0$) that are discussed in more detail in Supporting Information. Such effects diminish the dynamic range and are not visible in the steady-state fluorimetric measurements but are clearly discernible in the fluorescence decay profile measurements.

Strong differences in fluorescence lifetimes were observed in live cells incubated in a solution of **1**. TRF measurements were conducted using A549 epithelial cells which were cultured to 85% confluence on a glass microscope slide. The cells were thoroughly stained with **1** upon an overnight incubation. Sizable intracellular Zn^{2+} concentrations were achieved by treatment with the zinc complex of (2-mercaptopyridine)-*N*-oxide.[23] The slide with attached cells was placed diagonally across a cuvette and exposed to laser irradiation in the absence of solvent. The TRF spectrum obtained by this method displayed the same elements present in Figure 2, namely a short component corresponding to an uncomplexed ligand and a long component corresponding to [Zn(**1**)].

In conclusion, the selectivity of **1** for Zn(II) and the time-resolved fluorescence data not only provide for the accurate detection of this ion, but also can discriminate between other fluorescent forms of the probe and its metal complex. In biological systems, TRF may be useful for identifying the effects of localization of Zn(II) in different environments.

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Supporting Information Available: Synthetic procedures and characterization of **1**. Crystallographic data and CIF file for [Zn(**1**)]. Effects of aggregation on TRF of **1** and live cell fluorescence data. This material is available free of charge via the Internet at http://pubs.acs.org.

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