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COMMUNICATIONS

Published on Web 08/15/2002

A Novel, Cell-Permeable, Fluorescent Probe for Ratiometric Imaging of Zinc Ion

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Received April 6, 2002

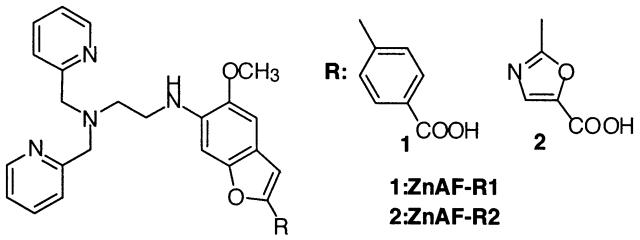
In all cells, zinc plays a critical role in the control of gene transcription and metalloenzyme function.[1] Chelatable zinc (Zn^{2+}) is present at particularly high concentration in the mammalian central nervous system,[2] and it is released in response to excitatory signals.[3] In addition to modulating neuronal transmission, Zn^{2+} is reported to contribute to neuronal injury in certain acute conditions,[4] to suppress or induce apoptosis,[5] and to induce the formation of β-amyloid,[6] which is reported to be related to the etiology of Alzheimer's disease.

Although Zn^{2+} has many important cellular roles, its physiological significance is little understood. Therefore, several chemical tools for measuring Zn^{2+} in biological samples have been developed. Fluorescent probes for Zn^{2+}, based on quinoline, which is excitable with UV light, or based on fluorescein, which is excitable with visible light, or based on other chemicals or proteins, have been reported.[7−15] Some of these probes can be used under physiological conditions, but they suffer from problems such as inadequate selectivity, insufficient sensitivity, dependence of fluorescence upon the dye concentration, and so forth.

We report here newly synthesized fluorescent probes for Zn^{2+}. These probes enable ratiometric imaging, which is a technique to reduce artifacts by minimizing the influence of extraneous factors on the fluorescence of a probe. Ratiometric measurement can provide precise data, and some probes allow quantitative detection.

We have already reported that ZnAFs,[16] which are based on fluorescein, are good candidates for Zn^{2+} probes. However, they signal Zn^{2+} by an increase of the fluorescence intensity without much shift of either excitation or emission wavelength. Unfortunately, fluorescence intensity is also dependent on many other factors such as illumination intensity, emission collection efficiency, environment around the probe, probe concentration, and effective cell thickness in the optical beam. It would be much better to have an indicator suitable for ratiometric measurement, that exhibits a large shift in its emission or excitation spectrum after it coordinates Zn^{2+}. The ratio of the fluorescences at two suitably chosen wavelengths then signals Zn^{2+}, while the influences of instrument efficiency, content of effective dye, and so forth, are largely or entirely canceled out.

To develop a Zn^{2+} probe for ratiometric measurement, we have employed the internal charge transfer (ICT) mechanism as a basis for probe design. When a fluorophore contains an electron-donating group (often an amino group) conjugated to an electron-withdrawing group, it undergoes ICT from the donor to the acceptor upon excitation by light. If the electron-rich terminal of the fluorophore (e.g., an amino group) interacts with a cation, a partial positive charge is photogenerated adjacent to the cation, and that affects the absorption or excitation spectral wavelength of the fluorophore with an ICT excited state. Therefore, a cation-induced blue shift is expected in the absorption or excitation spectra. Fluorescence emission spectra are less affected due to cation ejection during the excited state.[17] This approach is in wide use because of the success of low-molecular-weight sensors in physiological monitoring of H^+, Ca^{2+}, Na^+, and Mg^{2+}, for example, with the Ca^{2+} probe fura-2.[18] We therefore designed ZnAF-R1 and ZnAF-R2, utilizing benzofuran derivatives as the fluorophores and *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) derivative as the chelator of Zn^{2+}.



ZnAF-R1 and ZnAF-R2 were synthesized by conjugating *N*,*N*-bis(2-pyridylmethyl)ethylenediamine and 6-bromobenzofuran derivatives using palladium-catalyzed synthesis.[19] When these molecules formed complexes with Zn^{2+}, the wavelengths of the excitation maxima were blue-shifted, while the emission maxima remained essentially unchanged (Table 1). As ZnAF-R2 is more soluble and has a better fluorescence quantum yield than ZnAF-R1 in water, ZnAF-R2 should be more useful for biological applications. For ZnAF-R2, the quantum yield was decreased from 0.17 to 0.10 with saturating Zn^{2+}, but the wavelength of the excitation maximum shifted from 365 to 335 nm by complexation with Zn^{2+}. Thus, the ratio of fluorescece intensities (335 nm/365 nm) changed sufficiently, and it was possible to calculate the Zn^{2+} concentration by using the ratio of fluorescence intensities of ZnAF-R2. The apparent dissociation constant, K_d, was determined as 0.79 nM for ZnAF-R1 and 2.8 nM for ZnAF-R2 using Zn^{2+} and pH-buffered solutions (Table 1).[20] These values indicate that ZnAF-Rs can be used in the sub-nM range, which is the same as in the case of ZnAFs.[16] The detection limit of ZnAF-Rs was also in the sub-nM range, which affords sufficient sensitivity for application in mammalian cells.

Metal ion selectivity was also examined (Figure 1). ZnAF-R2 was not influenced by other cations, such as Ca^{2+}, Mg^{2+}, Na^+, and K^+, which exist at high concentration under physiological conditions, even at 5 mM. Thus, this molecule can be used even under biological conditions involving an increase of Ca^{2+} concentration. However, Cd^{2+} shifted the ZnAF-R2 spectrum in the same way as

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10.1021/ja026442n CCC: $22.00

Table 1. Chemical Properties of ZnAF-Rs with and without Zn^{2+} [a]

dye	apparent K_d for Zn^{2+} (nM)	absorption maxima (nm)		emission maxima (nm)		fluorescence quantum yield[b]	
		free base	Zn^{2+} complex	free base	Zn^{2+} complex	free base	Zn^{2+} complex
ZnAF-R1	0.79	359	329	532	528	0.088	0.031
ZnAF-R2	2.8	365	335	495	495	0.17	0.10

[a] All data were acquired in 100 mM HEPES buffer, pH 7.4. [b] Quantum yields were calculated using those of fluorescein (0.85) in 0.1 N NaOH as a standard.[21]

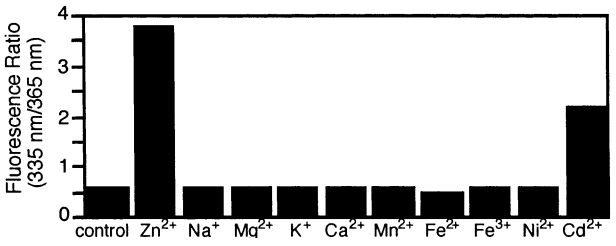

Figure 1. Selectivity of ZnAF-R2. All data were obtained at pH 7.4 (100 mM HEPES buffer, $I = 0.1$ ($NaNO_3$)) and are expressed as fluorescence ratio (335 nm/365 nm). Zn^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Cd^{2+} (5 μM) were added to 5 μM ZnAF-R2. Na^+, Mg^{2+}, K^+, Ca^{2+} (5 mM) were added to 5 μM ZnAF-R2.

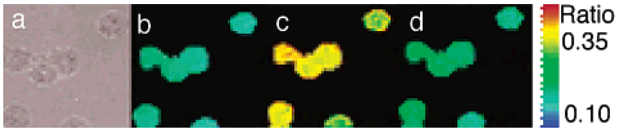

Figure 2. Fluorescence ratiometric images (340 nm/380 nm) of zinc in macrophages (RAW264.7) labeled with ZnAF-R2EE in PBS buffer, pH 7.4. (a) Bright-field transmission image. (b) Ratiometric image of (a). (c) 15 μM pyrithion (zinc ionophore) and 150 μM $ZnSO_4$ were added to (b). (d) 400 μM TPEN was added 15 min after the addition of pyrithione and Zn^{2+}.

Zn^{2+}. Cu^{2+} and Co^{2+} formed complexes with ZnAF-R2 and strongly quenched the fluorescence of this molecule, but these free cations would have little influence in vivo, since they exist at very low concentrations.[22]

To determine the cell permeability of ZnAF-R2, cultured macrophages (RAW 264.7) were incubated with phosphate-buffered saline (PBS) containing ZnAF-R2. The cells were not stained, indicating that ZnAF-R2 could not permeate through the cell membrane. Therefore, we prepared the ethyl ester derivative of ZnAF-R2 (ZnAF-R2 EE).[23] ZnAF-R2 EE is more lipophilic so that it should permeate into the cell where it will be transformed into ZnAF-R2 by esterase in the cytosol. Following incubation with PBS and 10 μM ZnAF-R2 EE at 37 °C for 1.0 h, RAW 264.7 cells were stained. The ratio of the fluorescences (340 nm/380 nm, using the imaging system for fura-2) was increased immediately by the addition of Zn^{2+} and 2-mercaptopyridine *N*-oxide (pyrithione), which is a zinc-selective ionophore, to the medium, and the increase was reversed by addition of TPEN. These data indicate that ZnAF-R2 is a good candidate for a sensitive, practically useful Zn^{2+} probe in biological applications (see Figure 2).

In conclusion, we have developed a new fluorescent probe for Zn^{2+} that is membrane-permeable and has a high sensitivity. Moreover, this molecule makes it possible to detect Zn^{2+} ratiometrically, thereby eliminating most or all of the possible variability due to differences in instrument efficiency and content of effective dye. Therefore, this molecule should be useful for studies on the biological functions of Zn^{2+}.

Acknowledgment. We thank Professor K. Yamaguchi and Dr. S. Sakamoto in Chiba University for HRMS measurement. We also thank Professor M. Iino in The University of Tokyo for donating RAW 264.7. This work was supported in part by the Ministry of Education, Science, Sports and Culture of Japan (Grants 11794026, 12470475, and 12557217 to T.N.; 13024217, 13558078, 1367232, and 14045210 to K.K.), by the Mitsubishi Foundation, and by the Research Foundation for Opt-Science and Technology.

Supporting Information Available: Synthesis, experimental details, and characterization of ZnAF-Rs (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Berg, J. M.; Shi, Y. *Science* **1996**, *271*, 1081−1085.
(2) Weiss, J. H.; Sensi, S. L.; Koh, J.-Y. *Trends Pharmacol. Sci.* **2000**, *21*, 395−401.
(3) Frederickson, C. J. *Int. Rev. Neurobiol.* **1989**, *31*, 145−238.
(4) (a) Koh, J.-Y.; Suh, S. W.; Gwag. B. J.; He, Y. Y.; Hsu, C. Y.; Choi, D. W. *Science* **1996**, *272*, 1013−1016. (b) Koh, J.-Y.; Choi, D. W. *Annu. Rev. Neurosci.* **1998**, *21*, 347−375.
(5) Truong-Tran, A. Q.; Ho, L. H.; Chai, F.; Zalewski, P. D. *J. Nutr.* **2000**, *130*, 1459S−1466S.
(6) Bush, A. I.; Pettingel, W. H.; Multhaup, G.; Paradis, M. D.; Vonsattel, J.-P.; Gusella, J. F.; Beyreuther, K.; Masters, C. L.; Tanzi, R. E. *Science* **1994**, *265*, 1464−1467.
(7) (a) Frederickson, C. J.; Kasarskis, E. J.; Ringo, D.; Frederickson, R. E. *J. Neurosci. Methods* **1987**, *20*, 91−103. (b) Savage, D. D.; Montano, C. Y.; Kasarskis, E. J. *Brain Res.* **1989**, *496*, 257−267.
(8) Zalewski, P. D.; Forbes, I. J.; Borlinghaus, R.; Betts, W. H.; Lincoln, S. F.; Ward, A. D. *Chem. Biol.* **1994**, *1*, 153−161.
(9) Budde, T.; Minta, A.; White, J. A.; Kay, A. R. *Neuroscience* **1997**, *79*, 347−358.
(10) Godwin, H. A.; Berg, J. M. *J. Am. Chem. Soc.* **1996**, *118*, 6514−6515.
(11) Walkup, G. K.; Imperiali, B. *J. Am. Chem. Soc.* **1997**, *119*, 3443−3450.
(12) Thompson, R. B.; Whetsell, W. O., Jr.; Maliwal, B. P.; Fierke, C. A.; Frederickson, C. J. *J. Neurosci. Methods* **2000**, *96*, 35−45.
(13) Kimber, M. C.; Mahadevan, I. B.; Lincoln, S. F.; Ward, A. D.; Tiekink, E. R. T. *J. Org. Chem.* **2000**, *65*, 8204−8209.
(14) Walkup, G. K.; Burdette, S. C.; Lippard, S. J.; Tsien, R. Y. *J. Am. Chem. Soc.* **2000**, *122*, 5644−5645.
(15) Pearce, D. A.; Jotterand, N.; Carrico, I. S.; Imperiali, B. *J. Am. Chem. Soc.* **2001**, *123*, 5160−5161.
(16) Hirano, T.; Kikuchi, K.; Urano, Y.; Higuchi, T.; Nagano, T. *J. Am. Chem. Soc.* **2000**, *122*, 12399−12400.
(17) Valeur, B.; Leray, I. *Coord. Chem. Rev.* **2000**, *205*, 3−40.
(18) Grynkiewicz, G.; Poenie, M.; Tsien, R. Y. *J. Biol. Chem.* **1985**, *260*, 3440−3450.
(19) See Supporting Information.
(20) Fahrni, C. J.; O'Halloran, T. V. *J. Am. Chem. Soc.* **1999**, *121*, 11448−11458.
(21) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 2nd ed; Kluwer Academic/Plenum: New York, 1999.
(22) Rae, T. D.; Schmidt, P. J.; Pufahl, R. A.; Culotta, V. C.; O'Halloran, T. V. *Science* **1999**, *284*, 805−808
(23) See Supporting Information.

JA026442N