

whereas for BSA, no apparent enhancement was detected. This is because SSB specifically binds to ssDNA with high affinity, while histone mainly binds to dsDNA. There has been no report suggesting any significant DNA binding to BSA. By intelligent molecular beacon design, this difference in fluorescence enhancement, which can be converted into a difference in bioassay sensitivity, might serve as the basis for the use of molecular beacons in specific and sensitive protein bioanalysis.

Our results demonstrate that there is a direct relationship between the restoration of molecular beacon fluorescence intensity and the DNA binding capability of the protein. Even though nonspecific DNA binding proteins are used here, the fact that different proteins do have different binding efficiencies with the molecular beacon in this first study is very encouraging. The molecular beacon used in this report lacks sequence specificity with regard to binding to particular proteins. Thus the next step is to develop MBs which can recognize certain proteins with high selectivity. There have been many recent studies aimed at designing functionalized single-stranded DNA or RNA molecules (aptamers) for selective protein binding studies and for enzymatic applications.^[18–20] Therefore, it is likely that aptamer-based molecular beacons can be developed for specific protein binding studies and for protein analysis with excellent selectivity.

In summary, we have opened a new way for studying protein–DNA interactions and for protein bioanalysis by using molecular beacons. The interaction between single-stranded DNA binding protein and molecular beacon results in significant restoration of the fluorescence of the molecular beacon. The fluorescence enhancement brought by SSB and by complementary DNA is very comparable. The molar ratio of the binding between SSB and MB is 1:1 with a SSB–MB binding constant of around $2.0 \times 10^7 \text{ M}^{-1}$. The detection limit of SSB is $2.0 \times 10^{-10} \text{ M}$. Preliminary results also show that there are significant differences in MB binding affinity by different proteins, which will constitute the basis for highly selective bioassay of a variety of proteins. The new approach is potentially useful for the study of protein–DNA/RNA interactions because of its high selectivity, speed, and convenience. The result also opens the possibility of using easily obtainable and designer DNA molecules for genomics and proteomics and for new drug development.

Received: June 21, 1999

Revised: December 10, 1999 [Z13592]

- [1] a) M. S. Wold, *Annu. Rev. Biochem.* **1997**, *66*, 61–92; b) A. A. Travers, *Annu. Rev. Biochem.* **1989**, *58*, 427–452; c) J. E. Cleaver, J. C. States, *Biochem. J.* **1997**, *328*(Part 1), 1–12; d) K. Ancelin, C. Brun, E. Gilson, *Bioassays* **1998**, *20*, 879–883.
- [2] M. Fried, D. M. Crothers, *Nucleic Acids Res.* **1981**, *9*, 6505–6525.
- [3] M. M. Garner, A. Revzin, *Nucleic Acids Res.* **1981**, *9*, 3047–3060.
- [4] T. M. Lohman, D. P. Mascotti, *Methods Enzymol.* **1992**, *212*, 424–458.
- [5] M. Argaman, R. Golan, N. H. Thomson, H. G. Hansma, *Nucleic Acids Res.* **1997**, *25*, 4379–4384.
- [6] S. Tyagi, F. R. Kramer, *Nat. Biotechnol.* **1996**, *14*, 303–308; b) S. Tyagi, D. Bratu, F. R. Kramer, *Nat. Biotechnol.* **1998**, *16*, 49–53.
- [7] a) G. Leone, H. Schijndel, B. Gemen, F. R. Kramer, C. D. Schoen, *Nucleic Acids Res.* **1998**, *26*, 2150–2155; a) R. Ehrlich, T. Kirner, T. Ellinger, *Nucleic Acids Res.* **1997**, *25*, 4697–4699.
- [8] D. L. Sokol, X. Zhang, P. Lu, A. M. Gewirtz, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 11 538–11 543.
- [9] B. A. Giesendorf, J. A. Vet, S. Tyagi, E. J. Mensink, F. J. Trijbels, H. J. Blom, *Clin. Chem.* **1998**, *44*, 482–486.
- [10] a) L. G. Kostrikis, S. Tyagi, M. M. Mhlanga, D. D. Ho, F. R. Kramer, *Science* **1998**, *279*, 1228–1229; b) S. R. Lewin, M. Vasanen, L. Kostrikis, A. Hurley, M. Duran, L. Zhang, D. D. Ho, M. Markowitz, *J. Virol.* **1999**, *73*, 6099–6103.
- [11] a) X. Fang, X. Liu, S. Schuster, W. Tan, *J. Am. Chem. Soc.* **1999**, *121*, 2921; b) W. Tan, X. Fang, J. Li, X. Liu, *Chem. Eur. J.* **2000**, *6*, in press.
- [12] R. E. Hurrington, I. Winicov, *Prog. Nucleic. Acid Res. Mol. Biol.* **1994**, *47*, 195–270.
- [13] T. M. Lohman, M. E. Ferrari, *Annu. Rev. Biochem.* **1994**, *63*, 527–570.
- [14] G. Krauss, H. Sindermann, U. Schomburg, G. Maass, *Biochemistry* **1981**, *20*, 5346–5352.
- [15] J. H. Weiner, L. L. Bertsch, A. Kornberg, *J. Biol. Chem.* **1975**, *250*, 1972–1980.
- [16] I. J. Molineux, A. Pauli, M. L. Gefter, *Nucleic Acids Res.* **1975**, 1821–1837.
- [17] E. S. Yeung, *Acc. Chem. Res.* **1994**, *27*, 409.
- [18] S. A. Benner, N. Trabesinger, D. Schreiber, *Adv. Enzyme Regul.* **1998**, *38*, 155–180.
- [19] S. E. Osborne, I. Matsumura, A. D. Ellington, *Curr. Opin. Chem. Biol.* **1997**, *1*, 5–9.
- [20] I. German, D. D. Buchannan, R. T. Kennedy, *Anal. Chem.* **1998**, *70*, 4540–4545.

Novel Zinc Fluorescent Probes Excitable with Visible Light for Biological Applications**

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Zinc is an essential component of many enzymes and transcription factors (for example, Zn^{II} in carbonic anhydrase and zinc finger proteins).^[1] Synaptic vesicles in excitatory nerve terminals contain high concentrations of chelatable Zn^{II} ,^[2] which is released by neuronal activity^[3] and may modulate the *N*-methyl-D-aspartate (NMDA) receptor.^[4]

Thus, Zn^{II} plays an important role in various biological systems. However, little is known about the cellular regulation of Zn^{II} in comparison with that of Ca^{II} . Chemical tools for measuring Zn^{II} in living cells (like fura-2 for Ca^{II}) are required. *N*-(6-Methoxy-8-quinolyl)-*P*-toluenesulfonamide (TSQ) was the first fluorescent probe to be developed for Zn^{II} : Its fluorescent intensity is increased by Zn^{II} , but not by Ca^{II} or Mg^{II} .^[5] TSQ is used for histochemical staining of Zn^{II} in tissue sections.^[6] Zalewski et al. developed a water-soluble TSQ derivative, Zinquin, which can, as an ethyl ester, be used to stain Zn^{II} in living cells.^[7] However, TSQ and derivatives are excited at UV wavelengths, which may cause cell damage, and is subject to interference by autofluorescence from pyridine nucleotides, among others.^[8]

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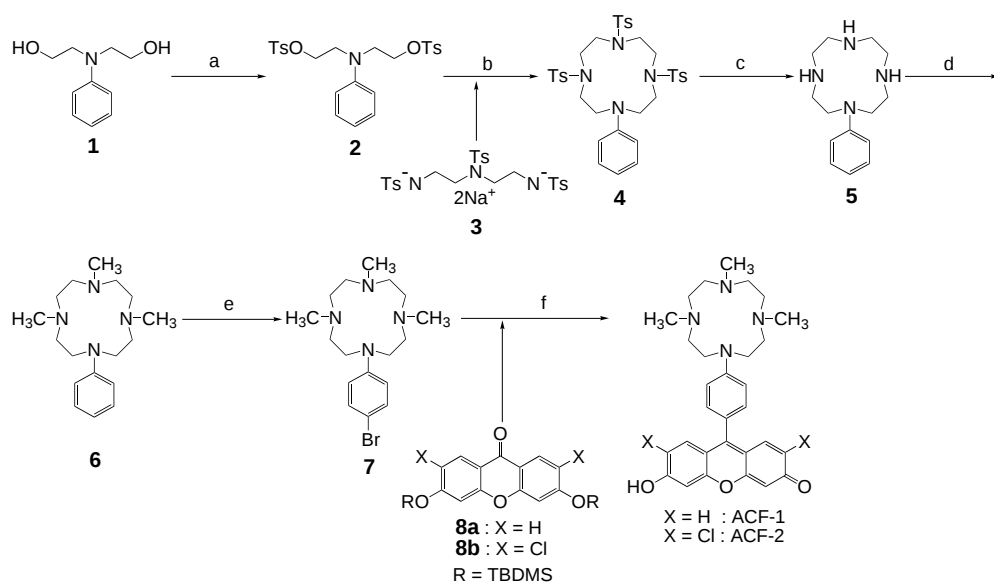
[**] We thank Prof. E. Kimura and Prof. T. Koike for advice on the chemistry of macrocyclic polyamines.

Fluorescein is one of the most widely used fluorophores in biology as, for example, a label in immunofluorescence studies. Fluorescein is advantageous in that it has a high quantum yield of fluorescence in aqueous solutions and its excitation wavelength is in the visible range. We have designed and synthesized two new probes for Zn^{II} , 6-hydroxy-9-[4-(4,7,10-trimethyl-1,4,7,10-tetraazacyclododecan-1-yl)]-phenyl-3*H*-xanthen-3-one (ACF-1; azacrownfluorone) and its 2,7-dichloride derivative (ACF-2), as shown in Scheme 1. The fluorophore of ACF-1 is a fluorescein derivative, 6-hydroxy-9-phenylfluorone. This fluorophore is directly linked to a macrocyclic polyamine, which strongly complexes with transition metals such as Zn^{II} and Cu^{II} .

When an amino group is attached to the phthalic ring of fluorescein, the fluorescence is quenched. This quenching is due to photoinduced electron transfer (PET), which is the intramolecular quenching mechanism of an amine group in fluorophore–amine conjugates.^[9] However, when the amino group is replaced by a weaker electron-donating group (e.g. an amido group), the molecule fluoresces because the PET is hindered. This hindrance is caused by a reduction of the HOMO energy compared to that of the amino moiety.^[10] On

the basis of this concept, our group has developed two types of fluorescent probes: diamino fluoresceins (DAFs) for nitric oxide (NO)^[11] and 9-[2-[3-(carboxy)-9,10-diphenyl]anthryl]-6-hydroxy-3*H*-xanthen-3-ones (DPAXs) for singlet dioxygen ($^1\text{O}_2$).^[12] When NO is treated with DAFs, the diamino group is transformed to a triazole, resulting in lowering the HOMO level and an increase in the fluorescent intensity. Similarly, when Zn^{II} binds to a macrocyclic polyamine, it too may hinder PET and enhance the fluorescence.

Excitation and emission spectra of ACF-1 ($5\text{ }\mu\text{M}$) at pH 7.5 (100 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer) in the presence of various concentrations of Zn^{II} are shown in Figure 1. Upon addition of Zn^{II} ($5\text{ }\mu\text{M}$), the fluorescent intensity of ACF-1 was increased 14-fold with no significant change in the position of excitation and emission maxima ($\lambda_{\text{ex}} = 495\text{ nm}$, $\lambda_{\text{em}} = 515\text{ nm}$). Under the same conditions, the fluorescent intensity of ACF-2 was



Scheme 1. The structure and synthesis of ACFs: a) TsCl , Py , 0°C , 91%; b) DMF , 100°C , 62%; c) Na , $n\text{BuOH}$, reflux, 67%; d) HCHO , NaBH_3CN , MeOH , rt, 26%; e) Br_2 , dioxane, 0°C , 90%; f) $t\text{BuLi}$, 2MeTHF , -150°C , (ACF-1 (22%) and ACF-2 (1.2%)). $\text{Ts} = \text{CH}_3\text{C}_6\text{H}_4\text{SO}_2$.

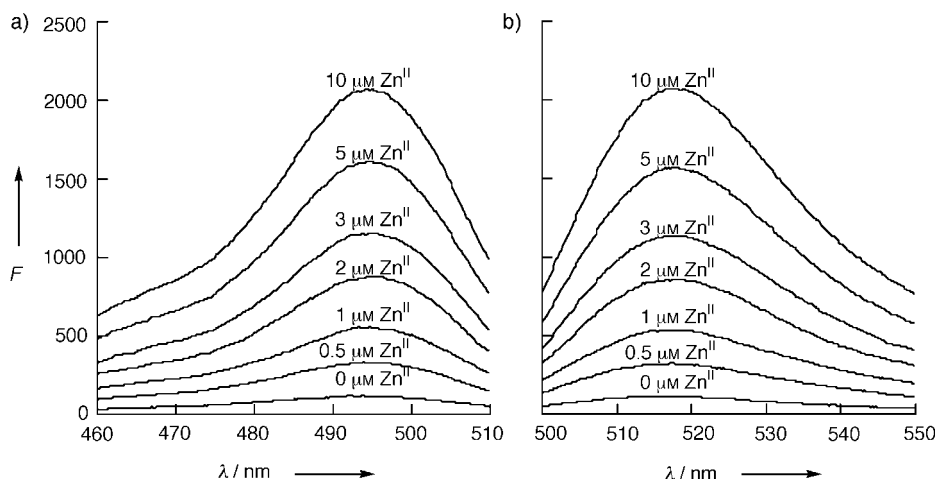


Figure 1. a) Excitation spectra (emission at $\lambda_{\text{em}} = 515\text{ nm}$) and b) emission spectra (excitation at $\lambda_{\text{ex}} = 495\text{ nm}$) of ACF-1 ($5\text{ }\mu\text{M}$) in the presence of Zn^{II} from 0–10 μM . These spectra were measured at pH 7.5 (100 mM HEPES buffer). F = Fluorescent intensity (arbitrary units).

increased 26-fold ($\lambda_{\text{ex}} = 505\text{ nm}$, $\lambda_{\text{em}} = 525\text{ nm}$). ACFs were more sensitive than the existing zinc fluorescent probe excitable with visible light, Newport Green,^[13] whose fluorescent intensity increased only 3.3-fold (Figure 2). The detection limit of the ACFs is 500 nM of Zn^{II} under these conditions, with a signal-to-noise ratio of 3.0, using $5\text{ }\mu\text{M}$ of ACFs. At pH 10.0 (100 mM 3-(cyclohexylamino) 1-propanesulfonic acid (CAPS) buffer), the fluorescent intensities of the ACFs increased linearly with Zn^{II} concentration up to 1:1 Zn^{II} :ACF mole ratio and remained at a plateau at increasing zinc levels (data not shown), indicating that ACFs and Zn^{II} form a 1:1 complex.

The fluorescent intensities of ACF-2 ($5\text{ }\mu\text{M}$) with various metal cations at pH 7.5 are shown in Table 1. The fluorescent intensities were not increased by addition of various cations found at high concentrations in cells, such as K^+ and Ca^{II} . These results were due to the poor complexation of alkaline

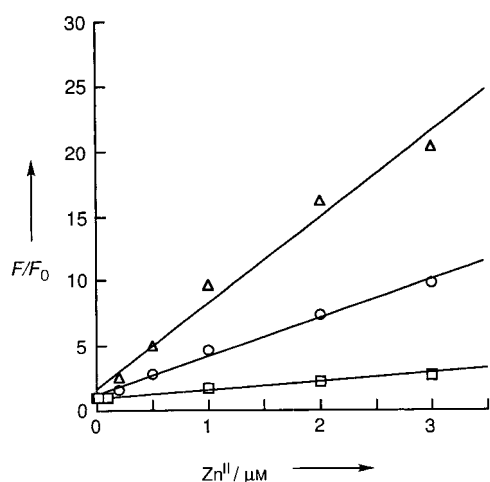


Figure 2. Fluorescent intensity of zinc fluorescent probes (5 μM) in the presence of various concentrations of Zn^{II}. The fluorescent intensities of ACF-2 (Δ: λ_{ex} = 505 nm; λ_{em} = 525 nm), ACF-1 (○: λ_{ex} = 495 nm; λ_{em} = 515 nm), and Newport Green (□: λ_{ex} = 505 nm; λ_{em} = 535 nm) were measured at pH 7.5 (100 mM HEPES buffer). *F* = Fluorescent intensity. *F*₀ = Fluorescent intensity of each fluorescent probe without metal.

Table 1. Relative fluorescent intensities of ACF-2 (5 μM) with various biologically important metal cations.

Entry	Metal cation	Relative intensity ^[a]
1	none	1.0
2	5 μM Zn ^{II}	24.3
3	5 mM Na ^I	1.2
4	5 mM K ^I	1.4
5	5 mM Ca ^{II}	1.0
6	5 mM Mg ^{II}	1.2
7	5 μM Fe ^{II}	1.1
8	5 μM Fe ^{III}	1.0
9	5 μM Cu ^{II}	0.3
10	5 μM Ni ^{II}	0.9
11	5 μM Co ^{II}	1.1
12	5 μM Mn ^{II}	1.1
13	5 μM Cd ^{II}	6.3
14	5 μM Zn ^{II} + 5 mM Na ^I	25.3
15	5 μM Zn ^{II} + 5 mM K ^I	25.4
16	5 μM Zn ^{II} + 5 mM Ca ^{II}	24.6
17	5 μM Zn ^{II} + 5 mM Mg ^{II}	23.1

[a] The fluorescent intensities (λ_{ex} = 505 nm, λ_{em} = 525 nm) were measured at pH 7.5 (100 mM HEPES buffer).

metals or alkaline earth metals with macrocyclic polyamines like the ACFs. Addition of transition metals such as Cu^{II} and Fe^{II} did not increase the fluorescent intensity. Although the binding constants of Cu^{II} to macrocyclic polyamines are generally larger than that of Zn^{II},^[14] the Cu^{II}–ACF complexes did not fluoresce possibly because of a quenching mechanism.^[15] Addition of Cd^{II} increased the fluorescence intensity slightly but its concentration in cells is much lower than that of Zn^{II}, so, in practice, Cd^{II} would not significantly affect the measurement of Zn^{II}. The fluorescence of ACF-2 (5 μM) and Zn^{II} (5 μM) was not affected by high concentrations (5 mM) of Na^I, K^I, Ca^{II} and Mg^{II}. Similar results were obtained with ACF-1.

Czarnik et al. developed fluorescent probes whose acceptors for Zn^{II} are also macrocyclic polyamines.^[16] At pH 10, the fluorescent intensity of their 9-(1,4,7,10-tetraazacyclododec-

yl)methylantracene increased 14-fold upon the addition of Zn^{II}. However, under neutral conditions the nitrogen atom in this molecule, which is linked with the anthracene moiety through a methylene bridge, is protonated and the fluorescent intensity increased without addition of Zn^{II}. This result was due to the elimination of the PET upon protonation.^[17] To avoid this, we bound the macrocyclic polyamine directly to the fluorophore in the design of our ACFs. The fluorophore–nitrogen atom binding results in lower p*K*_a values and consequently the fluorescent intensity of the ACFs at pH 7 was comparable to that at around pH 10. The ACF–Zn^{II} complex thereby fluoresces at a physiological pH.

In summary, our fluorescent probes for Zn^{II}, the ACFs, are excited by visible light, and can selectively detect Zn^{II} under physiological conditions. ACFs are expected to be a useful tool for measuring Zn^{II} concentrations in the living cells.

Received: October 5, 1999 [Z14112]

- a) B. L. Vallee, K. H. Falchuk, *Physiol. Rev.* **1993**, 73, 79–118; b) J. M. Berg, Y. Shi, *Science* **1996**, 271, 1081–1085.
- C. J. Frederickson, *Int. Rev. Neurobiol.* **1989**, 31, 145–238.
- a) S. Y. Assaf, S.-H. Chung, *Nature* **1984**, 308, 734–736; b) G. A. Howell, M. G. Welch, C. J. Frederickson, *Nature* **1984**, 308, 736–738.
- a) S. Peters, J. Koh, D. W. Choi, *Science* **1987**, 236, 589–593; b) G. L. Westbrook, M. L. Mayer, *Nature* **1987**, 328, 640–643.
- C. J. Frederickson, E. J. Kasarskis, D. Ringo, R. E. Frederickson, *J. Neurosci. Methods* **1987**, 20, 91–103.
- D. D. Savage, C. Y. Montano, E. J. Kasarskis, *Brain Res.* **1989**, 496, 257–267.
- a) P. D. Zalewski, I. J. Forbes, W. H. Betts, *Biochem. J.* **1993**, 296, 403–408; b) P. D. Zalewski, S. H. Millard, I. J. Forbes, O. Kapaniris, A. Slavotinek, W. H. Betts, A. D. Ward, S. F. Lincoln, I. Mahadevan, *J. Histochem. Cytochem.* **1994**, 42, 877–884; c) P. D. Zalewski, I. J. Forbes, R. Borlinghaus, W. H. Betts, S. F. Lincoln, A. D. Ward, *Chem. Biol.* **1994**, 1, 153–161.
- The problems of UV excitation are described in several books. For example, W. T. Mason, *Fluorescent and Luminescent Probes for Biological Activity*, 2nd ed., Academic Press, **1999**, pp. 108–113.
- A. P. de Silva, H. Q. N. Gunaratne, T. Gunnlaugsson, A. J. M. Huxley, C. P. McCoy, J. T. Rademacher, T. E. Rice, *Chem. Rev.* **1997**, 97, 1515–1566.
- C. Munkholm, D.-R. Parkinson, D. R. Walt, *J. Am. Chem. Soc.* **1990**, 112, 2608–2612.
- a) H. Kojima, N. Nakatsubo, K. Kikuchi, S. Kawahara, Y. Kirino, H. Nagoshi, Y. Hirata, T. Nagano, *Anal. Chem.* **1998**, 70, 2446–2453; b) H. Kojima, Y. Urano, K. Kikuchi, T. Higuchi, T. Nagano, *Angew. Chem.* **1999**, 111, 3419–3422; *Angew. Chem. Int. Ed.* **1999**, 38, 3209–3212.
- N. Umezawa, K. Tanaka, Y. Urano, K. Kikuchi, T. Higuchi, T. Nagano, *Angew. Chem.* **1999**, 111, 3076–3079; *Angew. Chem. Int. Ed.* **1999**, 38, 2899–2901.
- "Newport Green" was purchased from Molecular Probes, Inc., Eugene, OR, USA.
- R. D. Hancock, P. W. Wade, M. P. Ngwenya, A. S. de Sousa, K. V. Damu, *Inorg. Chem.* **1990**, 29, 1968–1974, and references therein.
- a) J. A. Kemlo, T. M. Shepherd, *Chem. Phys. Lett.* **1977**, 47, 158–162; b) G. D. Santis, L. Fabbri, M. Licchelli, C. Mangano, D. Sacchi, N. Sardone, *Inorg. Chim. Acta* **1997**, 257, 69–76.
- E. U. Akkaya, M. E. Huston, A. W. Czarnik, *J. Am. Chem. Soc.* **1990**, 112, 3590–3593.
- E. Kimura, T. Koike, *Chem. Soc. Rev.* **1998**, 27, 179–184.