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Review

Chemistry of zinc(II) fluorophore sensors

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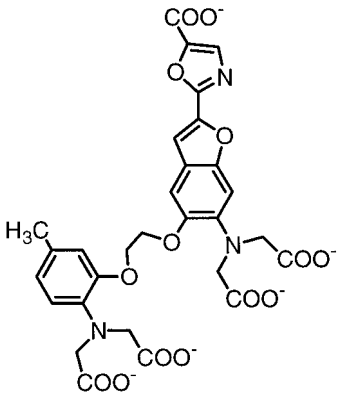
Abstract

The biological role of the zinc(II) ion has been recognized in DNA and RNA synthesis, apoptosis, gene expression, or protein structure and function. Therefore, development of useful zinc(II) sensors has recently been attracting much interest. Chemistry for selective and efficient detection of trace Zn^{2+} is a central issue. Recently, various types of zinc-fluorophores are emerging, comprising bio-inspired aromatic sulfonamide derivatives, zinc-finger peptides attached to fluorescent dyes, or fluorophore-pendant macrocyclic polyamines. The chemical principles, properties and limitations of these Zn^{2+}-fluorophores are discussed.

Introduction

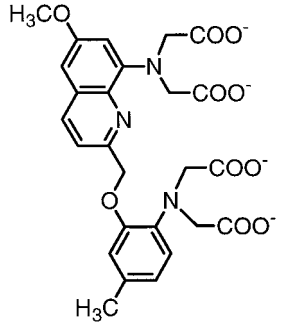
Qualitative and quantitative analyses of trace metal ions with selective analytical reagents have become extremely important for environmental and biological applications (Czarnik 1995). A remarkable development of fluorescent indicators has already been made for biologically important divalent metal ions, in particular Ca^{2+} and Mg^{2+}, with quite a few practical fluorophores such as Fura-2 (**1**), Quin-2 (**2**) and Mag-indo-1 (**3**) (Grynkiewicz *et al.* 1985; Tsien 1989; Tsien & Pozzan 1989; Haugland 1996).

The criteria for good sensors are (i) stability, (ii) metal selectivity, (iii) metal affinity, (iv) signal transduction, (v) fluorescent signaling, (vi) kinetically rapid sensitization, (vii) ease of delivery to target systems, and (viii) availability. For measurement of dynamic mechanisms of intracellular metal ions, the typical concentrations in resting cells should be known: for instance, $[Ca^{2+}] = 50$–200 nM. Therefore, for the metal affinity criteria, Ca^{2+}-selective biosensors should possess a K_d (dissociation constant) near the median concentration at physiological pH. When a normal median concentration gives a 50% sensing signal, sensors could most effectively detect both concentration increases and concentration decreases. Fura-2 (K_d = 145 nM) and Quin-2 (K_d = 60 nM), in this regard, are quite appropriate probes for measurement of intracellular Ca^{2+} concentrations (Haugland 1996). As for the desirable fluorescent signaling properties,



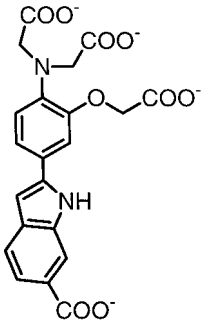
Fura-2
$K_d(Ca^{2+})$: 145 nM
Em: 512 nm (without Ca^{2+})
Em: 505 nm (with Ca^{2+})

Structure 1.



Quin-2
$K_d(Ca^{2+})$: 60 nM
Em: 495 nm (without Ca^{2+})
Em: 495 nm (with Ca^{2+})

Structure 2.



Mag-indo-1
$K_d(Mg^{2+})$: 2.7 mM
Em: 480 nm (without Mg^{2+})
Em: 417 nm (with Mg^{2+})

Structure 3.

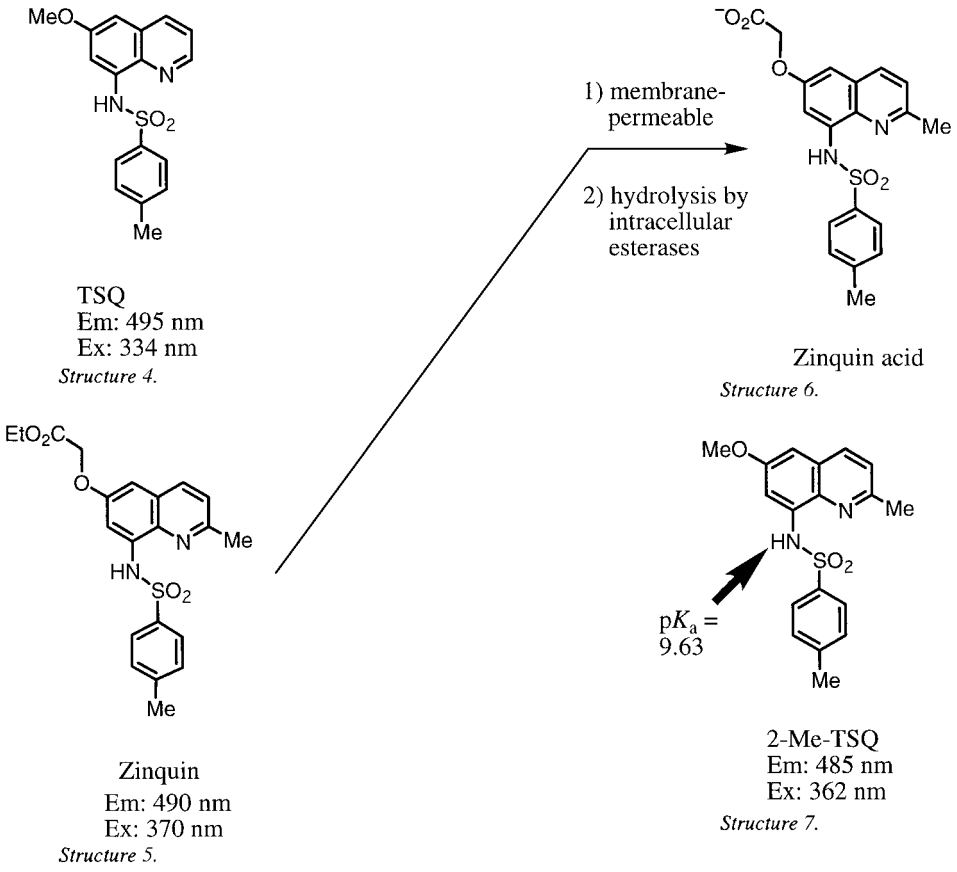
(a) intense fluorescence, (b) excitation wavelengths exceeding 340 nm (to pass through glass microscope objectives and minimize UV-inducing cell damage) corresponding to available laser sources, and (c) desirably, emission wavelengths to shift >80 nm before and after metal complexation, so that ratiometric titration can be utilized (for quantification) rather than mere intensity changes.

Development of classical zinc(II)-fluorophores

Zinc(II) is an essential metal ion in the active sites of more than 300 enzymes such as carbonic anhydrase, carboxypeptidase A, class II aldolase, β-lactamase, alkaline phosphatase, phosphotriesterase, and collagenase (Fraústo da Silva & Williams 1991; Kimura 1994; Kimura & Koike 1996; Lipscomb & Sträter 1996; Sträter *et al.* 1996). Moreover, the importance of zinc(II), which is critical for the growth and survival of cells, is becoming recognized in biology, physiology, and pathology (Vallee & Falchuk 1993; Lippard & Berg 1994); e.g., protein structure and function (Cox & McLendon 2000), DNA and RNA synthesis, gene expression (Greisman & Pabo 1997), transcription mediated by NO, apoptosis (Berendji *et al.* 1997), and brain metabolism or diseases (Frederickson *et al.* 1987; Cuajungco & Lees 1997; Choi & Koh 1998). The concentration of free Zn^{2+} within biological cells varies from about 1 nM in the cytoplasm of many cells to about 1 mM in some vesicles. The need for useful zinc-fluorophores to quantify trace Zn^{2+} in these biological mechanisms has become more urgent.

A zinc(II)-fluorophore 6-methoxy-8-*p*-toluenesulfonamido-quinoline (TSQ) **4** was first used as a histochemical stain for Zn^{2+} in various tissue sections of brain, heart, and some other tissues (Frederickson *et al.* 1987). While TSQ was the only available Zn^{2+}-specific fluorophore in the presence of much higher concentrations of Ca^{2+} and Mg^{2+}, the complex structures and stability constants of the TSQ-Zn^{2+} complexes were neither identified nor characterized. The complexation of TSQ with free Zn^{2+} probably occurs not only in a stoichiometry of 2:1 TSQ/Zn^{2+}, but also in a 1:1 complex that may equilibrate with protein-binding. The fluorescence intensity (i.e., quantum yield) of the complex(es) varies with the media. Accordingly, TSQ needed to be carefully studied for quantitative analysis of Zn^{2+}.

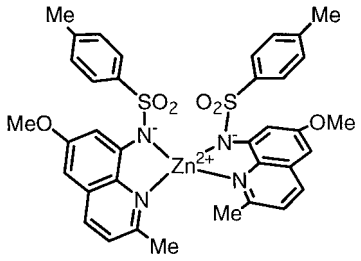
Zalewski's group developed Zinquin **5** and extensively used it for cellular physiological studies (Zalewski *et al.* 1993; Zalewski *et al.* 1994a, b). An ester group was incorporated at 6-position of **5**, so that after the neutral lipophilic probe **5** permeates into the cell, the ester would be hydrolyzed to a carboxylate anionic form **6** by intracellular esterases to stay within the cell. Thus, **5** became the first practical zinc-fluorophore to examine the role of Zn^{2+} in regulation of cell growth. Zinquin **5** could monitor loosely bound, labile intracellular Zn^{2+} (but not tightly bound Zn^{2+} in zinc-enzymes or zinc-finger proteins) by fluorescence video image analysis or fluorometric spectroscopy. For instance, the importance of cellular Zn^{2+} distribution in the process of apoptosis was first assayed by **5** (Zalewski *et al.* 1994a) in zinc-rich cells such as hepatocytes and pancreatic islet β-cells where the fluorescence was very intense



(Coyle *et al.* 1994; Zalewski *et al.* 1994b). Very weak fluorescence (at 490 nm) of a 2 μM solution of **5** at pH 7.4 was increased with subnanomolar free Zn^{2+} and was saturated at 1 μM Zn^{2+}. The fluorescence was enhanced 20-fold by 1 μM Zn^{2+}. Other biologically relevant metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Co^{2+}, etc.) did not affect the Zn^{2+}-dependent fluorescence of **5**, which empirically seemed to be a practical fluorophore for probing Zn^{2+} concentrations ranging 100 pM–10 nM. By fluorometric titration, **5** was shown to form 1:1 and the subsequent 2:1 complexes with Zn^{2+} with binding constants of 7.0×10^6 M^{-1} and 11.7×10^6 M^{-1} at pH 7.4 (Zalewski *et al.* 1993). However, the structures of these complexes were not referred to.

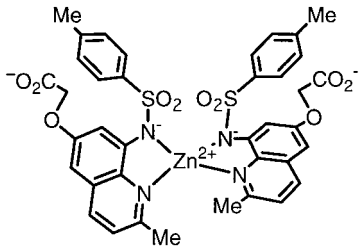
In addressing the basic chemistry of the TSQ fluorophores **4** and **5**, O'Halloran's group recently studied 2-methyl-6-methoxy-8-*p*-toluenesulfonamido-quinoline (2-Me-TSQ) (**7**) (Nasir, *et al.* 1999; Fahrni & O'Halloran 1999). The deprotonation constant of the sulfonamide in **7** was determined to be 9.63 in a 80:20 (v/v) mixture of DMSO/water (I = 0.1 ($KClO_4$)) at 25 °C by potentiometric pH titration. The formation constants, log β_1 of 8.43 ± 0.38 and log β_2 of 18.24 ± 0.24 for the 1:1 and 2:1 complexes of **7** with Zn^{2+} were established $\beta_1 = [(\mathbf{7}^-) - Zn^{2+}]/[\mathbf{7}]\,[Zn^{2+}]$ (M^{-1}) and $\beta_2 = [(\mathbf{7}^-)_2 - Zn^{2+}]/[\mathbf{7}]^2\,[Zn^{2+}]$ (M^{-2}). It was revealed that the 2:1 $\mathbf{7}^-$-Zn^{2+} tetrahedral complex (**8**) is the dominant species at neutral pH in DMSO/water, in which the deprotonated imide N^- and aromatic N atom of **7** coordinated to zinc(II), as confirmed by the X-ray crystal structure analysis. It was assumed that the adoption of the distorted tetrahedral geometry by the two methyl groups at 2-position of quinoline rings made **7** a Zn^{2+}-selective staining reagent in living cells.

Further, Zinquin **5** (ester form) and its carboxylic acid form **6** (Zinquin acid) have been more elaborately characterized (Hendrickson *et al.* 1997; Fahrni & O'Halloran 1999). Under physiological conditions (pH 7.2), the two forms of Zinquin **5** and **6** bind to Zn^{2+} to form 2:1 complexes with similar overall binding constants, e.g., log K_{app} of 13.5 for $(\mathbf{6}^-)_2$-Zn^{2+} complex (K_{app} =



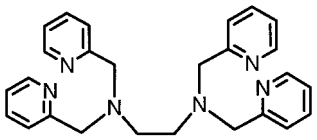
2:1 ($\mathbf{7}^-$)–Zn^{2+} complex
Em: 485 nm
Ex: 362 nm

Structure 8.



2:1 ($\mathbf{6}^-$)-Zn^{2+} complex
Em: 490 nm
Ex: 370 nm

Structure 9.



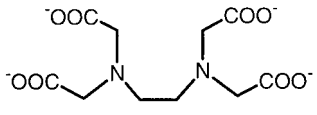
TPEN

Structure 10.

$[(\mathbf{6}^-)_2 - [(\mathbf{6})_2 - Zn^{2+}]/[\mathbf{6}]^2[Zn^{2+}]\ (M^{-2}))$ (Fahrni & O'Halloran 1999). In the presence of 50 μM Zinquin acid **6**, the lower detection of Zn^{2+} was ca. 4 pM and the fluorescence intensity reaches saturation above 100 nM Zn^{2+}. It was evident earlier, however, that the Zn^{2+}-Zinquin stability constants were apparently not large enough to permit interaction of Zinquin with extremely tightly bound ($K_d << 1$ nM) Zn^{2+} in metalloenzymes or zinc-finger proteins. A typical intracellular Zn^{2+} chelator *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylendiamine (TPEN) **10**, which apparently has a much higher affinity for Zn^{2+}, masked the Zn^{2+}-dependent fluorescence of Zinquin in lymphocyte cells (Zalewski *et al.* 1993). O'Halloran's group determined the affinity of various ligands to Zn^{2+} in terms of free $[Zn^{2+}]$ (M), referred to as -log$[Zn^{2+}]$ at [ligand] = 10 mM and [initial Zn^{2+}] = 1 mM at pH 7, 0.1 M ionic strength, and 25 °C: Zinquin acid (**6**) 9.3; TPEN (**10**) 16.0; EDTA (**11**) 14.3; carbonic anhydrase (CA) 12.4 (Fahrni & O'Halloran 1999). Thus, it was quantitatively confirmed that TPEN **10** and CA bind Zn^{2+} with much higher affinities and therefore Zinquins do not mobilize tightly bound Zn^{2+} from enzymes such as carbonic anhydrase (CA). It was suggested that once formed in the cell, the 2:1 complex **8**, like **9**, is not membrane-permeable, since these two fluorophores show similar staining pattern in the presence of Zn^{2+} in living cells (Nasir *et al.* 1999).

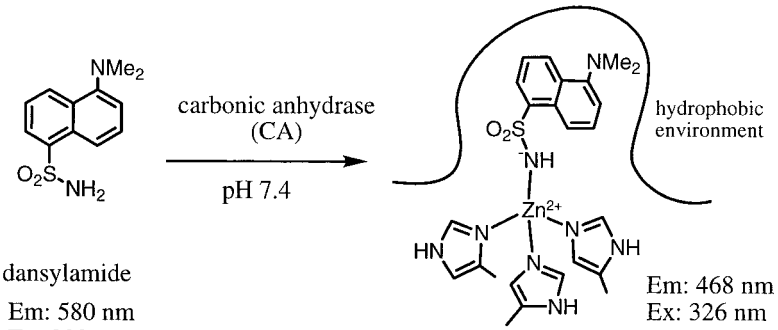
Development of zinc-fluorophores from the zinc-containing enzyme, carbonic anhydrase

Mann and Keilin first discovered that sulfonamides inhibit carbonic anhydrase (CA) (Mann & Keilin 1940). Chen and Kernohan presented evidence that bovine erythrocyte CA incorporates equimolar dansylamide **12** to form a highly fluorescent complex **13** with a dissociation constant K_d of 2.5×10^{-7} M at pH 7.4 (Chen & Kernohan 1967). For comparison, the K_d value for Zn^{2+} binding to apoCA is much smaller 4×10^{-12} M (Kiefer *et al.* 1993). The fluorescence of free **12** in water has an emission peak at 580 nm with a quantum yield of only 0.055, but the CA-bound dansylamide **13** dramatically shifted the emission maximum down to 468 nm with much higher quantum yield of 0.84 (excited at 326 nm). The large blue shift of emission was rationalized by the well-shielded and extremely hydrophobic dansyl binding site and in addition by the sulfonamide group losing a proton (to SO_2NH^-) upon binding to CA (Scheme 1). Thus, dansylamide **12** was thought to be a good fluorescent probe of CA or free Zn^{2+} in the presence of apoCA. The pK_a value of the SO_2NH_2 group in **12** was 9.8 either at ground state or excited state. Mere deprotonation of the free **12** (in the absence of CA) in alkaline solution shifted



EDTA

Structure 11.



dansylamide

Em: 580 nm
Ex: 320 nm

Structure 12.

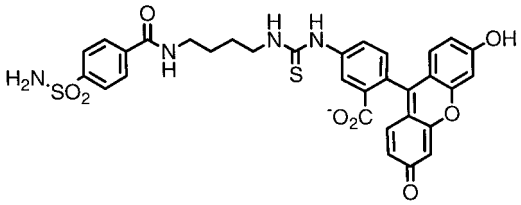
Structure 13.

Scheme 1.

the emission peak less dramatically from 580 nm to 540 nm with the quantum yield from 0.055 to 0.085.

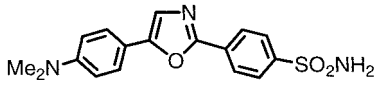
These chemical principles were adopted to a new CA-based fiber optic zinc-biosensor developed by Thompson's group (Thompson & Jones 1993; Thompson & Patchan 1995; Thompson & Maliwal 1998; Thompson *et al.* 1998). The concentration of Zn^{2+} is proportional to the ratio of fluorescence intensities (at 580 nm vs. at 480 nm) at 10–1000 nM (with 1 μM apoCA (metal-free CA) and 10 μM **12** in pH 7.4 HEPES buffer). An advantage with the CA-dansylamide system is that a great wavelength shift in fluorescence with or without Zn^{2+} in CA permits the ratiometric detection at two different wavelengths to be correlated with the analytical level. This linear range interestingly corresponds to the zinc(II) concentration range in the ocean. A fiber optic sensor constructed using this approach showed the zinc-detection limit 10-fold inferior (Thompson & Jones 1993). For practical application of this CA-based sensor to measure environmental Zn^{2+} (e.g., in sea water), a serious problem is reversibility. The off-rate of Zn^{2+} from CA is ca. 10^{-8} s^{-1}, which is too slow to permit taking continuous data. Another problem is fiber attenuation.

Further development from the CA inhibitors are sulfonamide-fluorescein conjugate **14** (Elbaum *et al.* 1996) and dapoxyl sulfonamide **15** (Thompson *et al.* 1999). The design of **14** was achieved by an iterative structure-based procedure in which the X-ray crystal structure of the CAII-arylsulfonamide complex was determined and analyzed for the optimal attachment point of fluorescein. The probe **14** bound tightly only to the Zn^{2+}-bound CAII ($K_d = 2.3$ nM) and exhibited fluorescence anisotropy that is proportional to the concentration of bound Zn^{2+} in the range of 10–100 nM. Dapoxyl sulfonamide **15** exhibited a large increase



λ_{em} = 520 nm (in CA)
λ_{ex} = 488 nm

Structure 14.



dapoxyl sulfonamide

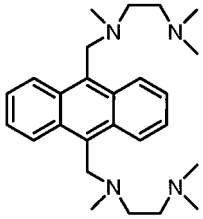
λ_{em} = 506 nm (in CA)
λ_{ex} = 466 nm

Structure 15.

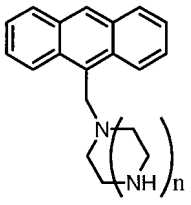
(90-fold) with blue shift (from 605 nm to 530 nm) in its fluorescence emission upon binding to holoCA (the dissociation constant is 0.3 μM). Ratiometric detection of Zn^{2+} was possible by measuring the emission intensity at 535 nm vs. 685 nm. The increase of emission seems to be due to the 20-fold increase in the fluorescence lifetime of **15** bound to Zn^{2+} in CA (τ changes from 0.22 ns to 3.80 ns upon binding to CA).

Zn^{2+} Chelation-enhanced fluorescence (CHEF)

The chelation-enhanced fluorescence (CHEF) was reported with anthracene derivatives having chelating moieties (e.g., 9,10-bis(2,5-dimethyl-2,5-diazahexyl) anthracene **16**) for Zn^{2+} in CH_3CN (Huston *et al.*



Structure 16.

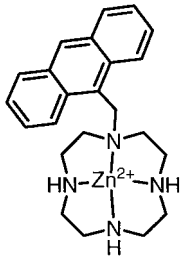


(n = 1 – 5)

Structure 17.

1988; Fabbrizzi 1997). It was extended to a macrocyclic system **17** (Akkaya *et al.* 1990; Huston *et al.* 1990; Czarnik 1992). A large CHEF effect by Zn^{2+} (14.4-fold) and Cd^{2+} (9-fold) was observed with **17** ($n = 2$) at pH 10 in aqueous solution. However, the protonation(s) and metal complexation at the macrocyclic polyamine moiety commonly inhibit the quenching process by the unprotonated benzyl nitrogen atom. The diprotonated ligand **17** ($n = 2$)$\cdot 2H^+$ at pH 7 (pK_a values for a monosubstituted cyclen are ~12, ~11, <2, and <2; Koike *et al.* 1996b; Kimura 1997) showed almost 120-fold larger fluorescence intensity than that of the unprotonated ligand **17** ($n = 2$) at pH 12. In fluorescence titration of **17** ($n = 2$) (10 μM) with Zn^{2+} (0–20 μM), impractically alkaline pH 12 buffer should be used, where **17** ($n = 2$) is unprotonated and hence competition between H^+ and Zn^{2+} for the macrocycle does not occur. Under these conditions, the emission maximum at 416 nm (excited at 335 nm) increases linearly till nearly 1:1 complexation (to **18**). Thus, **17** ($n = 2$) cannot be a practical zinc-fluorophore in aqueous solution under normal biological pH conditions.

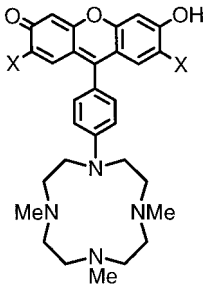
The drawback of **17** ($n = 2$) was the protonation of the macrocyclic polyamine part at neutral pH, which inhibits photoinduced electron transfer (PET) (Prasanna de Silva *et al.* 1997), resulting in the strong fluorescence emission even in the absence of Zn^{2+}. To remedy this monosubstituted macrocyclic tetraamine properties, Nagano's group devised ACF-1 (**19a**) and ACF-2 (**19b**), in which a fluorescein dye and three



Structure 18.

methyl groups are attached to four nitrogens of the macrocyclic tetraamine (Hirano *et al.* 2000). Fluorescein is advantageous in that it has a high quantum yield of fluorescence in aqueous solution and its excitation wavelength is in the harmless visible range. The tetraalkylations of the macrocyclic tetraamines in **19** lower the amine pK_a values (~8, ~6, <2, and <2) (Maumela *et al.* 1995), so that protonations would be less likely at neutral pH and Zn^{2+} would bind to **19** without competition against protons. As expected, the fluorescence of **19** (5 μM) was quenched at pH 7.5 due to PET. Upon addition of Zn^{2+} (0–5 μM), the fluorescence emission intensity of **19a** and **19b** linearly increased up to 14- and 26-fold, respectively, with little change in the position of excitation and emission maxima ($\lambda_{ex} = 495$ nm, $\lambda_{em} = 515$ nm for **19a** and $\lambda_{ex} = 505$ nm, $\lambda_{em} = 525$ nm for **19b**). The detection limit of **19** was noted as 500 nM of Zn^{2+} under these conditions. ACF-2 **19b** was selective only for Zn^{2+} and Cd^{2+} and the fluorescence of Zn^{2+} −**19** complexes was not interfered by other transition metal ions, Fe^{2+}, Fe^{3+}, Ni^{2+}, Co^{2+}, or Mn^{2+}. It is unfortunate, however, that the complexation constant with Zn^{2+} was not reported. Applicability to biological systems has yet to be determined.

For detection of trace Zn^{2+} in living cells, good permeability of the Zn^{2+}-sensors is crucial, e.g. Zinquin **5**. Recently, Lippard and Tsien *et al.* developed a membrane-permeable Zn^{2+}-fluorophore, Zinpyr-1 (**20**), which is a conjugate of 2′,7′-dichlorofluorescein with bis(2-pyridylmethyl)amine (DPA), a homologue to the membrane-permeable heavy metal chelator, TPEN (**10**) (Walkup *et al.* 2000). Zinpyr-1(**20**) (0.5 μM) has an excitation maximum at 515 nm in 50 mM PIPES (pH 7.0) with 100 mM KCl with a quantum yield of 0.39, and saturated with Zn^{2+} (25 μM), the resulting Zn^{2+}-complex showing λ_{ex} at 507 nm and a quantum yield of 0.89. The apparent dissociation constant, K_d, of the Zn^{2+}-**20** complex was determined fluorometrically to be 0.7 ± 0.1 nM

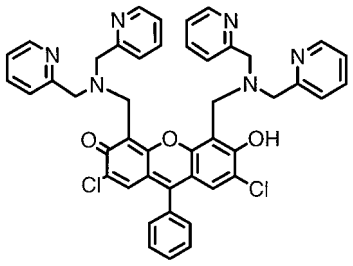


a: X = H **b**: X = Cl

ACF-1 ACF-2

λ_{em} = 515 nm λ_{em} = 525 nm
λ_{ex} = 495 nm λ_{ex} = 505 nm

Structure 19.



Zinpyr-1

λ_{em} = ca. 525 nm (with Zn^{2+})
λ_{ex} = 515 nm (without Zn^{2+})
λ_{ex} = 507 nm (with Zn^{2+})

Structure 20.

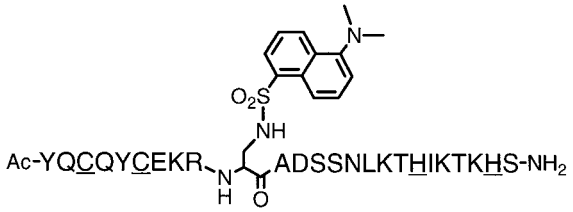
under the given conditions (emission was measured between 509 and 650 nm). The response of **20** to the concentration change of Zn^{2+} in COS-7 cells was demonstrated by influxed Zn^{2+} (exogeneous concentration of 50 μM) with the aid of a zinc ionophore 2-mercaptopyridine *N*-oxide (pyrithione, 20 μM). The enhanced fluorescence due to the complexation with Zn^{2+} was observed, which then was decreased by addition of TPEN **10** having a much higher affinity ($K_d = 10^{-13}$ M at pH 7). Preliminary results showed that Zinpyr-1 stains the Golgi and acidic cellular compartments.

Peptide fluorescent probes for Zn^{2+}

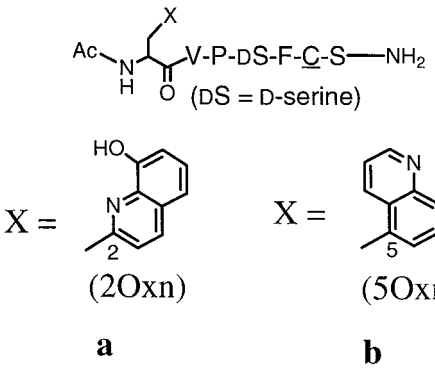
As a bio-inspired zinc-fluorophore, a peptide **21** (25 amino acids) containing a zinc-finger motif (a strong Zn^{2+}-binding site, Cys_2/His_2) covalently attached with a dansylamide residue, was synthesized (i) for selective and efficient Zn^{2+}-binding ($K_d = 1.4 \times 10^{-10}$ M at pH 7) and (ii) to create a hydrophobic environment around the encapsulated dansylamide residue upon its Zn^{2+} complexation (Walkup & Imperiali 1996). The addition of Zn^{2+} (0.1–1 μM) to the peptide **21** (1.4 μM) in pH 7 HEPES buffer resulted in a linearly increasing emission peak at 475 nm (excited at 333 nm) up to 2.4 fold (the emission maximum of the Zn^{2+}-**21** complex was 525 nm). In the absence of Zn^{2+}, the emission maximum was 560 nm. The dissociation constant, K_d, of the Zn^{2+}-**21** complex was $K_d = 1.4 \times 10^{-10}$ M at pH 7. The presence of 0.5 M Na^+, 50 mM Mg^{2+}, and 100 μM Co^{2+} did not interfere with the Zn^{2+} analysis. The covalently attached fluorescent reporter dansylamide is sensitive to metal-induced conformational changes of the supporting peptide framework and yet is remote from the Zn^{2+}-binding site. The enhanced emission is mostly due to the reporter to be placed in a hydrophobic environment. Problems with the zinc-finger peptide, in addition to its synthetic availability, would be vulnerabilities to air oxidation of cysteine residues and to redox active metal ions such as Cu^{2+}, despite the high affinity to Zn^{2+}. In application to the reductive environment of cells, this may not be problematic, but the digestion by proteases is another problem.

A more oxidatively robust peptidyl zinc-fluorophore was later synthesized by substitution of the peptide from Cys_2/His_2 to Cys/His_3 at the Zn^{2+}-binding site (Walkup & Imperiali 1997). However, the zinc affinity dropped by an order of magnitude ($K_d = 3 \times 10^{-9}$ M at pH 7) and the fluorescence response to Zn^{2+} became smaller (fluorescence enhancement was 1.3 fold upon addition of Zn^{2+}) for a sensitive zinc-fluorophore (the emission maxima are 552 and 548 nm in the absence and the presence of Zn^{2+}, respectively). Another modification of the zinc finger motif from Cys_2/His_2 to $Cys/Asp/His_2$ yielded a zinc sensor with enhanced oxidative stability, but with a further weakened affinity ($K_d = 6.5 \times 10^{-8}$ M at pH 7), although this one is responsive to submicromolar to micromolar concentrations of Zn^{2+} in the presence of redox active Cu^{2+} or Fe^{2+}.

In order to minimize the size of the peptidyl component for Zn^{2+} detection, the new heptapeptide **22**, in which unnatural amino acids having a chelating hydroxylquinoline (oxine) unit, were synthesized (Walkup & Imperiali 1998). The chiral amino acids



Structure 21.



Structure 22.

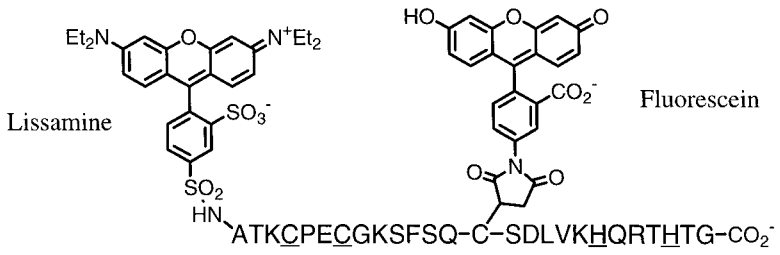
having an oxine unit, **22a** and **22b**, were prepared via diastereoselective alkylation of the pseudoephedrine glycinamide. Cysteine was incorporated for selective binding to Zn^{2+} ion. Although **22b** was found to form complicated metal complexes due to the formation of a disulfide dimer under normal conditions, **22a** showed 1:1 complexation with Zn^{2+}. By UV and fluorescence titrations of **22a** with Zn^{2+} in pH 7.0 buffer (50 mM HEPES with 150 mM NaCl), the dissociation constant, K_d, was determined to be 17 μM. The limit of detection was less than 250 nM.

Another type of a zinc finger consensus peptide **23** (Cys_2/His_2) was designed (Godwin & Berg 1996). The peptide was attached to two fluorescent dyes, fluorescein (F) as the energy donor and lissamine (L) as the acceptor, to visualize zinc binding. In the absence of Zn^{2+}, the peptide is unfolded and the dyes are relatively far apart (i.e., small intramolecular energy transfer occurs between F and L). Upon Zn^{2+} binding to the Cys_2/His_2 site, the peptide folds to bring the two fluorophores closer together, increasing the amount of intramolecular energy transfer. The binding of **23** (3.7 μM) to Zn^{2+} at pH 7.1 was monitored by increasing fluorescence (ca. 2.3-fold) at 596 nm (excitation at 430 nm) with an increase in $[Zn^{2+}]$, which provided the 1:1 and 1:2 Zn^{2+}-**23** complex formation (the estimated K_d for the 1:1 complex is 1×10^{-12} M at pH 7.1). Again, the complexation had to be carried out under a reductive atmosphere to avoid peptide oxidation.

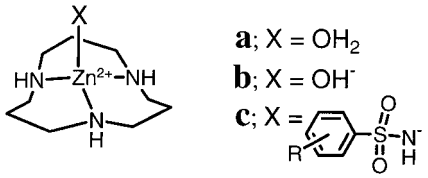
A bio-inspired macrocyclic fluorophore

While engaged in elucidating roles of Zn^{2+} in zinc enzymes (in particular carbonic anhydrase (CA)) by means of macrocyclic polyamine complexes (e.g., Zn^{2+}-1,5,9-triazacyclododecane (Zn^{2+}-[12]aneN_3) **24** and Zn^{2+}-1,4,7,10-tetraazacyclododecane (Zn^{2+}-cyclen) **25**, Kimura's group has discovered intrinsic acid properties of Zn^{2+} (Kimura 1992, 1994, 2001; Kimura *et al.* 1992, 1997a,b, 1999; Koike & Kimura 1991, 1998; Kimura & Shionoya 1994, 1996; Kimura & Kikuta 2000). One of the most outstanding properties of Zn^{2+} revealed was a strong affinity to aromatic sulfonamides, as illustrated by formation of coordination bonds between Zn^{2+} and deprotonated sulfonamide N^- anions at physiological pH. A new chemical model **24c** was presented to account for aromatic sulfonamide anions being good ligands for Zn^{2+} ion at the active center of CA to be strong inhibitors (Scheme 3) (Koike *et al.* 1992). The zinc enzyme models **24** and **25** form stable 1:1 complexes with deprotonated weak acids such as thymine derivatives (**25c**) (Shionoya *et al.* 1993, 1994; Kimura *et al.* 1998, 2000; Aoki *et al.* 1998a,b; Kikuta *et al.* 1999; Aoki & Kimura 2000) and barbital (**25d**) (Koike *et al.* 1996a; Fujioka *et al.* 1996; Aoki *et al.* 2000) in neutral aqueous solution, which results from the Zn^{2+}-bound OH^- species generated with pK_a values of 7.3 (for **24a** $\rightleftarrows$ **24b** + H^+) and 7.9 (for **25a** $\rightleftarrows$ **25b** + H^+) acting as bases to dissociate the acidic protons. The resulting conjugate bases strongly bind to Zn^{2+}, which compensate for the unfavorable deprotonations at neutral pH. It was further demonstrated that tosylamidopropyl[12]aneN_3 **26** yields a very stable four-coordinate, tetrahedral zinc(II) complex **27** under physiological pH (Scheme 2), where the aromatic sulfonamide N^- anion strongly binds to Zn^{2+} ion from the fourth coordination site (Koike *et al.* 1992).

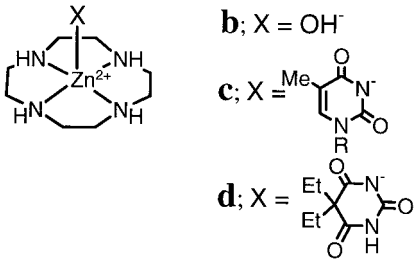
On the basis of these basic studies on the CA-model, a dansylamide-pendant macrocyclic tetraamine (dansylamidoethylcyclen) **28** was designed for a new type of selective and efficient zinc-fluorophore (Scheme 3) (Kimura 1997; Kimura & Koike 1998; Koike *et al.* 1996b). The 12-membered macrocyclic tetraamine, cyclen (L) forms a much more stable Zn^{2+} complex (K=[ZnL]/[Zn^{2+}][L] = $10^{15.3}$ M^{-1}) than [12]aneN_3 ($K = 10^{8.4}$ M^{-1}) in H_2O at 25 °C.



Structure 23.

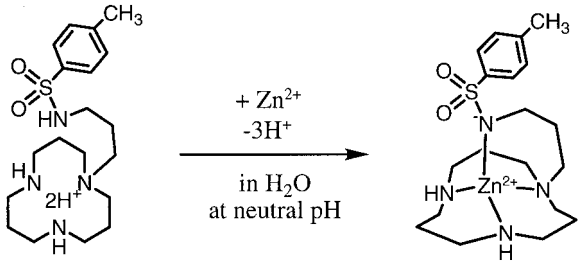


Structure 24.



Structure 25.

The complexation constant for **29** ($K = [Zn^{2+}\text{-}H_{-1}L]/[L \cdot 2H^+][Zn^{2+}]$) was established to be $10^{20.8}$ M^{-1} at 25 °C with $I = 0.10$ ($NaNO_3$) by potentiometric pH titrations. For comparison with other Zn^{2+}-fluorophores earlier described, the dissociation constants, K_d, of **29** are 1.4×10^{-10} M at pH 7.0 to 5.5×10^{-13} M at pH 7.8. The K_d values for **29** appreciably change because of competing protonation. As the pH is raised to 7.8, the K_d value for **29** becomes smaller than those for the zinc finger peptides (e.g., 5.7×10^{-12} M at pH 7.0), which do not so dramatically change at pH 7.8. Most remarkably, 1 μM of **28** (almost in $L \cdot 2H^+$ form) sequesters nearly 100% of Zn^{2+} (1 μM) in the form of stoichiometric Zn^{2+}-$H_{-1}L$ **29** at physiological pH. Such a strong and pH-dependent affinity to Zn^{2+} is one of the most characteristic properties of **28** (in comparison to Zinquin **5** and anthracene-pendant cyclen **17**($n = 2$)) and will be useful for quantifying trace amounts of free Zn^{2+} or bioligand-bound Zn^{2+} in environmental and biological systems.

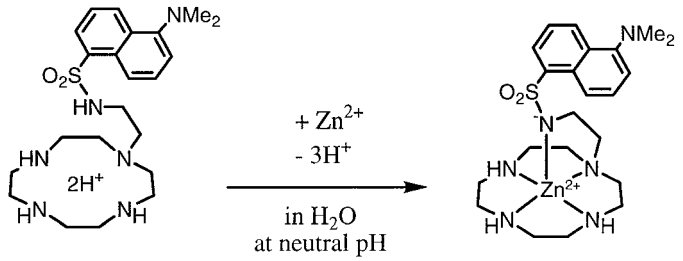


Structure 26. *Structure 27.*

Scheme 2.

The dansylamide deprotonation of **28** with Zn^{2+} at pH 7.8 increased the emission intensity by 4.9-fold at 540 nm and 10-fold at 490 nm, while the nonmetallated dansylamide deprotonation of L to $H_{-1}L$ without Zn^{2+} at high pH (>12) brought about only ca. 20% increase in the fluorescence emission intensity. To the contrary, the dansylamide deprotonation with Cu^{2+} completely quenched the fluorescence. The fluorescence maximum of H_2L (582 nm) at neutral pH blue-shifted upon zinc(II) complexation (Zn^{2+}-$H_{-1}L$) to 540 nm. A greater fluorescence blue shift (580 nm to 468 nm) and intensity enhancement (15.3-fold in quantum yield excited at 320 nm) were reported for the dansylamide complexation with CA, which were accounted for by the hydrophobic environment and the deprotonation of dansylamide on Zn^{2+} at the active center of CA.

The fluorescence changes of **28** (5 μM) with various metal ions (5 μM) at pH 7.3 (HEPES buffer) and 25 °C are summarized in Figure 1. The addition of various concentrations of Zn^{2+} (0–10 μM) resulted in increased emission upon excitation at 330 nm as shown in Figure 2. The response (at 528 nm) was linear between 0.1 and 5 μM until it reached a 1:1 [Zn^{2+}]/[**28**] ratio, and then became a plateau, indicating that the increase in fluorescence is due to the 1:1 Zn^{2+}-$H_{-1}L$ **29** formation, and moreover, Zn^{2+}-$H_{-1}L$ is stable even at subnanomolar concentrations. On the other hand, Cu^{2+} linearly diminished the fluorescence emission



dansylamidoethylcyclen
$L{\cdot}2H^+$
λ_{em} = 555 nm
Structure 28.

$Zn–H_{-1}L$
λ_{em} = 528 nm
Structure 29.

Scheme 3.

until complete quenching at $[\mathbf{28}]/[Cu^{2+}] = 1$, although Cu^{2+} forms the most stable five-coordinate complex at 25 °C. Other fluorescence-quenching metal ions (paramagnetic Co^{2+}) that tend to bind fairly strongly with cyclen also caused some quenching, although the effects were not so drastic as Cu^{2+}. Other metals such as Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, or Mg^{2+} gave negligible effect on the fluorescence of **29**. Preliminary experiments with **28** showed low toxicity against several cell lines and good cell-permeability. Further study of **28** and its homologues for both Zn^{2+} recognition and fluorescence signaling would find useful applications in Zn^{2+} biology.

Other types of zinc-fluorophores

Several other types of zinc-fluorophores have been reported. However, they are less practical than those earlier described. A tripodal ligand, tris[2-(5-dimethylamino-1-naphthalenesulphonamido)ethyl]-amine **30**, was synthesized (Prodi *et al.* 1999). A quantum yield of fluorescence emission of **30** enhanced with a blue shift upon addition of Zn^{2+} and Cd^{2+} in CH_3CN/H_2O (1:1 (v/v)) at relatively high pH 9.5 required to remove all of the sulfonamide protons (excitation at 340 nm), while Cu^{2+} and Co^{2+} quenched the emission. For comparison, an emission of the monomeric control compound **31** increased by Zn^{2+}, but the complexation was prevented by formation of metal hydroxide at the pH employed. Addition of Na^+, K^+, Ca^{2+}, Sr^{2+}, Ba^{2+}, Eu^{3+}, Ni^{2+}, Mn^{2+}, Pb^{2+}, Fe^{2+}, Fe^{2+}, and Cr^{2+} up to 50 mM caused negligible change of fluorescence spectra of **30** and **31**.

Recently, a lanthanide(III) complex **32** attached with a chelator for Zn^{2+} has been reported (Reany *et al.* 2000). While addition of Zn^{2+} induced negligible UV absorption spectral change of the Eu^{3+} complex **32a**, a small blue shift was observed in absorption of the Tb^{3+} complex **32b** from 255 nm to 250 nm upon complexation with Zn^{2+}. Fluorescence emission of **32a** and **32b** increased by 26% and 42%, respectively, upon complexation with Zn^{2+} (excitation at 262 nm), possibly due to inhibition of PET (from the benzylic nitrogen to the intermediate aryl singlet excited state). In a fluorescence emission spectra of **32b**, two bands were observed at 440 nm and 365 nm. By complexation with Zn^{2+}, the former band shifted from 442 nm to 430 nm with decrease of intensity and the latter band increased without a shift. Affinity constants, log β, of **32a** with Zn^{2+} determined by UV and fluorescence titrations were 5.4–6.0 and those of **32b** with Zn^{2+} are 5.5–6.4. Small changes of UV and emission spectra of **32a** and **32b** by addition of Ca^{2+} and Mg^{2+} were observed, from which log β values for Ca^{2+}-**32a**, Ca^{2+}-**32b**, Mg^{2+}-**32a**, and Mg^{2+}-**32b** were determined to be 3.9–4.0, 3.8–4.0, 1.9–2.1, and 2.0–2.5, respectively, suggesting the possible detection of these metal ions at submillimolar to millimolar order concentration. However, it is seen that the biological application for Zn^{2+} detection is limited.

Concluding remarks

As reviewed here, several useful Zn^{2+}-selective fluorescent probes are now developed. Some sensors are chelators with fluorescent dyes such as fluores-

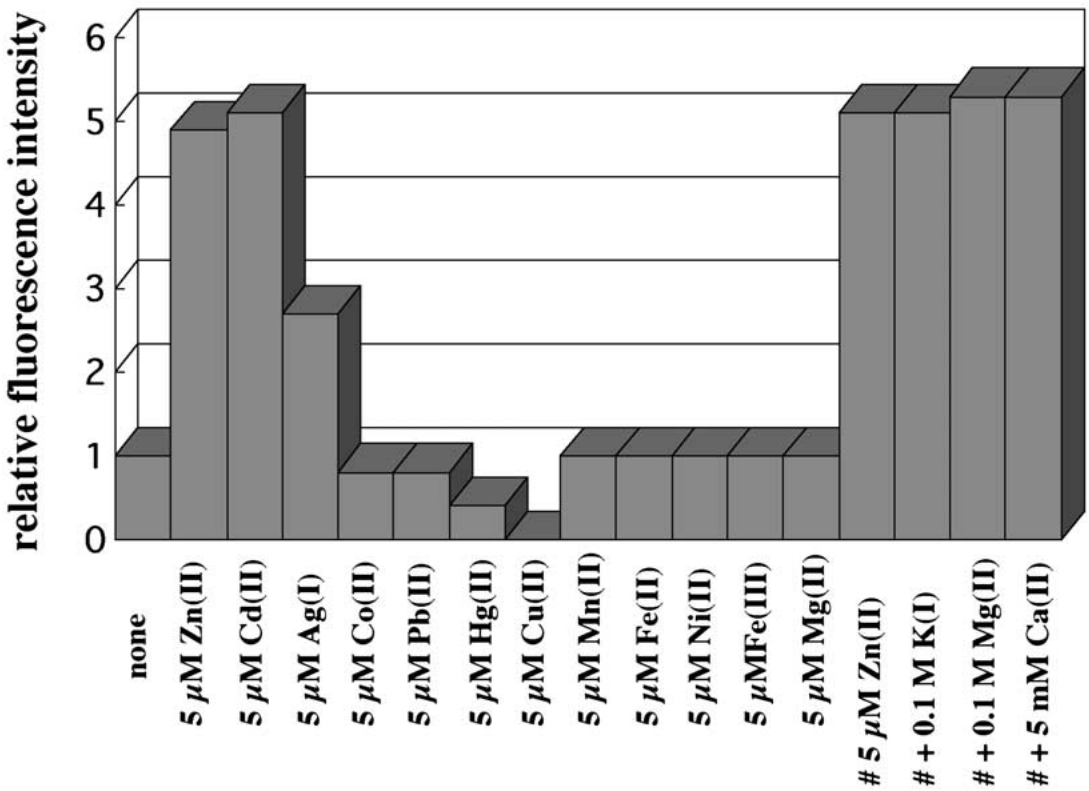


Fig. 1. Relative fluorescence intensity of **28** (5 μM) responding to 1 eq of various metal ions at pH 7.3 (1 mM HEPES with $I = 0.1$ ($NaNO_3$)) and 25 °C. The data marked with # were obtained without the supporting electrolyte.

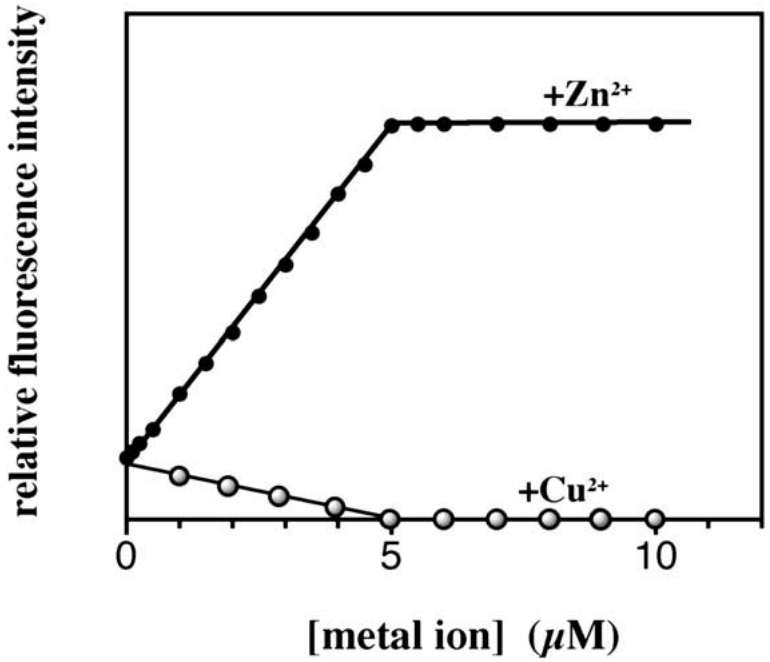
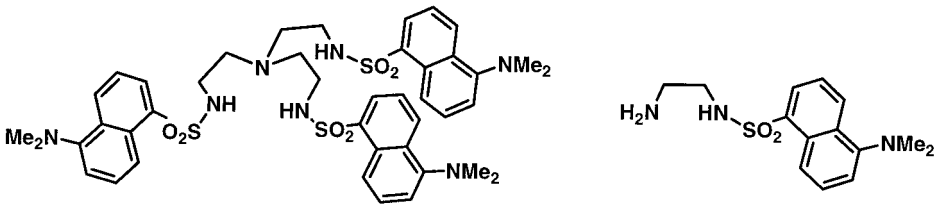


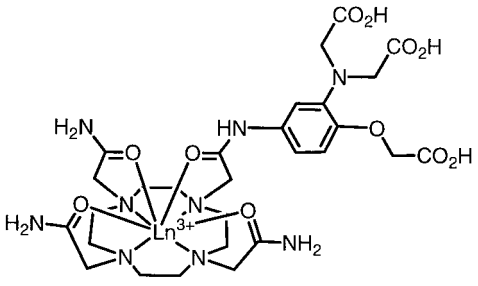
Fig. 2. Fluorescence titration curves of **28** (5 μM) with Zn^{2+} and Cu^{2+} at pH 7.3 (1 mM HEPES with $I = 0.1$ ($NaNO_3$)) and 25 °C.



Structure 31.

λ_{em} = 540 nm
λ_{ex} = 334 nm

Structure 30.



a : Ln = Eu
b : Ln = Tb λ_{em} = 440, 345 nm

Structure 32.

cein, whose microenvironments yields stronger fluorescence upon Zn^{2+}-complexation. Other sensors using dansylamide strongly fluoresce by the deprotonated sulfonamide binding to Zn^{2+} at neutral pH. Chemically ideal Zn^{2+} fluorophores will require the following criteria; (i) The ligands should be easy to make and chemically and biologically robust; (ii) the affinity to Zn^{2+} should be sufficiently high, capable of complexing with trace free Zn^{2+} or protein (e.g., zinc finger)–bound at physiological pH; (iii) the Zn^{2+} selectivity (either by Zn^{2+}-selective chelation or Zn^{2+}-selective fluorescence) should be high and the perturbation by other metal ions should be minimal (e.g., Hg^{2+}, Pb^{2+}). Further, for biological applications, one has to pay attention to (i) kinetic aspects (e.g., how fast is the Zn^{2+} chelation?) , (ii) permeability into cells and how long it stays before diffusion to be responsive to intracellular Zn^{2+}, (iii) excitation of probes with harmless irradiation wavelengths, and (iv) improvement in the fluorescence efficiency. It is evident that we still need to search for new fluorophores and study more biologically suitable Zn^{2+}-fluorophores as an extension of the present basic chemical knowledge.

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