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Ratiometric and Near-Infrared Molecular Probes for the Detection and Imaging of Zinc Ions

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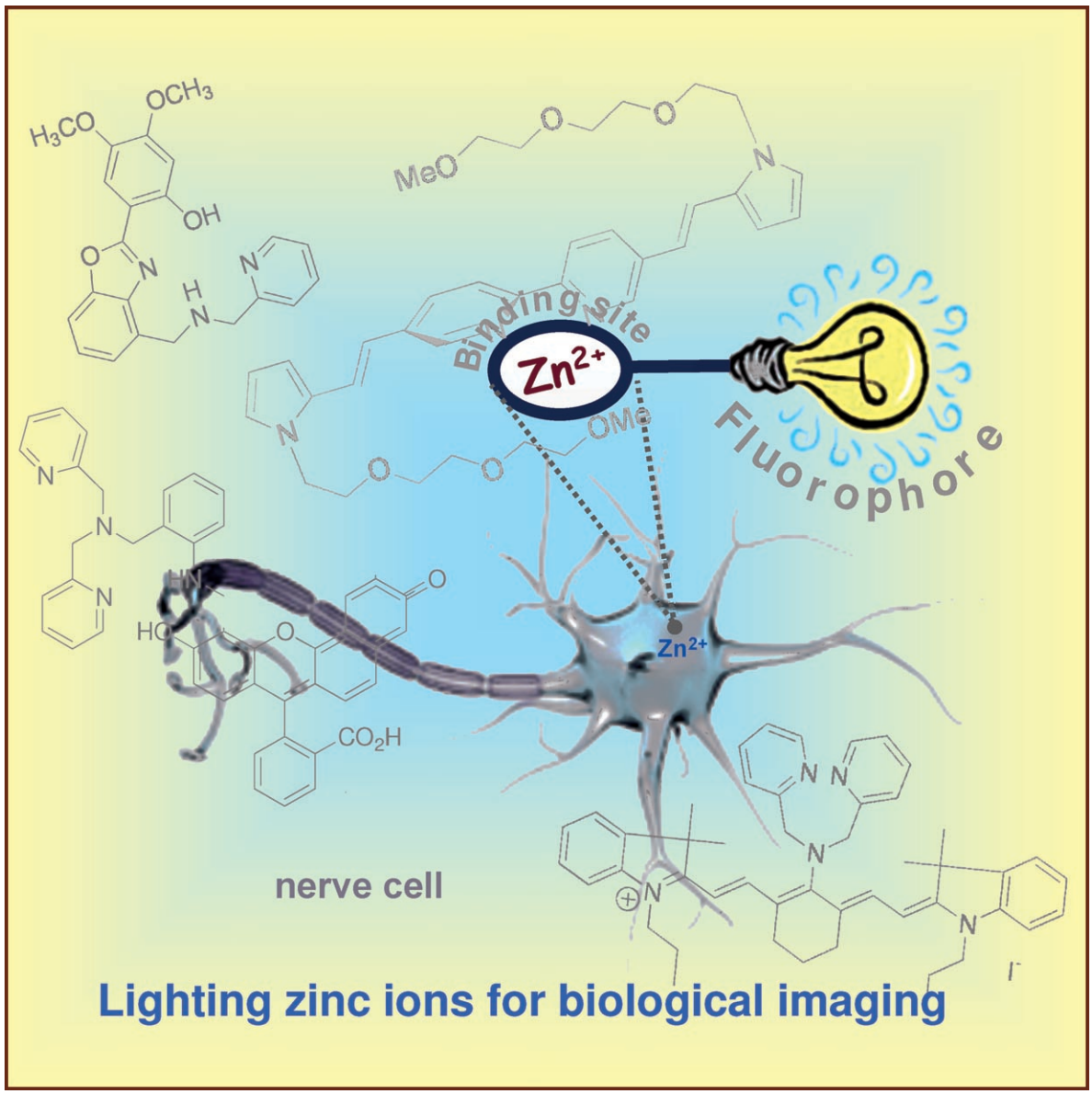

Abstract: The detection and imaging of Zn^{2+} in biological samples are of paramount interest owing to the role of this cation in physiological functions. This is possible only with molecular probes that specifically bind to Zn^{2+} and result in changes in emission properties. A "turn-on" emission or shift in the emission color upon binding to Zn^{2+} should be ideal for in vivo imaging. In this context, ratiometric and near-IR probes are of particular interest. Therefore, in the area of chemosensors or molecular probes, the design of fluorophores that allow ratiometric sensing or imaging in the near-IR region is attracting the attention of chemists. The purpose of this Focus Review is to highlight recent developments in this area and stress the importance of further research for future applications.

Keywords: chemosensors · fluorescent probes · molecular probes · ratiometric sensors · zinc

1. Introduction

Zinc is the second-most-abundant transition-metal ion in the human body, where it has multiple roles in both intra- and extracellular functions.[1–3] A large number of proteins and enzymes have been identified to contain Zn^{2+}. Zinc is reported to be responsible for neurological disorders such as Alzheimer's disease, amyotropic lateral sclerosis (ALS), Parkinson's disease, and epilepsy.[4] Furthermore, zinc plays a crucial role in insulin secretion and apoptosis. The World Health Organization estimates that more than 40% of the children in Africa and Asia have stunted growth associated with limited dietary zinc.[5] The extent to which conditions of zinc deficiency persist today is difficult to determine because of the lack of suitable biochemical markers for zinc.

Besides growth, numerous body functions are affected by zinc ions, including the immune, endocrine, and gastroenterological systems. The huge scope for the exploration of the diverse physiological roles of biological zinc demands sensitive and noninvasive techniques for real-time detection and imaging. The relative concentration of free Zn^{2+} within biological cells varies from 1 nM in the cytoplasm of many cells to 1 mM in the vesicles of presynaptic neurons in the human brain. Although the total concentration of zinc in a cell is relatively high, the concentration of free zinc, which is not strongly bound to proteins, is extremely low. The estimation of free zinc has proved to be difficult with classical methods. These concerns make it a top priority of chemists to develop selective and efficient probes or so-called chemosensors for zinc ions.

As Zn^{2+} is invisible to most analytical techniques, fluorescence techniques stand out as the method of choice. This method utilizes a probe molecule that recognizes Zn^{2+} and emits a specific wavelength upon binding, which in turn allows tracking of zinc ions in live cells with fluorescence microscopy. A fluorescent molecular probe consists of a fluorophore attached to a chelating agent or an ionophore with or without a spacer group.[2] A change in the fluorescence intensity or wavelength occurs as a result of analyte binding, which leads to signal output (Figure 1). The mechanism of signal transduction through change in fluorescence occurs by a process such as charge transfer,[6] electron transfer,[7] energy transfer,[8] excimer formation, or conformational change.[9] Among these mechanisms, photoinduced electron transfer (PET) is widely used in chemosensor design as it significantly influences the fluorescence emission.[7]

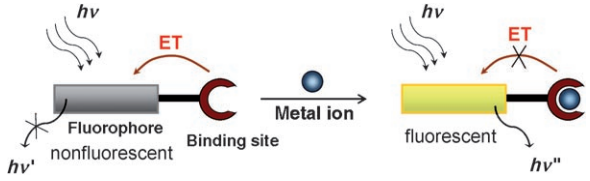

Figure 1. Schematic representation of a fluorescent metal-ion sensor. ET = electron transfer.

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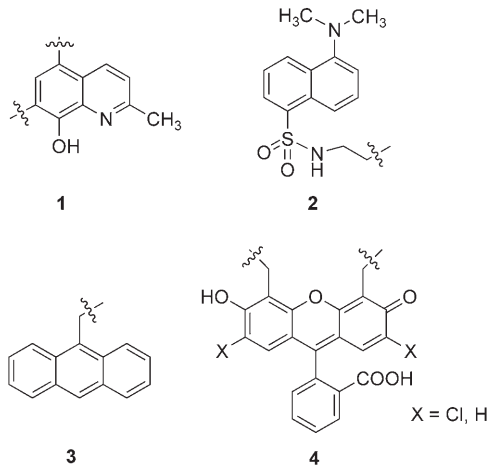
2. PET: The Preferred Mechanism of Signal Transduction

Many fluorescent sensors have been designed based on the principle of PET as the signaling process. At the initial state, due to electron transfer, the fluorophore becomes non-emissive. Metal coordination to the receptor unit makes it a less-efficient electron donor to the attached fluorophore. PET-type fluorescence quenching then becomes less probable or is interrupted altogether. Thus, the native fluorescence of the fluorophore is restored. This signaling is highly sensitive and can be made selective for a specific analyte. Sensors operating according to this principle are known as CHEF (chelation-enhanced fluorescence)-type sensors.[10,11]

An ideal chemosensor for Zn^{2+} should have good chemical and photostability, fluorescence selectivity, rapid sensitization, pH-independent fluorescence, fast target delivery, and good solubility. For biological applications, the excitation wavelength should be in the visible region. Recent progress in the area of fluorophore-based chemosensors has contributed significantly to the development of a variety of fluorescent probes for the detection of zinc ions. Most of these are based on quinoline (**1**),[12] dansyl (**2**),[13] anthracene (**3**),[14] and fluorescein (**4**)[15] derivatives, which function as the fluorophore. Among these, fluorescein-based probes have been extensively studied owing to visible absorption of the probe, water solubility, and permeability to biological tissues.



3. Di-2-picolylamine: The Best Ligand for Zinc Ions

Ionophores specific to Zn^{2+} are limited to certain groups such as quinolines, bis(2-pyridylmethyl)amine (or di-2-picolylamine, DPA), linear and cyclic polyamines, and some bioligands such as zinc-finger domains. Among these iono-

Abstract in Malayalam:

ജീവജാലങ്ങളുടെ ശാരീരിക പ്രവർത്തനങ്ങളിൽ Zn^{2+} അയോണുകളുടെ പ്രാധാന്യം, ജൈവമാധ്യമത്തിൽ അവയുടെ ആനുപാതിക നിർണ്ണയത്തേയും പ്രതിഫലനത്തേയും കുറിച്ചുള്ള ആധികാരിക പഠനത്തെ വളരെയേറെ പ്രാധാന്യം ഉള്ളതാക്കുന്നു. Zn^{2+} അയോണുകളെ പ്രത്യേകമായി ആകർഷിക്കുകയും തന്മൂലം പ്രത്യേക നിറത്തിലുള്ള പ്രകാശ വികിരണങ്ങൾ പുറപ്പെടുവിക്കുകയും ചെയ്യുന്ന രാസതന്മാത്രകളുടെ ആവിർഭാവത്തിലൂടെ മാത്രമേ ഇത് സാധ്യമാവുകയുള്ളൂ. ഇത്തരം തന്മാത്രകൾ ജൈവ കോശങ്ങളിലെ Zn^{2+} അയോണുകളുടെ അളവിനെ നിർണ്ണയിക്കാനും, പ്രതിഫലിപ്പിക്കാനും ഏറെ അനുയോജ്യമാണ്. ഈ സാഹചര്യത്തിലാണ് ആനുപാതിക നിർണ്ണയത്തിന് ഉതകുന്ന സമീപ ഇൻഫ്രാറെഡ് (near-IR) തരംഗങ്ങളുടെ ആഗിരണം സാദ്ധ്യമായിട്ടുള്ള തന്മാത്രകളുടെ സാധ്യത ഏറുന്നത്. തന്മൂലം രാസസൂചകങ്ങളുടെയും തന്മാത്രാപരിശോധിനികളുടെയും മേഖലയിൽ, സമീപ ഇൻഫ്രാറെഡ് (near-IR) രശ്മികളെ ഉപയോഗിച്ച്, ആനുപാതിക നിർണ്ണയത്തിനും പ്രതിഫലനത്തിനും സാധ്യമായ രാസതന്മാത്രകളുടെ രൂപകൽപനയും ആവിർഭാവവും ഏറെ രസതന്ത്രജ്ഞരുടെ ശ്രദ്ധ ആകർഷിച്ചിരിക്കുന്നു. ഈ അവസരത്തിൽ ഇത്തരം രാസസംയുക്തങ്ങളുടെ പ്രാധാന്യത്തെകുറിച്ചും, ഈ മേഖലയിലെ കാലിക പുരോഗതികളെക്കുറിച്ചും, ഭാവിയിലെ ഗവേഷണ സാധ്യതകളെക്കുറിച്ചുമുള്ള ഒരു അവലോകനം ഏറെ പ്രാധാന്യം അർഹിക്കുന്നു.

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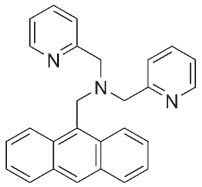
phores, DPA is the most widely used ligand for Zn^{2+} binding. The amine nitrogen atom of DPA is a good electron donor in the PET process. DPA-based sensor **5** is a typical



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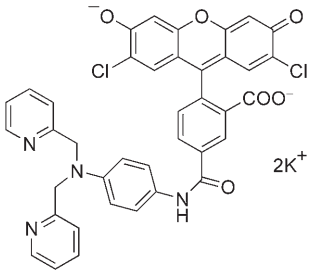

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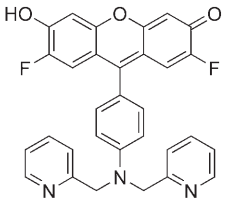

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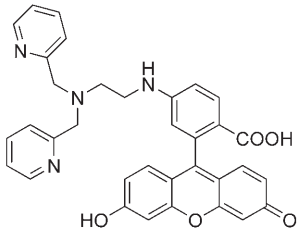
PET sensor for protons and Zn^{2+}.[16] Upon binding of the metal ion, the electron-transfer process of the probe is interrupted, and the fluorescence quantum yield is increased. Fluorescein derivatives **6** and **7** have the advantage of absorption in the visible region, which facilitates excitation at those frequencies. Only the anionic form of fluorescein emits strongly, which means that the pK_a values are an important factor for the pH-dependent performance. Attachment of electron-withdrawing groups renders better performance over a much-broader pH range. Newport Green DCF (**6**) and Newport Green DPX (**7**) are DPA-based sensors that show fluorescence enhancement upon binding to zinc.[17]

Recently, several new sensors that belong to the family of ZnAF2 (**8**) and Zinpyr-1 (**9** and **10**) were reported for the detection of Zn^{2+}.[18] The ZnAF2-type molecules were designed based on PET and are suitable for biological applications. Although these second-generation probes have a higher affinity for Zn^{2+} and are brighter than the first-generation UV-based probes, several issues such as easy synthesis, sensitivity, selectivity, optical compatibility with biological samples, and stability need to be improved upon. Some recent reviews discuss the merits, disadvantages, and use of these probes for Zn^{2+} detection and imaging in biological samples.[1,2,19] Herein we highlight recent developments in the design and application of ratiometric and near-infrared (NIR) Zn^{2+} probes.

4. Ratiometric Zinc-Ion Probes

The current interest in Zn^{2+}-specific sensors is based on ratiometric sensing. Ratiometric behavior can be expected



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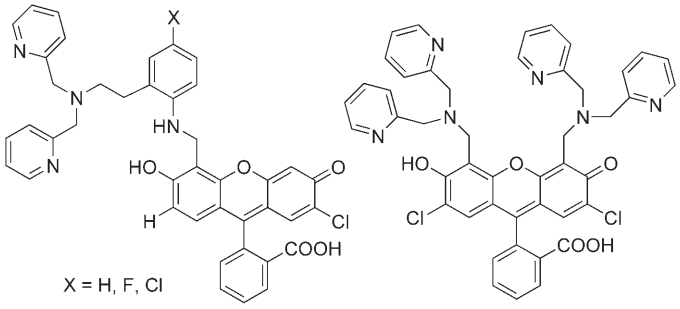
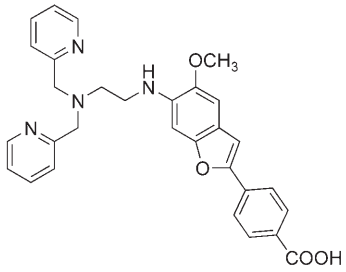


9 **10**

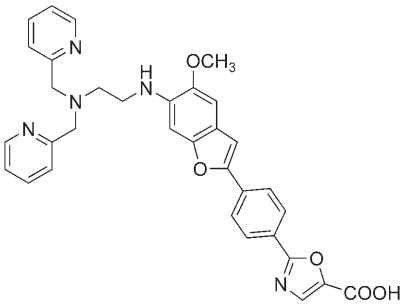
when analyte binding changes the electronic properties of a chromophore, resulting in absorption or emission at a different wavelength. Thus, a fluorescent ratiometric sensor responds to an analyte by a shift in its emission maximum, which may or may not be concomitant with the variation in intensity. This shift in the emission wavelength should be enough to distinguish the emission maximum of the coexisting free and bound Zn^{2+} species, thus allowing the determination of the ratio of emission maxima of the two species. Together with the known binding constant of the sensor, the unknown zinc concentration can be determined.[20] The ratiometric signal is internally calibrated, and neither the light source nor photobleaching affects the ratio of the signal of the bound sensor to that of the unbound.

Maruyama et al. made use of the internal charge transfer (ICT) mechanism in electron-donating DPA conjugated to an electron-withdrawing benzofuran derivative in the design of ratiometric Zn^{2+} probes.[6a] The probes **11** (ZnAF-R1) and **12** (ZnAF-R2), upon complexation with Zn^{2+}, exhibited a blue shift in the excitation maxima, whereas the emission maxima remained unchanged. Sensor **12** is more soluble and has a better fluorescence quantum yield than **11** in water, thus making it useful for biological applications as illustrated by the fluorescence ratiometric imaging of Zn^{2+} in macrophages. As **12** is not permeable through the cell membrane, the more-lipophilic ethyl ester derivative (ZnAF-R2 EE) was used to enter the cell and be transformed into **12** by esterase in the cytosol (Figure 2).

Lim, Brückner, and co-workers reported the coumarin-derived ratiometric sensor **13** for Zn^{2+} (Scheme 1).[21] The lactone oxygen atom of the coumarin moiety is a potential donor atom and, hence, participates in chelation with Zn^{2+}, resulting in a change in the electronic properties of the chromophore. Although a range of metal ions can bind to the



11 ZnAF-R1



12 ZnAF-R2

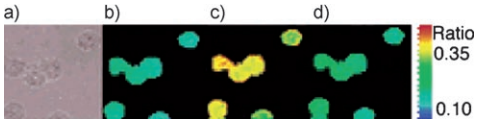
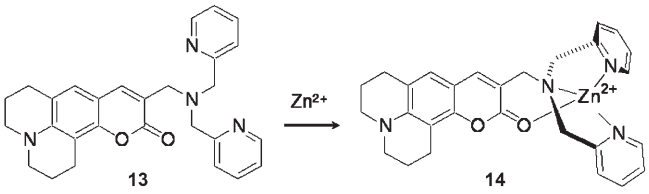


Figure 2. Fluorescence ratiometric images (340 nm/380 nm) of zinc in macrophages (RAW 264.7) labeled with ZnAF-R2 EE in PBS buffer, pH 7.4. a) Bright-field transmission image. b) Ratiometric image of a). c) Pyrithione (zinc ionophore; 15 μM) and $ZnSO_4$ (150 μM) were added to b). d) *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN; 400 μM) was added 15 min after the addition of pyrithione. (Reprinted with permission from Maruyama et al.[6a] Copyright (2002) American Chemical Society.)

sensor, most lose out when in competition with Zn^{2+}, thus making **13** a suitable probe for biological systems (Figure 3).

Woodroofe and Lippard made a significant contribution to the development of Zn^{2+} probes.[22] They synthesized a number of ratiometric Zn^{2+} probes by attaching DPA-chelating groups to different chromophores. An interesting case is the novel two-fluorophore approach with coumazin-1 (**15**), which is a nonfluorescent prosensor containing a Zn^{2+}-insensitive coumarin derivative attached to a Zn^{2+}-sensitive fluorescein moiety.[22] The two fluorophores, when conjugated through an ester linkage, become nonfluorescent. This



Scheme 1. Zinc-binding mode in DPA-substituted coumarin **13**.

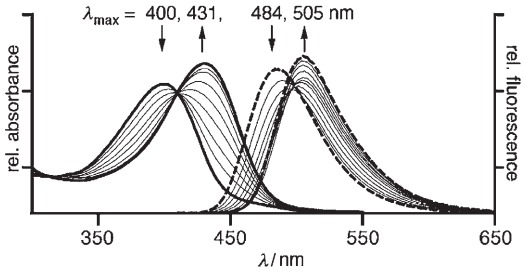
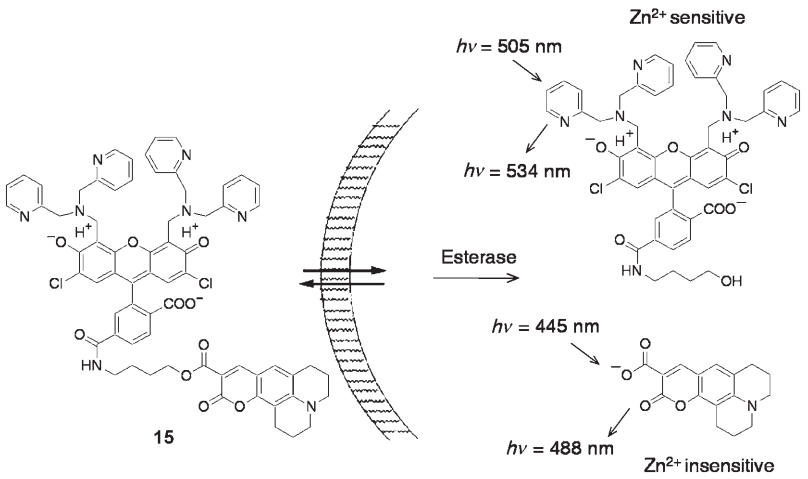


Figure 3. UV/Vis spectral titration (——) and fluorescence response (– – –) of **13** with Zn^{2+} (0–1 equiv). (Reprinted with permission from Lim and Brückner.[21a] Copyright (2004) The Royal Society of Chemistry.)

nonfluorescent compound can be hydrolyzed by esterase (Scheme 2), thus separating the Zn^{2+}-sensitive fluorescein chemosensor from the coumarin part, both of which are fluorescent.

Coumazin-1 is a cell-permeable latent fluorophore useful for the biological ratio imaging of Zn^{2+} (Figure 4). When it is injected into cells, the ester linkage is cleaved by esterase, and the fluorophores are activated. As both fluorophores have characteristic excitation and emission properties, they can be probed ratiometrically. Excitation of the coumarin at 445 nm and measurement of the emission intensity at 488 nm provides information about the cleaved sensor. Excitation of the fluorescein part at 505 nm and monitoring of the emission intensity at 534 nm gives information about the amount of zinc present. In the absence of Zn^{2+}, the ratio of the emission intensities I_{534}/I_{488} was 0.5, which increased to 4.0 upon saturation with Zn^{2+}.



Scheme 2. Proposed mechanism for the activation of coumazin-1.

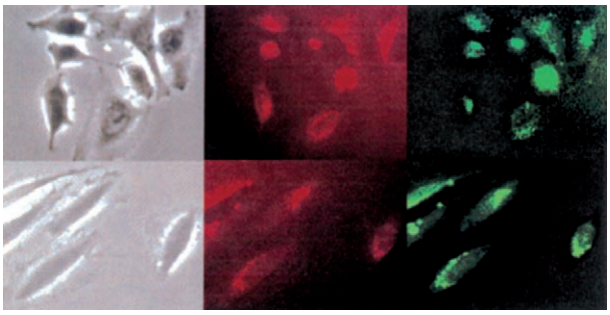
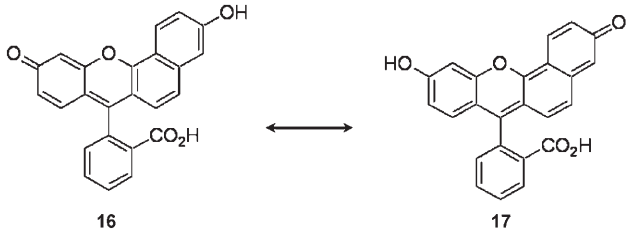


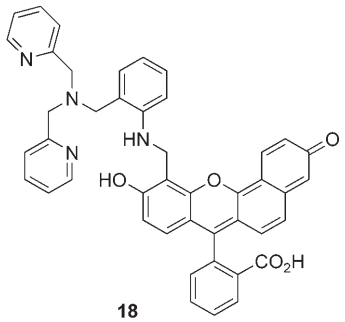
Figure 4. Phase contrast (left) and fluorescence (middle, right) microscopy images of HeLa cells incubated for 6 h with coumazin-1 (5 μM), without (top) and with (bottom) the addition of $ZnCl_2$ (5 μM) and sodium pyrithione (45 μM). Fluorescence images were acquired with excitation at 400–440 nm, bandpass of 475 nm (middle) or with excitation at 460–500 nm, bandpass of 510–560 nm (right). (Reprinted with permission from Woodroofe and Lippard.[22] Copyright (2003) American Chemical Society.)

Lippard and co-workers later reported ratiometric sensors based on the Zn^{2+}-induced shift of the phenoxynaphthoquinone–naphthoxyquinone tautomeric equilibrium (Scheme 3).[23] Coordination of Zn^{2+} to **18** increases the



16 **17**

Scheme 3. The two limiting tautomeric forms of the seminaphthofluorescein fluorophore. The naphthoxyquinone mesomer **16** has fluorescein-like optical properties, and the phenoxynaphthoquinone mesomer **17** shares optical characteristics with naphthofluorescein.



18

overall intensity of the sensor and shifts the spectrum to one dominant peak indicative of the phenoxynaphthoquinone tautomer. As **18** is impermeable to cell membranes, the non-fluorescent diacetate derivative of **18** was used to measure changes in the intracellular Zn^{2+} concentration in living mammalian cells by ratiometric fluorescence imaging.

The seminaphthofluorescein-based probe allows single-excitation, dual-emission ratio detection of Zn^{2+}-triggered switching between fluorescein- and naphthofluorescein-like tautomers. The probe exhibits excellent selectivity for Zn^{2+} over other competing biological cations (Figure 5). The

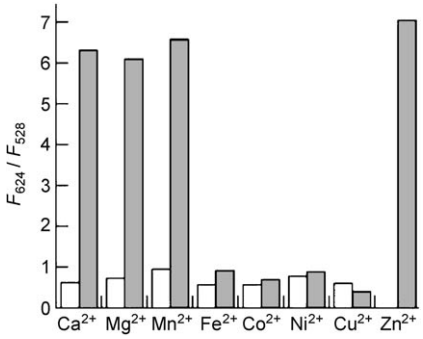
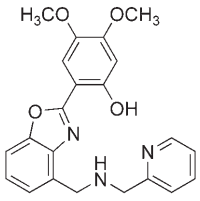


Figure 5. Ratiometric fluorescence spectroscopic response of **18** to various metal ions. Bars represent the ratio of fluorescence intensities collected at 624 and 528 nm (F_{624}/F_{528}). White bars: Addition of an excess of the appropriate metal ion to the solution of **18**. Gray bars: Subsequent addition of Zn^{2+} to the same solution (excited at 400 nm). (Reprinted with permission from Chang et al.[23] Copyright (2004) National Academy Sciences, USA.)

long-visible-wavelength excitation and emission profile of the probe minimizes cell and tissue damage and avoids interference of autofluorescence from native cellular species.

A benzoxazole-based fluorescent sensor (Zinbo-5) and its application in two-photon microscopy and emission ratiometric imaging of intracellular Zn^{2+} was reported recently by Taki et al.[24] Zinbo-5 (**19**) is cell-permeable, binds free Zn^{2+}, and shows significant zinc-induced changes in quantum yield. This molecule exhibited a characteristic band at 407 nm ($\Phi_f = 0.02$) that shifted to 443 nm ($\Phi_f = 0.10$) upon binding with Zn^{2+} (Figure 6). Except for Zn^{2+} and Cd^{2+}, all other transition-metal cations quenched the fluorescence of Zinbo-5. Neither high nor low concentrations of alkali- and alkaline-earth-metal ions influ-



19 Zinbo-5

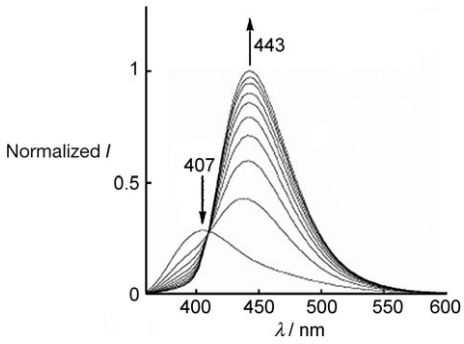


Figure 6. Emission spectra of Zinbo-5 (**19**), with excitation at 356 nm in Zn^{2+}-buffered system. (Reprinted with permission from Taki et al.[24] Copyright (2004) American Chemical Society.)

enced the emission response of Zinbo-5 to Zn^{2+}. This observation indicates that the probe may be useful in a wide range of biological and microscopic applications.

The affinity to Zn^{2+} of Zinbo-5 is much higher than that of DPA, thus indicating that the phenolate oxygen and benzoxazole nitrogen atoms probably chelate the metal as the third and fourth ligands. The utility of **19** in two-photon excitation fluorescence (TPE) microscopy of mammalian cells is demonstrated with the imaging of mouse fibroblast cells (Figure 7). These emission-ratio imaging experiments reveal that Zinbo-5 readily records changes in the availability of intracellular zinc ions.

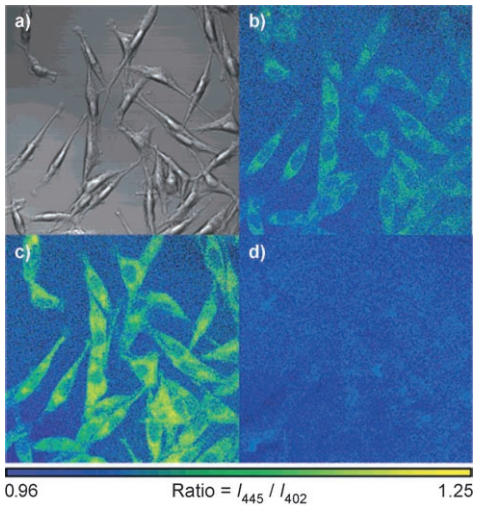
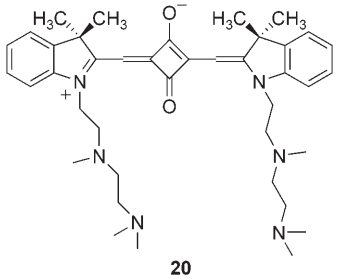


Figure 7. Emission ratio images of fibroblast [L(TK)$^-$] cells in Zinbo-5. a) Bright-field transmission image. b) Ratio images collected at $\lambda_{em}=445$ and 402 nm. c) Ratio image collected after treatment with Zn^{2+}/pyrithione followed by washing with Zinbo-5 stock solution. d) Ratio image of the same field after treatment with TPEN. (Reprinted with permission from Taki et al.[24] Copyright (2004) American Chemical Society.)

Squaraine dyes are excellent chromophores for the design of molecular probes for biologically relevant cations. Dilek and Akkaya synthesized squaraine dye **20**, which binds to Zn^{2+} to result in a three-state fluorescence response to a single input (Figure 8).[25]



20

In a rare example, Ojida et al. used the Zn^{2+} complex of the DPA-attached xanthone derivative **21** for the ratiometric sensing of phosphate anion under neutral aqueous conditions.[26] The ligand, upon titration with Zn^{2+} in aqueous

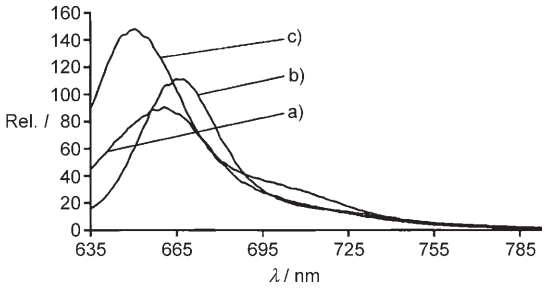
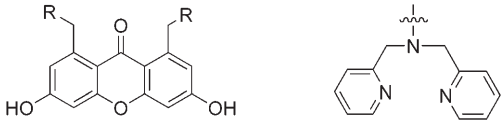


Figure 8. Changes in the emission spectrum of squaraine derivative **20** with increasing amounts of Zn^{2+}. a) $[Zn^{2+}]=0$; b) $[Zn^{2+}]=1.0$ μM, Zn^{2+}/ligand = 1; c) $[Zn^{2+}]=0.025$ M, Zn^{2+}/ligand = 25000. (Reprinted with permission from Dilek and Akkaya.[25] Copyright (2000) Elsevier.)

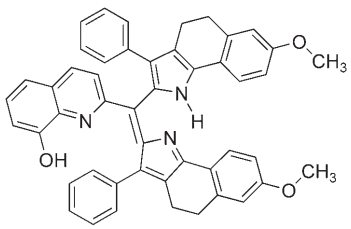


21: R = Dpa

Dpa

methanol, showed a stepwise spectral change. Addition of one equivalent of Zn^{2+} induced a decrease in absorbance at 328 nm and concomitant formation of a new band at 376 nm (Figure 9). Subsequent addition of up to 2 equivalents of Zn^{2+} caused an increase at 397 nm and a decrease at 335 nm through an isosbestic point at 376 nm. These changes are ascribed to the coordination of two Zn^{2+} ions with the molecule, which would perturb the electronic properties of the xanthone chromophore. The change in ratiometric excitation of the original Zn complex **22** upon phosphate-anion binding is ascribed to the rearrangement of the coordination mode (Scheme 4).

Recently, Mei and Bentley reported the ratiometric fluorescent sensor **24** for Zn^{2+}, which works on the principle of



24

ICT.[6b] The changes in the fluorescence spectrum of **24** upon addition of various quantities of Zn^{2+} is shown in Figure 10. The blue shift in the emission is attributed to the capture of Zn^{2+} by the dipyrrin moiety of **24**, leading to diminished electron-donating ability.

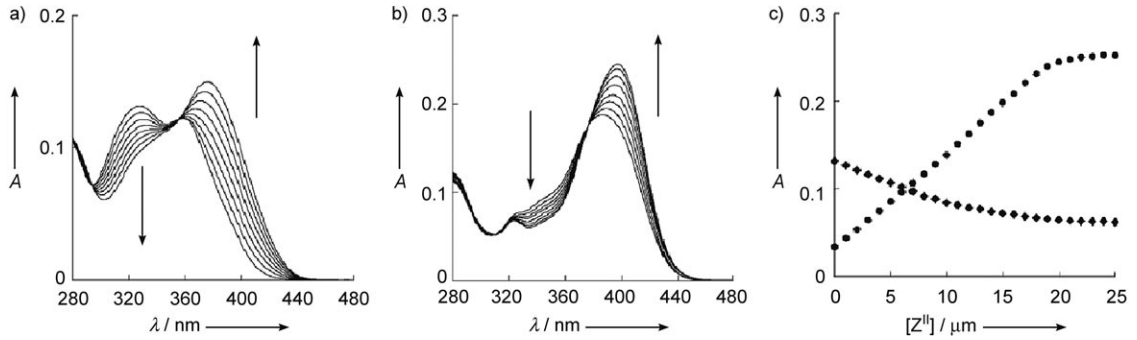
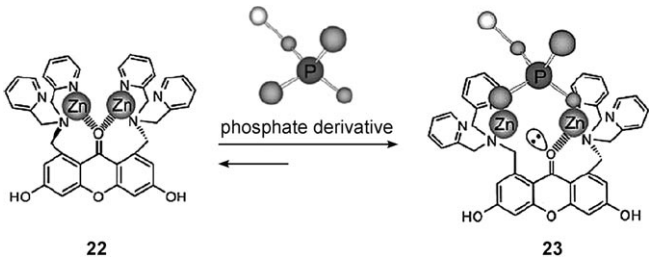
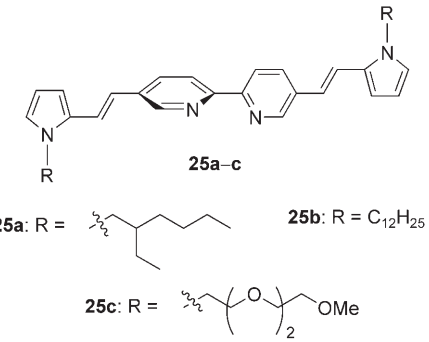


Figure 9. a–c) Zn^{2+}-induced changes in the UV/Vis absorption of ligand **21** (10 μM) upon addition of a) 0–0.7 and b) 1.3–2 equivalents of zinc. c) Plot of UV/Vis absorption at 397 (●) and 328 nm (◇) of **21** (10 mm) in the Zn^{2+} titration (50 mm HEPES buffer, 50 mm NaCl, H_2O/MeOH = 1:1, pH 7.2, 25 °C). (Reprinted with permission from Ojida et al.[26] Copyright (2006) Wiley-VCH.)



Scheme 4. Phosphate-anion-induced coordination rearrangement in zinc coordination complex **22.**



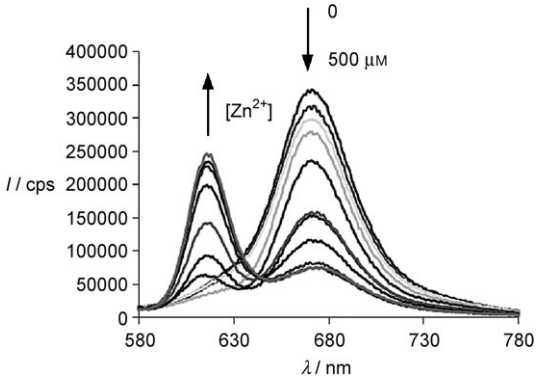


Figure 10. Fluorescence emission spectra of **24** (5 μM) in acetonitrile in the presence of different concentrations of Zn^{2+}. (Reprinted with permission from Mei and Bentley.[6b] Copyright (2006) Elsevier.)

enhancement was observed only for Zn^{2+}, which allows selective detection of the latter. Similarly among different transition-metal cations, only the Zn^{2+} complex exhibited fluorescence emission (Figure 11).

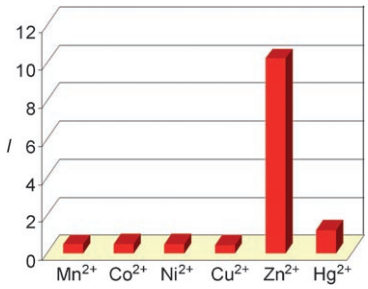


Figure 11. Plot showing variation of fluorescence intensity of **25c** monitored at 650 nm with different transition-metal ions.

4.1. Bispyrrole-Based Ratiometric Fluorescent Probe

Pyrrole-end-capped divinyl aromatic systems[27] are known to be strongly fluorescent substrates for the synthesis of electrochromic[28] and low-band-gap polymers.[29] Incorporation of a bipyridyl moiety into this system resulted in molecules **25a–c**, which are excellent ratiometric probes for the specific detection of Zn^{2+}.[30] Although these probes bind to a variety of biologically significant metal ions, fluorescence

When Zn^{2+} was added to a nonfluorescent Cu^{2+} complex of **25c**, the emission corresponding to the Zn^{2+} complex reappeared, which indicates the preferential binding of Zn^{2+} (Figure 12). Thus, the reported fluorophore allows ratiometric sensing of Zn^{2+} in the presence of other competing cations with a red shift of the emission in the visible region, under a physiological pH window. The remarkable difference in the emission color of the probe before and after Zn^{2+} binding is of advantage for imaging applications.

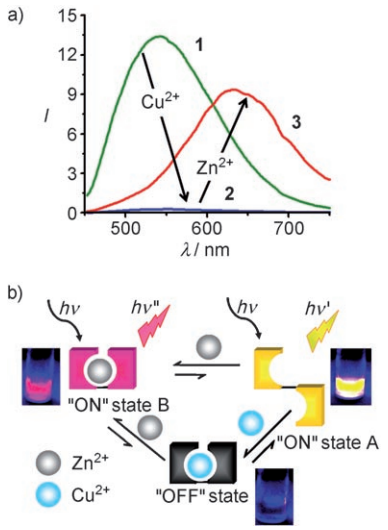
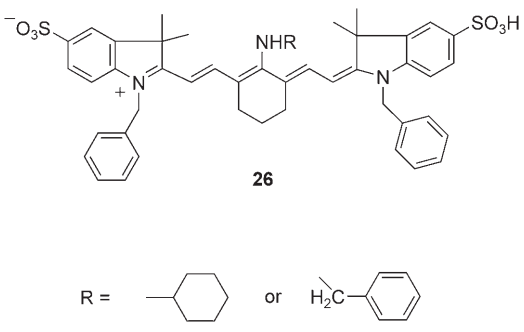


Figure 12. a) Emission spectra of **25c**: 1) blank; 2) **25c**+Cu^{2+}; 3) **25c**+Cu^{2+}+Zn^{2+}. b) Schematic representation of the binding and signaling events.[30]

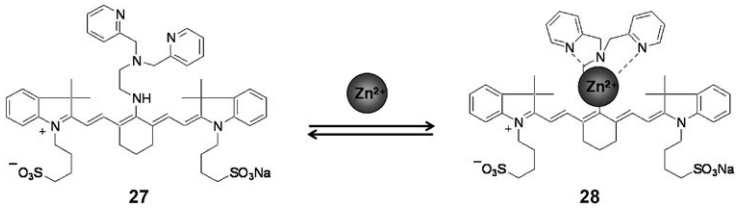
5. NIR Fluorescent Probes

Molecules that absorb and emit in the NIR region, when properly functionalized, are useful for the sensing and imaging of specific analytes, particularly for in vivo biological imaging. Low background noise and low scattering of NIR emissions result in a high signal-to-noise ratio, thus facilitating highly sensitive detection. NIR-emitting molecular probes have the advantage of allowing in vivo imaging and subcellular detection owing to low absorptivity and scattering. The NIR region around 650–900 nm is ideal because of good transmission and low autofluorescence in biological samples.

Several NIR fluorescent probes have been synthesized and used for biological imaging. However NIR probes for the detection of Zn^{2+} are rare. Among different NIR dyes, cyanine dyes have been widely used in fluorescence imaging of biological samples.[31] The major problem with most of NIR dyes is low photostability and fluorescence quantum yields. Amine-substituted tricarbocyanine dyes **26** are reported to have large Stokes shifts and strong fluorescence intensities, which is advantageous for the detection of a specific analyte under physiological conditions.[32]



Recently, Nagano and co-workers synthesized a series of carbocyanine dyes **27**, which are useful as ratiometric NIR probes for Zn^{2+}.[33] A specific example is shown in Scheme 5. The dipicolylethylenediamine moiety attached to the chromophore acts as the metal-ion chelator. The molecule has a high extinction coefficient ($7.0\times10^{-4}\,M^{-1}cm^{-1}$) and a large Stokes shift. Upon binding to Zn^{2+}, the electron-donating ability of the amine to the chromophore decreases, and the color of the solution changed from blue to teal (dark greenish-blue) owing to 1:1 complexation (Scheme 5). The formation of the 1:1 complex was confirmed by ESI-MS as well as changes in the absorption and emission spectra. At higher concentrations of Zn^{2+}, a 44-nm red shift of the absorption maximum occurred, thus indicating that the molecule is a ratiometric probe for Zn^{2+} (Figure 13). The metal-ion selectivity of **27** is shown in Figure 14.



Scheme 5. Preferred binding mode of the NIR probe **27** to Zn^{2+} ions.

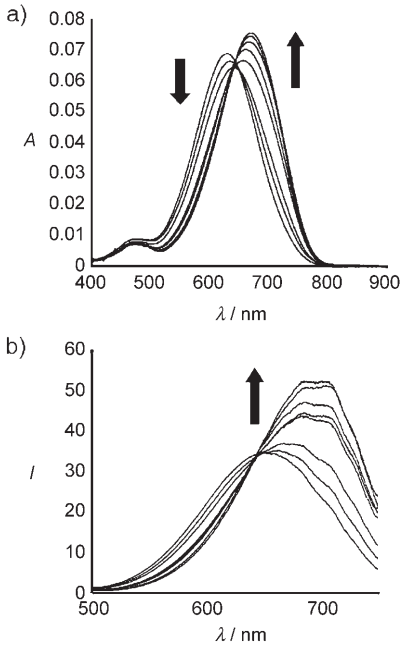


Figure 13. a) Absorption and b) excitation spectra of **27** (1 μM) at various Zn^{2+} concentrations (in 100 mm HEPES buffer, pH 7.4). (Reprinted with permission from Kiyose et al.[33] Copyright (2006) American Chemical Society.)

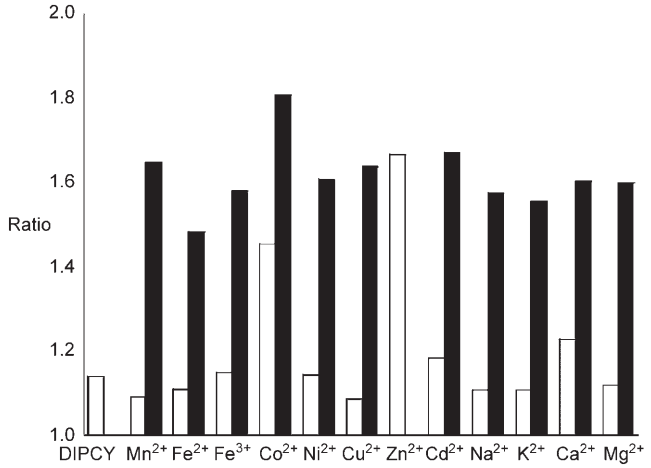
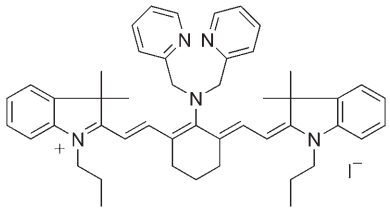


Figure 14. Metal-ion selectivity of **27**. Bars indicate the fluorescence ratio (671 nm/627 nm excitation, 760 nm emission). White bars: each cation was added. Black bars: each cation and zinc ions were added. DIPCY = dipicolylcyanine. (Reprinted with permission from Kiyose et al.[33] Copyright (2006) American Chemical Society.)

Although metal ions such as Co^{2+} and Cu^{2+} influence the emission, these ions would have little influence in vivo owing to their extremely low concentrations. The fluorescence changes of these amine-substituted carbocyanine dyes should be applicable to dual-wavelength detection of various biomolecules or enzymes.

Another variant of the tricarbocyanine dye that emits in the NIR region was reported by Tang et al.[34] This probe (**29**) can be easily prepared by a one-step reaction of tricarbocyanine with 2,2′-dipicolylamine. Compound **29** absorbs at 606 nm and emits at 800 nm in acetonitrile, with a large Stokes shift of 194 nm. The fluorescence emission of the probe increases upon binding to Zn^{2+}, whereas other cations such as Na^+, K^+, Ca^{2+}, and Mg^{2+} do not enhance the fluorescence (Figure 15). This probe works on the basis of the PET mechanism and gives a 20-fold turn-on response for detecting zinc ions. The dye is cell-permeable and can respond to Zn^{2+}, thus demonstrating that it is an excellent NIR fluorescent probe for zinc imaging in macrophage cells (Figure 16).



29

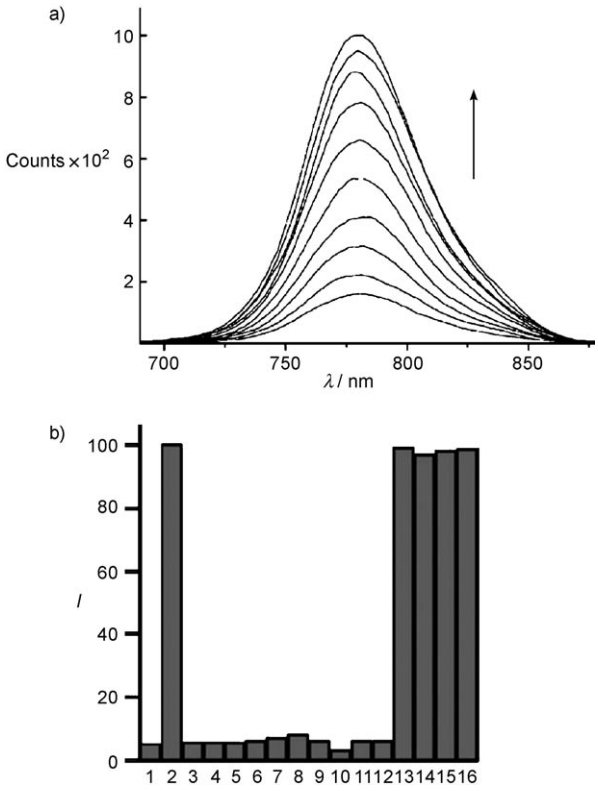


Figure 15. a) Emission spectra (excitation at 731 nm) of **29** (5 μM) in the presence of various concentrations of Zn^{2+} (0, 1–5 μM in steps of 0.5 μM). b) Relative fluorescence intensity of various cations: 1) none; 2) Zn^{2+}; 3) K^+; 4) Na^+; 5) Ca^{2+}; 6) Mg^{2+}; 7) Ni^{2+}; 8) Hg^{2+}; 9) Co^{2+}; 10) Cu^{2+}; 11) Fe^{2+}; 12) Fe^{3+}; 13) $Zn^{2+}+K^+$; 14) $Zn^{2+}+Na^+$; 15) $Zn^{2+}+Ca^{2+}$; 16) $Zn^{2+}+Mg^{2+}$. (Reprinted with permission from Tang et al.[34] Copyright (2006) The Royal Society of Chemistry.)

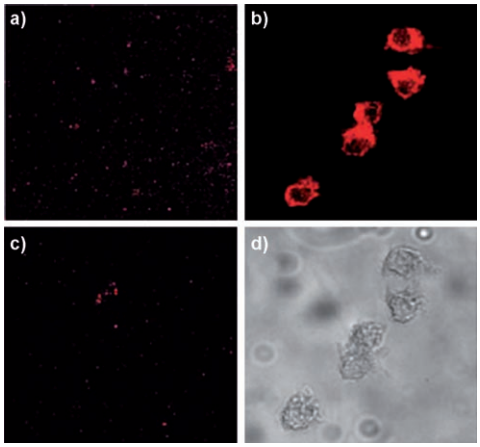


Figure 16. Confocal fluorescence images of live macrophage cells. a) Cells incubated with cyanine derivative **29** (5 μM) for 30 min at 37 °C. b) Cells supplemented with Zn^{2+} (100 μM) to macrophage cells treated with **29**. c) Treatment with TPEN (100 μM), a high-affinity membrane-permeable heavy-metal chelator, for 5 min at 37 °C. d) Bright-field image of the live macrophage cells shown in b), confirming their viability. (Reprinted with permission from Tang et al.[34] Copyright (2006) The Royal Society of Chemistry.)

6. Conclusions and Outlook

We have highlighted recent developments in the area of ratiometric and NIR Zn^{2+} probes. As stated in the Introduction, the design of molecular probes for the detection of biologically relevant molecules and imaging is an area of considerable importance. Although a significant amount of work has been done in this area, more remain to come. This is particularly true of NIR ratiometric probes for biological imaging. Although a large number of NIR dyes are known in the literature, they are currently underutilized in the design of ratiometric zinc-ion probes. We hope that this Focus Review will spread the importance of developing new NIR ratiometric probes for biological applications. This short review is expected to encourage new aspirants who are interested in the field of molecular probes to come up with novel systems that are stable and specific to the sensing and imaging of zinc ions under physiological conditions.

Acknowledgements

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