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Review

Fluorescent detection of zinc in biological systems: recent development on the design of chemosensors and biosensors

Pengju Jiang, Zijian Guo*

Coordination Chemistry Institute, State Key Laboratory of Coordination Chemistry, Nanjing University, Nanjing 210093, PR China

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Contents

Abstract

Zinc is an essential biological element for living organisms and fluorescent imaging has been proven to be the most suitable technique for its in vivo monitoring. From the late 1980s, many fluorescent probes have been designed and some have been used successfully in zinc neurochemistry. This paper will review the progress made in the past decade or so, focusing on sensor design strategy based on molecular structure and fluorescent mechanism.

Keywords: Fluorescent probe; Zinc(II) ion; Biological imaging; Ionophore; Fluorophore

Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; AM, acetoxymethyl; APTRA, *o*-aminophenol-*N*,*N*,*O*-triacetic acid; AQ, aminoquinoline; BAPTA, bis(*o*-amino-phenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; BPAMP, [2-{bis(2-pyridylmethyl)amino-methyl}-*N*-methylaniline]-phenol; CA, carbonic anhydrase; CEF, chelation enhancement of fluorescence; DCF, dichlorofluorescein; dansyl, 5-(dimethylamino)-naphthalene-1-sulfon-amide; DNSA, dansylamide; DFF, difluorofluorescein; DPA, di-2-picolylamine, bis(2-pyridylmethyl)-amine; DTPA, diethylenetri-aminepentaacetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; HOMO, highest occupied molecular orbital; HQ, hydroxyquinoline; ICT, intramolecular charge transfer; LUMO, lowest unoccupied molecular orbital; MBP, maltose binding protein; MMP, matrix metalloproteinase; MT, metallothein; MTP, metal-response element-binding transcription factor; NG, Newport Green; PCT, photo-induced charge transfer; PDX, 9-phenyl-2,7-difluoro-6-hydroxy-xanthen-3-one; PET, photo-induced electron transfer; RET, resonance energy transfer; RF, Rhodafluor; SOD, superoxide dismutases; TF, transcription factor; TPEN, *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine; TSQ, 6-methoxyl-8-*p*-toluenesulphonamidoquinoline; ZP, Zinpyr

* Corresponding author. Fax: +86-25-3314-502.
E-mail address: zguo@nju.edu.cn (Z. Guo).

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Nomenclature

Greek Letters

λ_{abs}	maximum absorption wavelength
λ_{em}	maximum emission wavelength
λ_{ex}	maximum excitation wavelength

1. Introduction

Zinc, always occurring as a divalent cation [zinc(II)] in biological systems, is the second most abundant transition metal following iron. In an adult human body, for example, there 2–3 g of zinc in total, while the content of copper is only 250 mg [1]. More interestingly, large amounts of zinc(II) are likely to concentrate in nerve tissues (0.1–0.5 mM for brain tissue) [2]. Although the majority of biological zinc ions are tightly sequestered by proteins [3,4], the presence of "free zinc pools" in certain cells may still be possible [5].

Though the low concentration delayed the recognition of its importance [6], zinc(II) ions are well known to play diverse roles in biological processes such as those shown in Fig. 1 [7]. The most important and best known role for zinc is as a structural cofactor in metalloproteins. Over the last half a century, hundreds of zinc proteins possessing one or more zinc-stabilized motifs have been identified and classified into several major families [8]. As initially found in transcription factor IIIA (TF IIIA), the Cys_2His_2 motif interacts with zinc(II) to provide a nucleic-acid-binding domains which are referred to as "zinc-fingers" [9]. Meanwhile, zinc(II) ions are directly associated with the regulation of gene expression through metalloregulatory proteins such as metal-response element-binding transcription factor-1 (MTF-1) that acts as a cellular zinc sensor [10]. Many hydrolytic enzymes contain zinc(II) ion in the active site, since zinc-bound water or hydroxide are excellent nucleophilic agents. For example, in the catalytic centre of human carbonic anhydrase II (CA II), zinc(II) is coordinated by three histidine residues and a water molecule [11]. Substantial in vivo and in vitro evidence has demonstrated that zinc can be an important regulator of cellular apoptosis [12]. In addition, zinc(II) ions are also present in most DNA or RNA polymerases [13].

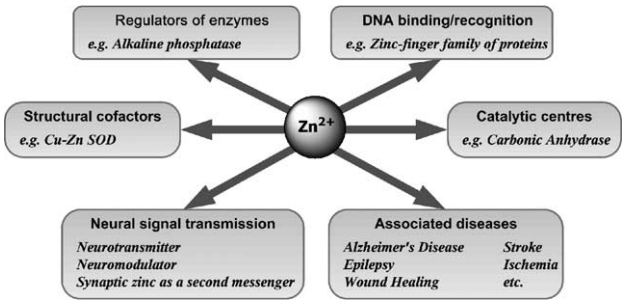


Fig. 1. Major biological roles of divalent zinc(II) ion.

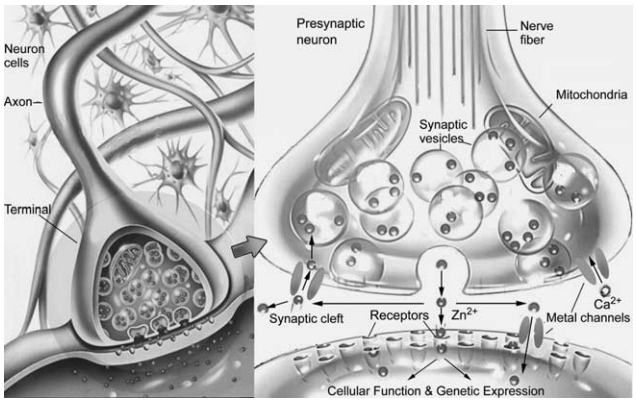


Fig. 2. Schematic diagram showing the zinc(II) signaling pathways between neurons. There are four parallel pathways for zinc ions released from a presynaptic neuron: (1) to bond to receptors of postsynaptic neuron to modulate it; (2) to enter postsynaptic neuron through zinc(II) permeable ion channels (i.e. voltage-gated Ca^{2+} channels); (3) to return to presynaptic neuron; (4) to diffuse away from the synaptic cleft.

The neurological roles of zinc have attracted much attention in recent years [14–16]. It has been postulated that the synaptically released zinc(II) modulates the excitability of the brain through the accommodation of amino acid receptors. During the process, zinc(II) ions not only functions as a conventional synaptic neurotransmitter or neuromodulator for the presynaptic neuron [17], but also as a transmembrane neural signal to traverse the postsynaptic neuron [18]. In this signalling pathway [19] (Fig. 2), zinc transporters (ZnTs) are responsible for the transfer of zinc(II) ions across the membrane and compartmentalizing them into synaptic vesicles [14]. The synaptically released zinc(II) may act as a second messenger1.6 like Ca^{2+} for long-term potentiation in the hippocampus [20]. Recently, it has been reported that metallothein-III (MT-III), the brain form of the MT family, might be responsible for converting NO signals into zinc(II) signal [21].

A disorder of zinc metabolism is closely associated with many severe neurological diseases such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Guam ALS-Parkinsonism dementia, Parkinson's disease, hypoxia-ischemia and epilepsy [22]. The zinc(II) ions released during neurotransmission may induce galvanization of β-amyloid (Aβ) plaques occurring in AD disease [23]. The correlation between copper–zinc superoxide dismutases (Cu–Zn SOD) and ALS disease has been known for almost a decade [24]. Additionally, clinical evidence showed that zinc nutrition aids wound healing through a family of zinc dependent endopeptidases named matrix metalloproteinases (MMPs) [25].

There is a huge scope for the exploration of the diverse physiological roles of biological zinc, especially the neural "free zinc" (often referred to as non-ligand bonded or weakly bonded zinc) [26]. Therefore, a sensitive and non-invasive technique is desired in order to accomplish real-time local imaging. Unlike other biological transition

metal ions such as Fe^{2+}, Mn^{2+} or Cu^{2+}, Zn^{2+} does not give any spectroscopic or magnetic signals due to its $3d^{10}4s^0$ electronic configuration. The common analytic techniques such as UV-Vis spectroscopy, Mössbauer spectroscopy, nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) spectroscopy cannot be applied to detect this atypical metal ion in biological systems, thus the fluorescence method stands out as a method of choice [27]. This technique employs a probe molecule that recognizes zinc(II) and emits specific fluorescence upon zinc(II) binding, which is recognized as a feasible method for real-time and real-space imaging of living cells without damaging them [28]. The design of an appropriate fluorescence ligand is the key step to make this method practical. It requires multidisciplinary knowledge in areas such as coordination chemistry, photophysics, organic synthesis, analytical techniques, cellular biology, histology, neurophysiology and clinical imaging. This review will summarize the recent development in the field, focusing mainly on the probes designed over the past decade or so.

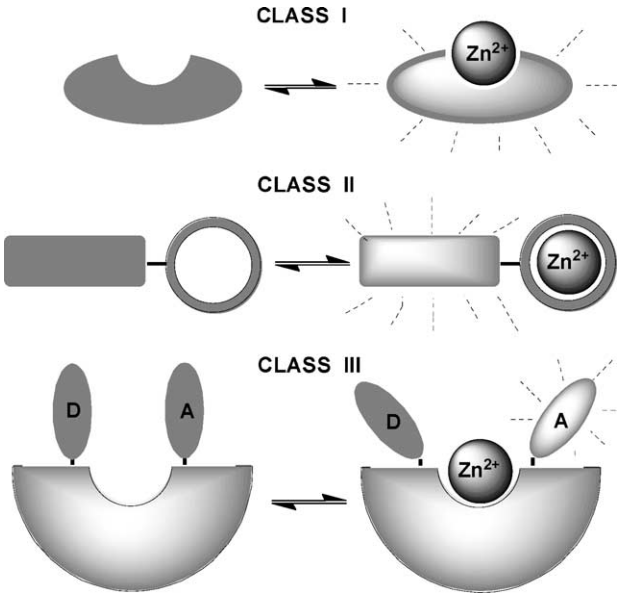


Fig. 3. Classification of zinc fluorescence probes: Class I, fluorogenic chelating agents; Class II, fluoroionophores; Class III, FRET sensors (D: donor, A: acceptor).

2. Design principles of zinc fluorescent probes

2.1. Classification and mechanism of fluorescence

Upon binding to zinc(II), the responsive moiety termed as zinc fluorescent probe forms a fluorescent complex, which can be used to stain and image free zinc(II) ions. Although fluorescence can be either enhanced or quenched upon zinc(II) binding, "chelation enhancement of fluorescence" (CEF) type should preferably be adopted so as to visualize zinc(II) ions in biological systems [29]. Three essential characteristics of fluorescence, including spectrum (shape of bands, λ_{ex}, λ_{em}), quantum yield (ϕ) and lifetime (τ), can provide information on the microenvironment of the excited states.

Fluorescent sensors can be classified into two major groups: chemosensors and biosensors according to the originality of the responsive moieties for analytes [30]. These sensors can also be divided into three classes (Fig. 3) based on their difference in ion sequestering pathways [31]. Class I are "fluorogenic" chelating agents (e.g. oxine), class II are fluoroionophores with separate fluorophore and ionophore linked with or without a spacer and class III are fluorescent sensors based on resonance energy transfer (RET, also called electronic energy transfer, EET) which is also frequently termed fluorescence resonance energy transfer (FRET) [32]. Fluorophore is the functional group responsible for emitting fluorescence, while the ionophore refers to receptor group for ion recognition.

Due to the feasibility for diverse manipulation, class II is the most widely employed strategy in probe design. According to the nature of the photo-induced process, fluorescent probes of this class can be further classified into two categories: photo-induced electron transfer (PET) probes and photo-induced charge transfer (PCT) probes [33]. In PET sensors, the metal ion receptor is an electron donor (e.g. amino-containing group) and the fluorophore acts as an electron acceptor. In the analyte-free form, upon excitation by photons the electron of the highest occupied molecular orbital (HOMO) of the fluorophore is promoted to the local lowest unoccupied molecular orbital (LUMO); subsequently, the electron seated on the HOMO of the donor transfers to the HOMO of the fluorophore synergistically (electron transfer), which causes fluorescence quenching [34]. When binding to a target cation, the energy gap between these two HOMO orbitals is changed from positive to negative, so the quenching process does not occur.

In PCT sensors, the fluorophore contains an electron-donating group (usually an amino group) conjugated to an electron-withdrawing group, which undergoes internal charge transfer (ICT) from the donor to the acceptor [35]. The consequent change of the dipole moment leads to a Stokes' shift, which is influenced by the microenvironment of the fluorophore. Therefore, it is expected that a cation like zinc(II) closely interacting with a fluorophore will cause an observable change in fluorescent intensity. Additionally, the ICT process may be accompanied by an internal rotation between the donor and acceptor leading to a twisted intramolecular charge transfer state, which will influence the fluorescence intensity to some extent. As a result, the most prominent features of PET and PCT sensors are the distinction between the fluorescence changes upon cation binding: fluorescence enhancement exhibited by PET, while obvious spectroscopic band shifts appear in PCT (Fig. 4).

Differing from class I and class II, the class III fluorescent probes are based on dipolar (Förster) long-range spatial

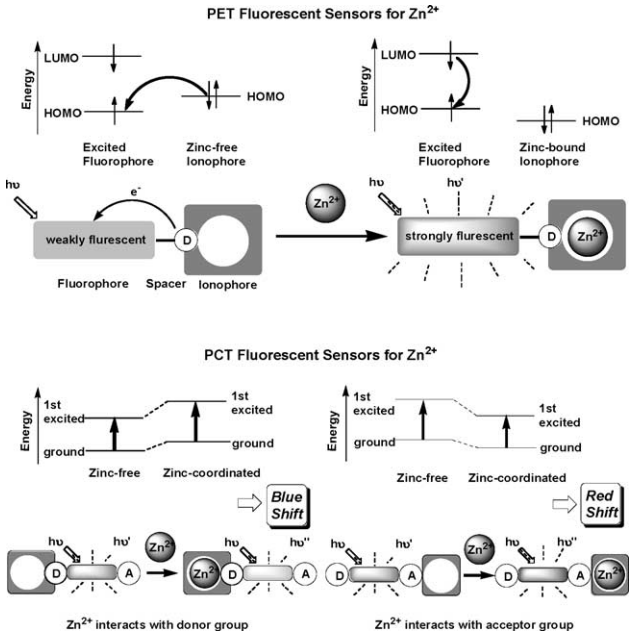


Fig. 4. Fluorescent mechanisms of PET sensors (upper) and PCT sensors (lower) for zinc detection (A: acceptor, D: donor) (modified from [32]).

interaction [32]. Excited by short wavelength light, the donor transfers energy to a spatially adjacent acceptor through dipole–dipole interaction. The acceptor may be either a fluorophore or a quencher leading to two different sorts of CEF application. If the acceptor is a fluorophore, the fluorescent ligand should chelate the analyte to draw the donor–acceptor (D–A) pair closer and thereby to allow stronger energy transfer. Whereas, if the acceptor is a quencher, the sensor is based on chelation-induced release of the fluorophore which then emits. Since RET relies on a rather large intramolecular displacement of donor and acceptor groups, it is more widely used in biosensors, though it has been employed in the design of small chemosensors [36]. In some cases, a change in fluorescence polarization has been employed in the detection of zinc(II) and other metal ions [37].

In addition to the steady-state fluorescence methods mentioned above, excited state lifetimes can also be measured and analyzed to detect zinc(II) ions. The steady-state methods are currently much more common than the time-resolved methods because of their relative simplicity. However, with the development of analytical techniques, the acquisition of temporal information has become much easier than before, and some trials based on time-resolved fluorescence methods for the detection of zinc(II) have been implemented [30]. Nowadays, high repetition rate picosecond lasers and microchannel plate photomultipliers have been used to achieve a time resolution of a few tens of picoseconds. More recently a fluorescence up-conversion technique has been developed for even better time resolution (100–200 fs, 10^{-12} s).

Furthermore, confocal configuration and multiphoton excitation have been realized in fluorescence microscopy so that even a single molecule could be detected. The newly developed near-field scanning optical microscopy technique has a higher resolution than that of confocal microscopy. To supply complementary information for conventional steady-state experiments, fluorescence lifetime imaging microscopy, using differences in the excited-state lifetime of fluorophores is evolving very rapidly as a contrast mechanism for imaging [32]. Therefore, it is anticipated that all these novel techniques will provide methods for more sensitive detection of zinc(II).

2.2. Criteria and design strategies

A reliable fluorescent zinc sensor should possess the following characteristics: chemical and photo-stability, fluorescence selectivity and sensitivity, rapid sensitization, fast target delivery, suitable solubility, and manoeuvrability [27,38,39]. In order to stain zinc ions in living systems, many other aspects should be considered. For examples, the excitation wavelength in the visible range ($\geq$400 nm) is preferred because UV light is known to impair living organisms [14]. Solubility and membrane-permeability should be emphasized for in vitro and in vivo assays, too. Though pH values would not diverge too much from the neutrality under physiological conditions, pH dependence of fluorescence should be avoided with full efforts for higher stability and greater reliability of quantitative monitoring in complex situations. A broad linear range of responsive bandwidth is also worthy of pursuing. Although quite a lot of fluorescent indicators for zinc(II) have been designed and synthesized, none can yet perfectly satisfy all the criteria mentioned above. Thus, efforts to design novel zinc probes should be encouraged.

With respect to fluorogenic sensors, the design work should be focused on the modification of chelating agents, since the types of ligands are limited (mainly substituted quinolines). The basic objective is to improve the fluorescence performance and solubility, but the opportunities for progress are rather limited. In general, hydrophilic groups are included to improve solubility.

The most common research involves class II sensors, the design of an appropriate combination of an ionophore and a fluorophore. Fluorophores control the fluorescence wavelength and sensitivity, whereas ionophores determine the affinity and selectivity for zinc(II) ion. In fact, the chelating groups specific for zinc(II) are limited to only several moieties such as bis(2-methylpyridine) amine, cyclen and some bioligands such as zinc-finger domains. The possible fluorophores are really diverse and those with high fluorescent efficiency such as fluorescein and dansyl sulfonamide are preferred. If there is a spacer (between ionophore and fluorophore), its length should also be taken into consideration, especially in PCT sensors, because it has great influence on the polarity of the probe.

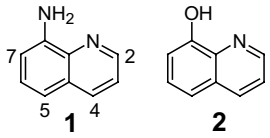
With respect to RET sensors, the individual adoption of donor–acceptor pairs strictly relies on the distance between the pair (in either proteins or nucleic acids), because Förster

type resonance energy transfer can be used as a spectroscopic ruler only in the range of 10–100 Å [32] depending on the extent of spectroscopic overlap between the donor and acceptor [28]. Thus, upon zinc(II) coordination, the distance between the donor–acceptor pair must be changed predictably within an appropriate range.

3. Chemosensors

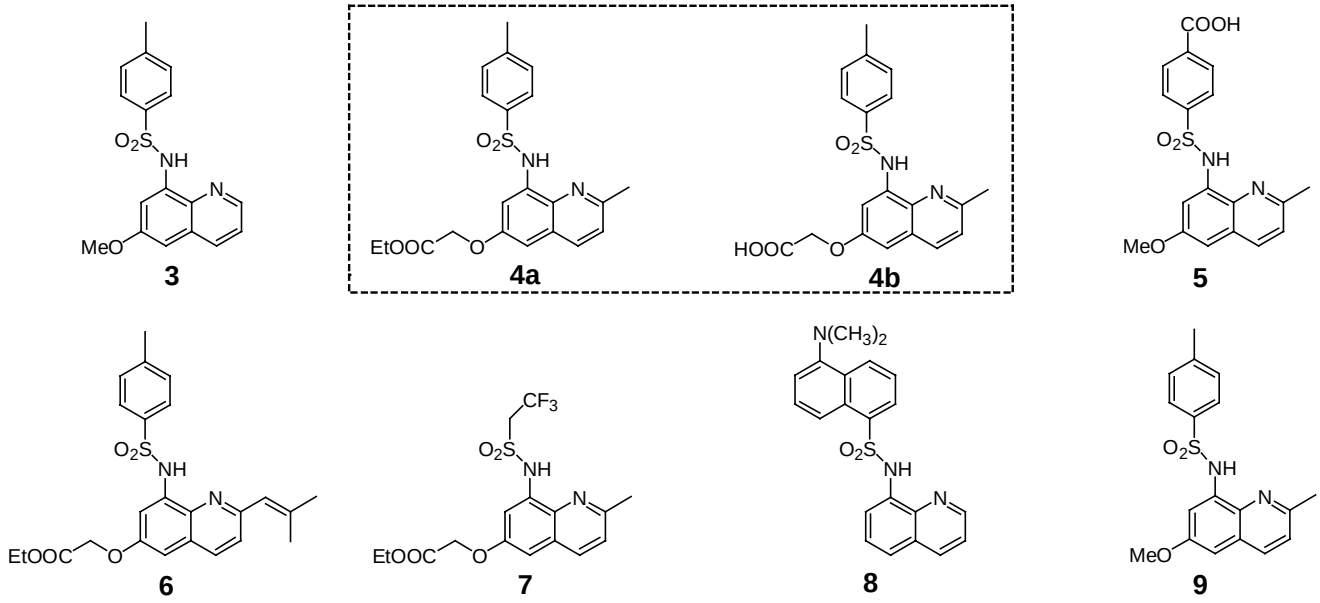
3.1. Quinoline-based sensors

Quinolines and their derivatives have been used traditionally as fluorogenic agents for the quantitative chemical assay of zinc(II) and other metal ions [40,41]. Among them, 8-aminoquinoline (8-AQ, **1**) and 8-hydroxyquinoline (8-HQ or oxine, **2**) are the most representative of the species studied (Scheme 1). Although oxine is regarded as the second most important chelating agent after EDTA for cation analysis, most indicators derived from it are not specific enough for the selective detection of specific metal ions. The 8-HQ, 8-AQ and their derivatives are non-fluorescent and are un-influenced by change in pH [42] because of the existence of an intramolecular hydrogen bond between the heterocyclic nitrogen atom and the 8-substituted group (i.e. –OH or $–NH_2$) [43,44]. In a strong basic aqueous environment 8-HQ and 8-AQ are likely to deprotonate and their intramolecular hydrogen bonds are broken, however, the fluorescence will still be suppressed by intermolecular hydrogen bonds formed with water. This hydrogen bond couples the photo-induced excited-state proton transfer with an intramolecular electron transfer. De-excitation of the resulting tautomer occurs mainly via a non-radiative pathway. However, when the quinolines and derivatives chelate zinc(II) and other metal ions, they exhibit intense yellow-greenish fluorescent emission, because the cations are able to quench the above electron transfer process. Interestingly, only zinc(II) and Ca^{2+} can interrupt hydrogen bonding in 8-AQ and its derivatives, and thereby stimulate fluorescence forming the basis for the selective detection of these two cations. Thus, 8-AQ is a more useful basis for sensor design than 8-HQ. However, 8-AQ and 8-HQ themselves are not suitable for biological applications since the active hydrogen atoms (i.e. NH or OH) are likely to react with some active species in the complicated biological environments to affect the fluorescence and selectivity (Scheme 2).



Scheme 1. Schematic drawing of substituted quinolines.

In the 1960s, Russian scientists initially introduced the quinoline sulfonamide moiety as a fluorescent chemosensor for zinc(II) or cadmium(II) ions. However, it was not until 1987 that one of the sulphonamido compounds, namely 6-methoxy-8-*p*-toluenesulphonamido-quinoline (TSQ, **3**), was first applied for the in vitro imaging of ionic divalent zinc [45]. This work is regarded as a milestone in the development of fluorescent probes for biological zinc(II). The fluorescence of the apo-ligand is too weak to be observed, while its zinc(II) complex, upon ultraviolet excitation at pH 7.4, emits strong fluorescence with a maxima at 495 nm with quantum yield of ca. 0.1 [46,47] (Table 1). As a pH-independent fluorescent probe, compound **3** has proven to be a selective, non-toxic fluorescent sensor for the detection of biological zinc(II), and will remain to be the most



Scheme 2. Schematic drawing of 8-AQ-based probes.

Table 1
Fluorescent properties of zinc complexes with 8-AQ-based ligands[a]

Zinc complex (ligand)	λ_{ex} (nm)	λ_{em} (nm)	log β[b]	Reference
3 (TSQ)	334	495	–	[47]
4b (Zinquin A)	370	490	9.65	[67]
5 (TFLZn)	360	498	–	[59]
6	352	467	–	[61][c]
7	382	488	–	[62]
8 (Danquin)	395	469	6.46	[63]
9 (2-MeTSQ)	362	485	8.43	[66]

[a] The data may vary in several nanometers, since the measurements were taken in different solvents due to their poor solubility in water.
[b] The log β value means association constants for 1:1 complexes.
[c] The data are taken from an analogue of **6**.

widely used compound in biological zinc stain [39]. As shown in Fig. 5, it has been widely used to localize zinc pools in the central nervous system [48,49] and to stain pancreatic islet cells (a zinc-rich cell line) selectively for their flow cytometric isolation [50,51]. However, compound **3** has several limitations, among them the poor water solubility is the most noticeable one, which requires the TSQ stock solution to be prepared in dimethylsulfoxide (DMSO) or ethanol.

In order to improve the solubility and membrane-permeability of TSQ, Zalewski and co-workers introduced carboxylic acid groups or ester groups to extend the 6-methoxyl group (Zinquin, **4a** ester form, **4b** acid form) [53,54]. This design is based on the acetoxymethyl esterification loading theory ("AM-loading") proposed by Tsien for calcium fluorescent probes (Scheme 3) [55]. Modifying the carboxyl groups to AM groups renders the probe, membrane permeable. Once the AM form diffuses into the cytoplasm, endogenous esterase cleaves the AM group, and then the free acid form of the probe acts as a fluorescent indicator for cytoplasmic zinc(II). This feature allows the real-time imaging of the dynamic activities of cellular zinc [56–58]. Another attempt to improve the solubility of TSQ is to replace the methyl group on the benzene ring with a carboxylic acid group (compound **5**, TFLZn) [59]. The probe also has two forms: free acid and ester derivative. However, this probe is

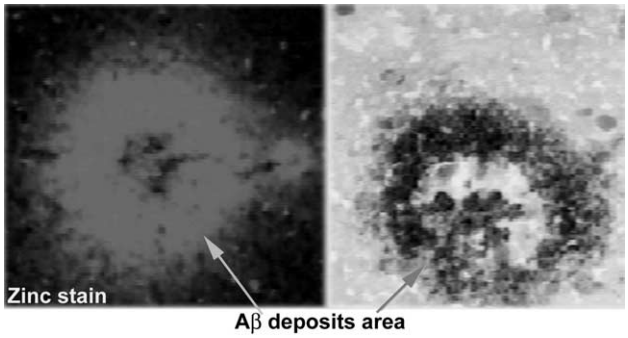
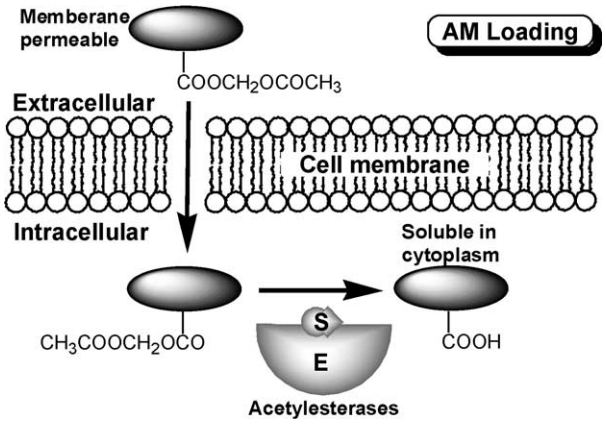


Fig. 5. Aβ deposits in adjacent sections of Alzheimer's disease-affected cortex visualized by Aβ immunohistochemistry (right) and stained with TSQ for zinc(II) (left) (adapted from [52]).

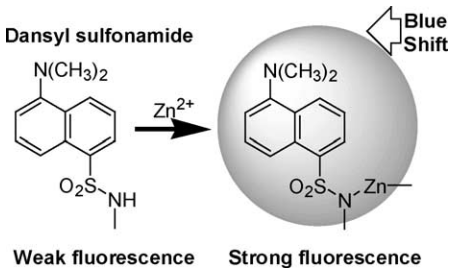


Scheme 3. The principle of "AM-loading" method.

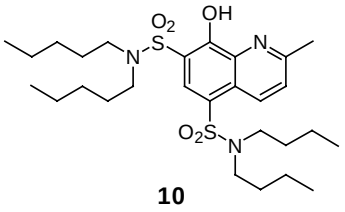
sparingly soluble in waterin neutral medium; therefore, it has not been widely adopted in bioassays. In fact, the sulphonic acid group may be a better solubility modifier than the carboxylic acid group. It has been reported that acid forms of both TFLZn and Zinquin can diffuse into the synaptic vesicles [60].

Ward and co-workers reported a series of Zinquin homologues and several of them have higher fluorescent sensitivity and selectivity than TSQ and Zinquin. The fluorescence of the isobutenyl analogue **6** is three times stronger than that of the Zinquin ester for an extension of the conjugate system [61], whereas the trifluoroethylsulfonyl analogue **7** is 1.5 times more efficient, but it is unstable to some degree [62]. Thus, compound **6** may be a suitable candidate for zinc(II) ion detection.

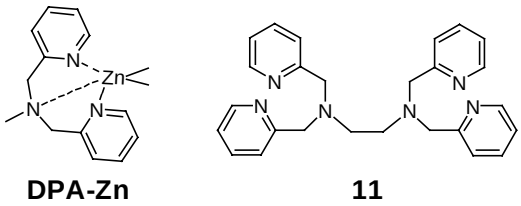
Another limitation of the quinoline-based sensors is the requirement of UV excitation, which may cause damage to living cells and bring artefacts to the dynamic activities of zinc(II) ions. Efforts to shift the excitation wavelength have been made by condensing a dansyl group with aminoquinoline to give a novel zinc fluorescent probe Danquin (**8**) [63]. Upon zinc(II) binding, the emission of compound **8** is blue-shifted with an intensity enhancement (Scheme 4). This can be ascribed to the formation of a complex containing the deprotonated ligand. In fact, complexation with d^{10} entities such as zinc(II) does not usually induce low-energy metal-centred or charge-separated excited states into the molecule, so that energy or electron transfer process



Scheme 4. Schematic drawing of dansyl amide and the fluorescent change upon binding Zn(II) ion.



Scheme 5. Schematic drawing of a potential 8-HQ-based probe.

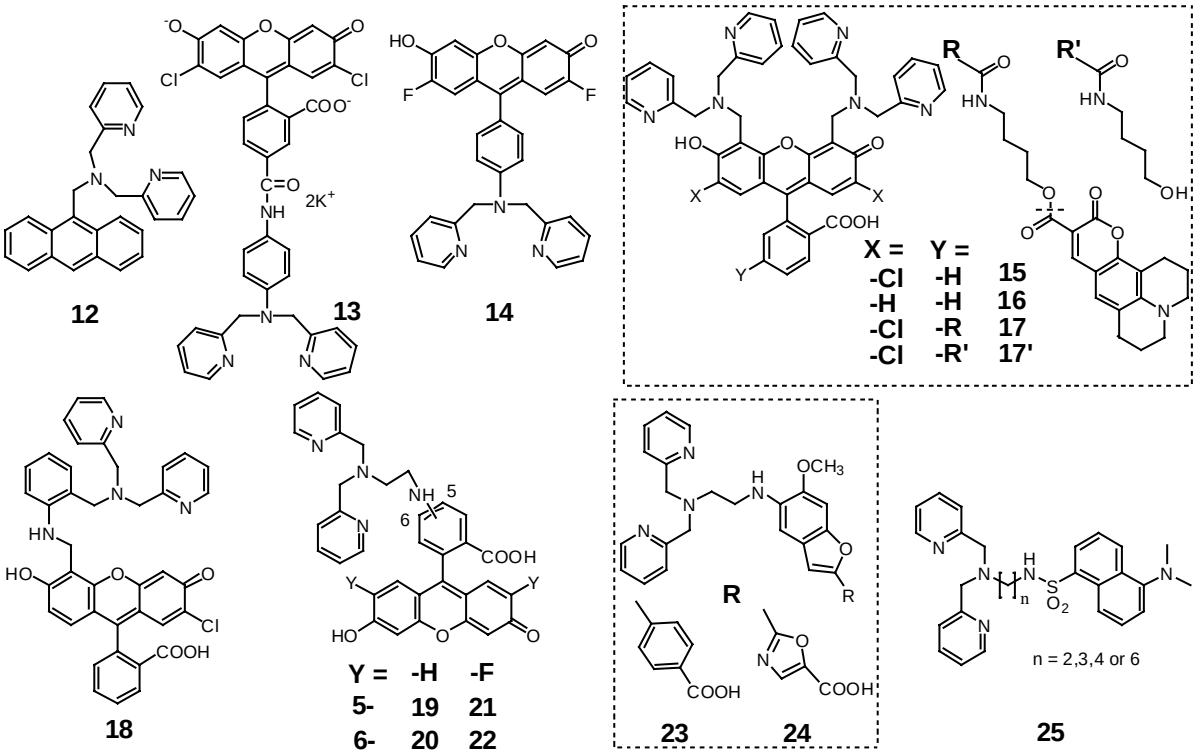


Scheme 6. Schematic drawing of DPA and TPEN groups.

cannot occur. However, the deprotonation or complexation increases the electronic density on the naphthalene ring, which makes the charge-transfer state more responsible for the dansyl luminescence towards higher energy [31]. Coupling of Zn(8-AQ) with Zn(dansyl-sulphonamide) indeed improved the fluorescent intensity of the zinc(II) complex. Although the apo-ligand is fluorescent (547 nm), the zinc(II) complex fluoresces at 469 nm with much stronger intensity, which will not be obscured by the original fluorescence. Meanwhile, the maximum excitation wavelength of the zinc(II) complex is on the borderline between UV and visible region (395 nm), therefore visible excitation can be utilized in practical applications with the quinoline-based sensor (Schemes 5–7).

The aryl substituents in sulphonamidoquinoline ligands (Scheme 2) are generally perpendicular to the quinoline rings [62–64]. However, owing to the complicated equilibria of zinc(II) species in aqueous solution (coordination number varies from 4 to 6) [65] and the reversible binding of water molecules, stable single crystals of the above probes cannot be easily obtained. In order to study the structure of the zinc(II) complexes, O'Halloran and co-workers designed and synthesized 2-Me-TSQ (**9**) [66]. This TSQ derivative has the same fluorescence properties as the original TSQ; however, its 2-methyl group hinders solvent access to zinc(II) ion, which allows the crystallization of a 2:1 stoichiometric complex in CH_2Cl_2 at low temperature. As shown in Fig. 6, the sulfonamide groups tilt away to accommodate the coordination of the amide nitrogen atoms, and the zinc(II) ion is coordinated in a tetrahedral geometry.

In fact, TSQ and its homologues form both 1:1 and 1:2 (zinc:ligand) complexes in solution [67] and the pK_a value of the amido proton is around 9.6. A detailed and systemic study of the stoichiometry and response of these probes was reported by Fahrni and O'Halloran [68]. Recently, Hendrickson et al. found that the anion of **4b** (ester form of Zinquin) forms ternary fluorescent zinc(II) complexes with several polyamines, and the fluorescence of the various complexes alter slightly [69]. Therefore, it is likely that



Scheme 7. Schematic drawing of DPA-based chemosensors.

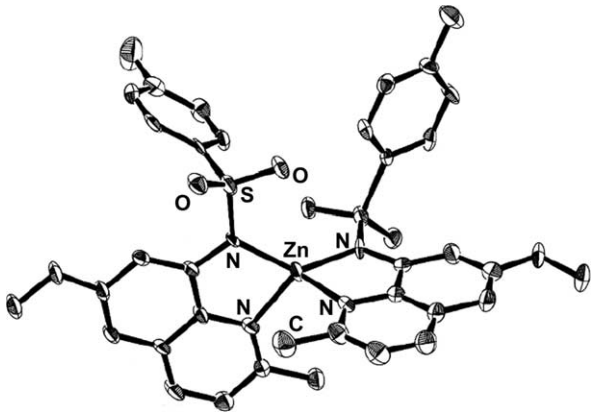


Fig. 6. Crystal structure of the complex $[Zn(\mathbf{9})_2{\cdot}2CH_2Cl_2]$ (hydrogen atoms and solvent molecules are omitted for clarity). The zinc ion is coordinated in a tetrahedral geometry (adapted from [66]).

intracellular imaging obtained from the 8-AQ-based probes can also arise from simultaneous coordination to zinc(II) by both sensor and a bioligand such as a MT protein. Consequently, these probes may only be useful for qualitative or semi-quantitative analysis for zinc(II). The 8-AQ and its derivatives have similar moderate formation constants (β for 1:1 complex) to zinc(II) as shown in Table 1. Nevertheless, this may not be a serious disadvantage because the relatively low binding capability will preclude the ligands to capture some protein-bound zinc(II) in addition to "free zinc". On the other hand, the sensitivity threshold of quinoline-based probes is relatively high compared to some other sensors to be discussed below, and the recommended lowest detection concentration of Zinquin is only 10 μM, though the detection limit could be as low as picomolar level [39].

In spite of several limitations, sulphonamide 8-AQ derivatives represented mainly by TSQ and Zinquin are by far the most practical and mature fluorescent probes for zinc(II). Recall that the sulphonamido group cannot be replaced by carboxamido in the probe design, because the latter can be easily decomposed via zinc-induced hydrolysis (vide infra).

Unlike 8-AQ, there is only one hydrogen atom on the oxygen of 8-HQ. In order to maintain the intramolecular hydrogen bond, the hydroxyl group should be kept unmodified. The general characteristics of these oxine-based ligands are very similar to those containing 8-AQ. For example, variable coordination numbers are accompanied by solution dynamics of the zinc(II) complexes [70,71]. Compared with 8-AQ-based probes, the 8-HQ type probes have rather low fluorescence selectivity for zinc(II).

Imperiali and co-workers have synthesized highly fluorescent ligands based on oxine for zinc(II) sensing by addition of diverse auxochromic groups at sites 5 and 7 (Scheme 1) [72]. The most hydrophobic derivative of the class, compound **10** has been tested for zinc(II) imaging [$Zn(\mathbf{10})_2$: $\lambda_{ex} = 369$ nm, $\lambda_{em} = 465$ nm, $\phi = 0.44$] in fibroblast and recommended as a fluorophore of choice in future peptide-based sensors. Many other oxine-based compounds have been shown to possess superior fluorescence sensitivity for zinc(II). However, the selectivity was rather poor or was untested [73]. Therefore, it is a critical challenge to design 8-HQ-based zinc probes with improved selectivity.

Some quinoline chemosensors based on supramolecular scaffolds will be discussed below. Peptide sensors containing an oxine group will be discussed in the biosensor section.

3.2. bis(2-Pyridylmethyl)amine as chelators

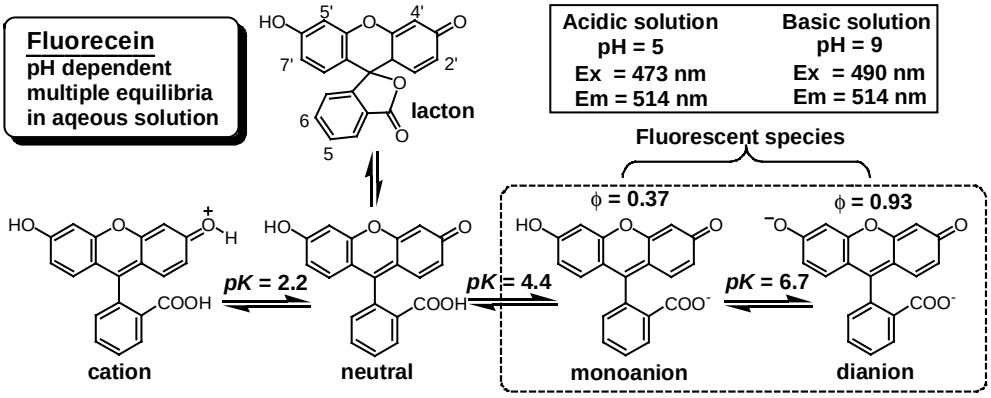
In general, the fluoroionophores (Class II sensors) are much more common and diversified in the design of fluorescent probes for cation recognition. They require a specific ionophore sensitive to zinc, as their basis. bis(2-Pyridylmethyl) amine (namely di-2-picolylamine or DPA) is a specific neutral chelator for zinc(II), providing three nitrogen donor atoms. The *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN, **11**) is a well known neutral sequestering reagent with very high affinity for zinc(II) ($K_d = 2.6 \times 10^{-16}$ M) but low affinity for Ca^{2+} ($K_d = 4.0 \times 10^{-5}$ M) [74,75]. This membrane-permeable compound is widely used to mask the fluorescence resulting from zinc(II) during TSQ staining [66]. It can be expected that the TPEN-like probes will high a selectivity for zinc(II) over Ca^{2+}. In these ligands, the amino nitrogen of the DPA group is a good candidate as an electron donor in either PET or PCT sensor. However, the same nitrogen atom can also be protonated in weak acidic environments, which makes the fluorescent sensors pH-dependent.

One DPA-based sensor which has been investigated is a conjugate of DPA and the anthrylmethyl group (**12**) [76]; this is a typical PET sensor and can be regarded as an "off–on" fluorescence switch (CEF sensor) for proton and post transition metal ions like zinc(II) [77]. Upon zinc(II) binding, the quenching process will be interrupted and in acetonitrile (CH_3CN) solution fluorescence yield increased by a factor of 77 (Table 2). However, UV titration revealed that this

Table 2
Fluorescent properties of zinc complexes with DPA-based ligands

Zinc complex (ligand)	λ_{ex} (nm)	λ_{em} (nm)	ϕ	K_d (nM)	Reference
12	350	370–550	0.77	5.0[a]	[77]
13 (NG DCF)	505	535	–	∼1[a]	[47]
14 (NG PDX)	495	520	–	∼30[a]	[84]
15 (ZP-1)	507	529	0.87	0.7	[85]
16 (ZP-2)	490	513	0.92	0.5	[86]
17′ (ZPA1)	505	534	0.64	0.20	[89]
18 (ZP-4)	495	515	0.34	0.65	[87]
19 (ZnAF-1)	492	514	0.23	0.78	[91]
20 (ZnAF-2)	492	514	0.36	2.7	[91]
21 (ZnAF-1F)	492	514	0.17	2.2	[91]
22 (ZnAF-2F)	492	514	0.24	5.5	[91]
23 (ZnAF-R1)	329	528	0.03	0.79	[93]
24 (ZnAF-R2)	335	495	0.10	2.8	[93]
25 ($n = 3$)	330	522	–	0.98	[94]

[a] Unit: μM.



Scheme 8. The pH dependent fluorescent properties of fluorescein.

single DPA-based sensor forms only an 1:1 complex with zinc(II) ion, and its binding affinity is moderate as shown in Table 2. Moreover, the fluorescence selectivity of zinc(II) of this UV-excitable compound is unknown.

In order to shift the excitation wavelength to the visible range, fluorescein and derivatives are employed as fluorophores; these are traditionally used as intracellular pH indicators. As shown in Scheme 8, fluorescein exhibits complex multiple step equilibria in aqueous solution [78]. It exists in several forms at different pH values and only the anionic forms fluoresces strongly, which shows that the pK_a value of fluorescein and analogues is an important factor for pH dependent performance. In order to achieve strong fluorescence in a much broader pH range (namely to lower the pK_a value), some electron withdrawing groups are introduced at sites 2′ and 7′ (labeled on lacton tautomer in Scheme 8). To reduce the electron density of xanthene, for instance, halides are introduced in dichlorofluorescein (DCF), difluorofluorescein (DFF), etc. When these fluorophores are connected to a DPA moiety, the electron transfer from the amino nitrogen lone pair of DPA will partially quench the fluorescence. However, the quenching process is interrupted when the nitrogen coordinates to zinc(II). Thus, all the DPA–fluorescein-conjugated probes belong to the PET class.

Newport Green DCF (**13**), which was developed by ‘Molecular Probes’, is the first practical DPA-based fluorescent sensor for zinc(II) [47,79]. This fluorescent probe consists of a modified DCF moiety and a DPA group connected through a *N*-phenyl-acetamide spacer like that in Calcium Green (a fluorescent probe for Ca^{2+}). Upon covalent binding to zinc(II), compound **13** exhibits a 3.3-fold enhancement of fluorescence intensity under physiological conditions, while it is insensitive to Ca^{2+}. This sensor is commercially available as a diacetate form, and has been widely adopted to detect zinc influx into neurons through voltage or glutamate gated channels [80,81]. Similar to TSQ, it has also been used to identify insulin-producing β-cells from human pancreatic islets [82] or used to image the zinc release from hippocampal slices [83]. Although this probe cannot be used ratiometrically towards free zinc(II) ions, quantitative assays can be realized at high concentrations ranging from nanomolar to micromolar level (10^{-6} M) by measuring the fluorescence lifetime (there is a large change from 0.88 to 2.93 nanoseconds upon zinc(II) binding). However, its low affinity hampers zinc(II) measurement for dialysis studies [39]. A subsequent ligand of **13**, Newport Green PDX (**14**), shows the same fluorescent mechanism as **13**. However, the loss of the spacer and the substitution of DCF by 9-phenyl-2,7-difluoro-6-hydroxy-xanthen-3-one (PDX) results in a more direct signal-transduction upon cation binding [47]. Although compound **14** has a larger fluorescence enhancement from a zinc-free state compared with **13**, its affinity for zinc(II) remains low or even weaker than **13** [84].

As discussed above, compounds **12**–**14** are not sensitive enough and the three nitrogen coordinating atoms of DPA can only generate a micromolar level affinity for zinc(II). Therefore, the incorporation of extra coordination donor atoms in the ligand molecules could improve the affinity to zinc(II) and result in more sensitive probes.

In the last few years, Lippard and co-workers synthesized several DPA-based fluorescent sensors (Zinpyr family) for zinc(II) detection. Representative compounds include Zinpyr-1 (ZP1, **15**) [85], Zinpyr-2 (ZP2, **16**) [86] and Zinpyr-4 (ZP4, **18**) [87], in which the fluorescein (or analogues) and DPA (or derivatives) are connected through site 4′ or 5′ of xanthenone. Compounds **15** and **16** structurally resemble Calcein (an early fluorescein-based sensor for Ca^{2+} in an alkaline environment [88] but rarely used now) and contain two DPA groups in one ligand, the fluorophore being DCF for **15** and fluorescein for **16**. The crystallographic data of 2:1 zinc(II) complex of ZP1 reveal penta-coordination of zinc(II) and participation of oxygen from fluorescein constrain the DPA group to move towards fluorescein (Fig. 7). Such binding results in a high affinity of **15** for zinc(II). Upon excessive zinc(II) addition, both **15** and **16** show a rather small excitation and emission shift (**15**: ex $+306\,cm^{-1}$, em $-144\,cm^{-1}$; **16**: ex $+165\,cm^{-1}$, em $+188\,cm^{-1}$) but a large enhancement of fluorescent intensity (**15**: 2.2-fold, **16**: 3.7-fold) under simulated physiological conditions [86]. In aqueous solution, owing to the complexity of fluorescence and dynamics of fluorescein, the

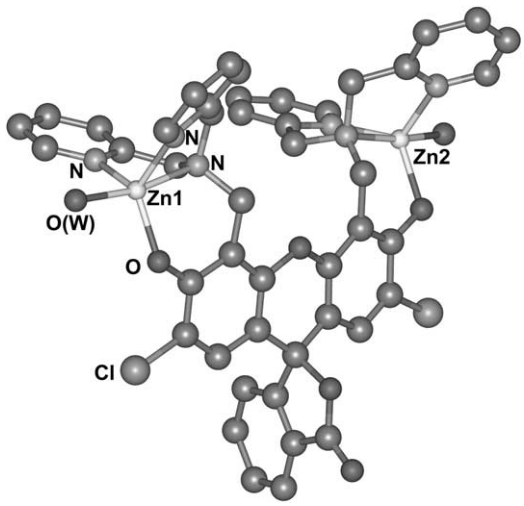
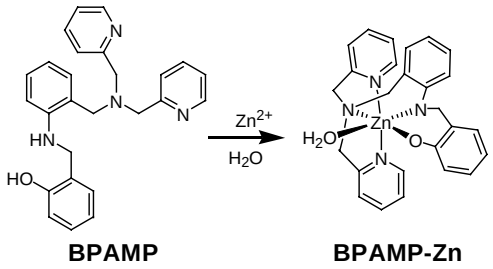


Fig. 7. Crystal structure of complex $[Zn_2(\mathbf{15})(H_2O)_2(ClO_4)_2]6H_2O$ (hydrogen atoms, counter ions and solvent water molecules are omitted for clarity). Both zinc ions coordinate with three nitrogen atoms of DPA, an oxygen of fluoroscein and a solvent water molecule in trigonal bipyramid geometries. Crystallographic coordinates are obtained from the supporting information of [86] on the website of ACS.

probes also show a pH dependent fluorescent behavior as expected. The pK_a values are 8.3 and 9.4 for compounds **15** and **16**, respectively, which suggests that the complexes will fluoresce much stronger in basic solution above the critical value. Although there is a two-step metallation procedure for these ligands, only the first one is accompanied by fluorescent enhancement. Therefore, the co-existence of 1:1 and 2:1 complexation and ambiguous change in fluorescence makes them rather unsuitable for quantitative analysis. Preliminary data demonstrated that ZP1 could be used for the intracellular zinc imaging in COS-7 cells and for staining the Golgi and other acidic compartments [85,86]. Most recently, a ZP1 derivative ZPA1 (**17′**) has been coupled to a coumarin 343 to form Coumazin-1 (**17**) [89]. This compound can be hydrolyzed by esterase into ZPA1 and a zinc(II) insensitive coumarin fluorophore, so it is expected to be utilized in ratiometric sensing system for Zn(II).

In order to avoid the coordination of two zinc atoms to a single ligand and the strong fluorescence interference of apo-ligands, ZP4 (**17**) was designed in which the single extended DPA arm was linked to [2-{bis(2-pyridylmethyl)-aminomethyl}-*N*-methylaniline]-phenol (BPAMP) moiety [87]. The phenol section is part of the fluorescein framework. BPAMP or compound **18** coordinates, in aqueous solution, to zinc(II) as a pentadentate ligand with a bound water (Scheme 9, the complex structure is drawn according to the crystallographic data of $[Zn(BPAMP)(H_2O)]$), and shows an enhanced affinity for zinc(II). The newly introduced aniline nitrogen donor is responsible for PET quenching, while the quenching process is accomplished with DPA's tertiary amino nitrogen in compounds 12–16. As a result, the apo-ligand fluoresces ($\phi = 0.03$) less weakly than its zinc(II) complex (with ~22-fold intensity enhancement).



Scheme 9. Schematic drawing of BPAMP and Zn(BPAMP).

Compared to that of its parent compounds, the pK_a value of the newly introduced nitrogen was also lowered to 10.0. Although ZP4 is membrane impermeable, it has been used to stain the seizure-damaged neurons of a rat brain [87].

Nagano and co-workers combined DPA with 5- or 6-aminofluorescein to give another family (ZnAF) of fluorescent ligands for zinc(II). Four analogues including ZnAF-1 (**19**), ZnAF-2 (**20**) [90], ZnAF-1F (**21**) and ZnAF-2F (**22**) [91] were reported. These "zero-spacer" compounds [92] contain a fourth additional coordination atom from the amino nitrogen of the fluorescein derivatives. The secondary amino nitrogen a strong electron transfer quencher which leads to a substantial fluorescence enhancement upon zinc(II) binding (10-fold for **19**, and 15-fold for **20**). Although the fluorescence enhancement for Zn(**19**) is smaller, the selectivity against Cd(II) was much higher for ZnAF-1 than for ZnAF-2. The methyl ester of **19** (Me-ZnAF-1) was also prepared which shows a similar selectivity to ZnAF-2. Therefore, the carboxyl group of compounds **19** and **20** coordinates to cadmium(II) but not to zinc(II) because of their different ionic radius. However, the fluorescence of their zinc(II) complexes decreases below pH 7.0 in aqueous solution (pK_a of ZnAF-1 and ZnAF-2 is 6.2). Thus, although ZnAF-1 or ZnAF-2 is relatively stable in the physiological pH range, the fluorescent signal will be affected under special acidic conditions such as in the case of acidosis. In compounds **21** and **22**, the DFF was introduced as a fluorophore [91], in which the two fluorine atoms decrease the electron density of the conjugated system. As a result, the pK_a value is reduced to 4.9 and the fluorescence quantum yield of both apo-ligands and zinc(II) complexes is lowered (Table 2) with respect to ZnAF-1 or ZnAF-2. Thus, **21** and **22** behave better under near neutral or even slightly acidic conditions. In addition, all these compounds have been proved to be kinetically appropriate for biological zinc ion detection. The diacetyl derivative form of **20** (ZnAF-2 DA) and **22** (ZnAF-2F DA), which are more lipophilic than **20** and **22**, respectively, were prepared and tested for in vitro experiments. Fig. 8 shows the fluorescent imaging of slices obtained from the hippocampus of a rat loaded with ZnAF-2F DA.

Generally speaking, the compounds derived from DPA fluorescein are highly selective and sensitive PET sensors, but their fluorescence intensity is pH dependent and difficult

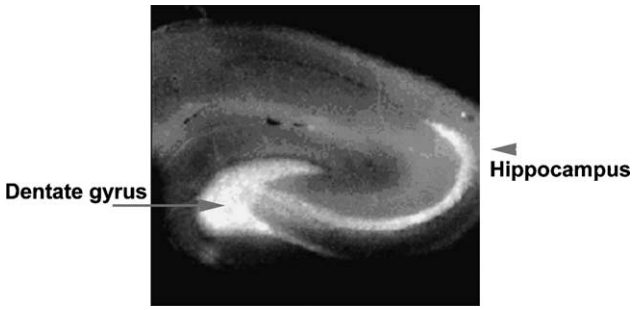


Fig. 8. Fluorescence imaging of rat hippocampus slices loaded with ZnAF-2F DA (adapted from [91]).

to quantify. For these reasons, PCT type probes based on DPA have been developed.

The fluorophore of ZnAF-R1 (**23**) and ZnAF-R2 (**24**) is benzofuran that is widely adopted in many well-known fluorescent probes for Ca^{2+} and Mg^{2+} (see Section 3.4) [93], while the chelating moiety was kept the same as with other ZnAFs. However, the electron-donating group in this case will not quench the fluorescence. Upon zinc(II) binding and irradiation at an appropriate frequency, a partial positive charge is photo-generated adjacent to the cation. The resulting ICT state causes a change in absorption and excitation spectrum, and a cation-induced blue-shift can be expected as found in the case of dansylamide. However, the emission wavelength is less affected because the positive charge repulsion between the cation and nitrogen keep them apart. Consequently, the energy gap between the excited state and the ground state is less disturbed (PCT model in Fig. 4). Of the two compounds, ZnAF-R2 is more soluble and fluorescent in aqueous solution, so may be a better candidate for biological zinc(II) detection. Compared to other PET sensors mentioned in this section, the emission wavelength remains unchanged (both are 495 nm) and the fluorescence yield (ϕ) decreased from 0.17 to 0.10 (excited at its own λ_{ex}) upon zinc(II) bonding. However, a relatively larger shift of excitation wavelength was observed ($\lambda_{ex} = 365$ nm, $\lambda_{ex} = 335$ nm) upon complexation to zinc(II) ions, which suggests its potential use as a zinc(II) sensor. This probe is reported to be suitable for ratiometrical assays; however, its pH dependency is unknown. The ethyl ester of **24** (ZnAF-R2 EE) was prepared and tested for intracellular ionic zinc staining [93].

The dansyl group was also introduced as a fluorophore in the design of DPA-based probes. Compounds **25**, where the spacer length varies from $n = 2$–6 (5 is absent), have been systematically studied [94]. The spacer length is found to be an important factor for fluorescent performance. The best selectivity was achieved with a spacer length of $n = 3$ suggesting a possible involvement of the sulphonamide nitrogen in coordination. Therefore, **25** is also pH dependent. At pH above 7, the fluorescent responses of the apo-ligands and their zinc(II) complexes do not show a consistent variation with the increase of pH value, leading possibly to an error of ca. 10%. The biological applications of compound **25** as fluorescent probes have yet to be evaluated.

Obviously, all the DPA sensors are highly selective for zinc(II) and insensitive for Ca^{2+} or Mg^{2+} due to the coordinating nature of DPA. Extra coordination sites can increase their affinity for zinc(II) to a nanomolar (10^{-9} M) or subnanomolar (10^{-10} M) scale as proven by compounds **15**–**25**. However, their disadvantages such as pH dependency, background interference and non-ratiometrical ability should be overcome or minimized.

3.3. Acyclic and cyclic polyamines as zinc receptor

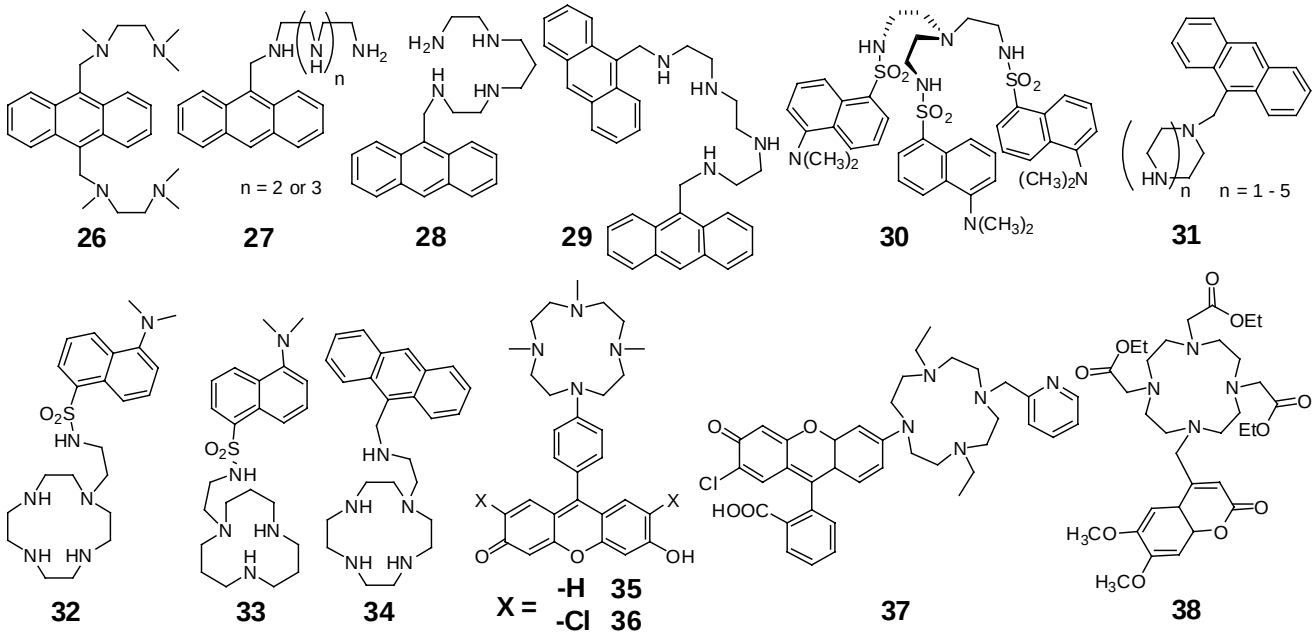
Polyamines are widely used as ionophores for zinc(II), because nitrogen donors exhibit a strong affinity for zinc. Moreover, the amino nitrogen atoms of polyamines are regarded to be good electron donors for adjacent fluorophores. Although these polyamin echaracteristics were well recognized a long time ago, their employment as chelators in zinc fluorescent sensing only started at the end of 80 s.

Czarnik and colleagues first combined two acyclic diamines with an anthracene fluorophore (**26**) for zinc sensing in CH_3CN [95]. Although the approach turned out to be unsuitable for biological zinc imaging due to the low affinity of the chelators to zinc(II), this preliminary trial of PET type fluorescent sensor suggested that “nitrogen-containing ligands with known specificities for certain ions may be utilized as fluorescent probes via a simple, flexible connection to a fluorescent compound” [95,96]. Thenceforth, various analogous, containing linear chain polyamine armed anthracene such as compound **27**, **28** and, etc., were synthesized as logic gates for transition metal ions [29,97–101]. A double anthracene-arm ligand (**29**) was also reported as an aqueous ratiometric fluorescent probe for zinc(II), which was supposed to be a PET-excimer based sensor [102]. It has been shown that Zn^{2+}/Cd^{2+} binding to the tetraamine ligands induces strong fluorescence enhancement while the binding of $Cu^{2+}/Ni^{2+}/Hg^{2+}$ induces fluorescence quenching [31]. These sensors show complicated solution behavior because of multi-protonation possibilities and low affinity to zinc(II) ion. zinc(II) complexes of variable stoichiometry (1:1, 2:1 or 2:2) may also be formed with these polyamine-based ligands [103], which hampers their potential application for zinc imaging under physiological environments (Table 3).

The dansyl group was also introduced into the polyamine fluorescent probes, also based on the PCT mechanism. Recently, a tri-dansylated branched polyamine (**30**) was reported for post-transition metal sensing [104,105]. Although this tripodal ligand was originally designed for copper(II) recognition, it forms a 1:1 complex upon zinc(II) binding accompanied by a blue-shift of 1480 cm^{-1} and a concomitant increase of fluorescence quantum yield (3.7-fold enhancement) in basic environments. The fluorescent properties of the probe in biological media remain to be elucidated (Scheme 10).

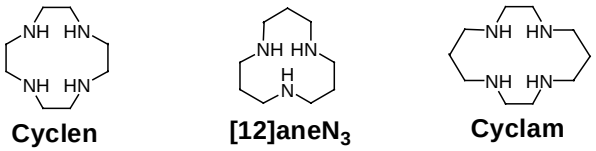
Table 3
Fluorescent properties of zinc complexes with polyamine-based ligands

Zinc complex (ligand)	λ_{ex} (nm)	λ_{em} (nm)	ϕ	K_d (μM)	Reference
26	335	427	–	–	[95]
29	335	495	–	–	[102]
30	326	500	–	–	[104]
31	335	416	–	–	[104]
			0.14 nM	pH 7.0	
32	323	528	0.11	0.55 pM	[107]
				pH 7.8	
33	320	538	0.19	0.9 μM pH 7.8	[112]
34	368	416	0.44	20 pM pH 7.4	[113]
35 (ZnACF-1)	495	515	–	~5.7	[114]
36 (ZnACF-2)	505	525	–	~5.7	[114]
37 (RF2)	510	539	0.56	13.5	[115]
38	345	448	0.26	<1	[118]



Scheme 10. Polyamine-based indicators.

In addition to the open-chain polyamine probes, macrocyclic polyamines with defined cavity size have been employed as ionophores for certain cations. A series of compounds (**31**) with different numbers of diamine subunits were compared by Akkaya et al. [106], among them a 1,4,7,10-tetraazacyclododecane (cyclen, Scheme 11) based compound which exhibited superior performance for zinc(II) recognition with ca. 14 time fluorescence enhancement in basic aqueous solution. However, a serious drawback for **30** is the protonation of the cyclic polyamine at neutral pH, which inhibits the PET process and results in strong fluorescence emission even in the absence of zinc(II).



Scheme 11. Cyclic polyamines as Zn(II) chelators.

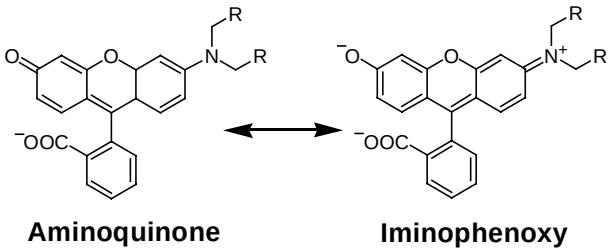
Since the radius of the divalent zinc ion fits the cavity of cyclen very well, the zinc(II)–cyclen complex is very stable and is used to mimic the active site zinc enzymes such as CA for comparable affinity [107]. In recent years, various substituted cyclen-type ligands have been synthesized [108] with great effort to incorporate a fluorophore group for signalling and to improve the selectivity for certain analytes. Kimura and colleagues conjugated a dansyl group to

aminoethylcyclen (**32**) [109,110], the synthetic procedure for which was later improved by Xue et al. [111]. X-ray crystallographic analysis reveals that zinc(II) coordinated to **32** in a distorted square-pyramidal geometry with the shortest Zn–N bond (1.97 Å) being that between zinc and the sulphonamide nitrogen, with other four Zn–N bonds of ca. 2.1 Å [109]. In aqueous solution, the formation constant of **32** for zinc(II) is pH dependent, being 0.14 nM at pH 7 and sub-picomolar level at pH 7.8, lower than that of a zinc-finger protein [112]. As previously discussed in dansyl amide model, this PCT exhibited a zinc(II)-induced blue-shift of the emission band from 582 nm in free ligand to 540 nm in zinc complex, a five-fold enhancement of fluorescence intensity, and a better selectivity for zinc(II) over Ca^{2+} or Mg^{2+}. However, the quantum yield of Zn (**32**) is only 0.11 and the excitation wavelength is far from the visible region (ca. 320 nm). These parameters are less than optimal for intracellular imaging because higher quantum yields and longer excitation wavelengths are desired [113].

As a comparison, the ionophore and fluorophore were replaced by the aminoethyl-[12]aneN_3 and the 9-methyl-anthracene group, respectively, to give two analogues **33** [114] and **34** [115]. Compound **33** showed a similar intensity enhancement (ca. 5.2-fold) and emission blue-shift (ca. 982 cm^{-1}) upon zinc(II) binding. However, the reduction of a coordinating nitrogen atom resulted in a significant decrease in affinity towards zinc(II) (0.9 μM at pH 7.8) [114]. Compound **34** showed a high affinity for zinc(II) compared with **32** and a strong fluorescence upon zinc(II) binding at nearly neural pH. It forms a stable 1:1 zinc(II) complex at pH greater than 6, allowing its application over a wider pH range [115]. Unfortunately, this probe also requires UV light for excitation.

To pursue visible excitation, two azacrownfluorone ligands ZnACF-1 (**35**) and ZnACF-2 (**36**) [116] were designed which adopt 6-hydroxy-9-phenylfluorone as fluorophore and a tetra-substituted cyclen as ionophore. Therefore, their affinity to zinc(II) was reduced compared with **32** and the detection threshold was ca. 500 nm due to the weaker binding ability of tertiary amino nitrogen donors rather than the secondary amines in **32**. As typical PET type sensors, compounds **35** and **36** showed a large fluorescence enhancement (14-fold for **35**, 26-fold for **36**) without significant emission band shift upon zinc(II) binding. Notably, owing to steric hindrance, these probes are kinetically very slow in metal ion binding (about 100 min is required for complete complexation) [90], which makes them unsuitable for real-time imaging in biological systems.

By employing a hybrid of fluorescein and rhodamine as fluorophore and a pyridine-appended cyclen as zinc(II) receptor, Lippard and co-worker developed a pH dependent fluorescent probe Rhodafluor-2 (RF-2, **37**) [117]. However, the penta-nitrogen coordination only gives a relatively low formation constant for zinc(II) with modest fluorescence enhancement (ca. 50% increase). The spectroscopic shift upon zinc(II) binding is negligible, which might be explained by



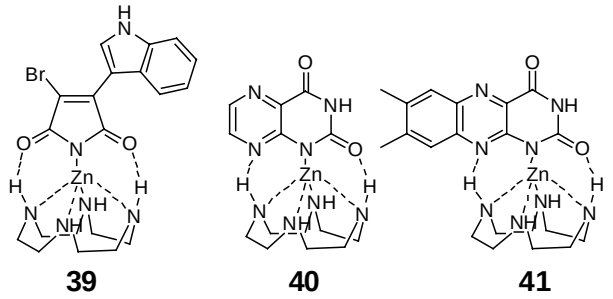
Scheme 12. Isomers of the hybrid fluorophore of fluorescein and rhodamine.

the predominant aminoquinone isomer of the fluorophore (Scheme 12). Therefore, the biological applications of this PET sensor will be largely limited by the strong background interference.

The coupling of a methylcoumarin group with DTPA forms another novel probe in this family (**38**) [118]. This PET sensor could be adopted for zinc imaging of live rat pituitary tumor cells. However, rather slow binding of **38** to zinc(II) was observed with a half life ($t_{1/2}$) longer than 60 min.

Some traditional fluorophores, such as 2-bromo-3-(1H-indol-3-yl-maleimide (**39**) [119], lumazin (**40**) and lumichrome (**41**) [120], have recently been shown to interact with zinc(II)–cyclen (Scheme 13) to form a tertiary cyclen-zinc(II)-fluorophore complex and induce a strong fluorescence enhancement. These fluorescent motifs have high selectivity for zinc(II) over other metal ions. Therefore, a combination of these mixed ligands might promise a potential fluorescent sensing reagent for zinc(II). Some special fluorescent ligands containing polyamine ionophores have been designed for zinc sensing. For example, a dendrimer with a cyclam (1,4,8,11-tetraazacyclotetradecane) core has been reported to exhibit a zinc(II)-induced fluorescence enhancement [121]. Bencini and co-workers reported several compounds with different phenanthroline containing macrocyclic polyamines as part of the ionophore [122,123]. The selectivity and other properties required for biological zinc imaging for these compounds remain unidentified.

As discussed above, the tetra-substitution of cyclen nitrogen results in relative low affinity to zinc(II), whereas with fewer than four *N*-cyclen substituents, there will be



Scheme 13. Tertiary zinc(II) complexes with cyclen and organic fluorophores.

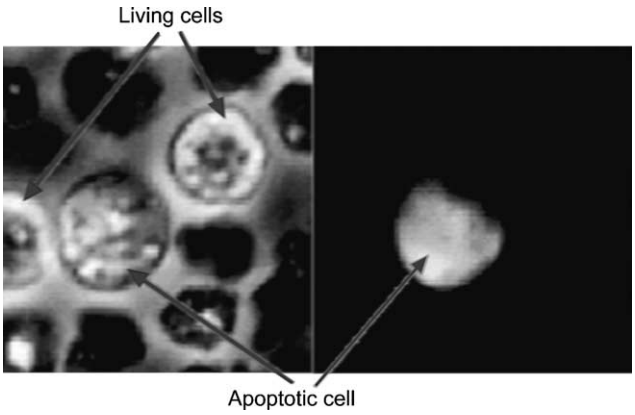


Fig. 9. Phase-contrast (left) and fluorescent (right) images of apoptotic HL60 human leukaemia cells stained with **32** (adapted from [125]).

several active hydrogen atoms, which will lead to complicated protonation equilibria and pH dependent fluorescence. As a result, although many fluorescent zinc(II)–polyamine complexes have been reported, they are rarely tested in biological systems aside from compound **31** which was recently used to stain tumor cells. Because of the significant increase of free zinc flux in the arly stages of apoptosis [124], apoptotic cells but not living cells can be selectively labeled with **31** (Fig. 9) [125].

3.4. Classical Ca^{2+} and Mg^{2+} fluorescent probes adopted for zinc(II) detection

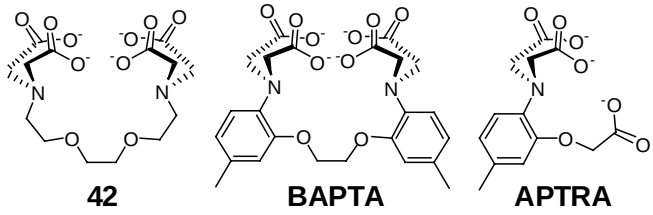
In the last few decades, the success of Ca^{2+} fluorescent indicators has led to the success of calcium neurophysiology [126]. In view of the comparability of calcium, magnesium (group IIA) and zinc (group IIB) in many aspects (Table 4), the chelators for Ca^{2+} or Mg^{2+} usually have a certain degree of affinity for zinc(II). In fact, some of them even showed higher affinity towards zinc(II) ions, therefore, TPEN is normally used to mask zinc(II) so as to avoid fluorescence interference [75].

As hard acids, Ca^{2+} and Mg^{2+} have a high affinity towards oxygen-containing moieties such as a carboxyl group. Therefore chelating agents, such as ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, 42, see Scheme 14) and its derivative bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), are widely used to determine Ca^{2+} [127]. The BAPTA moiety, specially designed for biological applications, is able to form an eight-coordinated cage such as the

Table 4
Divalent ionic diameter, coordination number of Ca, Mg and Zn[a]

M^{2+}	Ionic diameter (Å)	Coordination number
Ca^{2+}	1.98	7–9
Mg^{2+}	1.32	4–5
Zn^{2+}	1.48	4–6

[a] Data taken from [32].



Scheme 14. Schematic drawing of EGTA, BAPTA and APTRA.

Ca^{2+} complex shown in Scheme 14. Because Mg^{2+} is much smaller than Ca^{2+} and forms complexes with lower coordination number, an analogous chelating motif *o*-aminophenol-*N*,*N*,*O*-triacetic acid (APTRA) was developed as a specific receptor for Mg^{2+} [128]. As electron donors in these structural motifs, the tertiary aniline nitrogen atoms are able to contribute in either PCT or PET type fluorescent sensors.

Two representative PCT type calcium fluorescent probes Fura-2 (**43**) and Indo-1 (**44**) demonstrated a 100-fold higher affinity for zinc(II) than for Ca^{2+} [129,130]. Upon zinc(II) binding, **43** shows a blue-shift in excitation (from ca. 360 to 340 nm) based on the same mechanism as **23** and **24**, whereas **44** exhibits a blue-shift in emission (from ca. 480 to 400 nm) for a weaker PCT process [32]. Although fluorescent selectivity is rather poor [131,132], Fura-2 has been widely used to detect intracellular zinc(II) influx via voltage-gated Ca^{2+} channels [133] and to evaluate zinc(II) level in living neurons [79,134,135] and myocytes [136].

Magnesium probes Magfura-2 (furaptra, **45**) [137] and Magfura-5 (**46**) [138] have a high affinity to zinc(II) and are believed to be useful in zinc measurement. These two PCT type fluorescent ligands are structurally similar to Fura-2 with alteration of the ionophore from BAPTA to APTRA. Compound **45** exhibits slightly altered spectroscopic characteristics upon binding zinc(II), allowing zinc(II) to be measured in the presence of Ca^{2+} [139]. With similar fluorescent behavior and even lower affinity for Mg^{2+} or Ca^{2+}, **46** has been used to measure free zinc in living neurons [79,134,140]. Magnesium Green (**47**), a PET type probe for Mg^{2+} based on a similar ion-binding moiety [141], was reported to be sensitive enough to measure zinc(II) influx in human BOSC 23 cell [142].

Another moderate calcium probe BTC (**48**) shows an unexpected fluorescence enhancement (six-fold at the Ca^{2+} isosbestic point) in the presence of zinc(II), which suggested its potential as a zinc(II) indicator [131]. With similar fluorescent performance to **48**, two BTC derivatives BTC-5N (**49**) [143–145] and APTRA-BTC (**50**) [146] were developed and applied for the detection of zinc(II) at relatively high concentrations [47,79].

In order to achieve a higher selectivity of zinc(II) over Ca^{2+} or Mg^{2+}, novel probes **51**–**58** based on BAPTA or APTRA were designed by Molecular Probes [84]. Most of the species adopt half a BAPTA chelator as ionophore (except **55** and **57**), which leads to a reduction of “cage” size

Table 5
Fluorescent properties of zinc complexes with Ca^{2+} or Mg^{2+} fluorescent probes and the parallel ligands[a]

Zinc complex (ligand)	λ_{ex} (nm)	λ_{em} (nm)	K_d (μM)		Reference
			Zn^{2+}	Ca^{2+}	
43 (Fura-2)	340	510	2[b]	145[b]	[133]
44 (Indo-1)	330	401	0.16[b]	230[b]	[132]
45 (Mag-Fura-2)[c]	330	500	20[b]	25	[139]
46 (Mag-Fura-5)[c]	330	500	27[b]	20	[134]
47 (Mg Green)[c]	506	531	20[b]	6	[141]
48 (BTC)	400	525	20[b]	12	[131]
49 (BTC-5N)	459	517	210[b]	7	[47]
50 (APTRA-BTC)	380	530	1.4	–	[146]
51 (FuraZin-1)	330	510	3.4	40[d]	[84]
52 (IndoZin-1)	345	405	3.0	65[d]	[84]
53 (FluoZin-1)	491	520	7.8	8[d]	[84]
54 (FluoZin-2)	495	525	2.1	5[d]	[84]
55 (FluoZin-3)	494	516	15.0[b]	ND[e]	[84]
56 (RhodZin-1)	555	575	23	35[d]	[84]
57 (RhodZin-3)	545	575	65	–	[151]
58 (X-RhodZin)	575	604	11.0	15[d]	[84]

[a] Part of the spectra bands are taken from the similar Ca^{2+}-saturated fluorescence spectroscopic data.
[b] Unit: nM.
[c] $K_{d,\,Mg^{2+}}$ of magnesium probes: 1.9 mM for **43**, 2.6 mM for **44** and 1.0 mM for **45**, repectively.
[d] Unit: mM.
[e] ND: not-detectable.

and the number of donor atoms. This change induced a reduction of affinity by ca. three orders of magnitude for both Ca^{2+} and zinc(II) (Table 5). Similar to the parent ligands for Ca^{2+}, FuraZin and IndoZin series are all PCT type sensors, whereas the FluoZin and RhodZin series are PET type sensors with fluorescein and rhodamine as fluorophores, respectively. Upon zinc(II) binding, FuraZin-1 (**51**) exhibits a similar excitation blue-shift (from 378 to 330 nm) as Fura-2, while IndoZin-1 (**52**) shows a blue-shift of emission (from 480 to 395 nm) as Indo-1 [84]. The different behavior of these two PCT type ligands could be explained using a similar argument to that described for Fura-2 and Indo-1. Among the FluoZin series (**53**–**55**), compound **55** shows the highest selectivity and affinity (Table 5) for zinc(II), while calcium complexation does not result in a detectable response [84]. Structurally related to the calcium probes of Fluo-3 [147] and Fluo-4 [148], ligand **55** possesses a more BAPTA-like ionophore with the elimination of one of the four *N*-acetic acids. Consequently, seven donor atoms and a cage size between BAPTA and APTRA augment the affinity for zinc(II) but not for Ca^{2+}. Recently, this visible-light-excitable sensor has been applied for the imaging of zinc secretion from pancreatic β-cells [149,150]. Similarly, RhodZins, including RhodZin-1 (**56**), RhodZin-3 (**57**) [151] and X-RhodZin (**58**), also exhibit a large increase in fluorescence intensity with moderate affinity to zinc(II) [84].

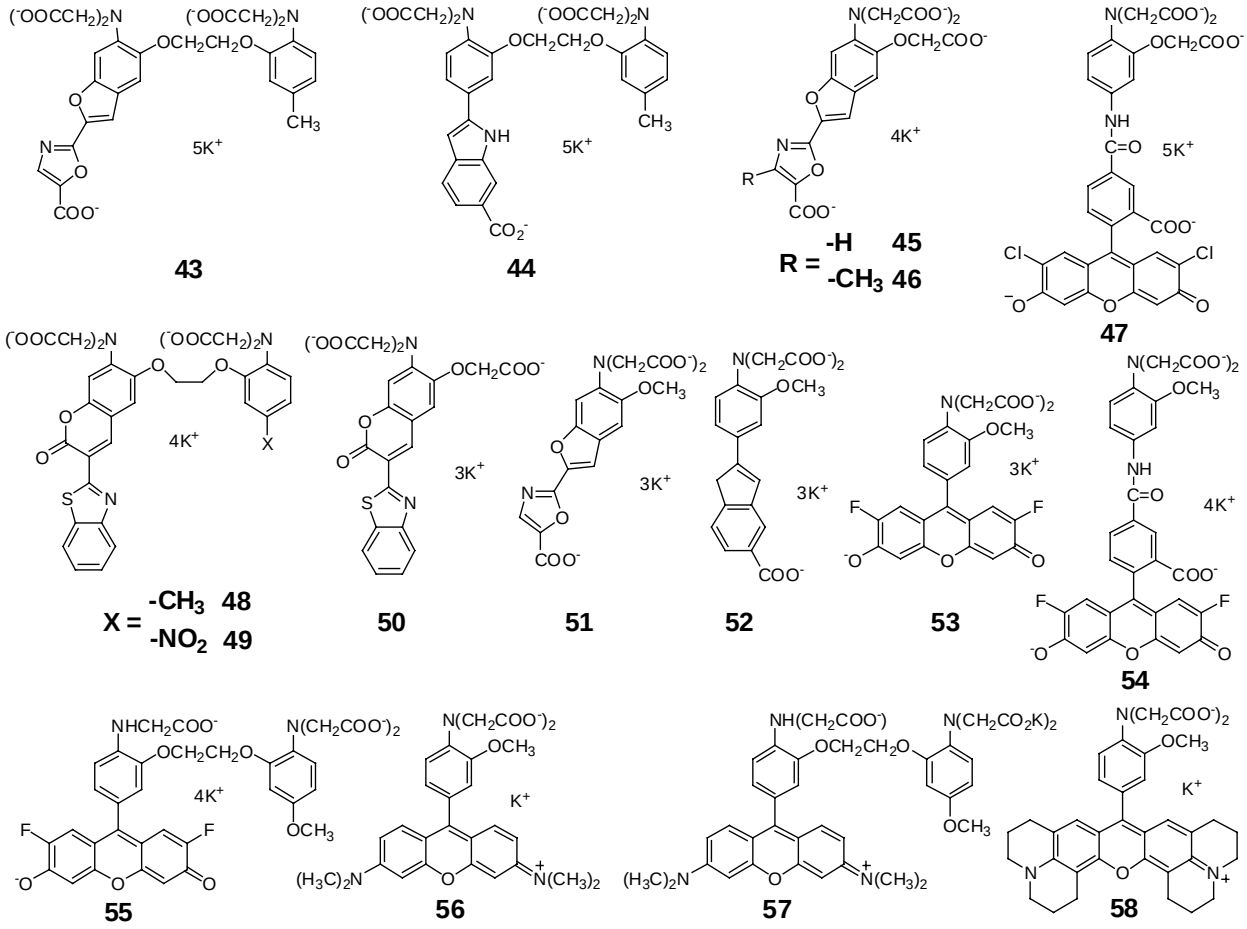
The AM forms of all these probes have been prepared for in vitro experiments. However, owing to the fluorescence interference by Ca^{2+} binding and the relatively low affinity for zinc(II), these probes are still insufficient for biological applications. Nevertheless, some of the novel zinc(II)-specific ligands showed improved fluorescent performance and could be suitable for detection of zinc(II) in the range of 1–100 nm.

3.5. Miscellaneous probes

There are many other types of fluorescent ligands that do not belong to the chemosensors discussed above. Here we mention some of the examples to illustrate the scope and potential of these probes.

Much effort has been expended to conjugate typical hosts such as crown ether, cyclodextrin, and calixarene with fluorophores to form luminescent ligands for zinc(II) sensing. Bradshaw and co-workers designed a series of double 8-AQ or 8-HQ derivatives and an azacrown skeleton, and compounds **59**–**62**, as UV-excitable fluorescent sensors for zinc(II) [152–154]. The two armed analogues of TSQ and Zinquin, **59a** and **59b**, can both form 1:1 or 2:1 (Zn:L) complexes with nanomolar affinity and show a large fluorescence enhancement (from $\phi = 0.004$ to 0.19 for Zn (**59b**)) upon zinc(II) binding [152]. Several oxine derivatives were condensed with different azacrowns [153–156], however, the specificity problem remains unsolved. For example, compound **57** could prevent fluorescent interference by Ca^{2+} or Mg^{2+} only in the pH range of 3–7 but accompanied by a linear decline of fluorescence intensity [153]. Compounds **61** and **62** exhibit 10-fold fluorescence enhancement compared to 8-HQ upon zinc(II) complexion, but the emission is largely blue-shifted at different stoichiometric ratios [154]. In addition to the crown ethers, other supramolecular scaffolds have also been introduced in this field. Recently, a porphyrin-β-cyclo-dextrin conjugate (**63**) has been tested to determine zinc(II) ion selectively based on a dual emission fluorescence ratio [157]. An anthracene appended calix[4]arene (**64**) was reported to give a fluorescent response towards zinc(II) in a mixed solution of acetonitrile and water [158]. However, the biological properties such as membrane-permeability of these ligands must be determined before further testing.

Pyridines and quinolines are good ligands for znic and can be employed in novel probe designs. Several fluorescent ligands bearing three pyridine groups (**65**–**68**) have been shown to be zinc(II)-specific. A BPAMP-like ligand **65**, provides four nitrogen donors to give a high affinity to zinc(II) [159]. A 30-fold fluorescence intensity increase ($\lambda_{ex} = 310$ nm, $\lambda_{em} = 378$ nm) can be achieved by **65** with zinc(II) complexation and exhibits a very high selectivity even against cadmium(II). Compound **66**, a terpyridine fluorescent ligand based on a PCT mechanism, provides a high fluorescent selectivity to zinc(II) over other metal ions and exhibits a large red-shift of emission (from free ligand 513 nm to zinc complex 642 nm) upon binding to zinc(II) in organic solvents [160]. A recent terpyridine ligand (**67**) also shows very similar fluorescent properties as its analogue [161]. Another tridentate pyridine **68**, has been reported to be zinc(II)-selective



Scheme 15. Schematic drawing of Ca^{2+} and Mg^{2+} fluorescent probes for zinc(II) detection.

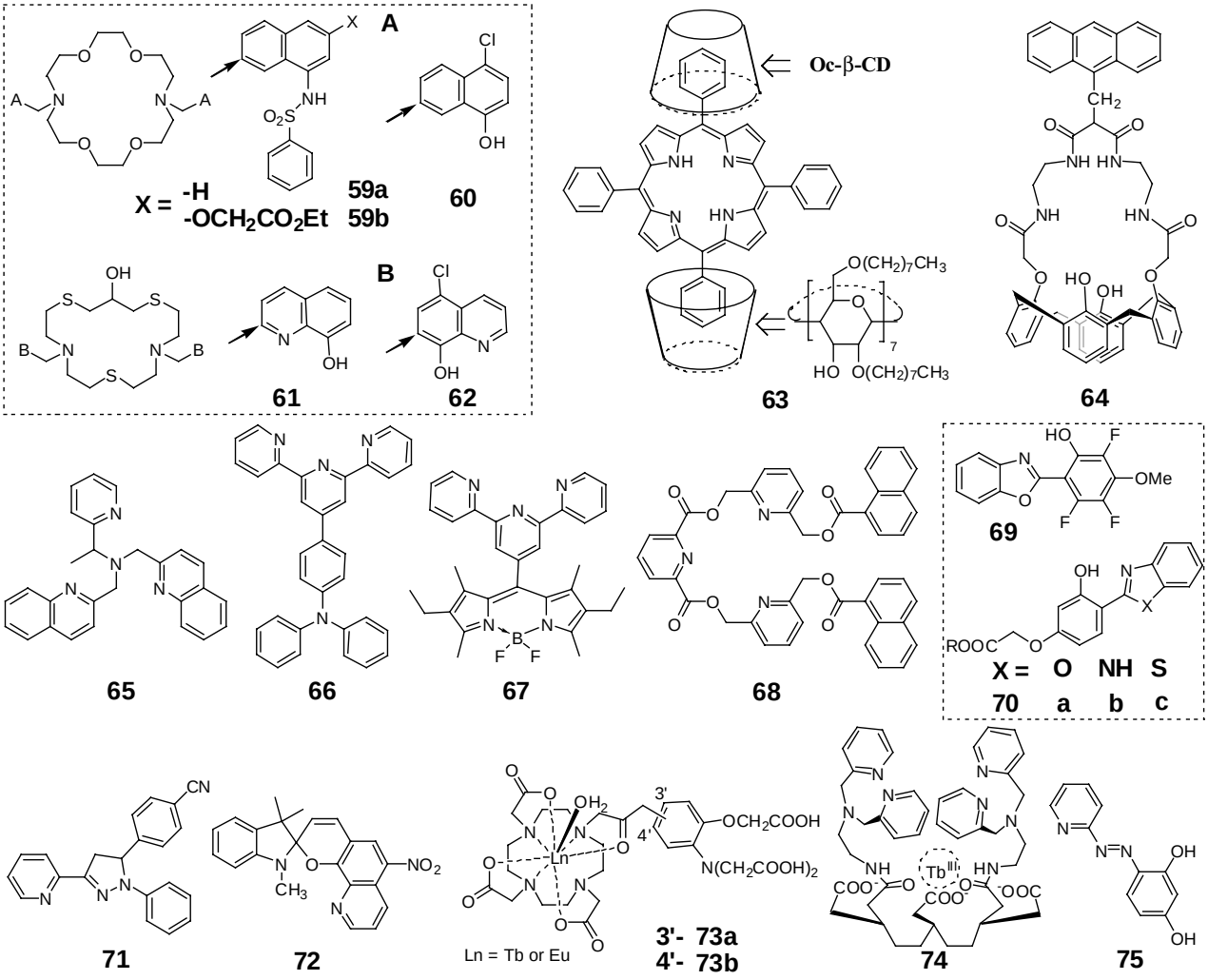
fluorescent chemosensor, based on an excimer effect. However, with an affinity of only 0.32 mM towards zinc(II), the compound may not be suitable for biological zinc detection [162] (Schemes 15 and 16).

Another representative fluorophore with zinc(II) and which involves heteroatoms is 2-hydroxy-phenyl-benzoxazole which fluoresces because of a metal-induced blockage of intramolecular proton transfer between the keto and enol tautomers (Scheme 17). A series of fluorescent ligands bearing this group or analogous groups (**69** [163], **70** [164]) have been developed for zinc(II) detection. Experimental data suggest that they form 1:1 complexes with zinc(II) accompanied by a significant fluorescence enhancement. However, the fluorescence of these ligands is very sensitive to solvent and changes in pH, and their affinity for zinc(II) is not high (117 μM for **70a** in aqueous solution) because only two donor atoms participate in coordination. Similarly, a pyridyl-pyrazoline derivative **71** was reported to be a novel zinc sensor in acetonitrile [165]. In addition, bipyridylol-based ligands may become another class of potential fluorescent chelating agents for zinc(II) [166].

In the presence of zinc(II), a spiro-pyrandinoline (**72**) [167] undergoes a reversible tautomerism equilibrium to give a strongly fluorescent [Zn(8-HQ)] moiety (Scheme 18). Although the solubility in aqueous solution is poor, its very high sensitivity (30 ppb) for zinc(II) in benzene provides a possibility to attach it to a fiber optic surface as the authors have suggested.

A series of lanthanide(III)–cyclen complexes attached through an APTRA chelator [Ln(**73**)] have been reported for zinc(II) signaling [168,169]. Upon zinc(II) addition, the [Tb(**73a**)] complex shows a fluorescence enhancement by 26% (λ_{ex} = 262 nm, λ_{em} = 545 nm) involving the PET mechanism. Complex **73a** shows a moderate affinity for zinc(II) (0.6 μM) with some degree of selectivity over Ca^{2+} and Mg^{2+} under physiologically relevant condition [168]. However, the short UV excitation wavelength obviously precludes the sensor from being used in biological systems. A similar design (**74**) has been accomplished by linking two DPA moieties to a popular MRI contrast agent Ln(III)–DTPA [170]. It is quite selective, but still requires a very short wave-length excitation (λ_{ex} = 260 nm, λ_{em} = 545 nm).

Although the selectivity towards zinc(II) is one of the most important parameters for fluorescent ligands to be of significant practical value, some less selective fluorescent chelators are occasionally used for studying biological zinc.

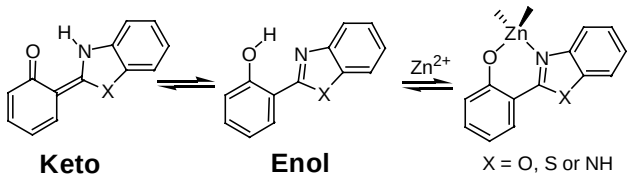


Scheme 16. Schematic drawing of miscellaneous chemosensors for zinc(II).

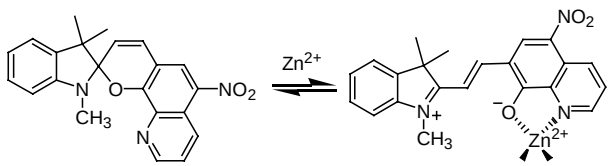
For example, 4-(2-pyridylazo)resorcinol (PAR, **75**), a commonly used dye for transition metals, can form a red adduct with zinc(II) and has been used for zinc(II) quantification [171–173].

Taken together, the chemosensors for biological zinc(II) developed over the last decade or so have shown their potential in revealing the nature of neural zinc. However, a great demand for effective sensors for general applications remains still to be met. Considering the feasibility for manipulation, histochemical staining will remain the most widely used method for zinc measurements in cellular samples [5]. Some experimental data should be interpreted with care because of the occurrence of artifacts, due to, for example, the interference of coordination competition from other biological species including metal ions and proteins [174].

A recently introduced nanocapsule technique, termed 'probe encapsulated by biologically localized embedding' [175], has been applied to zinc sensing [176]. In this method a polymer matrix with dye molecules was used to protect the chemosensors from complexation or digestion by protein and to protect cells from the cytotoxicity.



Scheme 17. Schematic illustration of keto and enol tautomers of 2-hydroxy-phenyl-benzoxazole.



Scheme 18. Tautomerism equilibrium of spiropyrandinoline.

4. Biosensors

4.1. Peptide sensors

Recent experimental data indicate that intracellular concentration of free zinc(II) ion can be as low as nanomolar level or even lower [30]. Although some chemosensors discussed in the last section can achieve this level of affinity, a greater selectivity and sensitivity is often required. Limited by their small size, low flexibility and insufficient screening, the specificity of chemosensors for zinc(II) could hardly be superior to that of the precise bioligands that were naturally selected through millions of generations. In fact, some wild type zinc proteins are able to achieve a picomolar or even femtomolar affinity for zinc(II).

Zinc-finger proteins with zinc binding motif Cys_xHis_y ($x = 2$–4, $y = 4 - x$) [177] have a high affinity towards zinc(II) (up to 10^{-12} M) [178]. Therefore, a synthetic peptide with zinc-finger consensus sequence (i.e. [F/Y]-X-C-$X_{2–4}$-C-X_3-F-X_5-L-X_2-H-$X_{3–5}$-H-$X_{2–6}$, here the amino acids are denoted as standard single character symbol and X means a certain variability [179]) might be adopted as a highly affinitive and selective ionophore for zinc(II) in fluorescent sensors. This idea has been considered by two independent groups.

Imperiali and co-worker first reported ZNS1 (FS03DNS, **76**) as a peptidyl fluorescent sensor for zinc(II) [180]. Compound **76** was constructed on the basis of a typical zinc-finger sequence with replacement of one "finger tip" amino acid by dansylated β-amino alanine. The dansyl group was employed to monitor the polarity change of microenvironment. Zinc(II) addition induces **76**-fold and embeds the fluorophore in a hydrophobic cage, which shields zinc(II) from polar solvent and causes the UV-excitable peptide sensor to shift emission from 560 to 525 nm.. A linear response of fluorescence intensity can be obtained in a zinc(II) concentration range of 0.1 to 1 μM. However, this Cys_2His_2 peptide is susceptible to oxidation through formation of an intramolecular disulfide bond and is thus incompatible with an aqueous environment containing oxidants such as oxygen and cupric ions. To enhance the oxidative stability of this family of sensors, two modified peptides FS04DNS (**77**) and FS02DNS (**78**) were synthesized [181] with an alternated coordination sphere of Cys_2AspHis for **77** and $CysHis_3$ for **73**. Though the enhanced oxidative stability was achieved, the affinity for zinc(II) was greatly sacrificed and lowered to a chemosensor level (Table 6). To reduce the size of previous sensors, two oxine-hybrid heptapeptides (**79a**, **79b**) were developed [182]. Although **79b** was found to suffer from rapid oxidation, **79a** showed an 8-HQ-resembling fluorogenic behavior upon zinc(II) addition at neutral pH, which suggested this probe as a proper design for further experiments. However, because only one cysteine and the 8-HQ unit may be involved in zinc(II) chelation, a very moderate (micromolar) affinity for zinc(II) is achieved. A recent development of this family of peptide probes is based on a newly synthesized artificial amino acid Sox (**80**) and a β-turn insertion [183]. Among the nineteen sequences that have been explored, the most prominent affinity towards Zn(II) can be as low as a tens of nanomolar magnitude.

Berg and co-worker have designed a similar peptide probe (**81**) based on zinc-finger consensus sequence [184]. In this peptide ligand, two strong fluorescent fluorophores, lissamine and fluorescein, and a Cys_2His_2 motif were employed and a picomolar level affinity for zinc(II) was achieved. Spectroscopic studies revealed that this visible-light-excitable sensor exhibits two distinct emission bands at 521 and 596 nm. Upon addition of zinc(II), the former band remains unchanged, whereas the latter gives a large fluorescence enhancement. As elucidated by the authors, this design was based on a FRET mechanism [185] (fluorescein as donor, lissamine as acceptor) and was interpreted as follows. Fluorescein ($\lambda_{abs} = 495$ nm) is excited at 430 nm and exhibits 521 nm emission. This fluorescence energy could intramolecularly excite the following lissamine ($\lambda_{abs} = 578$ nm), which leads to a longer wavelength emission at 596 nm. Upon zinc(II) chelation, the two fluorophores move closer as a consequence of zinc(II)-induced peptide folding and thus, the energy transfer efficiency can be enhanced. Compared to the original zinc(II) sensitive sequence, the affinity towards zinc(II) remained unchanged in the picomolar range. Like **76**, this peptide sensor is oxygen sensitive and should be handled under a N_2–H_2 atmosphere.

As noted in the above examples, a high affinity towards zinc(II) and a low oxidative stability are problems that need to be balanced in the design of zinc-finger-based peptide sensors.

Table 6
Fluorescent properties of zinc complexes with peptide-based ligands

Zinc complex (peptide)	Coordination sphere	λ_{ex} (nm)	λ_{em} (nm)	K_d (μM)	Reference
76 (ZNS1, FS03DNS)	Cys_2His_2	333	525	140[a]	[181]
77 (FS04DNS)	Cys_2AspHis	333	543	65[b]	[181]
78 (FS02DNS)	$CysHis_3$	333	548	3[b]	[181]
79a	Cys(8-HQ)	350	~500	17	[182]
80 (Sox)	8-HQ	360	500	–	[183]
81	Cys_2His_2	430	596	1[a]	[184]

[a] Unit: pM.
[b] Unit: nM.

4.2. Protein sensors

Protein-based sensors are more difficult to synthesize than peptides. Therefore, the design of novel zinc(II)-specific proteins relies mainly on protein engineering through gene expression [186]. The following two strategies have been adopted: (1) engineering a protein with appropriate zinc(II)-specificity and a signal-transduction fluorophore. Examples include biosensors based on carbonic anhydrase (CA) or maltose binding proteins (MBP); (2) identification of a protein with particularly well-behaved intrinsic signal-transduction function and well-constructed zinc(II) appropriate binding sites, such as green fluorescent protein (GFP) based sensors [187] (Scheme 19).

Pioneering work to incorporate bovine erythrocyte CA with dansylamide (DNSA) (**82**) was carried out in the 1960s [188] based on the fact that the aryl sulfonamides can inhibit CA [189]. Even though the affinity of **82** towards zinc(II) is moderate, zinc binding induces significant fluorescence enhancement (ϕ from 0.055 to 0.84) and a blue-shift in emission (4360 cm^{-1}). Kiefer et al. found that this fluorescent feature was quite different from that of apoCA and free DNSA, and therefore aryl sulphonamide fluorophores with apoCA were proposed for fluorometric detection of zinc(II) (Scheme 20) [190]. Crystallographic data revealed that in **82** three histidine imidazole ligands and DNSA were bound to zinc(II) (Fig. 10), namely the water molecule which functions as the nucleophile in hydrolysis was replaced by the sulphonamide [191]. The dansylamide fluorophore was surrounded by a hydrophobic pocket, which provides an explanation for the large change in fluorescence. Accompanied by

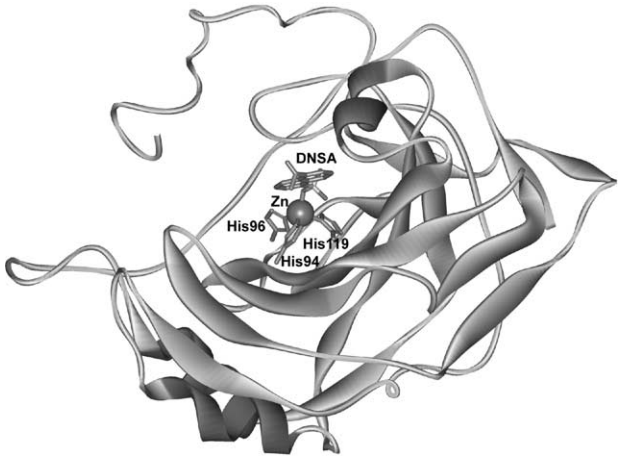
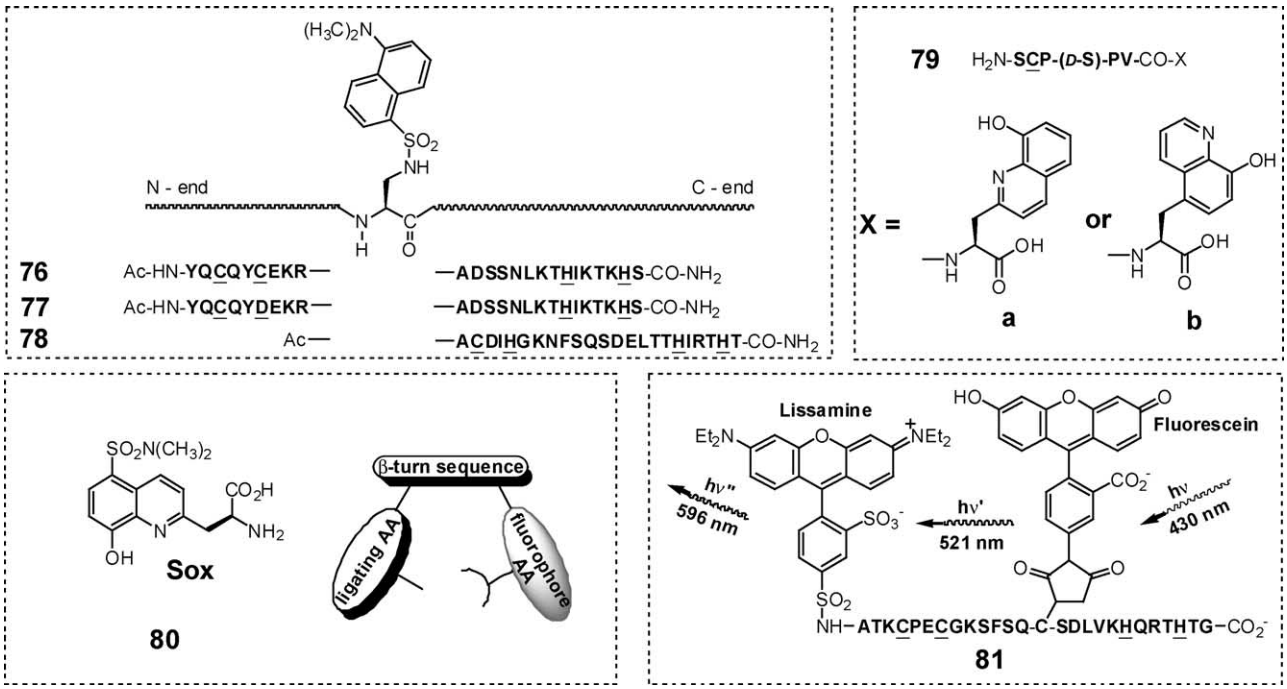
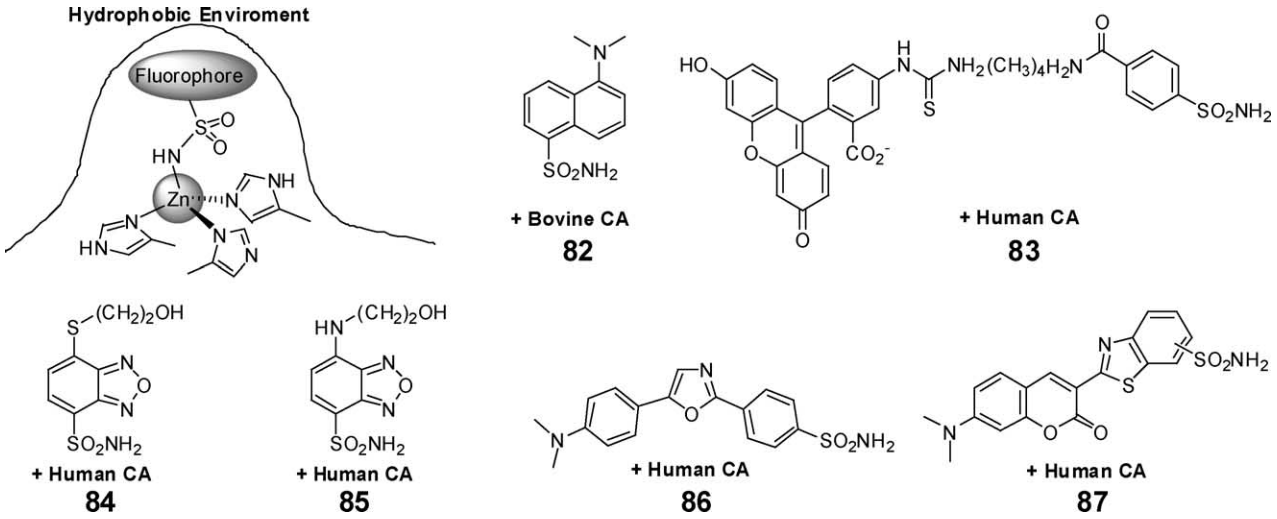


Fig. 10. Ribbon structure of zinc-loaded human CA II and DNSA in which zinc(II) is coordinated by three imidazoles (H94, H96, H119) and the sulphonamide [191] (data taken from Protein Bank).

the fluorescence enhancement, a change of fluorescence lifetime was also observed, which suggested another potential approach for the determination of zinc(II) [190,192]. A novel fluorescein-arylsulphoamide derivative (**83**) was synthesized [193], which demonstrated high affinity to zinc(II) and gives a 10-fold increase of fluorescent emission anisotropy upon CA binding because of the huge size and slow rotation of the proteins. (*Note*: *Emission anisotropy* is defined as the quotient of the difference between the vertical and horizontal polarized fluorescent intensity divided by the total fluorescence intensity.) The short excitation wavelength of the dansyl group has led to the development of ABD-M (**84**)



Scheme 19. Sequences and schematic structures of peptide sensors.



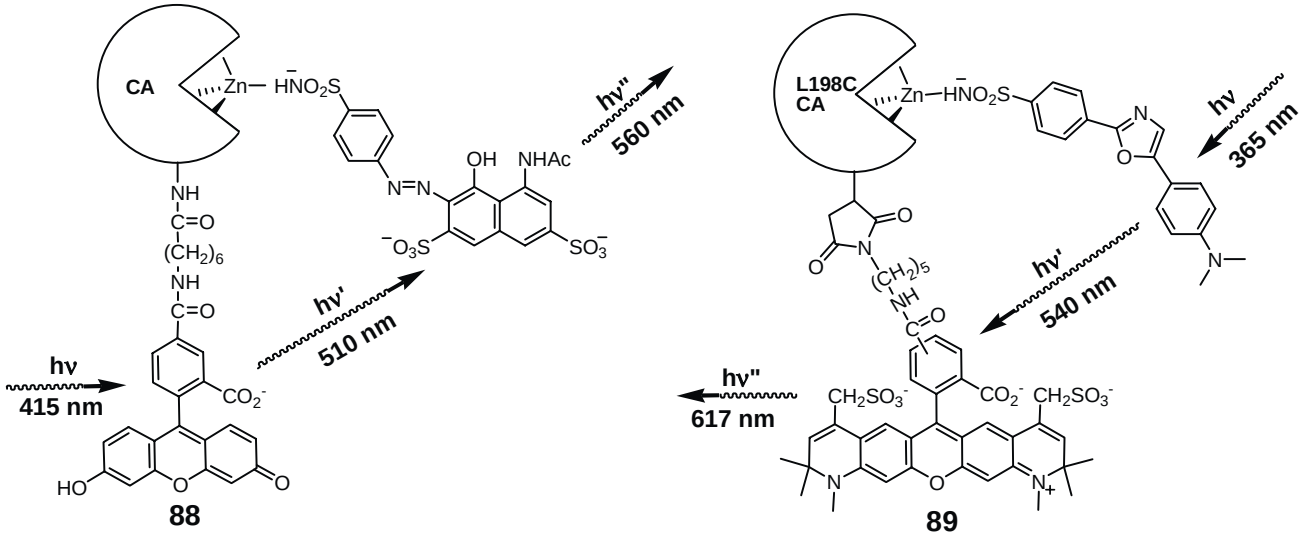
Scheme 20. Schematic illustrations of CA-based sensors.

Table 7
Fluorescent properties of sulphonamide fluorophores and their complexes with CA

Probe	Free/bound	λ_{ex} (nm)	l_{em} (nm)	τ (ns)	Aniso	K_d (μM)	Reference
82	F/B	330	560/450	3/22.1	–	0.25	[188,190]
83	F/B	495	505	4.0	0.01/0.10	0.002	[193]
84	F/B	390/380	528/492	0.8/1.53	0.03/0.32	0.3	[194]
85	F/B	435/420	602/558	0.34/4.98	0.09/0.23	0.9	[195]
86	F/B	365	615/535	0.22/3.60	–	0.13	[196]
87	F/B	466	504	2.53/2.71	0.05/0.25	–	[196]

[194] and ABD-N (**85**) [195] which have improved fluorescent properties and can be used to measure zinc(II) by the change in intensity, anisotropy or lifetime (Table 7). Successively, two other improved fluorophores with CA, dapoxyl sulfonamide (**86**) and BTCS (**87**), were developed and can be used for zinc(II) detection through a multiphoton excitation technique [196]. Upon binding to CA, compound **86** exhibits a dramatic increase and blue-shift in emission as well as a 20-fold increase of lifetime. However, the binding of CA increases five-fold the anisotropy of **87**. Therefore, compound **86** was suggested as a lifetime-based sensor and **87** as an anisotropy-based sensor.

A fluorescein-labeled CA with an azosulfamide fluorophore (**88**) was designed based on the FRET mechanism (Scheme 21) and used as a zinc(II) fluorescent sensor [197,198]. Upon zinc(II) complexation, an enhancement of



Scheme 21. Schematic illustrations of FRET-based protein sensors.

fluorescent intensity was achieved owing to the participation of azosulamide excited by energy transferred from fluorescein moiety [197]. Meanwhile, a decrease of fluorescent lifetime was also claimed being useful in zinc(II) detection [198]. Another FRET based system **89** has a detection limit of about 10 pM for zinc(II) employing sulphonamide as a donor instead of an acceptor in **88** [199]. Since the separate sulphonamide fluorophore and CA protein moieties have many different properties, it is difficult to achieve a synchronized signaling of zinc(II). Thompson and co-workers inserted a cysteine residue to replace certain amino acid residues in human CA II through gene mutation [200], and then covalently conjugated a thiol-reactive fluorophore, including ABD-F and ABD-T, to the CA protein variants [201,202]. No shift in emission or excitation of these fluorophore-integrated biosensors was observed due to only small changes occurring in the polarity of the fluorophore environment but the anisotropy and lifetime parameters could still be used to detect zinc(II). Recently, some of the CA-based biosensor systems have beenemployed for zinc imaging in the hippocampus [195]. However, their manipulation is much more complicated compared to that of the chemosensors.

Maltose binding protein (MBP), one of the periplasmic binding proteins from *Escherichia coli*, consists of a single polypeptide which is folded into two domains with a hinge connector. It can serve as a biosensor for certain analytes by the insertion of specific chelating residues [203]. Recently, an *N*-([2-(iodoacetoxy)ethyl]-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole [IANBD] labeled MBP has been converted into a zinc sensor by optimization of primary coordination sphere with an iterative approach called the DEZYMER algorithm [204]. The I329F mutant of MBP exhibits a high affinity for zinc(II) (0.35 μM) with the optimized binding site of His_2Glu_2 ($H63_{II}$, $H66_{II}$, $E144_I$, $E340_I$). This method is a step forward in the design of novel biosensors for zinc(II) and can be adapted for more complicated bioligands.

An intrinsic fluorescent protein GFP, originally isolated from the jellyfish *Aequorea victoria*, was first developed for calcium sensing by Tsien and co-workers through expression inside cells with the recombinant DNA technique [205]. A fusion protein consisting of a human type IIa metallothionein (hMTIIa) sandwiched between GFP variants, enhanced both the cyan fluorescent protein (ECFP) and the yellow fluorescent protein (EYFP), and has been used for nitric oxide signaling [206]. The two GFP variants act as donor (ECFP) and acceptor (EYFP), respectively, in a FRET process. Zinc chelation to the hMTIIa hinge induced the two GFP domains to fold more closely and exhibited enhanced fluorescent intensity. Jensen et al. constructed another fusion protein consisting of a GFP pair and inserted a multidentate zinc-binding site to the external surface of GFPβ-barrel [207]. Although these designs only offer millimolar (10^{-3} M) affinity for zinc(II), the design strategy may have a promising future in view of the success of analogous calcium biosensors [27,126]. Barondeau et al. designed and crystallographically characterized a series of GFP mutants with a porphyrin-like tridentate metal binding site (BFPms1) [208], which exhibit a fluorescence enhancement upon zinc(II) binding but a fluorescence quenching upon copper(II) binding owing to their differences in coordination geometry. Although the proteins have lower selectivity for zinc(II) than for copper(II), this work provided a prototype suitable for further evolution of metalloproteins with desirable properties.

Immunosensors, another major class of protein sensor [209], have been tested in zinc(II) detection [37]. Benkovic and co-workers created a CA-like zinc-binding site into antibody 43C9 and two mutants were obtained [210]. Unexpectedly, zinc(II) binding leads to an efficient quench of the antibody's fluorescence.

Many other zinc(II)-specific proteins such as Zur [3], ZntR [211,212], SmtB [213–216] and zinc(II) chelating peptides [217] are expected to be potentially applied for zinc detection if they are labeled by an appropriate fluorophore.

4.3. Nucleic acid sensors

Nucleic acids represent an emerging class of metal ion sensor [218,219]. A major advantage of nucleic acid sensors is that nucleic acids that can specifically recognize target analytes such as zinc(II) can be obtained through selective amplification from a large (up to 10^{15}) nucleic acid library without prior knowledge of how an effective sensor can be designed [220–224]. The resulting nucleic acids are called aptamers, if they recognize the target analyte, or called DNA/RNAzymes, if the resulting analyte recognition also helps catalyze chemical reactions. A number of studies have now shown that the aptamers and DNA/RNAzyme can often match the antibodies in the broad range of analytes they can recognize, and sometimes with a high affinity [209,225]. Several features make them excellent platforms for making sensors. First, the selection is in vitro and thus less time- and fund-consuming. Second, unlike antibodies, most aptamers, DNA/RNAzyme or aptazymes can be denatured and renatured many times without losing binding ability or activity. They can be used and stored under rather harsh conditions. Finally, the sensors can often be designed with minimum knowledge of the three-dimensional structures of the DNA/RNA molecules.

In the last few years, both aptamers [226–228] and DNA/RNAzymes [229,230] have been isolated in the presence of zinc(II). Although the selection process can result in nucleic acids with selectivity toward other metal ions, a "negative selection" strategy was employed to improve metal ion specificity (Fig. 11). The principle of using dual-labeled fluorophores in DNAzymesas FRET sensors has been demonstrated [231] and should be readily applicable for sensing zinc(II) if zinc(II)-specific DNA/RNAzymes are obtained. Interestingly a trifluorophore-labeled DNAzyme was found to undergo a two-step folding process in the

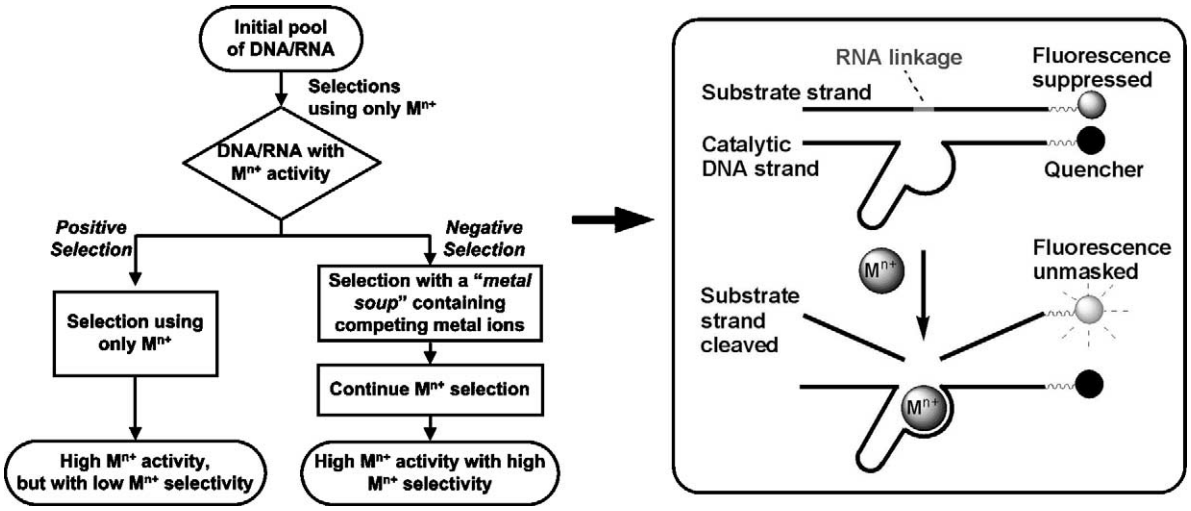


Fig. 11. In vitro selection of metal ion specific DNAzymes and their applications in fluorescent biosensors (adapted from [218]).

presence of zinc(II) through FRET fluorescence studies and may hold promise as a reversible fluorescent zinc sensor [232].

To sum up, the zinc(II) biosensors discussed in this section often show a better selectivity and higher affinity to zinc(II) than the chemosensors. However, their low stability (susceptible to oxidation or protease digestion) and the inconvenience in preparation, storage and in vivo transference are current problems to be solved before practical applications can be considered.

5. Concluding remarks

From the late 1980s, many zinc(II) fluorescent probes including chemosensors and biosensors have been synthesized. They fluoresce based on an electron transfer, charge transfer or energy transfer mechanism. Many probes exhibit higher selectivity and sensitivity over other biological essential metal ions in specific ranges of concentration. In chemosensors, the zinc(II)-specific chelating groups are limited to several moieties such as 8-substituted quinolines, DPA, cyclen, etc.; with respect to the biosensors, the bioligand scaffolds are also restricted to only a few proteins such as CA, MBP, GFP and so on. To date, some probes such as TSQ, Zinquin and Newport Green have successfully been used to elucidate the roles of biological zinc(II), especially in neural systems. In general, small chemosensors demonstrate a moderate specificity and sensitivity, while large biosensors often show higher selectivity although the latter are less widely used. It is now widely recognized that zinc(II) is important element after calcium in neurophysiology. The fluorescent method provides the basis for real-time monitoring of the zinc(II). Novel zinc sensors with high affinity (picomolar or even femtomolar level) and a large linear responsive range (from millimolar to picomolar level) are highly desired.

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