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Zinc complexes as fluorescent chemosensors for nucleic acids: new perspectives for a "boring" element

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Zinc(II) complexes are effective and selective nucleic acid-binders and strongly fluorescent molecules in the low energy range, from the visible to the near infrared. These two properties have been often exploited to quantitatively detect nucleic acids in biological samples, in both *in vitro* and *in vivo* models. In particular, the fluorescent emission of several zinc(II) complexes is drastically enhanced or quenched by the binding to nucleic acids and/or upon visible light exposure, in a different fashion in bulk solution and when bound to DNA. The twofold objective of this perspective is 1) to review recent utilisations of zinc(II) complexes as selective fluorescent probes for nucleic acids and 2) to highlight their novel potential applications as diagnostic tools based on their photophysical properties.

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Anna Maria Almerico graduated in chemistry with full marks and honours in 1977 at the University of Palermo. During her academic career, she has been visiting Professor at the School of Chemical Sciences, University of East Anglia (UK, 1983–1984), at the College of Pharmacy – Ohio State University (USA, 1986–1987) and at Masaryk University – Brno (Czech Republic, 1993). Currently she is Full Professor of Medicinal Chemistry in the Faculty of Pharmacy – University of Palermo. Her scientific interests are mainly devoted to the design and synthesis of new nitrogen heterocycles of biological interest. In particular, her most recent studies are devoted to the preparation and evaluation of the biological properties of compounds related to well-known anticancer/antiviral/antiparasitic drugs.

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1. Introduction

Metal complexes bind to nucleic acids through covalent and non-covalent interactions.[1–4] Furthermore, they often display photophysical properties that can be exploited for the design of diagnostic probes.[1,5,6] Moreover they undergo redox reactions with DNA or generate reactive oxygen containing species a property principally exploited in photodynamic therapy (PDT).[1,7–9]

Among the photophysical properties, fluorescence is the one most often exploited and metal complexes have been successfully used to selectively localize biological macromolecules in living cells and whole animals, in particular by imaging techniques. These latter have been mainly developed and applied for proteins,[10] and only rarely

and recently for nucleic acids.[11] Nevertheless, knowledge of the amount and kind of nucleic acids, e.g. DNA, RNA, and their constituents AMP, ATP, etc., can be important for monitoring of biological processes in cells and for the diagnosis of genetic diseases. In addition, if accompanied by a high binding affinity for nucleic acids, as many metal complexes show, such probes can be used as diagnostic tools for targeting and localising cancer cells in biological samples.

To be used as highly responsive and selective diagnostic fluorescent probe, the molecule-nucleic acid binding is usually followed by a higher fluorescence quantum yield and longer lifetimes in the excited state, compared to those of the free molecule. Low frequency excitation and emission wavelengths are preferred properties, allowing the penetration of tissues with low damage.[12–16] Many investigations on the interaction of fluorescent molecules with DNA have been carried out using either fluorescence quenching or enhancing mechanism.[14,15] Because of their stability in water solution under physiological conditions and of their peculiar photophysical properties, platinum and ruthenium complexes have been among the most employed fluorescent probes (with potential medical applications), exploiting their binding capabilities to DNA and to other nucleic acids.[17–24] In this field, a great contribution has been given by Barton and collaborators.[4,25–28] For example, it has been reported that ruthenium(II) complexes of polycyclic chelating ligands bind to DNA with a high affinity and, interestingly, they display intense metal mediated luminescence on the addition of DNA, whereas luminescence of the unbound complex is quenched. This phenomenon, which has become known as the DNA ‘‘light switch’’ effect, has been used extensively to study the interaction of a wide variety of metal polypyridyl complexes with DNA, as the binding process can be directly monitored through fluorescence spectroscopy.[4,25] However, in our opinion, limits of the application of ruthenium and platinum compounds can be ascribed to the fact that they are toxic and not normally present in living organisms. On the other hand zinc is a trace element which is essential for all living organisms[29–33] and, because of its higher bioavailability in comparison with other trace elements, it is involved in several catalytic, structural and regulatory functions.[34–37] In particular, zinc(II) is present in thousands of metalloproteins in humans, a number that exceeds that of any other metal cation, with a main role in the stabilization of their tertiary structure. Noteworthy, “Zn-finger” domains of proteins contain zinc(II) ions, coordinated by cysteine and histidine residues in a tetrahedral geometry,[38] are responsible of the interaction of these ones with other proteins and with DNA, RNA and lipids.[38,39] They are involved in DNA base recognition, allowing for specificity to a DNA sequence.[40] Despite all this, zinc has been defined as a “boring element” in the wet-laboratory,[32,33] because, together with many of its complexes, it is colourless and does not possesses exciting magnetic and redox properties. Nevertheless, according to Vahrenkamp, the “non-properties” of zinc could be the secret of its success in nature.[33]

Zinc(II) shows borderline hard–soft acid–base properties and its coordination number is typically four but easily expandable to five and six, with geometry ranging from tetrahedral to trigonal bipyramidal, square pyramidal and octahedral. Such a property allows tetra and pentacoordinated zinc(II) complexes to form additional stable bonds with the different O and N donor atoms of nucleic acids, of both the phosphate groups and the nitrogen bases. Another advantage is that zinc(II) is redox inert under biologically conditions, avoiding the production of reactive oxygen species.[32,33,41] It has been shown that zinc(II) is able to form selective and with high affinity complexes with several fluorescent ligands or metalloligands, which are stable and characterised by a fluorescence with higher quantum yield compared to that to the zinc-free ligands.[42–49] For this reason these ligands have been widely used to selectively detect traces of zinc(II) in several biological and environmental specimens, in nanomolar concentrations at physiological pH.[42] The stronger fluorescence of the zinc complexes, compared to those of their free ligands, is essentially attributable to the conformational rigidity imposed to the fluorescent ligands by the coordinated metal ion, precluding energy loss via bond vibration. The fluorescence decay of zinc(II) complexes usually comes from ligand-centred excited states because, being its external electron configuration the electronically saturated d^{10}, zinc(II) cannot be involved in ligand charge transfer to metal or in metal-centred transitions.[50] For the same reason, for example, zinc(II) also inhibits photoinduced electron transfer (PET) quenching pathways in its complexes of benzylic amines.[46] Intriguingly, Schiff bases zinc(II) complexes are highly efficient electroluminescent molecules that have been proposed as novel light emitting diode (LED) materials, to enrich the chemistry of full color red, green and blue (RGB) devices.[30] Finally, the strong fluorescence of zinc(II) salphen-like complexes can be quenched by PET through the new bonds formed with nitroalkanes and nitroaromatic compounds.[51,52] This indicates that the interaction with visible light generates a highly reducing excited state that leads to a ligand centred mono- or di-oxidated zinc(II) complex.[53] In our opinion, this latter phenomenon, depending on the structural and electronic properties of the ligands, is an extremely important property of zinc(II) complexes, still unexplored in the literature, that could open the way to new diagnostic applications in the near future. In fact, the irradiation with visible light of metal complexes in biological specimens, has been exploiting photophysical and/or photochemical properties, with diagnostic or therapeutic purposes, only for platinum and ruthenium complexes.[1,7]

This perspective focuses on the interaction of three categories of zinc(II) complexes with nucleic acids, that can be exploited for the selective and non invasive quantitative analysis of nucleic acids in biological samples, both *in vitro* and *in vivo*. In sections 2-4 the structure and properties as nucleic acid chemosensors of zinc(II) complexes of bis(2-pyridylmethyl)amine, or dipicolylamine (DPA), of macrocyclic tetradentate ligands and of N,N’-ethylene-bis(salicylideneimine) (salen) derivatives, respectively, are reviewed and their peculiar and promising properties highlighted and discussed.

2. Zinc(II) complexes of DPA ligands

Zinc(II) complexes of DPA ligands, see Fig. 1, are among the most commonly employed probes for anion sensing of double-stranded and single-stranded DNA, RNA and their hydrolysis products 3'-phosphate or 5'-phosphate nucleotides, including ATP, AMP, up to pyrophosphate ($P_2O_7^{4-}$),[5,54] cleavage products of polynucleotides after biochemical, chemical or photochemical oxidation. DPA ligands exhibit good selectivity for the zinc ion, which is tricoordinated by DPA and extends its coordination number to five or six by the coordination of an intraligand oxygen atom (such as in complexes **2**-**6**) and/or by labile water or chloride ligands in solution.[54] The solubility in aqueous solution of such complexes is essentially driven by their charges, induced by the presence of the zinc cation and and ranging from +1 to +8 for compounds **1**-**6**. The vacant coordination sites on the metal ion can be filled by the phosphate oxygen atoms of the anionic nucleic acids.[5,55] In fact, zinc(II)-DPA complexes typically show high selectivity for phosphates over other anions in aqueous solution. Such zinc(II) complex chemosensors for anion recognition have been designed with a conventional receptor-linker-fluorophore approach, which consists of three components: a zinc(II)-coordinated DPA ligand (the anion receptor), a signalling unit (the fluorophore) and a linker which covalently connects the binding and signalling moieties, i.e. fluorescent molecules.[56] The sensing mechanism relies on the change in the photophysical properties of the signalling moiety induced by the change in coordination of the zinc(II) center as a result of the strong anion binding. This change in fluorescence is proportional to the strength of zinc(II)-phosphate coordination, allowing direct quantification of the binding constants.[56] Interestingly, all of the zinc(II)-DPA chemosensors for nucleic acids show excitation wavelengths and fluorescence emission in the visible range.

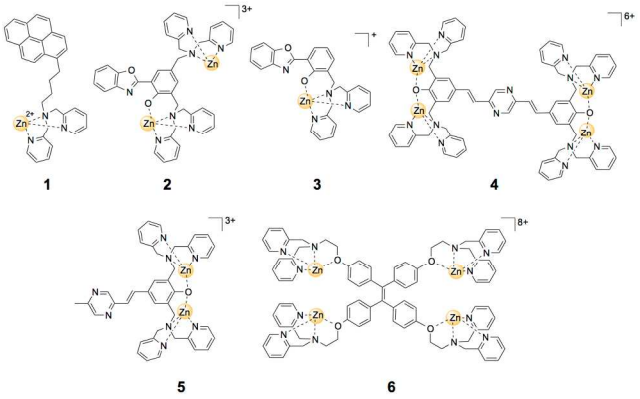

Fig. 1 Structures of zinc(II)-DPA complexes.[54,56–60]

For example, compound **1**, a fluorescent sensor bearing a pyrene substituent, shows selective detection for pyrophosphate in an aqueous solution via excimer emission through self-assembly.[57] In other words, two complexes **1** bind one pyrophosphate anion, exploiting a sandwich-type π-π stacking interaction of the pyrene groups, with a remarkable enhancement of the emission intensity. A similar but much less effective response was also observed upon binding to ATP. In detail, while less than 2 equivalents of pyrophosphate are enough to clearly show the excimer peaks, more than 10 equivalents of ATP are needed to produce a still weaker response.[57]

Most of the sensors developed for simple anions derived from nucleic acids, such as pyrophosphate and ATP, are based on binuclear zinc complexes, such as **2**, where two zinc centres are positioned at a certain distance in order to provide improved selectivity.[5,61,62] Such compound undergoes an unusually large spectral shift when zinc binds to pyrophosphate, with a blue emission shifted by about 100 nm toward lower frequencies, by turning on an excited state intramolecular proton transfer (ESIPT) from the pyrophosphate to the phenoxide oxygen.[58,63] On the other hand, when the phenoxide oxygen coordinates the zinc(II) ion ESIPT is disabled. Intriguingly, compound **3**, an analogue of **2** with only one zinc(II) site coordinated by a phenoxide oxygen,[54] responds selectively also to ADP with a bathochromic shift of about 60 nm. For the recognition of ADP, it has been proposed that ESIPT is supported by a π-π interaction between the 1,3-benzoxazole group and adenosine. In that way the phenoxide oxygen is separated from the zinc, with its consequent protonation and ESIPT turn-on event. Compound **4**, presenting four zinc(II)-DPA receptors and two bridging phenoxide oxygens, linked by a 2,5-diethylene-pyrazine group as fluorophore, shows enhanced fluorescence intensity by interacting with native DNA, denaturated DNA and RNA, due to the highly hydrophobic environment when it bound such macromolecules via intercalation mechanism.[59,64] The emission intensity increase is proportional to the nucleic acid concentration, and **4** was successfully used to determine the concentration of native DNA with a nanomolar detection limit. On the other hand, the recognition of ATP or pyrophosphate leads to fluorescence quenching due to the fact that zinc ion's ability to inhibit the PET process is weakened by electrostatic interactions.[59]

The fluorescence of compound **5**, similar but smaller in size than **4**, is significantly enhanced compared to that of the zinc-free ligand, while the binding to native DNA, by external electrostatic interaction, gave only rise to high fluorescence enhancement.[60] In a recent and interesting paper, it hase been demonstrated that probes based on coordination interactions with nucleic acids exhibited much higher sensitivity.[56] For example, **6**, a zinc(II)-DPA complex bearing a tetraphenylethene group, was developed to coordinate the zinc ion both to the oxygen atoms of phosphodiester backbone and to the donor atoms of the nitrogen bases of single-stranded DNA as well. This kind of ligands are highly emissive upon aggregation, because the non-radiative pathway is blocked by restrictions in the intramolecular rotations, following zinc-DNA binding.[56,65] The latter phenomenon is known as aggregation-induced emission (AIE).

3. Zinc(II) complexes of macrocyclic tetradentate ligands

Several zinc(II) complexes of macrocyclic ligands, typically presenting four amine or imine nitrogen donor atoms (see e.g. Fig. 2), have been used as probes for specific DNA or RNA secondary structures. The dicationic zinc(II) complex of

1,4,7,10-tetraazacyclododecane (cyclen), **7**, and its derivatives, have been proposed by Kimura and coworkers,[66] and by Morrow and coworkers,[41] for the selective molecular recognition of DNA and RNA oligomers and polymers, in single stranded, double stranded and non-canonical conformations, including cancer related structures named G-quadruplexes (G4s). These are G-rich sequences capable of forming 4-stranded structures organized in stacked guanine tetrads connected by looping DNA bases. They are enriched at chromosome telomeres, where they inhibit the activity of the telomerase, but are also found in promoter regions of a number of genes with important functions in transcriptional regulation.[67–69] The use of metal complexes as probes for such structures is particularly intriguing, considering the low abundance of G4s in cells together with the relatively homogeneous physicochemical properties of DNA and RNA molecules.[11]

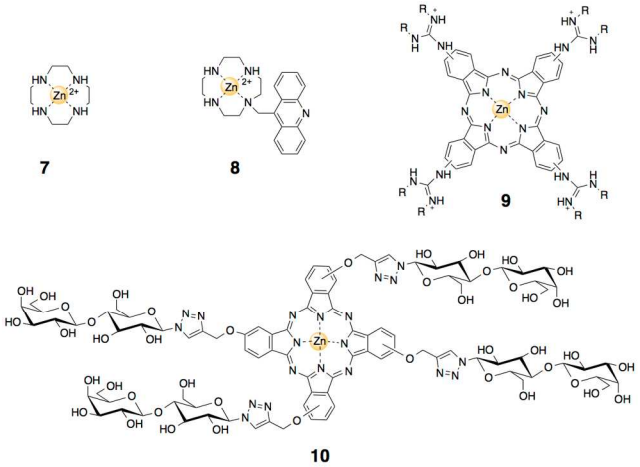

Fig. 2 Structure of zinc(II) complexes of macrocyclic N_4-tetradentate ligands.[41,66,70–72]

In cyclen complexes, the zinc(II) ion adopts a square pyramidal coordination geometry in solution, with the four donor nitrogen atoms of the ligand in the square plane and the apical position occupied by water or by nitrogen or oxygen donor atoms of ancillary ligands or of the nucleic acids. The presence of the positive charge favours the electrostatic attraction with the negative charge of the sugar-phosphate backbone, but the largest contribution to selectivity comes from the covalent bond formed between Zn and the N3 of thymine or uracil.[73] The attachment of aromatic pendent groups to the cyclen ligand, such as in compound **8**, considerably increases the fluorescence quantum yield of the zinc complexes,[74] and the binding affinity of the zinc(II) complex to uracil or thymine bases.[41,73] In particular, while the zinc(II) ion is coordinated by the N3 atom of thymine or uracil, the aromatic group can interact by π-π stacking with a nitrogen base of the nucleic acid, either by intercalation into double-helical DNA or by top stacking, for example over the G-tetrads of G4s. Such a bimodal and synergistic interaction was detected, for instance, in the binding to B-DNA of the zinc(II) complex **8**, showing a pendent acridine group.[70] Interestingly, it has been reported that multinuclear zinc(II)-cyclen complexes are also catalysts for the hydrolytic cleavage of RNA, (see ref. 41 and references therein), a known property of metalloenzymes with zinc(II) in the active site.[32] Notably, the fluorescence properties of aromatic pendent moieties of zinc(II) cyclen complexes allows for direct monitoring of the DNA binding. It is to be hoped that such class of molecules will be more extensively applied for the detection of specific isoforms of nucleic acids.

Another interesting class of macrocyclic tetradentate zinc(II) complexes, used as fluorescent probes for the detection of G4-DNA structures, is constituted by zinc(II) complexes of phthalocyanine derivatives.[11] Planar macrocycles, such as porphyrins, phthalocyanines and related derivatives, show excellent shape complementarity with the terminal G-tetrads of G4s. In fact, such molecules typically exhibit large binding constants, with 1:1 or 2:1 binding stoichiometries with G4-DNA.[11] Zinc phthalocyanine complexes are particularly interesting because of their long triplet lifetimes and high fluorescent quantum yields.[8] Although the binding constants of metal free cationic phthalocyanines toward G4s are generally already high, the presence of a zinc(II) ion considerably increases the fluorescence quantum yield of the complex, in particular following DNA-binding. Noticeably, the zinc(II) complex **9**, of a guanidinium-modified phthalocyanine, with R=isopropyl and bearing a positive charge +4, is one of the most promising fluorescence probes for G4-DNA. In fact, its fluorescence emission increases of more than 200 times upon DNA binding, in comparison to that of the metal-free phthalocyanine. Moreover, complex **9** exhibits up to 10000-fold higher affinity for a broad range of different G4 structures, such as those from *h-Telo*, *c-myc* and *c-kit* sequences, over duplex DNA and RNA, allowing selective detection of G4s at concentrations as low as 1 nm.[11] The turn-on fluorescence of compound **9** is finally associated to a good cellular uptake and negligible cytotoxicity, when delivered to living cells,[71] rendering such compound a promising candidate for targeting and localising cancer cells in living organisms. As a corollary, the zinc(II) complex **10**, with a lactose substituted phthalocyanine ligand, has been recently utilized as a fluorescence probe for liver cancer in cancer-bearing athymic nude mice as animal models.[72] It has been shown that the use of near infrared (NIR) fluorescence imaging allows deep penetration in biological tissues and organs, while the high fluorescence of **10** provides high sensitivity to the low-energy radiation. Moreover, the complex showed excellent cancer-targeting properties, with water solubility and biocompatibility increased by the presence of the four lactose groups. Finally, histological analysis showed that the lactose substituted zinc phthalocyanine did not damage the organs proving the safety of this fluorescence probe *in vivo*.[72]

4. Zinc(II) complexes of salen-like ligands

Salen ligands and their N,N'-phenyl-bridged analogues, generally named salphen, are N_2O_2-tetradentate coordinating systems obtained by the condensation reaction of diamines and salicylaldehyde derivatives.[52,75,76] Thanks to their peculiar properties, easily modulated in steric and electronic features, metal complexes of such ligands, are widely used in different

fields of chemistry including catalysis,[77] supramolecular,[78,79] photophysical[80] and bio-medicinal chemistry.[2,17,81] Schiff base ligands derived from salen and salphen have been extensively used in several DNA-binding zinc(II) complexes. Such complexes usually show strong affinity toward B-DNA, although in general weaker compared to analogous complexes of nickel(II) and copper(II).[2] In particular, salphen derivatives showed excellent intercalation properties toward B-DNA,[2,82–85] and top-stacking abilities toward G4s.[17,81,86] Banerjee and coworkers have recently reported on the fluorescence properties of zinc(II)-Schiff-base complexes,[43,44,87] although up to now little use has been made of them as fluorescent probes for nucleic acids. In this context, it is remarkable that ten Zn-salen derivatives (see for example complexes **11** and **12** in Fig. 3) were recently synthesized as optical probes in single and two-photon fluorescence microscopy images of living cells, to perform non-invasive organelle labelling *in vitro*. Such complexes present two cyano groups in the N,N' bridge and bear different lipophilic or cationic substituents on the salicylidene moieties, exhibiting photostability, low cytotoxicity and high subcellular selectivity.[88] Interestingly, two-photon fluorescence has the advantage that can be excited by NIR or longer wavelengths to reduce tissue damage.

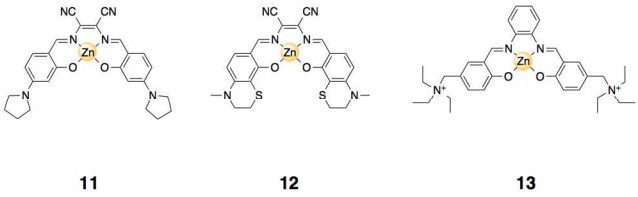

Fig. 3 Structure of zinc(II) salen-like ligands.[88-92]

Zhang and coworkers have recently synthesised several zinc(II)-salen derivatives as fluorescent probes for imaging in live cells.[91,92] In particular, compound **12** presents two thioether groups that can be oxidized to sulfoxides through one or two photon irradiation, switching on in this way the fluorescence of the zinc complex. In fact, thioether is an electron rich group that behaves as electron donor in a PET to the ligand centred first excited singlet state of the metal complex, and consequently quenches the fluorescence. On the other hand, sulfoxide is an electron deficient group that inhibits PET and consequently inducing a strong fluorescence emission.[91,92] Furthermore, a zinc-salen and zinc-salphen library, variously substituted, was employed as a colour palette for multicolour imaging of different biological targets in live cells.[93] The substituents on the N,N' bridge modulate the photophysical properties of the zinc complexes, while the substituents on the salicylaldehyde group control the lipophilicity of the whole molecule. The next step in such research line, in our opinion, should be taking advantage of the recognised high affinity of zinc-salen complexes toward both B-DNA and G4-DNA and of their interesting imaging properties for their localisation *in vivo*, to design improved fluorescent probes for particular conformations of nucleic acids.

Another important aspect, still insufficiently investigated, is the effect of photoactivation with visible light prior and after the binding with nucleic acids, which could be followed by fluorescence enhancing or by fluorescence quenching. For example, we have recently shown that the exposition to visible light of the dicationic zinc(II) complex **13** produces a dramatic enhancement of the fluorescence quantum yield.[89] Remarkably, when intercalated into B-DNA such process is inhibited, and the fluorescence intensity remains the same of the zinc(II) complex in bulk aqueous solution.[89] In agreement with the findings by Knapp and coworkers,[51–53] and with the support of DFT calculations, we have interpreted such phenomenon as a photoinduced two-electron oxidation process of **13**, a process which is inhibited by a protective action of DNA toward the intercalated zinc(II) complex, because in this case it is located in a hydrophobic environment.[89,90] Although this property diminishes the possibility of application in diagnosis, fluorescence quenching could nevertheless be used as a further analytic tool to detect the disposition of the probe in a hydrophobic region of the polynucleotide, for example after intercalation in double helical DNA.

Conclusions

At a first glance, zinc seems to be a non-aesthetically appealing element, because many of its compounds are not colourful, magnetic or redox active. Nevertheless, these "non-properties" of the zinc(II) ion confer in its complexes interesting qualities to organic ligands characterised by suitable structure and electronic configuration. In particular, very stable zinc(II) complexes are formed with the organic ligands discussed above, presenting conjugated and/or aromatic groups, the fluorophores, that may undergo low energy electronic transitions giving rise to absorption and emission spectra, ranging from the visible up to the near infrared frequencies. In such complexes, zinc(II) does not interfere with the electronic structure of the ligand, being not able to taking part to ligand-to-metal or to metal-to-ligand charge transfer, as a consequence of its d^{10} configuration. The only occurring electron transfer processes induced by light, may involve different portions of one ligand, which we may call intra-ligand electron transfer, or different neighbouring ligands attached to the metal ion, which we may call inter-ligand electron transfer. On the other hand, the rigidity of the structure, imposed by metal coordination, fixes the position in space and the intermolecular distances of the ligands in the zinc(II) complexes, consistently reducing to a minimum the energy losses associated with the photo-induced intraligand electron transfer and dramatically enhancing the quantum yield of the following fluorescence emission. This high intensity fluorescence can be either further enhanced or quenched by the binding to nucleic acids. Fluorescence enhancing follows the covalent binding of the zinc ion to donor atoms of the sugar-phosphate backbone or of the nitrogen bases, while quenching usually occurs when the environment of the zinc(II) complex changes from hydrophilic to hydrophobic, for example following DNA-intercalation. The use of zinc(II) chemosensors is in addition supported by the lower toxicity and higher biocompatibility of zinc(II) ions, compared to that of analogous transition metal ions. They can be eventually released in the biological environment where

they are delivered, as a consequence of the interaction with other biomolecules or after molecule degradation induced by light. For all these reasons it is very likely that zinc(II) nucleic acid chemosensors will find increasing ranges of applications in the near future.

Acknowledgements

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Abbreviations

ADP	Adenosine diphosphate
AIE	Aggregation-induced emission
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
Cyclen	1,4,7,10-tetraazacyclododecane
DNA	Deoxyribonucleic acid
DPA	bis(2-pyridylmethyl)amine, or dipicolylamine
ESIPT	Excited state intramolecular proton transfer
G4	G-quadruplex
LED	Light emitting diode
NIR	Near-infrared
PDT	Photodynamic therapy
PET	Photoinduced electron transfer
RGB	Red, green and blue
RNA	Ribonucleic acid
Salen	N,N'-ethylene-bis(salicylideneimine)
Salphen	N,N'-bis(salicylideneimine)-1,2-phenylenediamine

Notes and references

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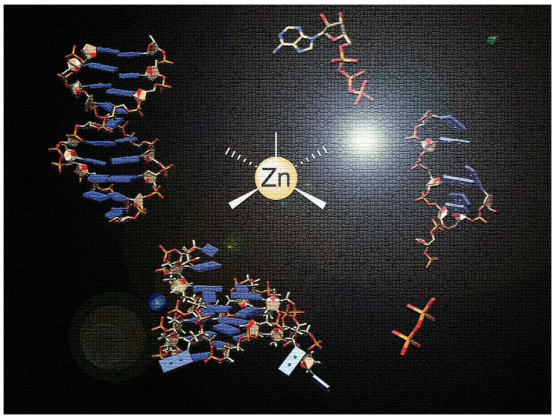

Recent applications of zinc(II) complexes as fluorescent probes for nucleic acids are described highlighting their potential as diagnostic tools