Artificial metallonucleases

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The development of synthetic agents able to hydrolytically cleave DNA with high efficiency and selectivity is a fascinating challenge that will show the way to obtaining artificial nucleases able to compete with the natural enzymes. This *Feature Article* highlights the progress reported toward the realization of synthetic nucleases with particular attention to the strategies that can be pursued to improve efficiency and sequence selectivity.

1 Introduction

The recent impressive progress in the field of genomic and biotechnologies has stimulated the search for effective tools to manipulate DNA. Among them, synthetic agents capable of hydrolytically cleaving nucleic acids are extremely important and have been attracting increasing attention in view of their applications.[1] On the one hand, artificial hydrolytic catalysts may be employed as biomimetic systems in elucidating the mechanisms of the corresponding restriction enzymes and nucleases, and, on the other hand, they could be used as conformational probes in the determination of DNA structure, as customized DNA restriction agents in molecular biology, and as antibiotic and chemotherapeutic drugs. They may even offer some advantage over natural enzymes, which cleave DNA with a sequence selectivity of 4, 6, or 8 bases. This is often too low to allow for a precise control of the manipulation of large DNA of higher species. At least in principle, in the case of synthetic hydrolytic catalysts a higher ''non-natural sequence specificity'' is attainable and could be transferred to obtaining chemotherapeutic agents able to block gene expression at the DNA level.

It is well known that DNA is particularly resistant to spontaneous hydrolysis: half-life times ranging from hundreds of thousands to hundreds of millions of years have been estimated for a single P–O bond cleavage at 25 °C and pH 7.[2] Such hydrolytic inertness, which ensures the preservation of the genetic information, makes quite difficult its manipulation, *e.g.* expression and duplication, repair of damage and elimination of foreign DNA. *In vivo*, such problems are resolved by a wealth of hydrolytic enzymes, nucleases and topoisomerase, which efficiently catalyze DNA scission.

Many such enzymes contain in their active sites metal ions, mainly Ca(II), Mg(II) and Zn(II), which play a fundamental role in their catalytic action.[3] Mechanistic studies on related model systems have shown that several metal ions can effectively accelerate the hydrolysis of phosphate esters. More recently, it has been reported that lanthanide and transition metal ion complexes can promote the hydrolysis of DNA.[4] As a consequence, much research effort has been directed to the development of artificial metallonucleases, namely, synthetic DNA hydrolytic agents containing metal ions. Remarkable accelerations of the rate of DNA hydrolysis have been achieved. Nevertheless many problems still need to

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be addressed. This paper highlights the progress reported toward obtaining synthetic nucleases; particular attention will be devoted to the strategies that can be pursued to increase efficiency and to the sequence selectivity of the systems.

2 DNA hydrolysis

The commonly accepted mechanism of hydrolysis of deoxynucleotide phosphates involves a nucleophilic attack of a water oxygen to the phosphorus to give a five-coordinate phosphate intermediate (Fig. 1).[1a,b] Subsequent cleavage of either the P–O3′ or P–O5′ (usually P–O3′ cleavage in the enzymatic process) causes a strand scission, yielding the R–OH and R–O–$PO_3(H_2)$ termini. In the case of DNA, the breakdown of the intermediate is rate limiting.

The hydrolysis is assisted by metal ions in different ways. Acting as Lewis acids, they can activate the phosphate group toward the attack of the nucleophile, increase the leaving group ability of the departing alcohol and activate a metal-coordinated water molecule as a nucleophile.[2,5] As observed in many enzymatic processes, several metal centers may act cooperatively to accelerate these mechanistic steps. Accordingly, multinuclear complexes are often more active than their mononuclear counterparts.

The exceptional high resistance of DNA toward hydrolysis makes it difficult to perform any mechanistic investigation by using it as a substrate. Supercoiled plasmid DNA is a more accessible substrate and, because of this, very popular for such studies.[1h] This particular form of DNA, which is commonly found in bacteria cells, is a cyclic supercoiled double strand made by several thousands of base pairs. One single strand scission unravels the supercoiled DNA (form I) to a relaxed circular one (nicked, form II), while a second scission on the complementary strand, within about twelve base pairs from the first one, generates a linear DNA form (form III). These three DNA forms can be easily separated and quantified by gel electrophoresis, thus allowing a simple and rapid kinetic analysis (Fig. 2). Moreover, supercoiled DNA is somehow more reactive than a short linear DNA and this makes the study of the reaction easier even in the presence of relatively poor catalysts. The increased reactivity is due: a) to the obvious reason that the cleavage of a single phosphodiester

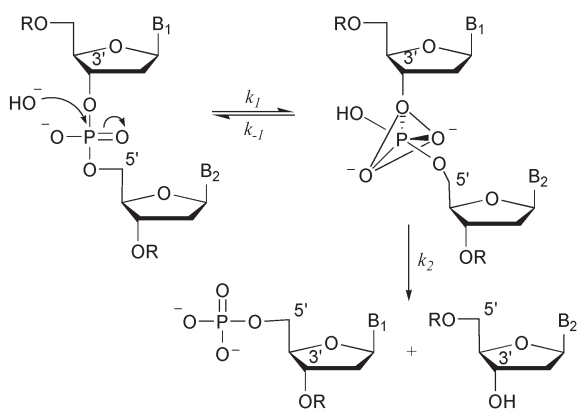

Fig. 1 Reaction pathway for DNA hydrolysis. The enzymatically promoted P–O3′ scission is shown.

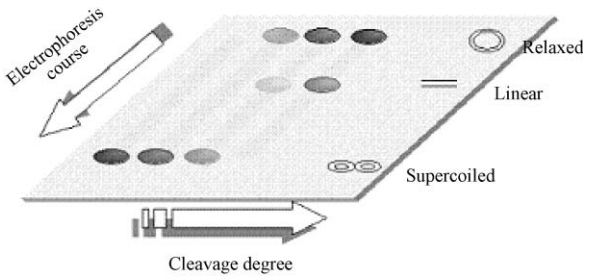

Fig. 2 Typical electrophoresis gel appearance in the cleavage of plasmid DNA.

bond in a large DNA molecule is statistically more likely than in a smaller DNA fragment; b) to the internal strain resulting in ground-state destabilization.[1h]

Peculiar and quite relevant features of this substrate are the possibility to enzymatically religate form II to give back supercoiled DNA and the possibility to discriminate between a single strand or a double strand cleavage simply by a statistical analysis of the relative abundance of forms II and III produced in the reaction. A successful enzymatic religation is often taken as evidence of a clean hydrolytic cleavage process. In spite of the fact that different commercial plasmid DNA samples may differ in kinetic behavior because of the amount of base pairs they contain and their natural origin, thus making the comparison between literature data not so straightforward, supercoiled plasmid DNA is the most popular substrate employed in investigations in the field and is surely the best choice when an initial rapid screening of different artificial nucleases is desired.

3 Free ions and mononuclear complexes

At the beginning of the last decade, it was demonstrated that aqueous trivalent lanthanide ions accelerate the hydrolysis of DNA.[6] Subsequent papers by Schneider and co-workers reported a systematic investigation on such ions aimed at determining relevant kinetic parameters for the nicking of plasmid DNA.[7] The different ions show similar maximum reactivity under saturation conditions (k_{max} 0.7–1.0·10^{-4} s^{-1}, 37 °C, pH 7.0) but different affinity for DNA. This latter increases along the series from La(III) (K_a = 5.0·10^2 M^{-1}) to Er(III) (K_a = 1.7·10^4 M^{-1}). The combination of the moderate reactivity with the low affinity for the substrate is such that substantial degradation of DNA can be obtained only at high metal ion concentrations. In contrast, in the case of late lanthanides such as Tm(III), Yb(III) and Lu(III), a remarkable reactivity was observed even at low concentrations in accord with a large binding constant of these ions with polyanionic DNA. A decrease in rate is however observed at high metal ion concentration, probably the consequence of their aggregation with formation of less active species.

Among lanthanides, cerium is peculiar for its ability to reach a stable tetravalent oxidation state. Ce(IV) is much more efficient in hydrolyzing DNA than the trivalent lanthanide ions. At pH 7.0 and 50 °C, the half life of the dinucleotide TpT is reduced to 3.6 hours, which amounts to more than 11 orders of magnitude rate acceleration over the spontaneous

hydrolysis reaction.[6c,8] As a result, Ce(IV) is 20 to 1000 times more efficient than any other trivalent lanthanide, with comparable high efficacy toward single and double strand linear DNA.[9] Komiyama and co-workers have attributed the source of such a remarkable reactivity to the great electron-withdrawing ability of this tetravalent ion.[1c] The reactivity of Ce(IV) ions is further enhanced by the use of mixtures with Pr(III).[10]

Aqueous lanthanide ions are toxic for biological systems and, more generally, free metal ions are not suitable candidates for preparing appealing catalysts. The formation of stable complexes with proper ligands is therefore mandatory. However, this task turns out to be quite difficult for two reasons: a) lanthanide ions undergo a very facile ligand exchange; b) catalytic activity requires an unsaturated coordination sphere for interacting with water and the substrate. Many of the complexing agents reported should be used in large excess to ensure complete formation of soluble complexes.[11] Stable complexes can be obtained by using polyaminocarboxylate derivatives, crown ethers or azacrown ligands.[1g] In many cases, however, the formation of the complex results in a lower reactivity than that observed with the free metal ions, particularly in the case of polyaminocarboxylate complexes, due to the decrease of the overall charge of the complex and to the saturation of the metal ion coordination sphere. One exception is represented by the Ce(IV)/EDTA complex which retains a good activity toward linear and supercoiled DNA.[9,12] Neutral ligands, such as azacrowns or crown ethers, are thus more suitable in order to retain the reactivity of the free metal ion, although their coordination strength is not so high.[11]

Metal ions other than lanthanides are scarcely active in promoting DNA hydrolysis. Nevertheless, a few mononuclear metal complexes have been reported to act as artificial nucleases. Co(III) complex of the polyamine ligand TAMEN (Fig. 3) cleaves plasmid DNA, at 37 °C and pH 7.6, with a first order rate constant of $5{\cdot}10^{-5}$ s^{-1}.[13] Although the concentration of complex required, 1 mM, is relatively high, suggesting a poor interaction with the substrate, the mechanism is fully hydrolytic as the nicked DNA can be religated. This complex is active also toward single strand DNA.

Because of their redox properties, Cu(II) complexes have been frequently used in the development of agents for the oxidative cleavage of DNA. However, there are several

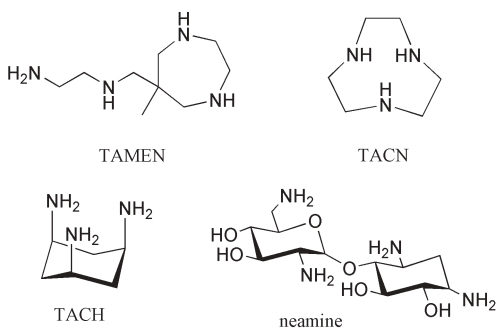

Fig. 3 Structures of some polyamine ligands used for the formation of mononuclear complexes active in DNA cleavage.

examples of Cu(II) complexes reported to cleave DNA with a hydrolytic mechanism. Burstyn and co-workers published the first example in 1996.[14] It was shown that at a 25 μM concentration the TACN/Cu(II) complex cleaves supercoiled DNA at 50 °C and pH 7.8 with an estimated rate constant of *ca.* $1.5{\cdot}10^{-5}$ s^{-1}. A decrease of reactivity was observed at higher complex concentrations, probably due to the formation of unreactive μ-hydroxo dimers.[14b] The complex is active also in the degradation of single strand DNA. Anaerobic experiments showed that the activity in the absence of oxygen is reduced by 30%, indicating the simultaneous occurrence of a prevailing hydrolytic cleavage and of an oxidative cleavage.

Later on, Fujii and co-workers reported that the TACH/Cu(II) complex promotes the oxygen independent cleavage of plasmid DNA.[15] Saturation kinetics were observed with a k_{max} value of $1.2{\cdot}10^{-3}$ s^{-1} and a K_a value of $1.3{\cdot}10^{4}$ M^{-1}. Such a reactivity is among the highest reported so far and corresponds to a half-life time, in the case of the supercoiled form, of 20 minutes in the presence of 75 μM Cu(II) complex. Several other complexes of macrocyclic and linear triamine ligands were tested and found to be scarcely reactive. On the basis of these results and of the relatively good affinity of the TACH/Cu(II) complex for DNA the authors suggest the possibility of a specific binding of the catalyst to the substrate.

Finally, Cu(II) complexes of natural aminoglycosides such as neamine have been shown to efficiently cleave plasmid DNA by Cowan and co-workers.[16] Also in this case saturation kinetics were observed with a k_{max} value of $5.2{\cdot}10^{-4}$ s^{-1} and a K_a value of $2.4{\cdot}10^{5}$ M^{-1}. The high affinity of the complex for the DNA is attributed to the tight binding of the positively charged aminoglycoside ligand to DNA: this apparently makes the system very effective even at low concentrations (the half-life time for the supercoiled form in the presence of 25 μM complex is 26 minutes). Evidence of a hydrolytic cleavage was provided because of the insensitivity of the reactivity to the absence of oxygen and by the successful enzymatic religation of the nicked DNA.

4 Bimetallic complexes

The extraordinary catalytic efficiency of natural metallonucleases most often relies on the cooperative action of two or more metal ions. Available X-ray structures of these proteins indicate that most of these metal ions are placed within a narrow range of distances between one and another. Accordingly, in a synthetic catalyst a precise spatial localization of the ions is mandatory to ensure multiple interactions with the substrate and, consequently, to take advantage simultaneously of all the different activation modes that metal ions can provide for the hydrolysis of phosphate esters (Lewis acid, leaving group and nucleophile activation). On the basis of these guidelines, a series of bimetallic complexes have been synthesized and investigated as catalysts for the hydrolytic cleavage of DNA.

In 1996, Schneider and co-workers studied a 30-membered azacrown macrocycle (Fig. 4, a) that can bind two Eu(III) or Pr(III) ions.[17] The Pr(III) complex was able to promote plasmid DNA nicking with a $k_{max} = 2.8{\cdot}10^{-4}$ s^{-1} and $K_a = 3.0{\cdot}10^{3}$ M^{-1} at 37 °C and pH 7.0. However, the activity

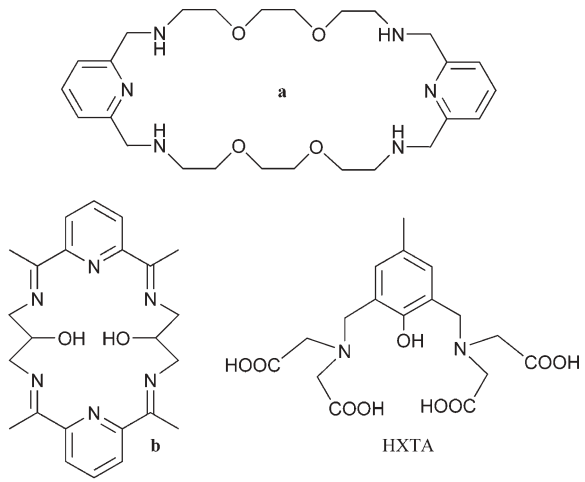

Fig. 4 Bimetallic ligands for lanthanide ions reported by Schneider (**a**), Zhu (**b**), and Que (HXTA).

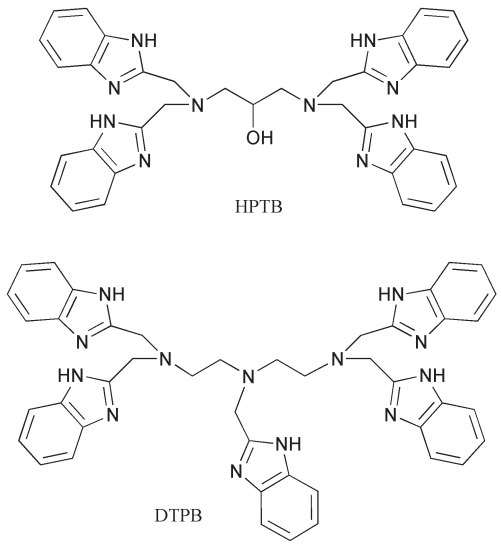

Fig. 5 Bimetallic ligand for Fe(III) reported by Schnaith and Que (HPTB), and Liu (DTPB).

of the binuclear complex was only twofold that of the free metal ion.

More effective are the $Er(III)_2$ complexes of a Schiff base-containing macrocycle (Fig. 4, b), proposed by Zhu and co-workers, which degrade supercoiled DNA at 37 °C, pH 7.0 with a maximum rate constant of $1.0 \cdot 10^{-3}$ s^{-1}.[18] Unfortunately such high reactivity is counterbalanced by a low affinity for the substrate ($K_a = 1.0 \cdot 10^3$ M^{-1}) so that high concentrations of complex are required to obtain a fast degradation of the DNA.

A very efficient dicerium complex of the polyaminocarboxylate ligand HXTA (Fig. 4) has been reported by Que and co-workers.[19] At a concentration of 10 μM, pH 8 and 37 °C, the Ce_2(HXTA) complex cleaves plasmid DNA with a rate constant of $1.4 \cdot 10^{-4}$ s^{-1}, which corresponds to a half-life of 1.4 hours for the nicking process. Interestingly, double strand scission is preferred to single strand scission and the complex cleaves also linear double strand DNA with high regioselectivity (more than 90%) favoring the scission of the P–O3′ bond.

Fe(III) is present in the active site of some phosphatases but it has been scarcely employed for obtaining artificial nucleases. Interestingly, the only two Fe(III) complexes reported in the literature with hydrolytic activity in DNA cleavage are binuclear. In both cases, the ligands are benzimidazolylmethyl derivatives, respectively of 1,3-diamino-2-hydroxypropane (Fig. 5, HPTB) and of 1,4,7-triazaheptane (Fig. 5, DTPB). The first complex, described by Schnaith, Que and co-workers in 1994,[20] shows a surprising reactivity: the system requires "oxidative" conditions, *i.e.* the presence of H_2O_2 or O_2 and a reductant (dithiothreitol or ascorbate), but yields DNA fragments that are consistent with a hydrolytic cleavage. In fact, the linearized plasmid DNA can be enzymatically religated with a quantitative conversion, indicating that only the products of the P–O3′ bonds scission are formed. This high regioselectivity is also supported by 3′- and 5′-end labeling studies. The cleavage of plasmid DNA is so efficient that, in the presence of 10 μM complex, at 25 °C and pH 8.0, the supercoiled form is completely degraded immediately after mixing. The extent of cleavage depends on the concentration of complex but, surprisingly, it does not increase after extended reaction times. To justify this unusual reactivity, the authors proposed a nucleophilic attack of a metal ion bound peroxide to the phosphodiester bond.[21]

The second, again very efficient, di-iron(III) complex has been recently investigated by Liu and co-workers.[22] In the presence of 100 μM of such complex, at 37 °C and pH 7.0, the supercoiled DNA is degraded with a rate constant of $2.1 \cdot 10^{-3}$ s^{-1}, which corresponds to a half-life of 5 minutes. This is the current "world record" for the cleavage of plasmid DNA under "hydrolytic conditions". The extent of cleavage does not depend on the presence of O_2 and the linearized DNA can be quantitatively religated thus confirming the occurrence of a hydrolytic mechanism with the sole cleavage of the P–O3′ bond. The complex shows a remarkable affinity for plasmid DNA ($K_a = 1 \cdot 10^5$ M^{-1}), probably related to the high positive charge of its bimetallic structure. The comparison with other bimetallic complexes indicates that a μ-oxo bridge between the two metal ions and the presence of a labile BF_4^- ligand play a key role in determining the reactivity: the first by ensuring the proper intermetallic distance and the second by allowing a facile substitution to yield free coordination sites for the interaction with the substrate.

Surprisingly the examples of bimetallic systems based on Cu(II) or Zn(II) are scarce and show a disappointingly low reactivity.[23] The only important exception is a binuclear Zn(II)-binding heptapeptide (Fig. 6), described by Scrimin and co-workers,[24] which cleaves plasmid DNA with a first order rate constant of $1.0 \cdot 10^{-5}$ s^{-1} at pH 7.0, 37 °C and 3.6 μM complex concentration. Due to the presence of several α-disubstituted amino acids, the peptide is folded to a relevant extent in a 3_{10}-helical conformation and the two metal chelating TACN moieties face each other at a distance of about 6 Å (the pitch of the 3_{10}-helix). Even if such a distance is larger than that found in bimetallic hydrolytic enzymes, the system is about 20 times more reactive than its monometallic counterpart. The mechanism is really cooperative and

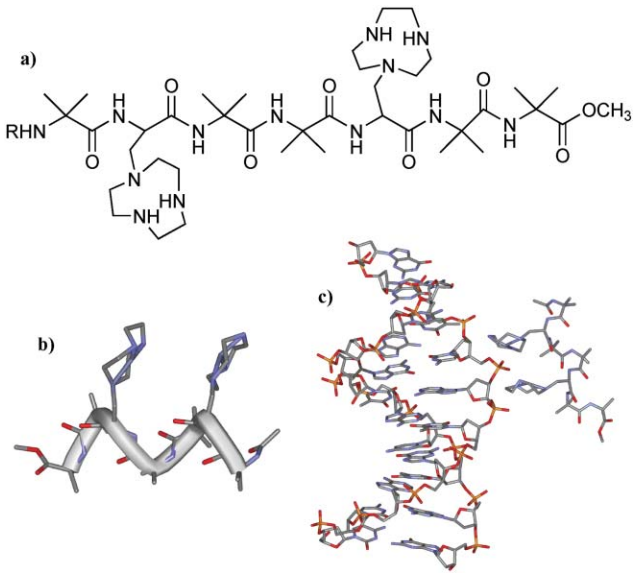

Fig. 6 Chemical structure (a), secondary structure (b), and proposed mode of binding to DNA (c) of Scrimin's Zn(II)-heptapeptide complex.

cooperativity between the two metal centers has been clearly demonstrated with Zn(II)-concentration-dependent experiments.

To justify the high cooperativity observed, the authors proposed the mode of binding to DNA shown in Fig. 6c. Since the distance between two consecutive phosphate groups in a B-DNA strand is *ca*. 7 Å, the heptapeptide may bind to it by inserting the two macrocycles within three adjacent phosphate groups. Such an arrangement forces the central phosphate to interact with both metal centers, thus taking full advantage of their complementary roles for its hydrolytic cleavage.

5 Conjugation with DNA-affine subunits

Simple considerations, supported by the results obtained with oxidative DNA cleaving agents,[25] suggest that the reactivity of hydrolytic systems should be strongly enhanced when their structures comprise groups with high DNA affinity. Furthermore, such groups could also provide sequence specificity to the system and thus open the way toward obtaining real artificial restriction enzymes.

The obvious choice among the different families of compounds able to increase DNA affinity is the utilization of intercalators. Quite surprisingly, examples of metal complexes appended to intercalating groups as hydrolytic agents are rare, and the effects of such elements on the reactivity of the systems are not always straightforward. For example, in the case of the Scrimin's peptide discussed above, the introduction of an acridine moiety at the N-terminus of the heptapeptide resulted in a slightly higher activity at low catalyst concentration compensated by a decrease at higher concentrations.

In 1987, Barton and co-workers published one of the first examples of an artificial system with DNA hydrolytic cleavage activity.[26] It was based on a ruthenium intercalator with two arms having the role of metal binding moieties (Fig. 7a). The

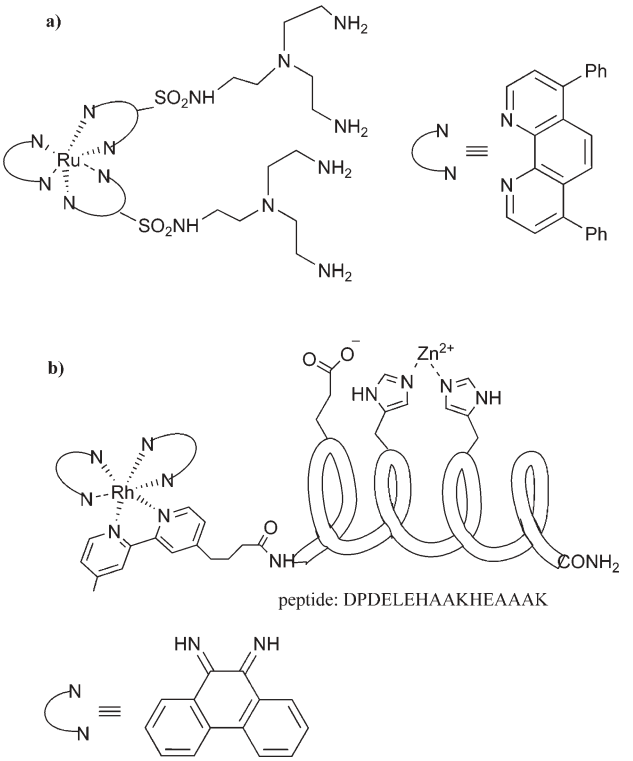

Fig. 7 Artificial nucleases based on a rhodium intercalator reported by Barton and coworkers.

Zn(II) and the Cd(II) binuclear complexes of this ligand cleaved plasmid DNA with high efficiency. At 37 °C and pH 8.5, 40% of the supercoiled form is degraded in the presence of a 7 μM concentration of the complex after 5 hours of incubation (a first order rate constant of $3{\cdot}10^{-5}$ s^{-1} can be roughly estimated). Religation experiments showed that the hydrolysis occurs randomly at the P–O3′ and P–O5′ bonds. However, the affinity of the ligand for the metal ions seems to be quite low and a large excess of metal ion is required. Unfortunately, no evidence concerning the role played by the intercalator was reported.

Later on, Barton and co-workers studied the reactivity of a mononuclear Zn(II)-binding peptide, tethered to a rhodium complex as major groove intercalator (Fig. 7b).[27] The system promotes plasmid DNA cleavage with a rate constant of $2.5{\cdot}10^{-5}$ s^{-1} at pH 6, 37 °C and in the presence of 5 μM complex with a similar activity also toward linear double strand DNA. Analysis of the fragments produced showed that the cleavage occurs only at the P–O3′ bonds with a modest sequence selectivity for 5′-Pu–*Py*–Pu–Py-3′sites (with cleavage at *Py*), presumably the result of the Rh complex binding selectivity. The reactivity decreases as the pH approaches neutrality. The peptide is scarcely structured and this is probably the source of the weak binding of the Zn(II) ion. Although the presence of the rhodium complex intercalator is of paramount importance for the activity of the complex, its substitution with a different rhodium based intercalator, which preferentially binds at mismatches, or with the ethidium intercalator led to unreactive systems.[27b]

Schneider and co-workers appended two naphthalene groups to an azacrown ligand *via* C_6 alkyl spacers (Fig. 8a)

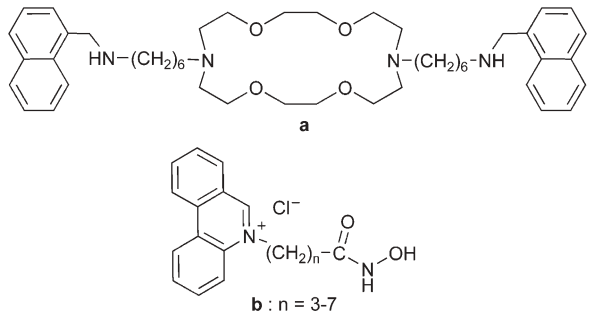

Fig. 8 Lanthanide ions ligands conjugated to intercalator reported by Schneider (**a**) and Nakamura (**b**).

and used it as a cofactor for Eu(III) promoted DNA cleavage.[28] The results are puzzling because the conjugation with the intercalating units led to an increase of the intrinsic reactivity with regard to the free metal ion but to a decrease of the DNA affinity. Finally, an earlier report of Nakamura and Hashimoto,[29] who have investigated the reactivity of a hydroxamic acid linked to a phenanthridine intercalator (Fig. 8b) in the presence of different lanthanide ions, draws attention to the length of the spacer which tethers the intercalating unit to the catalytic group as a key element for the cleavage activity.

This result is confirmed by the reactivity of a series of *cis-cis*-triaminocyclohexane Zn(II) complex–anthraquinone intercalator conjugates, linked by alkyl spacers of different length, reported by Tonellato and co-workers (Fig. 9).[30] At the concentration of 5 μM, the complex of the derivative with a C_8 alkyl spacer cleaves supercoiled DNA with a rate of $4.6{\cdot}10^{-6}$ s^{-1} at pH 7 and 37 °C. Saturation kinetics have been observed with a binding constant (K_a) of about $1{\cdot}10^{-4}$ M^{-1}, in agreement with the reported DNA affinity of anthraquinone.

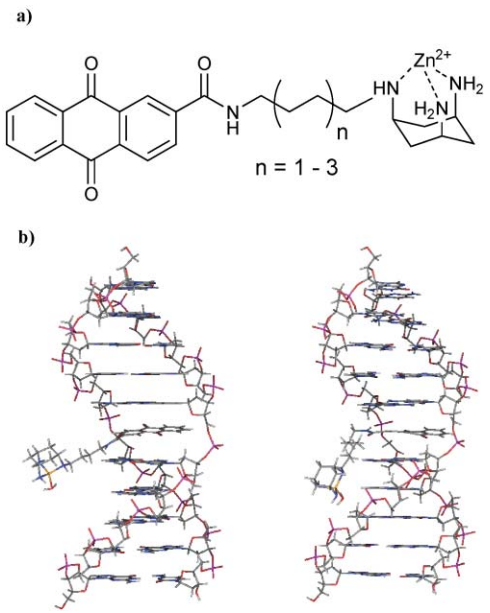

Fig. 9 Anthraquinone Zn(II) complex conjugates described by Tonellato (a) and calculated mode of DNA binding of the shortest (b, $n = 1$, left) and longest analog (b, $n = 3$, right).

Thus, the conjugation of the metal complex with the intercalating group led to a 15-fold increase of the cleavage efficiency when compared with the Zn-triaminocyclohexane complex lacking the anthraquinone moiety. Comparison of the reactivity of the different complexes showed a remarkable increase of DNA cleaving efficiency due to the increased length of the spacer. In the case of the shortest spacer (C_4) no cleavage was observed, indicating that the advantages derived by the increased DNA affinity may be cancelled out by the incorrect positioning of the reactive group. Such an interpretation was confirmed also by Molecular Modeling calculations of the structures of the DNA–hydrolytic agent complexes (Fig. 9b).

DNA affinity elements other than intercalators have also been used. Positively charged ammonium or peralkylammonium groups have a good affinity toward the polyanionic DNA. Schneider and co-workers[31] studied the reactivity of the Co(III) complexes of derivatives of cyclen bearing side chains of different lengths and terminating with a trimethylammonium group. At 37 °C and pH 7.0, they have observed saturation kinetics both with the Co(III) complex of cyclen and with the complexes of the alkylammonium conjugates. Interestingly, while the maximum reactivity remains constant within the series (k_{cat} *ca.* $2{\cdot}10^{-4}$ s^{-1}), the binding constant increases with the length of the spacer (from $1.0{\cdot}10^{3}$ M^{-1} in the case of $Co(cyclen)^{3+}$ to $5.5{\cdot}10^{3}$ M^{-1} for the complex with a C_6 spacer). Further increments of the activity could be obtained by adding a second alkylammonium and Co(III)/cyclen groups.

More sophisticated and biologically minded systems have been reported by the groups of Franklin and Sugiura. The first designed a 33-mer peptide incorporating a DNA recognition sequence and a metal ion binding site.[32] The peptide folds in a helix–turn–helix (HTH) motif where the two helices have been derived from the DNA binding engrailed homeodomain and the turn reproduces the sequence of the calcium binding site of calmodulin. Addition of Ce(IV) or Eu(III) leads to the formation of a peptide/lanthanide ion complex which retains the HTH tertiary structure. The Ce(IV) complex (and to a minor extent also the Eu(III) complex) promotes the cleavage of plasmid and linear double stranded DNA and the reactivity of the complex is similar to that of the free ion. However, while the uncomplexed ion cleaves randomly both the P–O3′ and P–O5′ bonds, the peptide complex is regioselective for P–O3′ bonds. Furthermore, modest sequence selectivity for T/C rich sites was also observed.

In a similar approach, Sugiura and coworkers have exploited the DNA-binding ability of zinc-finger motifs to prepare a sequence-selective DNA cleavage catalyst.[33] The authors have obtained zinc-finger peptides with hydrolytic ability by mutating the amino acid residues which are coordinated to the zinc ion. Tandem alignment of three of these zinc-finger mutants, like native finger protein Sp1, resulted in a system with high affinity for the DNA duplex containing a GC box (K_d = 85 nm). The cleavage is moderately efficient and occurs with good selectivity at the GC box.

6 Conjugation with sequence selective elements

The more exciting and fascinating application of the DNA hydrolytic catalysts is obtaining artificial restriction enzymes

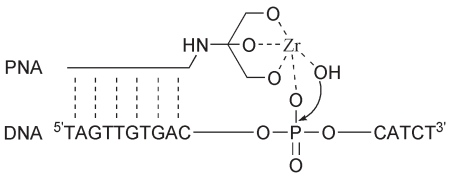

Fig. 10 PNA Zr(IV) complex conjugate described by Krämer.

with higher or different sequence specificity than natural systems. However, such a goal requires highly specific sequence recognition and most of the systems so far described produce a random cleavage of DNA or show only modest sequence selectivity.

A much higher selectivity is required and, in principle, this can be obtained by systems comprising conjugation with DNA oligonucleotides (for both antisense recognition or triple helix formation) or PNA fragments. At the present time, only very few examples of such systems are known. Komiyama and co-workers[34] first reported in 1995 a conjugate in which an iminodiacetate metal binding group was appended to a 19-mer DNA oligonucleotide. In the presence of Ce(IV) ions, the DNA–iminodiacetate conjugate efficiently cleaves a single strand 40-residue DNA at the linkage between residues 30 and 31, according with the predicted selectivity, with scission of the P–O5′ bond. Noticeably, the cleavage of the single strand DNA by the conjugate is much more efficient than hydrolysis of the dinucleotide TpT by iminodiacetate/Ce(IV), suggesting that the DNA moiety has also a role in delivering the metal ion close to cleavable phosphate groups.

Krämer and co-workers described a family of PNA–metal chelating group conjugates whose Zr(IV) complexes selectively cleave single strand DNA oligonucleotides (Fig. 10).[35] The cleavage is less efficient than in the case of the Komiyama DNA conjugate, probably due to the use of the less reactive Zr(IV) ion. Moreover, the employment, under the conditions used, of a large amount of free Zr(IV), due to the low metal affinity of the ligand (TRIS) subunit, led to a substantial non-specific random cleavage. At any rate, the fragments formed were consistent with the anticipated selectivity and the scission occurred with remarkable regiospecificity at the P–O3′ bond.

Very recently, Komiyama and co-workers described a new approach to the selective cleavage of DNA based on the known preference of Ce(IV)/EDTA complex to cleave DNA in gap-sites.[36] Gap-like structures were formed at the desired sites in single and double strand DNA by addition of monophosphate-bearing oligonucleotides[37] or by invasion of two pseudo-complementary PNA additives.[38] Upon treatment of these substrate-DNA/additive complexes with Ce(IV)/EDTA the substrate-DNA is cleaved at the target site with good selectivity but moderate efficiency. The cleavage is hydrolytic and the DNA fragments obtained can be religated and connected with foreign double strand DNA by using DNA ligase to provide recombinant DNA.

7 A critical survey and new perspectives

The research on artificial DNA hydrolytic agents is moving fast and in several directions, but the recognition of the most promising guidelines is still not easy. The fundamental features required for an artificial nuclease are high cleavage efficiency, DNA affinity, and sequence selectivity. Other requirements may vary depending on the final application: for an artificial restriction agent double strand cleavage at the P–O3′ site is a requirement for subsequent religation, while for an antisense drug the absence of cofactors, activity at very low concentrations and at physiological pH and temperature are important. For all the possible applications, the occurrence of an oxidative cleavage, both as main or concurrent pathway, is highly undesired.

Hence, the first problem one has to face is that of hydrolytic efficiency. A comparison of the different artificial DNA nucleases is rather difficult because of the large variety of reaction conditions used. However, a rough evaluation of the most reactive agents is tentatively shown in Table 1, which reports the plasmid DNA degradation rate at a fixed agent concentration (5 μM, k_ψ') and agent to DNA (base pair) ratio (0.125, k_ψ'').

If an acceptable minimum rate constant for practical applications is set at $1{\cdot}10^{-4}$ s^{-1} (which corresponds to a half-life time for the cleavage of the supercoiled form of about

Table 1 Kinetic parameters for some of the most active artificial metallonucleases

	k_{cat} (s^{-1})	K_a (M^{-1})	k_ψ' (s^{-1})[a]	k_ψ'' (s^{-1})[b]	pH	[bp][c]	Reference
Eu(III)	$7.0{\cdot}10^{-5}$	$2.6{\cdot}10^{4}$	$8.0{\cdot}10^{-6}$	$4.1{\cdot}10^{-6}$	7.0	19	28
Eu(III)-naphthocrown	$5.8{\cdot}10^{-4}$	$1.7{\cdot}10^{3}$	$4.9{\cdot}10^{-6}$	$2.3{\cdot}10^{-6}$	7.0	19	28
Er(III)	$1.0{\cdot}10^{-4}$	$1.7{\cdot}10^{4}$	$7.8{\cdot}10^{-6}$	$3.8{\cdot}10^{-6}$	7.0	19	7
Co(III)-cyclen	$2.2{\cdot}10^{-4}$	$1.0{\cdot}10^{3}$	$1.1{\cdot}10^{-6}$	$5.2{\cdot}10^{-7}$	7.0	19	31
Co(III)-cyclenC_6ammonium	$2.7{\cdot}10^{-4}$	$5.5{\cdot}10^{3}$	$7.2{\cdot}10^{-6}$	$3.5{\cdot}10^{-6}$	7.0	19	31
Cu(II)-TACH (35 °C)	$1.2{\cdot}10^{-3}$	$1.3{\cdot}10^{4}$	$7.3{\cdot}10^{-5}$	$1.3{\cdot}10^{-5}$	8.1	7	15
Cu(II)-neamine	$5.2{\cdot}10^{-4}$	$2.5{\cdot}10^{5}$	$2.8{\cdot}10^{-4}$	$3.1{\cdot}10^{-4}$	7.3	50	16
$Pr(III)_2$-azacrown	$2.8{\cdot}10^{-4}$	$3.0{\cdot}10^{3}$	$4.1{\cdot}10^{-6}$	$2.0{\cdot}10^{-6}$	7.0	19	17
$Er(III)_2$-Schiff macrocycle	$1.0{\cdot}10^{-3}$	$1.0{\cdot}10^{3}$	$5.0{\cdot}10^{-6}$	—	7.0	—	18
$Ce(IV)_2$-HXTA	—	—	$1.4{\cdot}10^{-4}$ (10 μM)	$1.4{\cdot}10^{-4}$ (r = 0.07)	8.0	150	19
$Fe(III)_2$-DTPB	—	—	$2.1{\cdot}10^{-3}$ (100 μM)	—	7.0	77	22
$Zn(II)_2$-peptide	—	—	$1.0{\cdot}10^{-5}$ (3.6 μM)	$1.0{\cdot}10^{-5}$ (r = 0.3)	7.0	12	24
Zn(II)-Rhpeptide	—	—	$2.5{\cdot}10^{-5}$ (5.0 μM)	$2.5{\cdot}10^{-5}$	6.0	40	27
Zn(II)-TACHanthraquinone	—	$1.0{\cdot}10^{4}$	$4.6{\cdot}10^{-6}$ (5.0 μM)	$1.9{\cdot}10^{-6}$	7.0	12	30

[a] Pseudo-first-order rate constant for the cleavage of plasmid DNA at a fixed concentration of metal complex (5 μM). [b] Pseudo-first-order rate constant for the cleavage of plasmid DNA at a fixed metal complex to DNA (base pair) ratio (0.125), unless otherwise stated. [c] Concentration of plasmid DNA reported as base pairs.

2 hours), inspection of Table 1 shows that only three agents satisfy such a requirement: Cu^{2+}-neamine, $Ce^{4+}{}_2$-HXTA and $Fe^{3+}{}_2$-DTPB (although in the last case the rate data were measured using a much higher complex concentration). However, several other agents have the potential to reach a good reactivity provided their affinity for DNA is increased (see k_{cat} column).

The analysis of these data indicates Cu(II), Fe(III) and Ce(IV) as the most promising candidates as metal centers in artificial hydrolytic agents. This list shares only Fe(III) with that of the metal ions most frequently occurring in natural nucleases, *e.g.* Ca(II), Mg(II), Fe(II), Fe(III) and Zn(II). As pointed out by Burstyn in his excellent review,[1a] the main features for an ideally suited metal ion in order to promote hydrolysis are hardness (to bind phosphate oxygen atoms), Lewis acidity (to polarize both the phosphate group and the nucleophile) and rapid ligand exchange (to ensure catalytic turnover). Nature's choice was limited by the environmental availability of the metal ions, while chemists have the possibility of finding the right metal over the whole periodic table. Among the metal ions that best fulfill the above criteria, essentially trivalent lanthanide ions, Zn(II), Cu(II), Fe(III), and Ce(IV), the lanthanides have probably not been used in natural nucleases because of their scanty availability and their toxicity, while Cu(II) has been discarded because of its redox behavior. As a matter of fact, oxidative cleavage pathways have been detected for all the hydrolytically active Cu(II) based systems so far reported both in the presence and even in the absence of reducing agents. The adventitious presence of reducing agents either in DNA samples obtained from natural sources or in the *in vivo* environment, makes the use of Cu(II) based systems as hydrolytic agents rather discouraging, notwithstanding its excellent activity.

As inspired by natural enzymes, the development of bimetallic systems appears to be a promising strategy in order to increase the activity of the artificial hydrolytic agents. In these systems, not only may the intrinsic reactivity increase as a result of the cooperative action of the two metal centers, but also the DNA affinity should increase due to the larger positive charge of the complex and to the possibility of multi-point interaction with the DNA phosphate backbone. As a matter of fact, the Fe(III) and Ce(IV) catalysts discussed above are bimetallic. This strategy is particularly important in the case of a Zn(II) based system, as the intrinsic hydrolytic activity of this metal is somewhat lower than that of the other ions, and in the case of lanthanide systems, since obtaining non labile metal complexes with free coordination sites usually leads to the use of polycarboxylic ligands which, as a drawback, reduce the reactivity of these ions. Nevertheless, the binuclear agents so far reported are relatively few and, in many cases, the reactivity gains are not so impressive when compared to the corresponding mononuclear complexes. Studies on model phosphate esters clearly indicate that the design of such systems is more delicate than the simple synthesis of ligands capable of binding two metal ions. The intermetallic distance and the rigidity of the complex are crucial points to allow a full cooperation between the metal centers. At least in the case of Cu(II) and Zn(II) based agents, the formation of μ-hydroxo bridges between the two ions can dramatically decrease the reactivity and must, accordingly, be strictly avoided.[14,39]

A second main route toward obtaining efficient artificial nucleases relies on the increase of the affinity of the hydrolytic complexes for DNA. The mechanism of the metal ion catalyzed hydrolysis of phosphate esters involves, as a crucial initial step, the coordination of the substrate to the metal ion. This coordination is an essential requisite in order to deliver the metal ion-coordinated hydroxide nucleophile close to the phosphate group, thus offsetting the electrostatic repulsion between the two negatively charged species, and allowing the activation of the phosphate toward the nucleophilic attack. The metal ion binding ability of phosphate diesters is rather poor and it is only slightly favored by the polyanionic nature of DNA. It is not accidental that all the most reactive systems so far reported feature DNA affinity elements such as two metal ions, intercalators, positively charged ammonium groups, aminosugars. Again, the results reported indicate that the simple conjugation of a metal complex to a DNA binding unit is not sufficient and that a very careful design of the catalyst structure is required. In fact, in order to obtain the desired activity, binding must lead to the correct geometry favoring a close contact between the metal complex and the DNA phosphate backbone. In this context, it appears that a factor of great importance is the design of a linker that can connect the metal ion complex to the DNA binding subunit and, in particular, its length and flexibility. This is not an easy task since these features may easily change depending on the DNA binding site (major groove, minor groove, mismatches) and need to be tailored accordingly.

Short DNA oligonucleotides (for both antisense recognition or triple helix formation) or PNA fragments are probably the most promising DNA binding subunits available to chemists. These systems conjugate tight binding to DNA with high selectivity and such features are ideally suited for obtaining a truly useful artificial nuclease. Up to now, only very few examples of such systems have been reported and they have been tested only on short single strand DNA fragments, while the problem of double strand cleavage of DNA has still to be addressed. However, these results are encouraging and are stimulating new research efforts.

8 Conclusions

Since the first report on the ability of metal ions to promote DNA hydrolysis, tremendous progress toward obtaining efficient synthetic DNA hydrolytic agents has been attained. At present, chemists have at their disposal catalysts that cleave DNA in a few tens of minutes and, in some cases, can display an interesting selectivity. However, several other aspects need to be addressed before a catalyst may reach the stage of practical application. Among the most important are: selectivity, double strand scission, and cleavage efficiency. The last point is of particular importance in the case of intrinsically less reactive but biologically relevant metal ions such as, for example, Zn(II). The use of multinuclear complexes as catalysts and of selective DNA binding subunits supported by a careful design of the system appear as the main routes which have been successfully followed up to now. At present, the problem

is the integration of these different features into a single system in order to obtain a really effective model of metallonuclease. This is not an easy task but the rapid progress recorded so far is encouraging and indicates that the final goal is not out of reach.

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