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Review

# Mechanistic aspects of the interaction of intercalating metal complexes with nucleic acids

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## Contents



**Abstract**

Since the discovery of the intercalative binding mode, almost half a century ago, intense efforts have been devoted to design, synthesize and test new small molecules that can bind nucleic acids with improved recognition and affinity. Among them, metal bearing compounds play a principal role. Despite the plethora of different metal complexes which have been designed to react with DNA and which have been tested, the binding mechanisms have often not been analysed. This is unfortunate, considering the importance of understanding of the binding features in depth in order to optimise their biological effects. This review covers articles where an analysis of the kinetic aspects of the interaction between the target metal compound and nucleic acids has been carried out and details of the reaction mechanism are provided. Flat metal complexes (porphyrins), spherical complexes with protruding intercalating residues, azamacrocycle metallo-intercalators and intercalators with metal bearing pendant arms are the classes of molecules that have been taken into account. The limits of the SDS method, employed to measure the rates of drug dissociation from polynucleotides, are also discussed.


*Keywords:* DNA; RNA; Intercalation; Metallo-intercalators; Kinetics; Porphyrins; Polypyridines; Aminoacridines; Azamacrocycles; SDS

## 1. Introduction

In the early 1960s Lerman discovered that dyes of the acridine family (Fig. 1A) are able to bind to nucleic acids inserting

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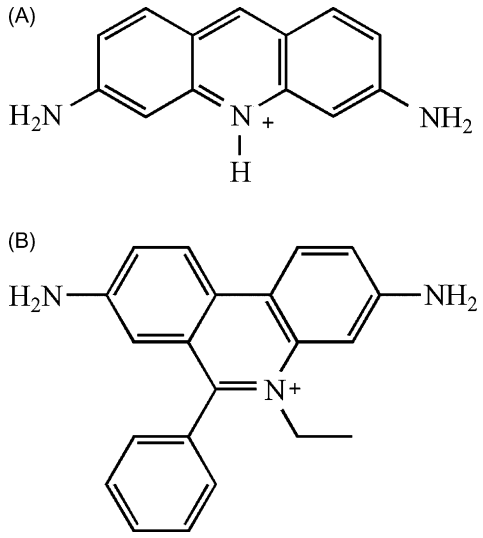



Fig. 1. Early intercalators: (A) proflavine and (B) ethidium.

themselves between the base pairs of the polynucleotide, being there stabilised by dye–base non-covalent interactions [1].

This process, denoted as intercalation, can produce deep alterations in the nucleotide secondary structure [2–4], with major consequences for DNA replication and transcription. In some cases intercalation can occur in a selective way, that is at a particular base sequence [5]. Therefore, many efforts have been devoted to design, synthesize and test small molecules that could specifically target polynucleotide sites or sequences, in order to generate new drugs displaying efficient pharmacological properties or acting as new sensitive diagnostic agents for novel applications. The tested molecules are often condensed aromatic compounds, since the presence of a planar hydrophobic residue is an essential requirement for intercalation. Moreover, metal complexes able to form non-covalent bonds with polynucleotides have also been considered. Actually, the use of coordination compounds as biological probes indeed represents one of the most successful applications of bioinorganic chemistry. Despite the fact that relatively few coordination compounds have been developed as drugs (due in part to the reluctance of pharmaceutical industries to convert their synthetic background from organic to inorganic) these compounds are increasingly employed in revealing structural features and functions of nucleic acids, as staining agents and as promoters of cleavage processes [6]. The number of metallo-intercalators that have been synthesized and tested is already very high and accordingly the number of scientific publications concerned with metal complexes binding to nucleic acids has sharply increased in the last years. Due to the massive amount of experimental work on the different aspects of polynucleotide interaction with metal complexes, a review of all the studies which have appeared in the literature would be hardly possible and is beyond the scope of the present paper. However, understanding the mechanisms by which drug molecules interact with nucleic acids and correlating them to biological effects has been a focus of interest for a long time. For instance, for a drug to be efficient as a cancer therapeutic agent, a slow rate of dissociation from DNA is considered one of the most important properties [7]. This example makes it clear that in studies of the interaction of DNA (and other poly/oligonucleotides) with small molecules, the association/dissociation kinetics are of great diagnostic importance. On this basis, we have considered it useful to present a review of those articles where the kinetic aspects of the interaction between nucleic acids and intercalators have been investigated and information about the details of the reaction mechanism is provided. In particular we shall focus on four classes of ligands: flat metal complexes (porphyrins), spherical complexes with protruding intercalating residues, azamacrocycle metallo-intercalators and intercalators with metal bearing pendant arms. *Cis*-platinum like complexes, carboplatinum and similar complexes that covalently attack the nucleobases have been the object of exhaustive reviews [8–10] and will not be considered here.

## 2. Basic reaction mechanisms

The usefulness of producing intercalating drugs with enhanced nucleotide sequence selectivity was recognized a long time ago [7,11,12]. Intercalating ligands of appropriate structure, as bifunctional quinoxaline antibiotics [13–15] and acridine or phenanthridine dimers [16], were synthesized and tested. Kinetic experiments show that intercalation in some cases [17–19] can be depicted according to the simple one-step model shown in Scheme (1)

$$\mathrm{D} + \mathrm{S} \underset{k_\mathrm{d}}{\overset{k_\mathrm{f}}{\rightleftharpoons}} \mathrm{DS} \tag{1}$$

where D represents a drug molecule (alternatively defined as dye), S is a reaction site on the polymer lattice and $DS_I$ is an occupied site (which can be seen as a complex) where D and S are held together by non-covalent bonds; $k_f$ and $k_d$ are the rate constants of $DS_I$ formation and dissociation, respectively.

Drug binding to nucleic acids is generally a fast process whose temporal course is in most cases investigated using stopped-flow or/and chemical relaxation methods (mainly temperature-jump). The relaxation time associated to reaction (1) is

$$\frac{1}{\tau} = k_\mathrm{f} F(C) + k_\mathrm{d} \tag{2}$$

Under conditions of polymer large excess, the concentration variable $F(C)$ corresponds to the total polymer concentration, $C_P$. On the other hand, when the concentrations of the reaction partners are similar, or under conditions of drug excess ($C_D > C_P$), $F(C)$ becomes a rather complex function of the extent of binding, $r$ ($r = [DS]/C_P$). Under these circumstances, the concentration variable assumes the form $F(C) = C_P \times f(r) - [D]f'(r)$, where [D] is the drug equilibrium concentration, $f(r)$ and its first derivative $f'(r)$ are functions of $r$ and site size [20]. The analysis of the dependence of $1/\tau$ on $F(C)$ according to Eq. (2) allows $k_f$ and $k_d$ to be simultaneously obtained. Such an analysis requires the values of [D] and [DS] at equilibrium. Iterative procedures have been devised to evaluate the above concentrations, which make use of the relationship $k_f/k_d = [DS]/[D][S]$.

However, early studies on the interaction of acridine [21] and ethidium [22] derivatives with DNA inferred that the process of drug binding to polynucleotides could be more complex than that represented by Scheme (1). Actually, the drug spectra recorded for different concentrations of added nucleic acid did not display a well-defined isosbestic point [23], thus suggesting the binding process is not simple.

The first detailed kinetic investigation on drug intercalation was performed by Li and Crothers [21] on the DNA/Proflavine (PR) system. T-jump experiments revealed the occurrence of two relaxation effects that were interpreted on the basis of the sequential mechanism shown in Scheme (3), in which fast formation of $DS_I$ is followed by the relatively slow penetration of the chromophore between base pairs to form the intercalate complex ($DS_{II}$)

$$D + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} DS_I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} DS_{II} \quad (3)$$

The process represented in Scheme (3) displays two kinetic effects; these can be separated on the time scale their relaxation times, $\tau_f$ (fast) and $\tau_s$ (slow), change with reactant concentrations according to Eqs. (4) and (5)

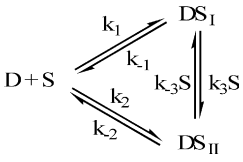
$$\frac{1}{\tau_f} = k_1 F(C) + k_{-1} \quad (4)$$

$$\frac{1}{\tau_s} = \frac{K_1 k_2 F(C)}{1 + K_1 F(C)} + k_{-2} \quad (5)$$

where $K_1 = k_1/k_{-1}$ [24].

Subsequent investigations have shown that not only acridines [24–27] but cyanines as well [28,29] interact with DNA according to the sequential mechanism, although the details of the kinetic behaviour have revealed that the step leading to $DS_I$ formation is preceded by an ever faster step where a precursor complex is formed. Differently from other systems where the nature of the precursor complex was believed to be essentially electrostatic, in the DNA/cyanine systems electrostatic interactions play only a partial role in the stabilization of the precursor complex [30].

Also very important among the early studies of drug intercalation kinetics is a T-jump investigation of the binding of ethidium ion (EB) to calf thymus DNA (ct-DNA) by Bresloff and Crothers [22]. As in the case of the DNA/PR system, the DNA/EB system as well displays two relaxation effects, but the features of the kinetic behaviour lead the authors to propose the cyclic mechanism represented by Scheme (6)




(6)

where the complexes $DS_I$ and $DS_{II}$ do form according to two parallel steps, and undergo inter-conversion according to a "site catalysed" step (direct transfer mechanism).

The expressions for the relaxation times associated to Scheme (6) are somewhat more complex compared to those associated to Scheme (3). The cyclic scheme involves three reactions, two of them being thermodynamically independent; hence, process (6) as well will show two relaxation effects. The sum and the product of the relaxation times are given respectively by Eqs. (7) and (8)

$$\frac{1}{\tau_f} + \frac{1}{\tau_s} = (k_1 + k_2)F(C) + (k_3 + k_{-3})[S] + k_{-1} + k_{-2} \quad (7)$$

$$\frac{1}{\tau_f} \times \frac{1}{\tau_s} = [k_1 F(C) + k_3[S] + k_{-1}] \times [k_2 F(C) + k_{-3}[S] + k_{-2}] - [k_1 F(C) + k_{-3}[S]] \times [k_2 F(C) + k_{-3}[S]] \quad (8)$$

where $[S] = C_P \times f(r)$ [20].

The two different modes of binding depicted in Schemes (3) and (6) could not be differentiated by using static spectroscopic methods, but they can be distinguished by kinetic methods. Actually, both mechanisms are characterized by two relaxation effects, but in the sequential mechanism the product of the corresponding fast and slow relaxation times ($1/\tau_f \times 1/\tau_s$) shows a concentration dependence which tends to become linear at the highest reactant concentrations (Fig. 2A), whereas in the case of the direct transfer mechanism the dependence of ($1/\tau_f \times 1/\tau_s$) on the reactant concentration increases according to a parabolic trend (Fig. 2B).

The direct transfer mechanism was postulated to explain the features of the binding of ethidium ion to DNA [22,31,32]. The first explanation advanced to interpret the behaviour of ethidium, so different from that of proflavine, was based on the inferred bifunctional character of the former drug. When one of the two aromatic residues of ethidium is intercalated ($DS_I$), the second stays outside of the cavities formed by adjacent base pairs and can directly react with a site of another polymer molecule to form $DS_{II}$ without passing through the free reactant state. Subsequent investigations [25] have shown that removal of the phenyl group of ethidium does not suppress the direct transfer step; moreover, mono-functional acridines, which usually display the sequential binding mode (Scheme (3)), when properly substituted (10-methyl-9-aminoacridine), appear to intercalate according to Scheme (6). To interpret these results it was proposed that $DS_I$ and $DS_{II}$ represent molecules intercalated via one or other of the DNA grooves and that the transfer pathway involves drug exchange between the major groove of one helix and the minor groove of another.

The direct exchange mechanism has been recently found to operate for the binding of ethidium to RNAs like poly(A)·poly(U) [23] and also to triple stranded poly(A)·2poly(U) [33]. Since in the latter polynucleotide the major groove is occupied by the third strand, ethidium can intercalate only through the minor groove, but the direct transfer is still operative. So, the hypothesis that $DS_I$ and $DS_{II}$ represent two molecules intercalated via either of the grooves is hardly tenable; hence, the results have been interpreted assuming that $DS_I$ and $DS_{II}$ are two intercalated forms differing in the extent of intercalation. Mention should also be made of the fact that in the DNA/EB system the step $D + S \leftrightarrows DS_{II}$ is a minor process and in the poly(A)·poly(U)/EB system this step can be neglected, so

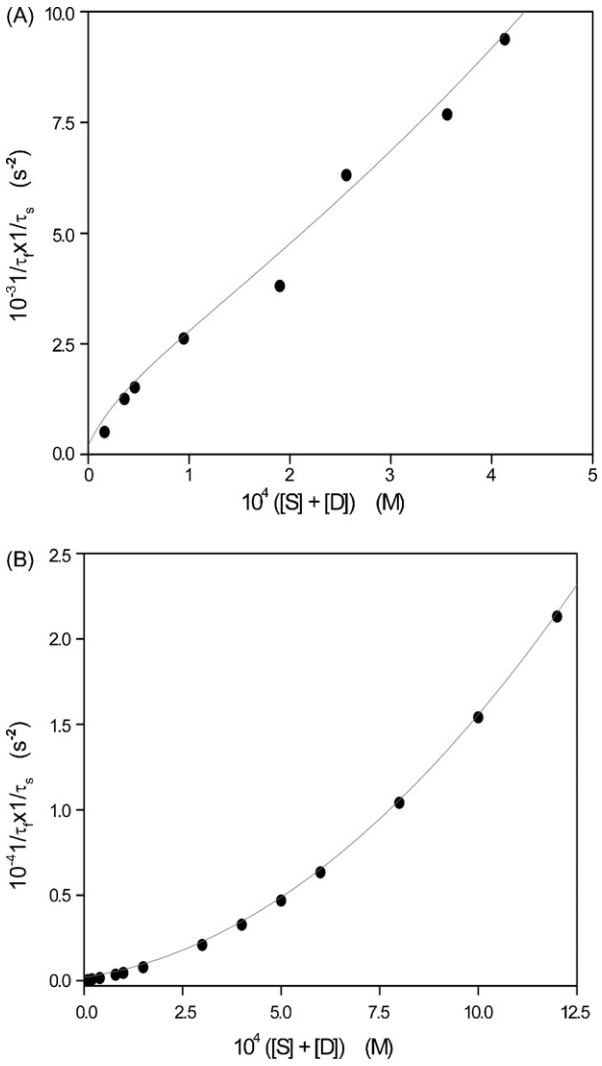



Fig. 2. Plots showing the reactant concentration dependence of the product of the two reciprocal relaxation times for the poly(A)·poly(U)/PR system (A) and for the poly(A)·poly(U)/EB system (B); $I = 0.1$ M (NaCl), 25 °C. PR reacts with the polynucleotide according to the sequential mechanism (Scheme (3)), whereas EB reacts according to a direct transfer mechanism (Scheme (6)).

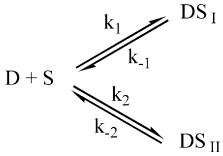
that the cyclic character of the mechanism disappears [23] and Scheme (6) is reduced to a sequential mechanism.

It is not easy to distinguish a priori between a drug that could bind *via* the direct transfer pathway from another that prefers to interact with double (or triple) strands by the sequential mechanism. If one assumes that $DS_I$ formation involves groove interactions coupled with partial intercalation, then the energy penalty required for mono-molecular conversion of $DS_I$ into the fully intercalated complex $DS_{II}$ is rather low and that drug will bind by the sequential pathway. Proflavine and cyanines seem to belong to this class of drugs. By contrast, phenanthridines and 10-methyl-9-aminoacridine, which cannot easily form hydrogen bonds with the groove components, are expected to bind according to the direct transfer model.

Perusal of the literature shows that systems following Scheme (6) are rather limited, while most intercalators bind to nucleic acids according to the sequential mechanism. In the case of metallo-intercalators the features of the binding could be more complex than that shown in Scheme (3) but, as will be shown below, the sequential character of the reaction mechanism is preserved.

The basic reaction mechanisms shown in Schemes (3) and (6) are derived from the analysis of the concentration dependence of reaction rates. Additional information on the polynucleotide–drug mechanism has been derived from a study of the dependence of rate constants on salt concentration. A system reacting according to Scheme (3) has been considered [34–36]; assuming that the drug, D, is positively charged and that the polymer is surrounded by a cloud of positive ions (e.g. $Na^+$), then the first step of Scheme (3) could be seen as a substitution step where D replaces a given number of $Na^+$ ions. The rate constant for $DS_I$ formation, $k_1$, should then depend on $Na^+$ concentration. Moreover, since $DS_I$ and $DS_{II}$ differ for the degree of polymer unwinding and for the number of $Na^+$ ions bound per site, also the forward and backward rate constants for the $DS_I \leftrightarrows DS_{II}$ step, $k_2$ and $k_{-2}$, are both expected to display a (different) dependence on $Na^+$ concentration. However, concerning DNA/cyanine systems, the dissociation rates appear to be scarcely dependent on the salt concentration [28].

The early studies on the binding of ethidium and proflavine to DNA and RNA had the merit of enlightening the basic features of the intercalation mechanisms, but not much attention was paid in these studies to the use of drugs for biomedical applications. As mentioned in Section 1, it is supposed that the therapeutic efficiency of a DNA bound drug will increase as the rate of its dissociation from the nucleic acid decreases. In effect, this issue was investigated using intercalating agents of more complex structure as natural antibiotics (daunomycin, actinomycin [7]) and, more recently, using cyanine dimers such as YOYO and finally dimers of Ru(II) complexes that bind very tightly to DNA according to a biphasic process involving formation and interconversion of two complexes [37]. Since the inter-conversion between $DS_I$ and $DS_{II}$ is very slow, it is assumed that the parallel mechanism shown in Scheme (9) is operative for these drugs [38]




(9)

The relaxation times expressions related to Scheme (9) are the same found for Scheme (3) (Eqs. (4) and (5)); therefore, the above parallel mechanism cannot be kinetically distinguished from the series mechanism of Scheme (3).

## 3. Interaction of porphyrins with nucleic acids

Interactions of synthetic and natural nucleic acids with porphyrins have been intensively investigated since the discovery in 1979 that these compounds are able to intercalate into DNA [39]. Fig. 3 shows the structure of different methylpyridinium porphyrins that have been employed in intercalation studies. The flat aromatic structure of porphyrins, both free and in form of metal complexes, coupled with their high absorptivity and intensity of fluorescence emission, make these substances specially

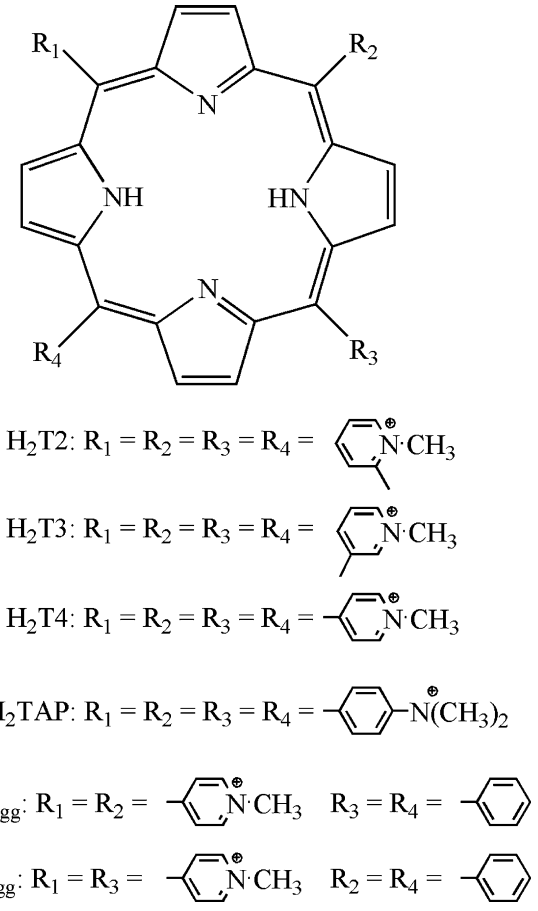



Fig. 3. Structure of meso-substituted porphyrins. The central group intercalates between the DNA base pairs while the positively charged substituents stay outside of the DNA cavities where they can interact with the phosphate groups.

suitable for investigations on the mechanisms of drug binding to nucleic acids. Actually, DNA/porphyrin systems have been analysed using different methods; among them we recall spectroscopy [40], viscometry [41], dialysis equilibria [42] and resonance light scattering (RLS), the latter being introduced by Pasternack and his school [43]. Kinetic investigations on the DNA–porphyrin interaction are rather scarce [19] and often confined to the analysis of drug dissociation induced by SDS [44]. However, these studies, besides enabling the interaction mechanism to be elucidated, also have demonstrated the importance of the electrostatic contribution to the rates of both the association and dissociation steps.

The central moiety does constitute the basic intercalating residue; however, further properties are offered by the peripheral residues that, if positively charged, provide additional stability to the bound form because of their interaction with the phosphate groups of DNA. Introduction of a metal ion converts a porphyrin into a metallo-intercalator with additional properties.

Most of the knowledge on the behaviour of porphyrins and metallo-porphyrins comes from the Pasternack school [43,45] and from the studies of Strickland et al. [42]. Besides employing all static methods suitable to define the modes of porphyrin binding to DNAs, these authors have made intelligent use of the kinetic method of analysis not only to corroborate static studies, but also to obtain information on the details of the binding mode that could not be gained only from the analysis of static experiments.

### 3.1. Porphyrin binding modes

Studies on porphyrins binding to DNAs demonstrated the importance of the peripheral residue geometry and of the metal centre in driving the binding towards one particular mode. The binding features of the multi-charged cationic porphyrins $H_2T4$, $H_2T3$ and $H_2T2$ (Fig. 3) and the corresponding Cu(II) complexes to DNA have been investigated [42]. The above molecules are isomers in which the position of the charge centre is varied. Equilibrium, dialysis, stopped-flow kinetics, flow dichroism, viscometric and NMR experiments were performed to investigate the binding modes of both the free ligands and their Cu(II) complexes. The results have shown that $H_2T2$ and CuT2 bind externally and only to polydA·polydT homopolymer, the more charged $H_2T3$, $H_2T4$ bind tightly to either polydG·polydC, poly(dG-dC)·poly(dG-dC), polydA·polydT or poly(dA-dT)·poly(dA-dT). The dependence of the equilibrium constant on $Na^+$ concentration suggests three-charge interaction upon binding of $H_2T3$, CuT3, $H_2T4$ and CuT4 to A-T and G-C double strands. These experiments reveal the major role played by the electrostatic contribution to the DNA/porphyrin complexes.

The role of the metal centre in driving the binding towards one particular mode was elucidated by Pasternack et al. [19,46]. It was found that Ni(II) and Cu(II) complexes of the $H_2T4$ porphyrin with no out of plane ligands and also the free porphyrin are able to intercalate into the polydG·polydC homopolymer. By contrast, porphyrin metal complexes with ligands axially bound to the central metal ion (Mn(II), Fe(III), Co(III) and Zn(II) metallo-porphyrin) are not able to find a place between base pairs and thus undergo external binding; on the other hand, all the above mentioned porphyrin derivatives undergo groove binding with the more flexible polydA·polydT.

### 3.2. Kinetic aspects

The kinetic behaviour of $H_2T4$ and related metal complexes indicates that these compounds exhibit significant selectivity for the G-C base pairs over A-T base pairs. Interestingly, T-jump experiments on the binding of the intercalating porphyrins to ct-DNA display multiphasic relaxation effects. The kinetic curves could be fitted by three relaxation times which are concentration-independent. Moreover the dependence of the overall relaxation amplitudes on the DNA to porphyrin concentration ratio ($C_P/C_D$) displays an asymptotic increase, reaching a plateau for high values of this ratio. The resulting profile is markedly different from the bell-shaped profile displayed by polydG·polydC/porphyrin systems. These observations suggest the occurrence of dye redistribution among the A-T and G-C DNA sites. Experiments of porphyrin transfer between polydA·polydT and polydG·polydC show that the transfer of dye from A-T to G-C sites occurs according to a dissociation-reassociation mechanism, but the relaxation times are much

slower than those observed for the ct-DNA/$H_2T4$ systems. This observation indicates that site redistribution in the latter system occurs through a direct internal transfer, rather than through the state of free reactants.

Concerning the kinetics of the above cited multi-charged cationic porphyrins $H_2T4$, $H_2T3$ and $H_2T2$ and the corresponding Cu(II) complexes, studies on dye dissociation from polynucleotides, carried out by using SDS to extract the dissociated dye, revealed the occurrence of a biphasic process in most cases [42], consistent with the kinetic behaviour observed for most of the DNA/dye interactions (Scheme (3)). In the case of polydA·polydT the plot slopes $\delta \log k_d/\delta \log[Na^+]$ are similar to the values of the plot slopes $\delta \log K_{app}/\delta \log[Na^+]$ (2.2–2.9) whereas in the case of polydG·polydC it turns out that $\delta \log k_d/\delta \log[Na^+] = 0.5–1.2$ differs from $\delta \log K_{app}/\delta \log[Na^+] = 1.9–2.7$. Similar slope values of the kinetic and thermodynamic di-logarithmic plots, observed with the A-T double strand, are taken as indicative of one-step binding modes (Scheme (1)), while a slope value of $\log k_d$ *versus* $\log[Na^+]$ plots lower than that obtained from the $\log K_{app}$ *versus* $\log[Na^+]$ plots, as observed for the G-C double strand, is suggestive of a series two-step binding modes (Scheme (3)). The latter mechanism implies that the amount of $Na^+$ ions displaced in the formation of $DS_I$ is only a fraction of the total.

The interaction of penta-coordinate porphyrin [TPPCo(Trp)] (TPP = tetraphenylporphine and Trp = 1-tryptophan) with ct-DNA was investigated by NMR, voltammetric and spectrophotometric methods. The authors, based on the large hypochromic effect displayed by the absorbance spectra upon DNA addition to the drug, suggest an intercalative mode of binding [47]. However, the low rate of the interaction process and the presence of out of plane ligands contrast with an interpretation of the results based on intercalation.

## 4. Metal–polypyridine complexes

Metal–polypyridine complexes can be profitably used as probes for nucleic acid sites and structure [48]. The central metal ion can intercalate between base pairs and/or bind to groove components by surface interaction, depending on the flatness and extension of the ligand geometries. Moreover, the electrostatic interaction can play a remarkable role in both binding modes. These complexes are almost non-luminescent in aqueous solution because of quenching by water, but fluorescence is excited in the presence of double-stranded DNA owing to water stripping out of the intercalating ligand (light switch effect) [49]. By virtue of this property, fluorescence methods are best suited to investigate the interactions between metallo-polypyridines and DNA. It should be taken into account that the metal–polypyridine complexes, as for instance $[Ru(phen)_3]^{2+}$, do exist as a mixture of enantiomers and that chirality can be an important property for systems where particular geometries, as those of nucleic acids, are involved. Studies on resolved enantiomer species have indeed shown that, if complexes as $[Ru(phen)_2dppz]^{2+}$ (dppz = [dipyrido 3,2-*a*:2′,3′-*c*] phenazine) show similar affinity towards DNA either in the Λ-$[Ru(phen)_2dppz]^{2+}$ or Δ-$[Ru(phen)_2dppz]^{2+}$ form [50], other complexes show differences in the binding efficiency of the enantiomers and that binding mode modulation is possible. For instance Δ-$[Ru(bpyMe_2)_2dpq]^{2+}$ ($bpyMe_2$ = 4,4′-dimethyl-2,2′-bipyridine and dpq = dipyrido[3,2-*a*:2′3′-*c*]-quinoxaline) binds to DNA by intercalation, whereas insertion between base pairs for Λ-$[Ru(bpyMe_2)_2dpq]^{2+}$ is restricted [51].

### 4.1. Binding modes of octahedral transition metal complexes

Early studies, based on the binding of tris-(phenanthroline) complexes of zinc, cobalt and ruthenium to DNA [52–60] suggest that the cationic complexes interact non-covalently with DNA according to three basic modes: (a) electrostatic, (b) minor groove binding, (c) partial penetration of one of the three phenanthroline residues between DNA base pairs through the major groove. Concerning chirality, some preference of Δ-isomers for binding mode (c) and of Λ-isomers for binding mode (b) could be observed but the differences found were in any case small with these complexes. The absence of lengthening of DNA observed with $[Ru(phen)_3]^{2+}$ has been explained in terms of semi-intercalation (only one edge of the intercalation pocket opened) and quasi-intercalation (indenture of a base-pair allowing stacking of adjacent bases with the intruding phenanthroline ligand) [61,62].

Compounds expressly designed to optimally match the symmetry of the double helix display an enhanced ability toward chiral discrimination [48,63]. Replacement of one of the coordinated ligands with an aromatic ligand of extended surface will favour the intercalating properties of the resulting metal complex. Many polypyridine metal complexes, differing in ligand structure and metal nature, have been tested to probe DNA. Among these $[Ru(phen)_2dppz]^{2+}$ is the most widely employed (Figs. 4 and 5).

The remarkable light switch effect, produced upon interaction of $[Ru(phen)_2dppz]^{2+}$ with double stranded DNA (ds-DNA), is widely exploited to investigate the spectrophysical properties of the nucleic acid/dye complex, and is ascribed to the de-shielding effect produced by intercalation. However, it has been recently shown that light switch of $[Ru(phen)_2dppz]^{2+}$ is excited as well in the presence of single stranded DNA (ss-DNA) and ss-oligonucleotides [64]. Fluorescence and

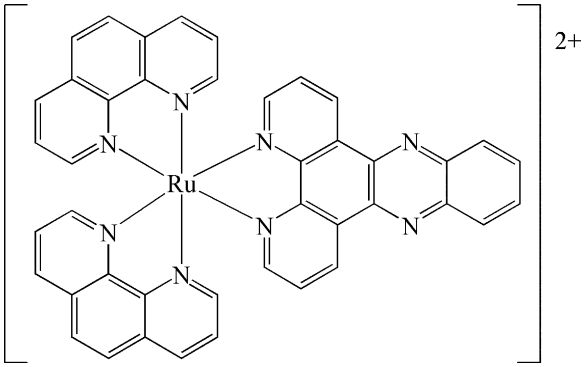



Fig. 4. $[Ru(phen)_2dppz]^{2+}$ molecular formula.

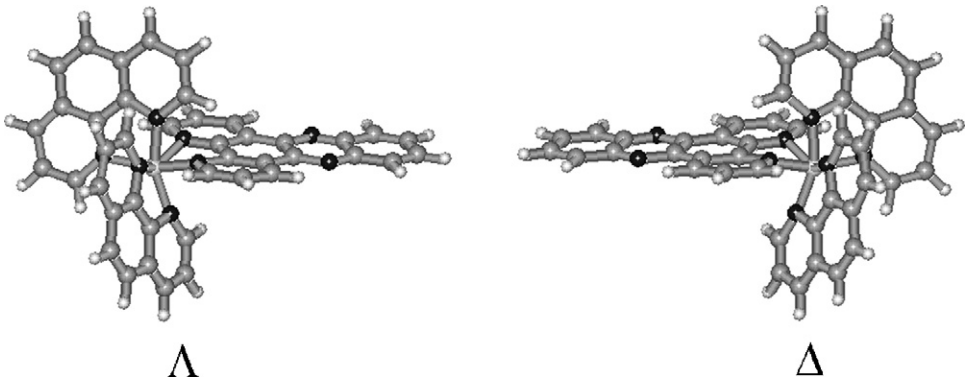



Fig. 5. The structure of Λ-[Ru(phen)$_2$dppz]$^{2+}$ and Δ-[Ru(phen)$_2$dppz]$^{2+}$ isomers.

absorption spectroscopy, isothermal calorimetry and viscosity measurements have been used to characterize the interaction of [Ru(phen)$_2$dppz]$^{2+}$ with ct-DNA [50]. Salt dependence of the equilibrium binding constant indicates that there is a significant electrostatic contribution to the binding process for both the Δ and Λ isomers. Relative viscosity measurements with rod-like DNA and fluorescence energy transfer experiments indicate that the [Ru(phen)$_2$dppz]$^{2+}$ complex intercalates into ct-DNA [50]. Whether [Ru(phen)$_2$dppz]$^{2+}$ intercalates from the major or from the minor groove has been a matter of debate. $^1$H NMR NOE data seem to indicate major groove intercalation [65], whereas spectral properties, including absorption, circular dichroism (CD) and linear dichroism (LD) of the poly(dT·dA-dT) triplex/[Ru(phen)$_2$dppz]$^{2+}$ system appear to favour intercalation from the minor groove [66]. However, when the minor groove of DNA is blocked by a minor groove binder such as DAPI (4′,6-diamidino-2-phenylindole), then intercalation of [Ru(phen)$_2$dppz]$^{2+}$ occurs from the major groove [67]. This conclusion is strengthened by experiments on the interaction of benzo-dppz with DNA in the presence and absence of DAPI [68]. While the selectivity of [Ru(phen)$_2$dppz]$^{2+}$ is rather modest, being only slightly oriented toward A-T base-pairs [69,70], a ruthenium dipyridophenazine complex has been synthesized, [Ru(tpm)(py)dppz]$^{2+}$ (tpm = tris-(1-pyrazolyl) metane), that exhibits a pronounced preference for G-C sequences [71]. This complex binds to poly(dG)·poly(dC) and poly(dA)·poly(dT) homopolymers, displaying in both cases biphasic binding modes, which depend on the polymer to dye concentration ratio.

The conclusion that can be derived from the analysis of the numerous static studies performed on this topic [72–75] is that the details of the binding of polypyridine complexes to DNA are still unclear and in some cases even the binding mode is uncertain since the assignments of the binding features are often based on subtle photophysical differences. For example, those observed in the case of the DNA/[Os(phen)$_2$dppz]$^{2+}$ system are interpreted in terms of two different intercalative modes of reaction: the "head on" and the "side on" intercalation [76,77].

The instability of many organometallic complexes in water has, in the past, discouraged DNA binding studies similar to that on polypyridyl complexes, but more recently bioorganometallic iridium, rhodium and ruthenium complexes have been synthesized that show high solubility and stability in water. In these complexes the metal centre is coordinated at one side to a $\eta^5$-cyclopentadiene or a $\eta^6$-arene, at the other to an intercalating moiety such as dppz. Stable intercalative binding is indeed found to occur for cationic complexes of the type [($\eta^5$-$C_5Me_5$)$Ir^{III}$(Aa)(dppz)]$(CF_3SO_3)_n$, [($\eta^5$-$C_5Me_5$)$Rh^{III}$(Aa)(dppz)]$(CF_3SO_3)_n$ ($n=1$, Aa = AccysOH; $n=2$, Aa = AcmetOMe; $n=3$, Aa = $H_2$metOMe and HMetOH = methionine, HcysOH = cysteine) and [($\eta^6$-arene)$Ru^{II}$(Aa)(dppz)]$(CF_3SO_3)_n$ ($n=1$, Aa = $AcH_{-1}$cysOH; $n=2$, Aa = AcmetOH; $n=3$, Aa = $H_2$metOMe) containing S-coordinated amino acids [78,79], the iridium complex having also shown plasmid DNA cleavage ability under high pressure Hg lamp irradiation. Stable coordination at the $\eta^6$ site of mono- and dinuclear peptides by organometallic half sandwich fragments was found to occur [80], suggesting difunctional complexes of longer peptides might be suitable as novel bis-metallo-intercalators.

### 4.2. Kinetic aspects

In contrast with the large amount of static investigations on the polypyridine complex interaction with DNA only one study is available where the kinetics of the direct binding of [Ru(phen)$_2$dppz]$^{2+}$ to DNA and ds-poly(A) have been investigated [81]. A combination of kinetic (stopped-flow) and static spectrophotometric and spectrofluorometric measurements reveals that the binding is rather complex. For a concentration ratio $C_{DNA}/C_{complex}$ higher than three intercalation of the phenazine residue between base pairs occurs according to the excluded site model ($n>1$) for both DNA and ds-poly(A). For $C_{DNA}/C_{complex}$ ratios lower than 3 a different process is observed. Both equilibrium and kinetic data could be fitted according to equations based on the Schwarz theory [82], which demonstrates the cooperative nature of the binding. The features of the cooperative binding mode are discussed in terms of prevailing surface interactions of the ancillary ligands coupled with partial dppz-base pair interaction. Both the intercalation and the cooperative binding modes display a remarkable dependence on ionic strength, suggesting that electrostatic attraction plays an important role in both binding modes.

The kinetics of ligand redistribution, occurring after the binding step, have been investigated in the case of raceme $[Fe(phen)_3]^{2+}$, $[Fe(phen)_2(dip)]^{2+}$, $[Fe(phen)(dip)_2]^{2+}$, $[Ru(phen)_3]^{2}$, $[Ru(bipy)_3]^{2+}$ mixtures (where dip = 4,7-diphenyl-1,10-phenantrolyne and bipy = 2,2′-bipyridyl). A very slow kinetic effect was found to take place upon interaction with DNA. Important changes in the CD spectra occurring in the time range of hours were ascribed to equilibration of the diastereomers of the DNA bound metal complexes in favour of the more stable Δ-$[ML_3]^{2+}$/DNA form [83,84].

### 4.3. Binuclear complexes

Several articles have been published on the binding of binuclear phenazine-linked $[Ru(phen)_2dppz]^{2+}$ dimers to ds-DNA. The equilibria and kinetics of binding to DNA of $[\mu\text{-}C_4(Cpdppz)_2(phen)_4Ru_2]^{4+}$ dimer, where the two metal containing subunits are held together by a flexible linker (Fig. 6A), have been measured [85].

The experiments reveal, interestingly, that the Δ-Δ and Λ-Λ enantiomers of this binuclear complex exhibit dynamic groove discrimination, in contrast with enantiomers of the monomer, which behave similarly in terms of groove discriminatory ability.

Both the Δ–Δ and Λ–Λ enantiomers form very strong complexes with ct-DNA with similar affinities, but, by contrast, they display considerable variation in their binding kinetics. The Δ–Δ enantiomer has higher affinity for ct-DNA compared to poly(dA-dT)·poly(dA-dT) and the association kinetics is biphasic for both nucleic acids. The dissociation kinetics, on the other hand, is described by a single exponential effect for poly(dA-dT)·poly(dA-dT) whereas, in agreement with the association kinetics, two exponentials are required to fit the data obtained with ct-DNA. The Λ–Λ enantiomer dissociates about one order of magnitude faster than the Δ–Δ enantiomer and the salt dependence of the dissociation rate constant is stronger for Λ–Λ than for Δ–Δ. A reaction mechanism, apparently described by Scheme (3), is proposed where the first step corresponds to threading intercalation of one of the two subunits of the metal complex ($DS_I$ thus corresponds to a mono-intercalated species). This step (relatively fast) is followed by a slower process where the second subunit also intercalates by threading through nearby base pairs (leading to $DS_{II}$, the bis-intercalated species). This second process is multi-step and seems to involve profound conformational rearrangements of the intermediate forms, as inferred by the slowness of the dissociation reaction (Scheme (10))

$$D + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} DS_I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \cdots \underset{k_{-n}}{\overset{k_n}{\rightleftharpoons}} DS_{II} \quad (10)$$

Salt effects on both the association and dissociation paths support the proposed mechanism. Spectroscopic and kinetic analyses have shown that $[\mu\text{-}C_4(Cpdppz)_2(phen)_4Ru_2]^{4+}$ intercalates its extended aromatic dppz moieties, placing the two ruthenium subunits in the same groove and the aliphatic linker in the opposite groove [86].

When the flexible linker is removed, so that the two $[Ru(phen)_2(dppz)]^{2+}$ subunits are directly connected through the two dppz moieties, as in $[\mu\text{-}(11,11'\text{-bidppz})(phen)_4Ru_2]^{4+}$ (Fig. 6B) which also has high affinity for DNA, the kinetic features of binding to DNA display a radical change. The process is also composite and in the first phase a groove bound intermediate is formed [87]. In subsequent investigations [38,88] it has been shown that the Δ–Δ, Λ–Λ and Δ–Λ (meso) stereoisomers of $[\mu\text{-}11,11'\text{-bidppz})(phen)_4Ru_2]^{4+}$ bind to ct-DNA, poly(dG-dC)·poly(dG-dC) and poly(dA-dT)·poly(dA-dT) giving a final complex where the bi-dppz residue is intercalated between base pairs while the two $Ru(phen)_2$ moieties are located each in one groove. Experiments with the meso Δ–Λ isomer showed that the Λ moiety prefers the minor groove and the Δ moiety the major groove. With poly(dA-dT)·poly(dA-dT) all stereoisomers

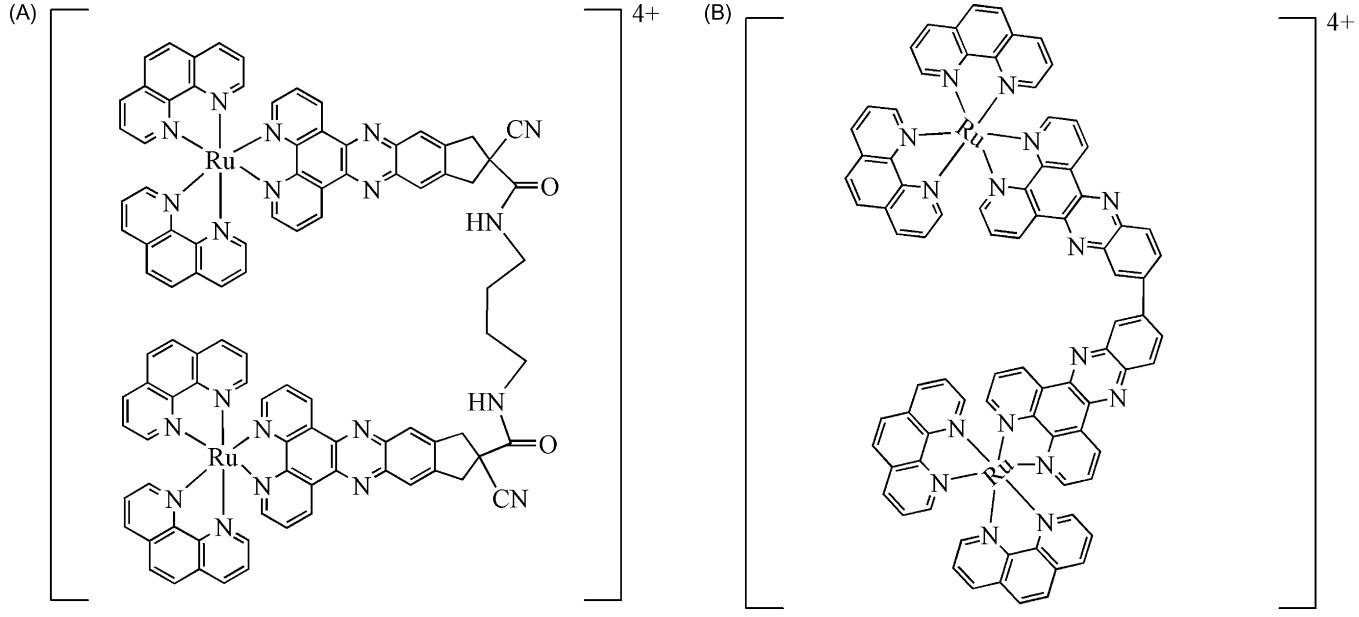
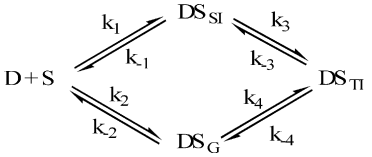



Fig. 6. Structure of $[\mu\text{-}C_4(Cpdppz)_2(phen)_4Ru_2]^{4+}$ (A) and of $[\mu\text{-}11,11'\text{-bidppz})(phen)_4Ru_2]^{4+}$ (B).

seem to react by one-step threading intercalation (Scheme (1)), whereas with ct-DNA, poly(dG-dC)·poly(dG-dC) and poly(dI-dC)·poly(dI-dC) an intermediate complex is formed (Scheme (3)), where the metal containing subunits are both located in the major groove. The threading intercalation mechanism has been investigated for both the Λ–Λ isomer of [μ-(11,11′-bidppz)(phen)$_4$Ru$_2$]$^{4+}$ (ΛΛ-P$_4$) and for its analogue ΛΛ-B$_4$ where the phenanthroline ligands are replaced by bipyridine molecules [89]. The binding mechanism for the DNA/ΛΛ-B$_4$ system has been interpreted in terms of a bimolecular interaction step leading to formation of a semi-intercalated species which, by slow threading intercalation converts to the final complex (Scheme (3)). On the other hand, the binding of ΛΛ-P$_4$ to DNA, biphasic as well, has been interpreted assuming the first phase to be composed of two parallel paths, as in Scheme (9), one of them leading to formation of a semi-intercalated complex (DS$_{SI}$), as with ΛΛ-B$_4$, and the other to a groove aligned complex (DS$_G$). In the second phase the conversion of the semi-intercalated species to the final threaded complex (DS$_{TI}$), as shown in Scheme (11) is slightly disfavoured owing to the steric hindrance of the larger phenanthroline residues




(11)

By contrast, the structure of the groove aligned state can favour an opening of the base stacks, thus facilitating threading through the DNA helix. The important role played by the ancillary ligands in directing the binding modes points out the potentiality of semi-rigid binuclear ruthenium complexes as selective DNA probes.

Concerning the Δ–Δ enantiomer, crucial evidence for threading intercalation of ΔΔ-P$_4$ into DNA is provided by the observation that the SDS-induced dissociation of the final complex needs several hours at elevated temperature (50 °C) to occur [38] (see Section 7). The details of the binding kinetics of Δ-Δ [μ-(11,11′-bidppz)(phen)$_4$Ru$_2$]$^{4+}$ (ΔΔ-P$_4$) to DNA have been investigated by exploiting the energy transfer fluorescence (FRET) emission from added DAPI [90]. The FRET experiments suggest that the binding step involves formation of a rather unstable threaded intercalate which slowly rearranges to a more stochastically favourable bound form, according to Scheme (10). This rearrangement can occur by a dissociation/re-association process, according to a sort of shuffling already suggested to explain the (relatively) slow binding to DNA of the natural antibiotic nogalomycin [7,91,92]. Alternatively, the complex remains threaded and moves within DNA according to a mechanism which requires sequential base-opening/base-closing. A similar mechanism has been proposed for the DNA/porphyrin system [19].

## 5. Azamacrocycle metallo-intercalators

Macrocycles made by a polyamine (or polyether) bearing an aromatic fluorophore inserted in the ring constitute an interesting class of metal receptors. These systems, besides enabling selective sensing of metal ions [93], can be employed as probes for nucleic acids [94], both as free ligands and bound to metal ions. As the luminescence of the system depends on the coordinated metal and on the substrate with which the macrocycle undergoes interaction, this property makes these metallo-receptors especially suitable to react with nucleic acids by intercalation of the aromatic fluorophore between base pairs [95,96]. However, the action of azamacrocycle metallo-intercalators is not limited to probing polynucleotides; in some cases, once the complex anchored in the vicinity of the nucleic acid by the intercalating residue, there is the possibility of cleavage of the phosphodiester bond by the metal centre [97–100].

### 5.1. Metal–macrocycle complex binding to polynucleotides

Polyamine complexes of Cu(II), Fe(II) and Zn(II) are able to tightly bind to polynucleotides [101]. Complexes containing a planar chromophore display hypochromic and bathochromic shifts of the UV–vis spectra upon addition of DNA, interpreted in terms of drug intercalation between the base pairs [97,98]. The intercalative mode of binding of cyclic or open polyamine metal complexes containing a 1,10-phenanthroline residue (Fig. 7) is demonstrated by Scatchard analyses of the binding isotherms for Cu(II)Neotrien, Zn(II)Neotrien, Cu(II)Neotetren and Cu(II)$_2$Neotreten [102,103]; such analyses yield site sizes higher than one, in agreement with the excluded site model [1].

Non-intercalative binding modes are also possible, as oxa-azamacrocycles that do not contain any planar intercalating

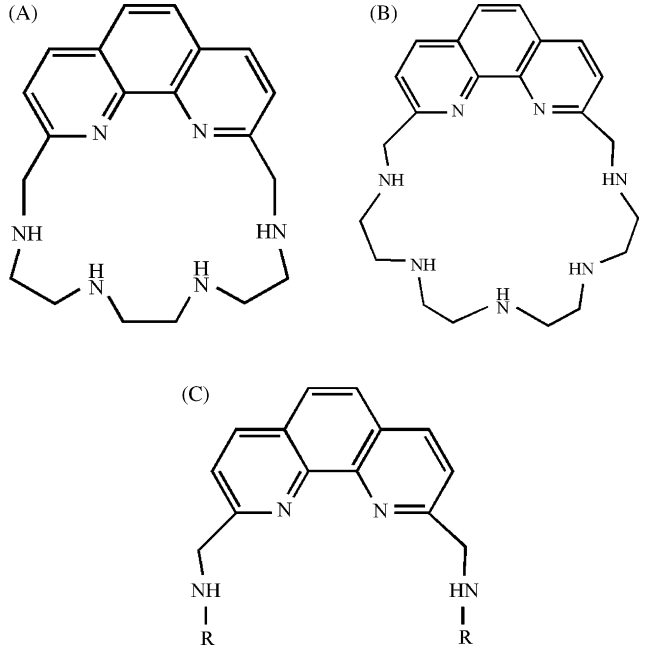



Fig. 7. Molecular structures of macrocyclic ligands in their unprotonated forms: (A) 2,5,8,11-tetraaza[12]-[12](2,9)[1,10]-phenanthrolinophane (Neotrien), (B) 2,5,8,11,14-pentaaza[15]-[15](2,9)[1,10]-phenanthrolinophane (Neotetren), (C) *N*,*N*′-dialkyl-1,10-phenantroline-2,9-dimethanamine, R = CH$_3$, CH$_2$CH$_3$, (CH$_2$)$_2$CH$_3$, (CH$_2$)$_3$CH$_3$, CH(CH$_3$)$_2$ or C(CH$_3$)$_3$.

residue inserted in the chain are also able to stabilise the double helix of DNA [95].

Electrostatic attraction is an important driving force for the binding of metallo-intercalators to nucleic acids. In principle, the contribution to the total charge of the complex is provided not only by the metal ion but, also by the ligand that could be protonated even at pH 7. Two metal centre hosting is also possible, depending on the size of the macrocycle, to give binuclear highly charged forms. An increase in the global positive charge borne by the metal/ligand complex is reflected in a higher affinity towards the polynucleotide [102]. However, the electrostatic interaction is not the only property to be taken into account; actually, cycle dimension and rigidity also play a role, as enhanced flexibility will give an increased number of contacts with the nucleic acid grooves. This is for instance confirmed by the higher binding constant observed for the binding to DNA of the open Cu(II)-*N*,*N*′-dialkyl-1,10-phenanthroline-2,9-dimethanamine in comparison to the cyclic Neotetren and Neotrien copper complexes [102,104]. If flexibility yields higher affinity, more bulky and rigid complexes may be more suitable to provide selectivity. For instance, some discrimination between RNA and DNA in favour of the former seems to be possible in the case of binuclear copper complexes of Neotetren [95].

### 5.2. Kinetic aspects

Kinetics studies of the binding of metallo-azamacrocycles complexes to DNA are rather scarce. The few studies available in the literature are concerned with the binding kinetics both of the complexes Zn(II)Neotrien, Cu(II)Neotrien, Cu(II)Neotetren and $Cu(II)_2$Neotetren to ct-DNA [102,103] and with the kinetics of ligand exchange, as in the case of the Cu(II) complexes with the open ligands shown in Fig. 7C. The latter complexes interact with a polynucleotide saturated by ethidium bromide [104]; as intercalated ethidium is fluorescent whereas free dye is not, a decrease in the fluorescence emission provides evidence for ethidium removal from the double helix. Both direct binding and ligand displacement kinetics reveal that, for all the analysed systems, two kinetic effects well separated in time are present.

The fast effect, in the sub-second time range, was ascribed to the intercalative binding of the metal complex. Experiments carried out on the direct binding to DNA of Zn(II)Neotrien, Cu(II)Neotrien, Cu(II)Neotetren and $Cu(II)_2$Neotetren yielded mono-exponential traces, whose reciprocal relaxation time was found to be linearly dependent on the DNA concentration. This behaviour is apparently in agreement with a simple, single step, reaction mechanism (Scheme (1)). However, the values of the forward rate constant, $k_f$, found for the Neotrien and Neotetren systems are orders of magnitude lower than those expected for a diffusional encounter of the reaction partners, thus suggesting that the intercalation step is preceded by a very fast (and therefore indirectly observable) step that is related to diffusion controlled formation of an external metal complex/DNA adduct stabilised mainly by electrostatic interactions. The $k_f$ values found for Cu(II)Neotrien ($2 \times 10^5\ M^{-1}\ s^{-1}$) and Cu(II)Neotetren ($2 \times 10^6\ M^{-1}\ s^{-1}$) are rather close together, although displaying a modest increase with ligand flexibility. This observation suggests that the common intercalating moiety plays the main role in this binding step. On the contrary, major differences are observed in the dissociation rate constants values, $k_d$. For instance low values of $k_d$ are found for the binuclear $Cu(II)_2$Neotreten complex dissociation from DNA with respect to Cu(II)Neotreten, suggesting that the second metal ion also plays a significant role.

Concerning the slow kinetic effect, which occurs in the minute time range, the analysis of the kinetic data does not decide if this effect should be ascribed to slow conformational changes, likely related to the aza-macrocycle accommodation into the polynucleotide grooves, or to a covalent attack of the metal centre to the nucleic acid, leading eventually to phosphodiester bond cleavage. However, it was observed, by means of HPLC experiments, that the copper complexes of Neotrien and Neotetren are not able to break DNA under the experimental conditions of the kinetic experiments and, therefore, the slow effect was ascribed to binding-induced conformational changes of DNA; hence, it can be concluded that the binding of Cu(II)Neotrien, Cu(II)Neotetren and $Cu(II)_2$Neotetren to DNA occurs according to the mechanism represented by Scheme (3). However, in the presence of $H_2O_2$ it was found that the above complexes induce DNA cleavage. On the other hand, gel electrophoresis experiments [95] revealed that DNA cleavage by the complexes is in effect possible, but only after several hours of incubation. Interestingly, the cleavage is effective only for those complexes where the cycle contained the intercalating phenanthroline moiety, whereas no cleavage ability could be observed for oxa-azamacrocycles that do not contain any intercalating residue [95]. It has been observed that nuclease activity decreases as the number of the repeating $–NH–(CH_2)_n–NH–$ ($n = 2$ or 3) units is increased, indicating that coordinative unsaturation of Cu(II) ion favours the activity of the complex. Moreover, the nuclease activity is found to be higher for the binuclear complexes, thus showing the essential role of the metal, which was confirmed by experiments with EDTA. Actually, coordination of the metal centre by EDTA resulted in a dramatic reduction of the cleavage efficiency [95]; moreover, rate measurements on bis-*p*-nitrophenyl-phosphate (BNPP) hydrolysis confirm reactivity increases with decrease of coordinative saturation [95]. The importance of coordinative unsaturation is also demonstrated by a study of BNPP hydrolysis by the triisopropyltriazacyclononane copper(II) $(Cu(iPr_3[9]aneN_3))^{2+}$ complex (Fig. 8) [105]. This reaction displays a fist order rate law dependence on the BNPP concentration and a half-order dependence on the metal complex concentration, the latter observation being consistent with a monomer-dimer equilibrium (Fig. 8), where only the monomer possesses cleavage ability.

This half-order dependence was also found in the case of the interaction between DNA and $Cu([9]aneN_3)^{2+}$, $Cu([10]aneN_3)^{2+}$ and $Cu([11]aneN_3)^{2+}$ complexes [106], these complexes showing a lower cleavage efficiency with respect to $Cu(iPr_3[9]aneN_3)^{2+}$.

A slow kinetic effect was also observed for the binding to DNA to an asymmetric copper(II) *N*,*N*-ethane bridged macro-

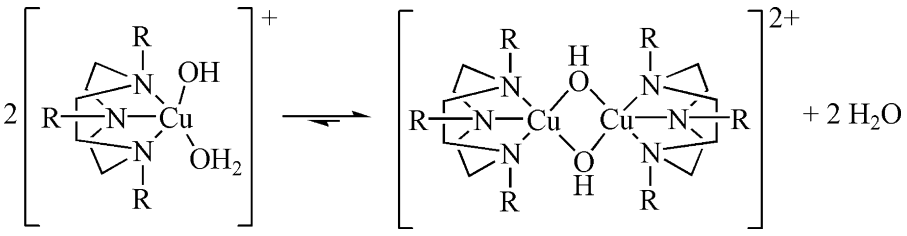


Fig. 8. Triisopropyltriazacyclononane copper(II) dimerisation: R = *i*-Pr.

cycle containing both nitrogen and sulphur atoms in the cycle [107].

## 6. Proflavine derivatives with metal-bearing pendant arms

Molecules displaying two different functionalities can be synthesized, where one moiety is especially designed to provide an anchor on the polynucleotide double helix by means of either intercalation or groove binding, and the second residue has a pendant arm of suitable length that it is able to exert a given chemical function. In particular, bifunctional molecules able to cut the phosphodiester bond with high selectivity are the objects of intensive studies.

Concerning molecules with metal-bearing pendant arms, Fitzsimons and Barton [108] synthesized a rhodium-peptide conjugate, $[Rh(phi)_2bpy']^{3+}$-peptide (Rh-P, phi = phenanthrenequinone diimine, bpy′ = 4-butyric acid-4-methyl-2,2′-bipyridine). This was designed to mimic the active site of metal-containing hydrolases and create a zinc coordination site on the peptide helix. The only species efficient for DNA cleavage is Zn(II)Rh-P. The rate constant, $k_{obs}$, for the cleavage of plasmid DNA by Zn(II)Rh-P at pH 6.0 is found to be $2.5 \times 10^{-5}\ s^{-1}$, three orders of magnitude higher than that for uncatalyzed DNA hydrolysis, and comparable to Eu(III) and Co(III) complexes [109,110]. However, unlike the Eu(III) and Co(III) complexes, efficient DNA cleavage is obtained with micromolar concentration of Zn(II)Rh-P. Cleavage was found to occur hydrolytically and stereospecifically.

### 6.1. Proflavine derivative binding modes

Proflavine derivatives bearing additional functional groups have been synthesized for many years. The target property to be optimised concerns both the dye photophysics and the biologic activity. An example of the former is acridine orange, a molecule very similar to proflavine ($N(CH_3)_2$ residues replacing $NH_2$ ones). This is a selective cell-permeable nucleic acid probe useful for cell cycle determination and epifluorescence microscopy, which interacts with DNA and RNA by intercalation or external binding [111,112]. The first attempts to modulate the platinum antitumour drug binding to DNA by linked acridines (or other potential intercalating residues) were made by Lippard and his school [113–116]. Other important derivatives are 9-aminoacridine. This class of molecules is the object of extensive investigations due to a biological activity that ranges from anti-malarial properties [117], enzyme inhibition [118–120], anticancer activity [121] that led to chemotherapeutics development, photo-affinity labels [122] and fluorescent probes for cancer cell detection [123]. Despite the numerous studies on aminoacridine derivatives, mechanistic aspects of these molecules binding to polynucleotides have been seldom analysed.

An organometallic compound has been synthesized [124] in order to combine two properties: intercalation ability into polynucleotides (owing to the proflavine residue) and anti-tumour activity of *cis*-platinum (Fig. 9). The metal centre is not simply coordinated but platinum is bound to a carbon atom of the ligand.

The features of the interaction of Pt-PR and of proflavine (PR) with ct-DNA [27], poly(A)·poly(U) [24], and poly(A)·poly(A) [26] have been investigated by both spectrophotometric and spectrofluorimetric methods. These studies can indicate differences in binding features on going from DNA to RNA and the role played by the metal containing residue in the reaction mechanism.

Analysis of the binding isotherms shows that in the case of poly(A)·poly(U) the site size value ($n = 1.8$) is the same for PR and Pt-PR, suggesting intercalation of the proflavine residue in both systems while the Pt residues stay outside the base pairs of poly(A)·poly(U). By contrast, in the cases of DNA and poly(A)·poly(A) the site size for binding of Pt-PR ($n = 2.4$) is twice that measured for binding of PR ($n = 1.3$), indicating that in these nucleic acids the Pt containing residues are also able to penetrate between base pairs. This conclusion is supported by reaction enthalpy measurements in the case of DNA;

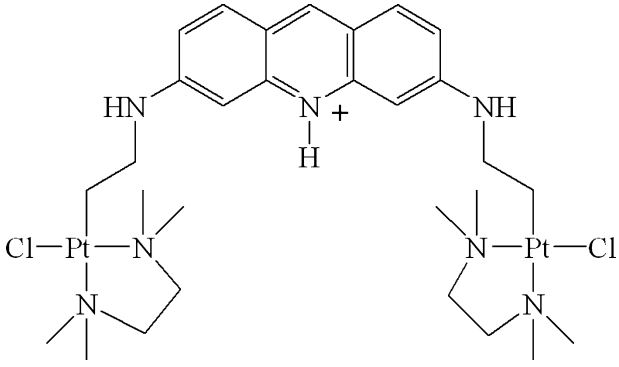


Fig. 9. Molecular formula of the *cis*-platinum derivative of proflavine $[\{PtCl(tmen)\}_2\{HNC_{13}H_7(NHCH_2CH_2)_2\}]^+$ (Pt-PR; $NC_{13}H_7(NH_2)_2$ = proflavine, tmen = *N*,*N*,*N*′,*N*′-tetramethylethylenediamine).

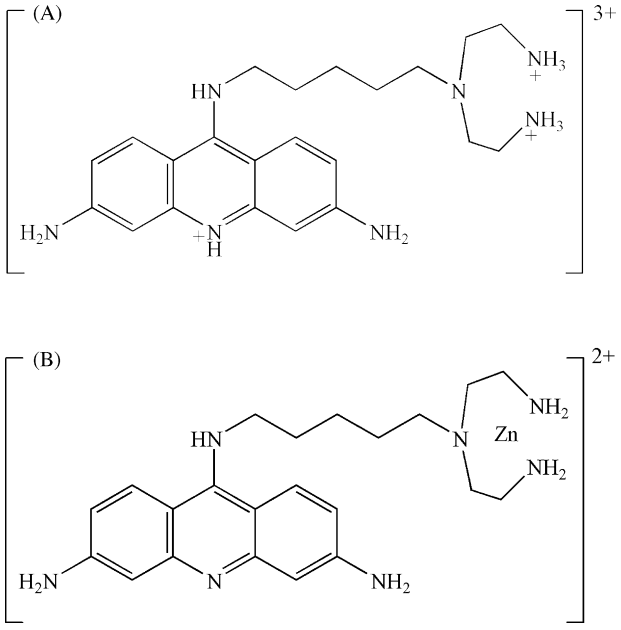



Fig. 10. Molecular formula of 3,6 diamine-9-[6,6-bis(2aminoethyl)-1,6-diaminohexyl] acridine (AzaPR) at pH 7.0 (A) and its Zn(II) complex at pH 8.0 (B).

the value for PR ($\Delta H^\circ = -5.9\,\text{kcal mol}^{-1}$) corresponds to the heat developed for drug penetration into a simple cavity, while the value for Pt-PR ($\Delta H^\circ = -10\,\text{kcal mol}^{-1}$) suggests occupation of two cavities. The analysis of the dependence of the equilibrium constants on NaCl concentration, according to the Record equation [125], shows that the binding of Pt-PR displaces one $Na^+$ ion from the surface of DNA but 1.7 $Na^+$ ions from the surface of poly(A)·poly(U). This observation could support the hypothesis made above, that the Pt containing residues tend to stay on the surface of poly(A)·poly(U), thus displacing a number of sodium ions higher that that displaced from DNA, where the Pt-containing residues fully penetrate between base pairs.

Another proflavine derivative is shown in Fig. 10 where a poly-aza pendant arm has been inserted on the C9 atom of the proflavine intercalating unit. The thermodynamics and kinetics of binding to DNA of Aza-PR, both in the free form and as a Zn(II) complex, have been investigated [126].

The presence of the flexible metal bearing poly-aza open chain confers to the dye properties that are different to those of proflavine alone and of Pt-PR. The binding isotherms observed for both the AzaPR/DNA and Zn(II)AzaPR/DNA systems are biphasic, suggesting the presence of two different binding modes. The first one, occurring at relatively low polymer to dye ratios and noticeably dependent on the presence of the metal centre ($K_1(\text{AzaPR}) = 1.3 \times 10^6\,\text{M}^{-1}$ and $K_1(\text{ZnAzaPR}) = 5.7 \times 10^6\,\text{M}^{-1}$ at $I = 0.1$ M), is interpreted in terms of external binding with an important contribution by the polyamine chain. The second binding mode, occurring at higher polymer to dye ratios, is invariant with respect to the presence of zinc (($K_2(\text{AzaPR}) = 6.9 \times 10^4\,\text{M}^{-1}$ and $K_2(\text{ZnAzaPR}) = 5.6 \times 10^4\,\text{M}^{-1}$ at $I = 0.1$ M), suggesting this is related to the intercalation of the proflavine moiety. The latter assertion is further confirmed by the fact that the value of the equilibrium constant of this binding mode is very close to that of proflavine under the same experimental conditions ($K(\text{PR}) = 6.6 \times 10^4\,\text{M}^{-1}$ at $I = 0.1$ M). The intercalative nature of the second binding mode is confirmed by supercoiled DNA unwinding experiments and by the CD spectra of DNA/AzaPR and DNA/ZnAzaPR that turn out to similar to those of the proven intercalator PR.

### 6.2. Kinetic aspects

The kinetics of the *cis*-platinum derivative of proflavine (Fig. 9) binding to nucleic acids have been analysed by the T-jump relaxation technique with both absorbance and fluorescence detection. The results of the kinetic experiments, as well as confirming the hypotheses advanced in the light of the static experiments, also provide a deeper insight into the details of the binding process. Concerning the binding of proflavine, both the poly(A)·poly(U)/PR and poly(A)·poly(A)/PR systems display two relaxation effects. The DNA/PR system exhibits a single relaxation effect. However, the curved concentration dependence of the relaxation time reveals the presence of a fast relaxation step coupled with the observed kinetic effect. Hence, the kinetic behaviour of the three investigated systems is interpreted according to Scheme (3) where $DS_I$ and $DS_{II}$ are respectively a partially intercalated and a fully intercalated complex.

The presence of the platinum containing residues introduces a further relaxation effect in the DNA/Pt-PR and poly(A)·poly(A)/Pt-PR systems. The time constant of this additional effect, being far below any time constant measured for intercalation of simple drugs into nucleic acids, suggests a wide alteration of the polymer cavity in order to introduce the metal containing residues. The alternative explanation based on groove binding can be discarded on the basis of the kinetic results, as these binding modes have been shown to be nearly diffusion controlled [127]. Therefore, the proposed reaction mechanism for both DNA/Pt-PR and poly(A)·poly(A)/Pt-PR systems is a three-step sequential pathway (Scheme (12))

$$\text{D} + \text{S} \leftrightarrows \text{DS}_\text{I} \leftrightarrows \text{DS}_\text{II} \leftrightarrows \text{DS}_\text{III} \qquad (12)$$

where $DS_I$ represents a partial intercalate, that converts into a complex ($DS_{II}$) where the proflavine residue fully penetrates between the base pairs. The last step is related to the platinum residue passing from the outside to the inside of the cavity. The third step produces a destabilising effect on the double helix, since the value of the $DS_{II} \leftrightarrows DS_{III}$ inter-conversion equilibrium constant is found to be lower than one.

The kinetics of the poly(A)·poly(U)/Pt-PR system are completely different. Whereas the binding of PR shows bi-exponential behaviour, with Pt-PR single-exponential kinetics are observed. This observation has been explained assuming that the Pt-containing residues bound to the RNA surface hinders full penetration of the proflavine residue between base pairs; hence, the sequential mechanism shown in Scheme (12) stops at the level of $DS_I$.

Studies on the salt concentration dependence of the kinetic effects have shown that ionic strength only influences the very fast step, in agreement with the hypothesis that electrostatic attraction plays an important role in the first act of the binding process.

With poly(A)·poly(U)/Pt-PR and poly(A)·poly(A)/Pt-PR the occurrence of a very slow kinetic effect, demonstrated by a blue shift of the absorbance spectra over a time range of 60 h, was observed and ascribed to slow covalent attack of the platinum atom to one of the nitrogen atoms of the nucleic acid bases.

Concerning the kinetics of AzaPR binding to DNA, stopped flow experiments have been carried out under polymer to dye ratio conditions where the intercalative binding mode is active. These yielded good mono-exponential kinetic traces for both free and Zn(II) complexed forms. However, the kinetic constant values found (magnitude order of $10\,s^{-1}$ at 25 °C for both AzaPR and ZnAzaPR, $I = 0.1$ M) are orders of magnitude lower than for diffusion-controlled reactions; this observation suggests the presence of a diffusion controlled pre-equilibration step representing an external binding that precedes intercalation. The reciprocal of the measured relaxation time does not depend on the reactant concentration, indicating that the equilibrium constant of the pre-equilibration step is high. The similarity of the results obtained for the free ligand and for the zinc(II) complex agrees with the thermodynamic observations and with the hypothesis that the second binding mode concerns intercalation of the common proflavine residue.

## 7. The use of surfactants in kinetic studies of ligand dissociation: the SDS sequestration technique

Sequestration of drugs released from DNA/drug complexes by using surfactant micelles, such as SDS (sodium dodecyl sulphate) is a well-established method to study the kinetics of dissociation of cationic, hydrophobic molecules from DNA. This technique, first described by Müller and Crothers [7], is based on the assumption that the micelles are able to sequestrate instantaneously the free drug molecules in equilibrium with the bound drug molecules, thus inducing further dissociation of the drug from the polymer/drug complex. Such a slow dissociation step is supposed to be not influenced by the surfactant nature and concentration, so that the measured dissociation constant should represent the "true" constant of the dissociation process. Further to its introduction, the sequestration method has been employed to investigate the dissociation kinetics of different polymer/drug systems [19,36,92,128–136]. However, some studies have revealed a dissociation rate dependence on the surfactant concentration [92,131–134] but no attempts were made to give a quantitative explanation of these observations until Westerlund et al. [37] demonstrated, by systematic studies, the possible enhancement of the rate of dissociation of cationic dyes intercalated into DNA. This rate enhancement depends on both the surfactant concentration and length of the surfactant tail and is favoured by increasing the positive charge and the hydrophobic nature of the intercalator.

Stopped-flow experiments of Ethidium, YOYO, Δ-Δ-[μ-11,11′-bidppz)(phen)$_4$Ru$_2$]$^{4+}$ (ΔΔ-P$_4$) and [μ-C$_4$(Cpdppz)$_2$(phen)$_4$Ru$_2$]$^{4+}$ dissociation from ct-DNA were performed at different concentrations of sodium dodecylsulphate (SDS), sodium decylsulphate (SDeS) and sodium octylsulphate (SOS) [37]. An increase of dissociation rate was observed which, for a given drug, depends of the length of the hydrophobic tail of the detergent, being higher for SDS and lower for SOS. Also, an increase of rate was obtained by increasing the surfactant concentration, which was much higher than expected on the basis of the ionic strength effect.

More interestingly, the rate enhancement effect on dissociation of Δ-Δ-[μ-11,11′-bidppz)(phen)$_4$Ru$_2$]$^{4+}$ (ΔΔ-P$_4$) from poly(dA-dT)·poly(dA-dT) is observed also for SDS concentrations below the cmc, thus providing evidence that surfactant monomers as well can accelerate the dissociation rate.

In order to explain the rate enhancement observed both below and above the cmc, a dynamic model was proposed. This model assumes that the rate acceleration effect does not involve the micelles as such, but rather local monomers which form as fluctuating entities during the process of micelle explosion and reforming. The surfactant molecules participate to the formation of the transition state as a transient plaque aggregated to the cationic drug bound to DNA. According to this model the total dissociation rate constant results from the contribution of $(N+1)$ reaction paths as

$$k_{\text{total}} = k_0 + \sum_{i=1}^{i=N} k_i [\text{monomer}]^i_{\text{local}} \tag{13}$$

where $k_0$ is the rate constant in the absence of surfactant and $i$ is the number of monomers participating to the formation of the $i$th-transition state.

In order to measure the "true" dissociation constants, the authors propose to extract the ligand from the solution using a different nucleic acid in large excess and a 50-fold excess of ct-DNA has proved to be sufficient to fully extract the dye from poly(dA-dT)·poly(dA-dT). It should also be noted that "true" dissociation constants can be obtained from experiments where the dissociation process is started by ionic strength jumps [19].

## 8. Conclusions and future developments

Since the discovery of the possible use of intercalators in biology and medicine, thousands of potential intercalating molecules have been synthesized and tested to probe nucleic acid structure and to understand the details of drug binding to DNA. Increased understanding of DNA structural changes and interactions will make easier the design of drugs with optimal characteristics for a given application. In this respect, the kinetic method, allowing the anatomy of a reactive process to be investigated, does constitute a tool of paramount importance.

Approaches to site recognition based on metal-nucleobase binding should not be forgotten. The recognition of nucleic acid derivatives by ruthenium (II) arene anticancer complexes like [$\eta^6$-arene Ru(II)(en)Cl]$^{2+}$ has been investigated [137]. The abil-

ity of these pseudo-octahedral Ru(II) complexes to discriminate between guanine and adenine bases is much higher than that displayed by square-planar Pt(II) complexes. The kinetic method has shown that the metal complexes bind to nucleobases according to a mechanism where the ligands around the metal centre play a remarkable role in the recognition process, demonstrating an important tool for the design of new site-specific DNA reagents.

Finally, the use of chemical relaxation methods allows the dynamic analysis to be generalized for application to multiphasic processes where it allows for simultaneous measurements of equilibrium and rate parameters of individual steps, thus yielding information about the fine details of nucleic acid–drug interaction that could not be provided by the static techniques ordinarily employed to investigate the equilibria.

Reduction of the dead-time of the stopped-flow technique to the microsecond scale [138,139] would enable to analyse the mechanisms of additional drug-nucleic acid processes which are scarcely sensitive to relaxation techniques and too fast to be measured with the presently employed fast mixing methods.

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