Formation of DNA Triple Helices by an Oligonucleotide Conjugated to a Fluorescent Ruthenium Complex

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A conjugate of a triple helix forming oligonucleotide (TFO) and the Λ and Δ enantiomers of the ruthenium diphenanthroline dipyridophenazine complex $[Ru(phen)_2dppz]^{2+}$ was synthesized. The ruthenium complex was attached to the 5′-end of the TFO through the dppz moiety. This conjugate formed a stable triple helix with the polypurine tract (PPT) sequence from HIV proviral DNA. The thermal denaturation temperature of the triplex was increased by 12 °C. One remarkable property of the Δ-$[Ru(phen)_2dppz]^{2+}$ complex is a strong increase in its fluorescence when it intercalates into DNA. While the fluorescence of the oligonucleotide conjugate was very weak, the formation of a duplex with a complementary sequence or of a triple helix with a target duplex resulted in a large increase in fluorescence of the Δ enantiomer. The increase in fluorescence allowed us to follow the kinetics of duplex and triplex formation by fluorescence spectrometry. In contrast, the Λ enantiomer gave a much smaller fluorescence change when a triplex was formed, even though the stability of the triplex was comparable to that of the Δ enantiomer. The property was ascribed to intercalation of the dipyridophenazine moiety of the Δ enantiomer into DNA and subsequent threading of the ruthenium complex through the DNA double helix. Salt effects were consistent with the involvement of DNA breathing in the formation of the intercalating complex.

KEYWORDS:

conjugates · fluorescence spectroscopy · oligonucleotides · ruthenium · triple helices

Introduction

Oligonucleotides capable of selectively recognizing specific DNA or RNA sequences and of forming complexes with RNA through complementary base pairing (antisense strategy)[1–4] or with DNA by triple helix formation (antigene strategy)[5–9] represent a promising class of potential therapeutic agents acting directly and selectively on gene expression.

In order to circumvent problems due to the low stability of oligonucleotides in biological media, their poor penetration into cells, and the dissociation of complexes, especially triplexes, conjugation of oligonucleotides to different functional residues has been employed. In the context of complex stability, the attachment of intercalating agents such as acridine,[10] benzopyridoindole,[11] and benzopyridoquinoxaline[12] to oligonucleotides has been shown to increase the stability of their double- or triple-helical complexes significantly.[13]

During the last decade, intercalating complexes of ruthenium(II) and polycyclic aromatic compounds have attracted special attention in studies of DNA properties such as long-distance electron transfer and photocleavage.[14–17] Good DNA-binding properties were found for chiral complexes of Ru^{II} containing 1,10-phenanthroline (phen) and dipyrido[3,2:a-2′3′:c]phenazine (dppz). The binding of the Δ and Λ enantiomers (Figure 1) of the $[Ru(phen)_2dppz]^{2+}$ complex to double-stranded DNA has been investigated.[18–22] The free complex is not fluorescent in aqueous solution, but exhibits strong fluorescence once bound to double-helical DNA (an increase by a factor of more than 10^4).[18, 19] This "light-switch" effect in the presence of DNA has been explained in terms of protection of the dppz moiety from water upon intercalation of the ligand. Comparison of the Δ and Λ enantiomers of $[Ru(phen)_2dppz]^{2+}$ revealed a quantum yield

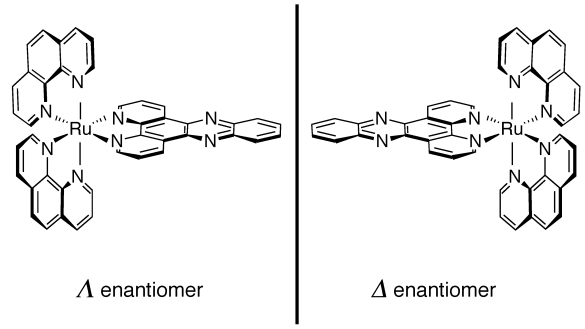

Figure 1. The Δ *and* Λ *enantiomers of the* $[Ru(phen)_2dppz]^{2+}$ *complex.*

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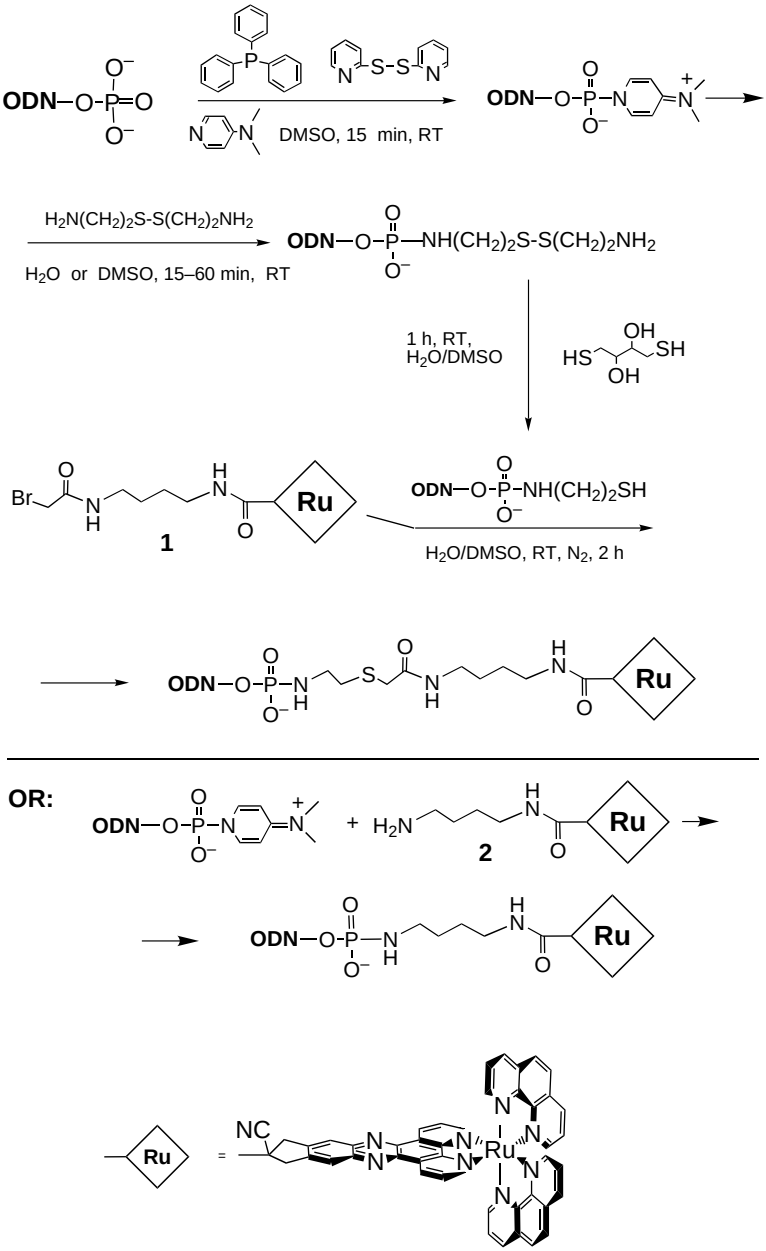
6 – 10 times higher for the Δ enantiomer. This strong variation in fluorescence was explained in terms of a slightly different intercalation geometry; this results in a different degree of protection from quenching by water and differences in the stacking interactions between enantiomers closely bound to contiguous sites on DNA.[20] A sequence-specific molecular "light switch" was synthesized by conjugation of an oligonucleotide to a $[Ru(phen)_2dppz]^{2+}$ complex attached to the 5′-phosphate group of the oligonucleotide through a linker tethered to one of the phenanthroline ligands.[23] The dppz ligand could then intercalate between base pairs after duplex formation with a complementary sequence.

In this study, we conjugated both the Δ and Λ enantiomers of the Ru^{II} complex $[Ru(phen)_2dppz]^{2+}$ to the 5′-end of a triple helix forming oligonucleotide through the dppz ligand. Our goal was to investigate whether the Ru^{II} complex could thread through the double helix at the triplex – duplex junction, despite the bulkiness of the two phen ligands, and allow for intercalation of the dppz moiety. In addition, we intended to exploit two main advantages of these conjugates. On the one hand, intercalation properties should result in stabilization of DNA triple helices, as shown before for other attached intercalators.[11, 12, 24, 25] On the other hand, the "light-switch effect" should offer the possibility to study the kinetics of DNA duplex- and triplex-formation by a highly sensitive fluorescence method.

In addition, other potential applications of the Ru^{II} complex conjugates can be envisaged. For example, dependence of the fluorescence intensity of the complex on the lipophilicity of the environment should provide a sensitive probe for studies of the internalization of conjugated biomolecules (oligonucleotides, proteins) through cell membranes,[26] as well as for detection of their interaction with nucleic acids inside cells.



Scheme 1. *Synthesis of oligonucleotide – ruthenium(II) complex conjugates. ODN = oligodeoxyribonucleotide, DMSO = dimethylsulfoxide.*

Results and Discussion

Synthesis of oligonucleotide – $[Ru(phen)_2dppz]^{2+}$ conjugates

Several methods for oligonucleotide 5′-phospate group functionalization have been described in a previous paper.[27] The general approach of the two alternative routes for the oligonucleotide – ruthenium complex conjugate synthesis is shown in Scheme 1; details are given in the Experimental Section. We used both direct coupling of an amino-containing ruthenium complex to an activated 5′-phosphate group of the oligonucleotide (shorter linker, compound **2** in Scheme 1) and conjoining of an alkylating ruthenium complex derivative with a thiol-modified oligonucleotide (longer linker, compound **1** in Scheme 1). Both methods were equally effective, but thiol alkylation provided a slightly better yield from the coupling reaction. In all comparative experiments the shorter linker gave results similar to those obtained with the longer one. We used this shorter linker for comparison of Δ and Λ enantiomers conjugated to oligonucleotides; all other experiments were carried out with the longer linker.

Stability of double- and triple-stranded DNA formed with Δ-HIV-T-Ru

The formation of a DNA triple helix by the HIV-T – Δ-$[Ru(phen)_2dppz]^{2+}$ conjugate (Δ-HIV-T-Ru) and its double-stranded target (Figure 2) was demonstrated first by nondenaturing polyacrylamide gel electrophoresis. The results are shown

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5'-CCACTTTTTAAAAGAAAAGGGGGGACTGG-3'   (HIV-D1)
3'-GGTGAAAAATTTTCTTTTCCCCCCTGACC-5'   (HIV D2)
        5'-pTTTTMTTTTMMMMMMT-3'       (HIV-T)

3'-CCACTTTTTAAAAGAAAAGGGGGGACT-5'     (HIV-comp)
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Figure 2. *Sequences of oligonucleotides used in this study. The target duplex HIV-D1:HIV-D2 is a conserved oligopurine – oligopyrimidine fragment existing in the* pol *and* nef *genes of HIV proviral DNA (the polypurine tract (PPT)). The triple helix forming oligonucleotide HIV-T was used for conjugation of the Ru^{II} complex. The oligonucleotide HIV-comp was used as a complementary target for Watson – Crick base pair formation with HIV-T. 5′-p = a 5′-phosphate group, M = 5-methylcytosine.*

in Figure 3. At pH 6 and low temperature (10 °C), both unmodified and modified oligonucleotides form a triplex that migrates in a gel as a distinct retarded band (Figure 3, lanes 3 and 4). When the pH value was changed to 8.0, no triplex formation at all was detected with the nonconjugated oligonucleotide. However, the triplex formed by the Ru^{II}-conjugated oligonucleotide was still detectable but its band was diffuse (data not shown); this indicates low stability of this triplex at this pH value. A similar picture was observed when the temperature of the gel was increased from 10 to 35 °C at pH 6.0 (data not shown).

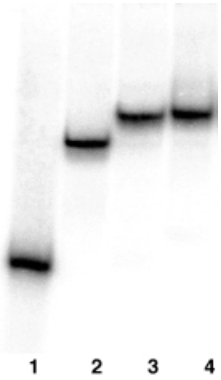

Figure 3. *Gel electrophoresis of oligonucleotides at 10 °C in 20% nondenaturing polyacrylamide gel. Lane 1: The 5′-^{32}P-oligonucleotide HIV-D1; lane 2: double-stranded DNA HIV-D1:HIV-D2; lane 3: the triplex formed by double-stranded DNA HIV-D1:HIV-D2 and nonmodified HIV-T; lane 4: the triplex formed by double-stranded DNA HIV-D1:HIV-D2 and conjugate Δ-HIV-T-Ru. The concentrations of HIV-D1 and HIV-D2 were 50 nM and of HIV-T and Δ-HIV-T-Ru were 20 μM. The electrophoresis buffer was 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES; pH 6.0) with 50 mM NaCl and 5 mM $MgCl_2$.*

Triplex stabilization was also demonstrated in thermal denaturation experiments. Figure 4 shows the denaturation profiles of double and triple helices formed by the oligonucleotide and its Ru^{II}-complex conjugate. The attachment of the $[Ru(phen)_2dppz]^{2+}$ complex to the oligonucleotide terminus increased the denaturation temperature of the duplex (HIV-T:HIV-comp) by $\Delta T = 8$ °C, and the stabilization of the triple helix reached $\Delta T = 12$ °C.

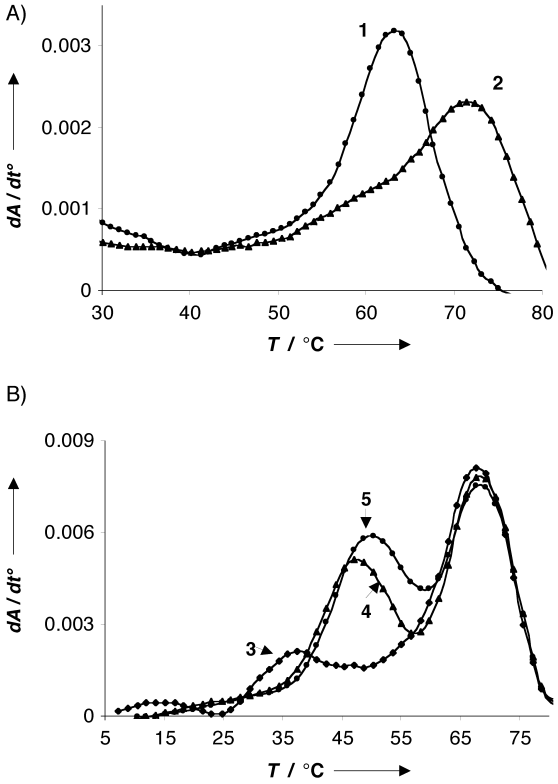

Figure 4. *Thermal denaturation of the complexes of HIV-T and its ruthenium conjugate with complementary strand HIV-comp (A) and with a duplex HIV-D1:HIV-D2 (B). The first derivatives of the melting curves are shown. The concentration of each strand was 1.3 μM in 10 mM cacodylate buffer (pH 6.0, 0.2 M NaCl, 10 mM $MgCl_2$). 1) Duplex (HIV-T:HIV-comp); 2) duplex (Δ-HIV-T-Ru:HIV-comp); 3) triplex (HIV-T:HIV-D1:HIV-D2); 4) triplex (Δ-HIV-T-Ru:HIV-D1:HIV-D2); 5) triplex (Λ-HIV-T-Ru:HIV-D1:HIV-D2).*

Comparison of the Δ and Λ enantiomers of the Ru^{II} complex conjugated with HIV-T (Δ-HIV-T-Ru and Λ-HIV-T-Ru)

It has previously been shown that the fluorescence of the Δ enantiomer of the ruthenium complex upon binding to double-stranded DNA is 6 – 10 times higher than that of the Λ enantiomer.[20–22] In this work, we also compared the fluorescence properties of the Δ and the Λ enantiomers conjugated to the oligonucleotide HIV-T. Although neither the Δ nor the Λ enantiomer of the free Ru^{II} complex was fluorescent in aqueous solution, their oligonucleotide conjugates Δ-HIV-T-Ru and Λ-HIV-T-Ru exhibited a temperature-dependent fluorescence that decreased significantly upon heating. The Λ conjugate was about twice as fluorescent as the Δ conjugate (Figure 5). Upon addition of double-stranded DNA, in contrast, the fluorescence of the Λ conjugate did not increase markedly, even when the DNA was added in excess and even though triplex formation was clearly detected by electrophoresis, whereas the fluorescence of the Δ enantiomer was increased by 6 – 10 times (Figure 5).

It should be noted that similar behavior was observed when a complementary 27-mer oligonucleotide (HIV-comp) was added to the Δ and Λ conjugates to form double helical complexes. These results show that the difference in fluorescence behavior of the enantiomers does not depend on their conjugation to

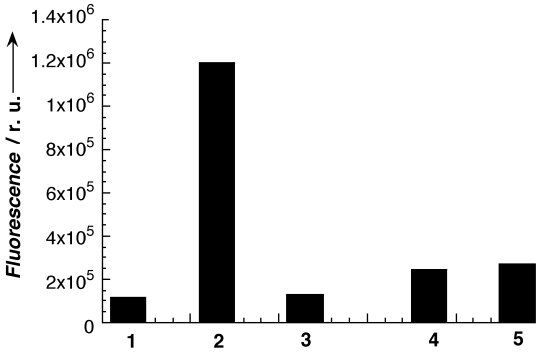

Figure 5. *Fluorescence of 2.7×10^{-7} M Δ-HIV-T-Ru (1 – 3) and Λ-HIV-T-Ru (4 – 5) in 50 mM MES buffer (pH 6.0, 50 mM NaCl, 5 mM $MgCl_2$) after incubation overnight at 30 °C. 1) and 4) with no additive; 2 and 5) after addition of one equivalent of double-stranded DNA (HIV-D1:HIV-D2); 3) after addition of one equivalent of a noncognate double-stranded DNA of sequence:*
CAATCGGATCGAATTCGATCCGATTG
GTTAGCCTAGCTTAAGCTAGGCTAAC

oligonucleotides. This difference was previously explained in terms of a slightly different geometry of complex intercalation and stacking interaction between the ligands in the complex. Our data show that stacking interactions are not so important, as in this case only one Ru^{II}-dppz moiety is bound to DNA.

The melting temperatures of the triplexes formed by Δ-HIV-T-Ru and by Λ-HIV-T-Ru were not very different, with the Λ conjugate exhibiting a slightly greater stabilization of the triplex than the Δ conjugate (Figure 4). In all the following experiments only the Δ enantiomer conjugates were used.

Fluorescence properties of Δ-HIV-T-Ru upon interaction with DNA

As mentioned above, the free ruthenium complex is not fluorescent in aqueous solution (Figure 6 A, curve 1), unlike the complex in an organic solvent (Figure 6 A, curve 3). After conjugation to an oligonucleotide, however, it showed fluorescence upon excitation with 380 nm light (Figure 6 A, curve 2), a feature that appeared to be strongly temperature-dependent. Upon heating from 10 to 60 °C the fluorescence intensity decreased about sixfold, increasing again upon cooling (data not shown). When the samples were kept at 30 °C the fluorescence remained fairly low and stable over 24 h incubation at this temperature. We explain the fluorescence of the conjugate by nonspecific inter- or intramolecular stacking interactions of the dppz unit of ruthenium complex with the oligonucleotide bases, facilitated by the positive charge of the ruthenium complex. To minimize these self-interactions, all the fluorescence studies were carried out at 30 °C.

As already observed for the free Ru^{II} complex, the fluorescence increases significantly when a duplex is formed with a complementary single strand (Figure 6 B) or when a triplex is formed with the double-stranded DNA target (HIV-D1:HIV-D2) (Figure 6 A, curve 4 and Figure 6 C). On titration of the Δ-HIV-T-Ru conjugate with a complementary strand or a cognate duplex,

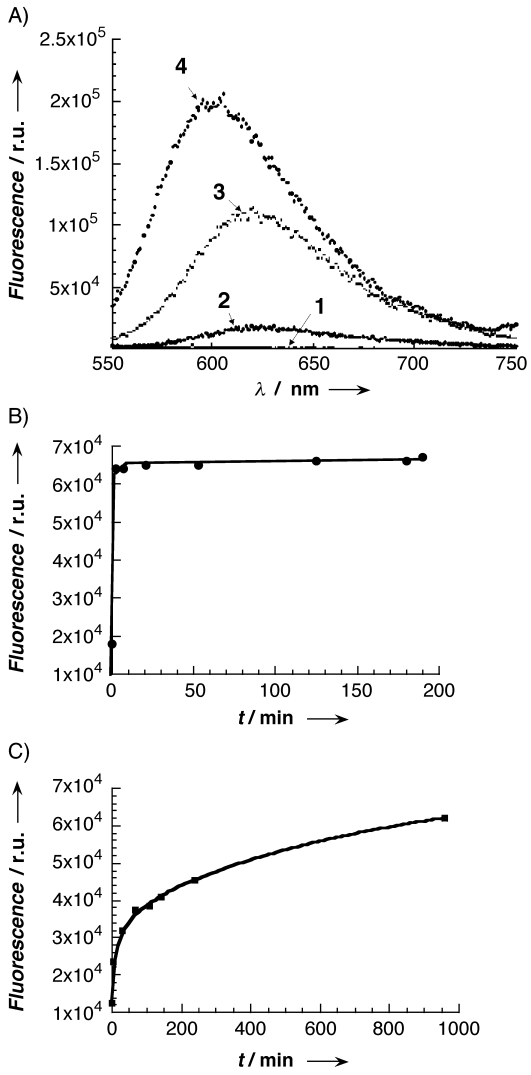

Figure 6. *A) Fluorescence spectra ($\lambda_{exc} = 380$ nm) at 20 °C of: 1) 8×10^{-7} M free ruthenium complex in aqueous buffer; 2) 8×10^{-7} M free conjugate Δ-HIV-T-Ru; 3) 8×10^{-7} M free ruthenium complex in dioxane; and 4) 8×10^{-7} M conjugate Δ-HIV-T-Ru after addition of 1.6×10^{-6} M (2 equiv) double-stranded DNA HIV-D1:HIV-D2. 1), 2), and 4) were measured in 50 mM MES buffer (pH 6.0, 50 mM NaCl, 5mM $MgCl_2$). B) and C) Kinetics of increase in fluorescence of Δ-HIV-T-Ru at 30 °C: B) on addition of 0.2 equiv of complementary strand HIV-comp; C) on addition of 0.2 equiv of duplex (HIV-D1:HIV-D2).*

the fluorescence levels out at a 1:1 molar ratio. This increase in the fluorescence can in the first case be attributed to the formation of a complementary duplex, and in the second case to that of a triple helix, with intercalation of the Ru^{II} complex moiety and consequent shielding of the Ru^{II} complex from water. Titration experiments demonstrated that the plateau level of fluorescence for the oligonucleotide conjugate upon addition of the duplex was 2.5 times higher than that for the free nonconjugated complex at the same duplex:Ru^{II} complex ratio in the same buffer (data not shown). The fluorescence increase kinetics differed significantly for the duplex and the triplex. Upon addition of the complementary strand, the fluorescence increased and leveled out immediately, while slow kinetics were observed when the duplex HIV-D1:HIV-D2 was added; several

hours were necessary to reach the plateau fluorescence level (Figure 6B and C). This result is compatible with slow formation of triple helix at pH 6, which contrasts with rapid duplex formation.[28]

The sequence specificity of the fluorescence increase on triplex formation was confirmed in experiments with a double-stranded DNA fragment that did not contain the target sequence. When the 26-mer noncognate duplex was added to the oligonucleotide – Ru^{II} conjugate, no increase in fluorescence compared to that of the target sequence was observed, even after several hours of incubation (Figure 5). A slight increase observed with calf thymus DNA could be explained by the possible existence of oligopurine/oligopyrimidine tracts partially homologous to the HIV polypurine tract (PPT; data not shown). This sequence-specific fluorescence increase provides a powerful tool to detect, characterize, and follow the kinetics of duplex and triplex formation by fluorescence measurements.

Comparison of duplex and triplex stability by competition between Δ-HIV-T-Ru and HIV-T

We compared the stability of duplexes and triplexes formed by HIV-T and its conjugate with ruthenium complex by the fluorescence method. Since the unmodified HIV-T forms thermodynamically less stable duplexes and triplexes (see above) than the modified Δ-HIV-T-Ru, the latter should be able to displace the unmodified oligonucleotide from preformed duplexes or triplexes. This was indeed the case. The kinetics of fluorescence increase and the plateau level at room temperature were not changed when we added either the complementary strand HIV-comp or a preformed duplex (HIV-T:HIV-comp) to a conjugate Δ-HIV-T-Ru. Displacement of the third strand in the triplex also occurred. The same kinetics of fluorescence increase were observed when one equivalent of Δ-HIV-T-Ru was added to the preformed triplex (HIV-D1:HIV-D2:HIV-T; data not shown). In contrast, addition of as much as ten equivalents of nonconjugated HIV-T to a preformed fluorescent duplex (Δ-HIV-T-Ru:HIV-comp) or triplex (HIV-D1:HIV-D2:Δ-HIV-T-Ru) did not reduce the fluorescence of the preformed complex, a fact clearly indicating a better affinity of the conjugate both for single-stranded and for double-stranded DNA. The mixture obtained in this last experiment was also heated to 90 °C and slowly cooled to room temperature in order to provide equal renaturation conditions for both conjugated and nonconjugated oligonucleotides. In all cases the same intensity of fluorescence at the end of the cycle as at its beginning was observed; this again indicates preferential formation of complexes by ruthenium conjugates.

Studies of intercalation of oligonucleotide-conjugated ruthenium complexes into DNA

As suggested previously, a large increase in fluorescence indicates protection of the dppz moiety from water by intercalation into DNA.[20–22] However, it is not evident that a conjugated complex with a limited extent of freedom also intercalates into DNA after triplex formation. The two bulky phenanthroline units would have to cross the DNA in order to give the dppz unit a possibility to intercalate. This would only be possible if local opening of the double helix ("breathing") were able to take place. In order to understand this process better, we compared the fluorescence increases on formation of triplex by a conjugate with: i) a 29 base pair (bp) target, and ii) a much longer 162 bp fragment containing only one target 16-mer sequence in the center of the fragment (positions 72 – 87).

As can be seen in Figure 7, the increase of fluorescence for the conjugate was much slower and less effective with the 162 bp fragment than with the 29 bp fragment. We explain this difference by "breathing" of the terminal double-stranded region

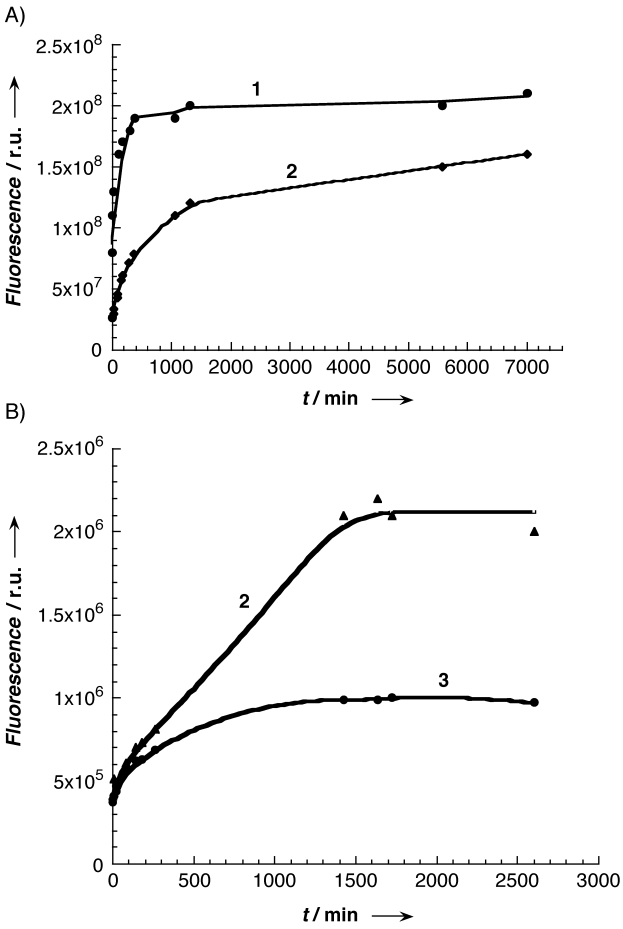

Figure 7. Fluorescence increase of 1.0×10^{-7} M Δ-HIV-T-Ru on addition of 1.0 equiv of double-stranded DNA containing one copy of the 16 bp target sequence for triplex formation: A) fragment length effect; B) salt concentration effect. 1) 29 base pair fragment in 50 mM MES buffer (pH 6.0, 5mM $MgCl_2$, 50 mM NaCl); 2) 162 base pair fragment in 50 mM MES buffer (pH 6.0, 5mM $MgCl_2$, 50 mM NaCl); 3) 162 base pair fragment in 50 mM MES buffer (pH 6.0, 5 mM $MgCl_2$, 1 M NaCl).

of the 29 bp target in order to permit threading of the bulky ruthenium complex through the double helix. Indeed, the distance between the intercalation site and the end of the duplex is only 9 base pairs for the 29 bp target, while this distance in the longer duplex is 72 base pairs and the opening of the double helix in the intercalation region is more difficult.

In order to rule out the possibility that the lower fluorescence yield and the lower rate of its increase obtained with a long target was due to nonspecific interaction between ruthenium complex moiety and noncognate DNA, an additional control was carried out with a noncognate sequence. Triplex formation with the short double-stranded DNA (29 bp) was carried out in the presence of two molar equivalents of a double-stranded DNA fragment that did not contain the target sequence:

5′-AATTCGAGCTCGCCCGGGGATCCTCTAGAGCTCGCTCAATCTCTTTCCTCTTCTCCCTTAACTCGGGCGCCCGGGAA-3′

3′-AGCTCGAGCGGGCCCCTAGGAGATCTCGAGCGAGTTAGAGAAAGGAGAAGAGGGAATTGAGCCCGCGGGCCCTTCGA-5′

Its length (77 bp) roughly corresponds to the size of the flanking sequences of the target in the 162 bp fragment. The presence of a noncognate double-stranded fragment did not influence the kinetics of association (data not shown). We therefore concluded that hampered opening of double-stranded DNA at the site of intercalation was responsible for the slow fluorescence increase upon interaction between Δ-HIV-T-Ru and a long target.

We therefore investigated the effect of addition of NaCl on the kinetics of fluorescence increase. In our experiments in the presence of 5 mM Mg^{2+}, addition of NaCl stabilized the triple helix formed by HIV-T with the 29 bp duplex—the melting temperature of a triplex was 10 °C higher at 1 M NaCl than at 0.2 M NaCl. An increase in NaCl concentration is also expected to decrease DNA strand "breathing".[29–31] The fluorescence increase was smaller and much slower (a fluorescence intensity 2 times lower was reached after 2.5 hours of incubation) for the triplex formed by Δ-HIV-T-Ru with the 162 bp fragment in the 50 mM MES buffer (pH 6.0) in the presence of 1 M NaCl and 5 mM $MgCl_2$ than it had been with the same buffer without NaCl. In contrast, the effect of NaCl addition was much less marked when the 29 bp fragment was used instead of the 162 bp fragment. An additional control experiment demonstrated that no effect of salt concentration on the fluorescence of the conjugate alone was observed in the same buffer.

These data strongly support the hypothesis that formation of a triple helix by Δ-HIV-T-Ru is accompanied by intercalation of the dppz moiety of the $[Ru(phen)_2dppz]^{2+}$ complex into the DNA molecule, giving rise to an increase in its fluorescence and a stabilization of the triplex. This intercalation is facilitated by "breathing" of double-stranded DNA in the vicinity of the complex and implies threading of the bulky complex through the double helix.

Thus, the fluorescence enhancement reflects the kinetics of two mutually dependent processes: triple-helix formation and intercalation of the ruthenium complex into the DNA double strand. Intercalation of the conjugated ruthenium complex is not possible without triplex formation, but, in cases of rigid DNA structure, the intercalation process might take a longer time than the triplex formation itself. However, the intercalated ruthenium complex stabilizes the triple helix and increases its lifetime.

Conclusions

In this work we have developed a simple synthetic method for conjugation of an intercalating ruthenium complex with oligodeoxyribonucleotides. Our studies show that the ruthenium complex $[Ru(phen)_2dppz]^{2+}$, conjugated to an oligonucleotide (to form Δ-HIV-T-Ru), binds sequence-specifically to DNA. The formation and stabilization of the triple helix by this conjugate has been demonstrated. In competition experiments with the unmodified oligonucleotide HIV-T, only the modified one binds. The Λ enantiomer conjugate (Λ-HIV-T-Ru) binds to double-stranded DNA with the same affinity; however, the fluorescence increase is much less important in this case.

The strong increase in fluorescence upon triplex formation with Δ-HIV-T-Ru, together with the significant decrease in the rate of fluorescence enhancement when a longer double-stranded DNA fragment is used as a target or when the "breathing" of DNA is inhibited by high salt concentration, are in agreement with intercalation of the dppz moiety of the ruthenium complex into DNA.

Such ruthenium complexes conjugated to oligonucleotides could be usable as photosensitizers for directed photodamage of DNA through triple-helix formation, as well as for studies of electron transfer along DNA chains, as reported previously for double-helical complexes[14–17] and more recently for a triple-helical complex involving an intercalating dibenzophenanthroline photosensitizer conjugated to a triple helix forming oligonucleotide.[32]

The drastic increase in fluorescence of the conjugate Δ-HIV-T-Ru upon formation of duplexes and triplexes offers the potential to study the kinetics of DNA binding of the conjugate by fluorescence spectroscopy. Alternatively, the modified oligonucleotide could provide a sensitive probe to test the hydrophobicity of its environment, which might be applicable in cellular systems. For example, the fluorescence approach might be used to follow cellular penetration of the conjugates and their interaction with nucleic acids inside cells.

Experimental Section

Reagents: Organic solvents and the majority of reagents were purchased from Sigma/Aldrich/Fluka. Phosphodiester oligonucleotides were synthesized by Eurogentec; their sequences are shown in Figure 2. Phosphorylation of oligonucleotides was carried out enzymatically with T4 polynucleotide kinase (New England BioLabs) as described previously.[33] Concentrations of oligonucleotides were determined spectrophotometrically.

A 162 bp fragment containing the polypurine target sequence of HIV-1 was obtained by a standard polymerase chain reaction (PCR) method with specific primers from plasmid PSPF47 containing fragments of HIV provirus and kindly provided by Dr. C. Giovannangeli.

Synthesis of functionalized Δ and Λ ruthenium complexes: The synthesis of $[Ru(phen)_2dppz]^{2+}$ and the separation of its Λ and Δ

enantiomers was carried out according to procedures published previously.[20, 34] The bromoacyl group was introduced by acylation of the aminobutyl substituent with bromoacetic acid and *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate in acetonitrile in the presence of *N*,*N*-diethyl-*N*-isopropylamine. The ruthenium complexes were purified by chromatography as their hexafluorophosphate salts on neutral alumina (Brockmann activity grade III) with acetonitrile as the eluent.

Synthesis of oligonucleotide – $[Ru(phen)_2dppz]^{2+}$ conjugates: For covalent coupling of $[Ru(phen)_2dppz]^{2+}$ to the 16-mer oligonucleotide, the bromoacetyl-substituted ruthenium complex and the oligonucleotide with a terminal thiol group were used (Scheme 1). Briefly, the 5′-phosphorylated oligonucleotide was first precipitated with 8% cetyltrimethylammonium bromide (CTAB, cetyl = hexadecyl). The dry CTAB salt was dissolved in dry DMSO (50 μL). Dimethylaminopyridine (5 mg, 40 μmol) in DMSO (25 μL) was added, together with dipyridyldisulfide (6.6 mg, 30 μmol in DMSO (25 μL)) and triphenylphosphine (7.9 mg, 30 μmol in DMSO (25 μL)). After 20 min incubation at room temperature, triethylamine (5 μL) and cystamine (20 μmol) were added. After 30 min, the oligonucleotide was precipitated with 3% $LiClO_4$ in acetone, washed with acetone, dried, and dissolved in water.

The reduction of the disulfide bond was performed by incubation of the conjugate in an aqueous solution of dithiothreitol (DTT; final concentration of 0.3 M) for 2 h at room temperature. The thiol-containing oligonucleotide was then precipitated with degassed ethanol/sodium acetate under nitrogen. Triethylamine (10 μL) and complex **1** (Scheme 1) as a hexafluorophosphate salt (90 μL of a 7 mM solution, 5 equiv) in degassed DMSO (270 μL) were added to a solution of the modified oligonucleotide in degassed water (30 μL). The solution was incubated under nitrogen for 2 h at room temperature. The reaction mixture was analyzed on a 20% denaturing polyacrylamide gel. The conjugate, which was obtained in about 90% yield, was observed as one distinct, retarded, and weakly fluorescent band.

The product was purified by HPLC on a C-18 Lichrosorb column (Interchrom, 5 μm, 250 × 4.6 mm) with an acetonitrile linear gradient (5 – 80%) in 0.02 M ammonium acetate, by using an Agilent Technologies 1100 chromatography system. The retention times were 11 min for the starting material and 15 min for the product. The absorption spectrum of the product was compared to the sum of those of the oligonucleotide and free ligand. A 1:1 ratio of oligonucleotide and ligand absorption was obtained.

Direct attachment of the amino-functionalized ruthenium complex **2** (Scheme 1) to a 5′-activated phosphate group of the oligonucleotide[27] gave a 50 – 60% yield of conjugate.

Gel retardation assay: For triple-helix gel retardation assays, one of the two target duplex strands (Figure 2) was 5′-end-labeled with [γ-^{32}P]-adenosine triphosphate (ATP) by T4 polynucleotide kinase. The two strands were mixed at equimolar concentrations in a 50 mM MES buffer (pH 6.0) containing 50 mM NaCl and 5 mM $MgCl_2$, heated to 90 °C, and slowly cooled. The third strand, in the same buffer, was added. The final concentrations of the duplex and the third strand were 50 nM and 20 μM, respectively. For triplex formation, the samples were incubated overnight at 4 °C. Electrophoresis was performed at 8 – 10 °C on a 20% nondenaturing polyacrylamide gel in the same MES buffer (pH 6.0). The bands were visualized with a Molecular Dynamics PhosphorImager instrument and treated with ImageQuant software. To test the stability of the triplex at higher pH values, the investigation was also performed in a standard TBE buffer (89 mM tris(hydroxymethyl)aminomethane (Tris)-Borate, 0.05 mM ethylenediaminetetraacetate (EDTA)) at pH 8.0.

Thermal denaturation assay: Thermal denaturation and renaturation studies of duplexes and triplexes were carried out on a Kontron Uvikon 940 spectrophotometer with thermostated cuvettes of 1 cm optical pathlength. The concentrations of the duplex and the third strand in each sample were 1.3 μM and 1.7 μM, respectively, in 10 mM cacodylate buffer (pH 6.0, 0.1 M NaCl, 5 mM $MgCl_2$). The sample temperature was changed by 0.2 °C/min, and the absorption at 260 nm was recorded every 200 s. The treatment of the melting curves was carried out by using KaleidaGraph and Microsoft Excel software.

Fluorescence studies: All fluorescence spectra were recorded on a Spex Fluorolog DM1B instrument in 0.2 cm × 1 cm quartz cuvettes. The slit width was usually 2.5 mm. The excitation wavelength was 380 nm, at which the maximum absorption by the ruthenium complex was observed. Fluorescence emission was recorded between 550 and 750 nm. The temperature of the cuvette was 30 °C, unless otherwise stated.

Under standard conditions, each sample contained $1 - 8 \times 10^{-7}$ M HIV-T-Ru conjugate, 50 mM MES buffer (pH 6.0), 50 mM NaCl, and 5 mM $MgCl_2$. Specific conditions for each experiment are given in the figure legends.

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