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Recognition of DNA Three-Way Junctions by Metallosupramolecular Cylinders: Gel Electrophoresis Studies

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Abstract: The interaction of metallosupramolecular cylinders with DNA three-way junctions has been studied by gel electrophoresis. A recent X-ray crystal structure of a palindromic oligonucleotide forming part of a complex with such a cylinder revealed binding at the heart of a three-way junction structure. The studies reported herein confirm that this is not solely an artefact of crystallisation and reveal that this is a potentially very powerful new mode of DNA recognition with wide scope. The cylinders are much more effective at stabilizing three-way junctions than simple magnesium dications or organic or metallo-organic tetracations, with the *M* cylinder enantiomer being more effective than *P*. The recognition is not restricted to three-way junctions formed from palindromic DNA with a central AT step at the junction; non-palindromic three-way junctions and those with GC steps are also stabilised. The cylinder is also revealed to stabilise other Y-shaped junctions, such as that formed at a fraying point in duplex DNA (for example, a replication fork), and other DNA three-way junction structures, such as those containing unpaired nucleotides, perhaps by opening up this structure to access the central cavity.

Keywords: DNA · gel electrophoresis · metallosupramolecular cylinder · supramolecular chemistry · three-way junctions

Introduction

Metallosupramolecular chemistry has been used to generate nanoscale synthetic agents, such as the tetracationic supramolecular cylinder $[Fe_2L_3]^{4+}$ (for a definition of L see Figure 1a), a triple-stranded helicate formed from three bispyridylimine organic strands wrapped around two Fe^{2+} ions.[1–3] The binding of this agent to natural polymeric DNAs and the intramolecular DNA coiling that results have been described previously[1,2] and appears to be through major-groove recognition.[4,5] Recently, the agent in a complex with the palidromic hexanucleotide 5′-d(CGTACG)-3′ was crystallised.[6] Surprisingly, molecular recognition not of duplex DNA, but rather of a three-way junction was observed. The DNA formed a Y-shaped three-way junction and the central hollow cavity at the branch point of the junction was occupied by the tetracationic supramolecular compound (Figure 2). Importantly, the structure of the cylinder agent did not suffer any significant conformational change upon binding to DNA and fitted perfectly into the central hydrophobic cavity of the three-way junction.

To gain more information about the scope and potential of this new and unprecedented mode of DNA recognition,[7] in the present work we examine the recognition of different three-way junctions by supramolecular cylinders and other tetracations with the aid of polyacrylamide gel electrophoresis (PAGE). This method allows us to use only very low concentrations of the cylinders and DNA so that the potential that three-way junction recognition is merely an artefact arising from crystal packing can be excluded. We performed experiments that confirm that the three-way junction assembled from palidromic sequences is also stabilised by $[Fe_2L_3]^{4+}$ in solution and also from longer nonpalindromic oligonucleotides than those that were used in the crystallographic study.[6] In addition, our results using PAGE support the view that the size and shape of the helicate are crucial for recognition and stabilisation of the three-way junction. Excitingly, the studies reveal that the cylinders can recognise and stabilise a variety of different three-way junction structures (and indeed other Y-shaped structures) and that the scope of this mode of DNA recognition might be considerable.

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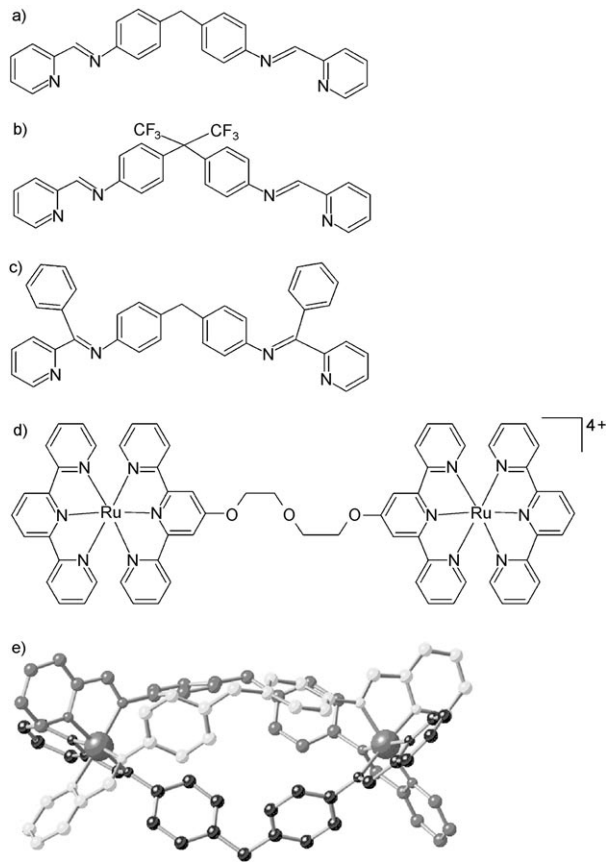

Figure 1. Structures of the ligands in the metallosupramolecular cylinders used in this work. a) $[Fe_2L_3]Cl_4$ ($L=C_{25}H_{20}N_4$). b) $[Fe_2(L\text{-}CF_3)_3]Cl_4$. c) $[Fe_2(L\text{-}Ph)_3]Cl_4$. d) Structure of the tetracation [(tpy)Ru(dtdeg)Ru(tpy)]Cl_4. e) Structure of the unsubstituted cylinder $[Fe_2L_3]^{4+}$ illustrating its three-dimensional structure.

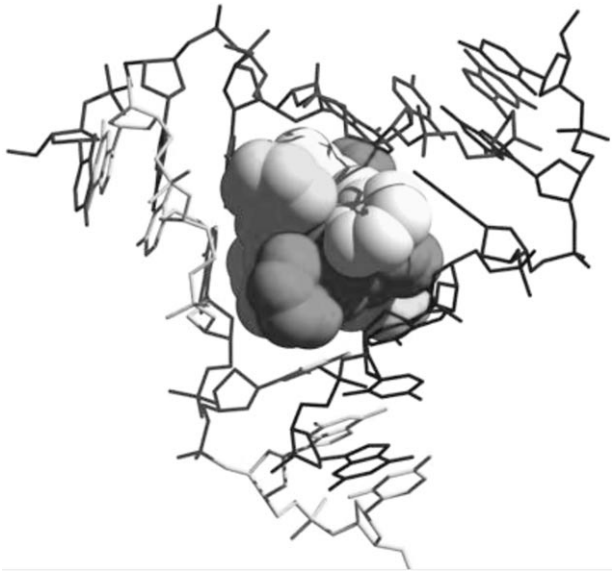

Figure 2. Illustration of the structure of a cylinder recognizing a DNA three-way junction by binding in its central cavity, based on the X-ray crystallographic results described previously.[6]

Results and Discussion

The helicate $[Fe_2L_3]^{4+}$ ($L=C_{25}H_{20}N_4$) can recognise and stabilise a three-way junction assembled from nonpalindromic oligonucleotides: The initial crystallographic study described recognition of a DNA three-way junction formed by a palindromic hexamer; a junction with short arms [three base pairs (bps)]. We chose to initiate our studies with a DNA three-way junction that was formed from three distinct DNA strands, each of which is a 14-mer nonpalindromic oligonucleotide (Figure 3A). The resulting three-way junction has seven bps per arm. Such a three-way junction has been shown previously[8] to be the minimum length of DNA required for a three-way junction lacking unpaired bases to stay stable during electrophoresis on a native gel at low temperatures ($\approx 5\,°C$).

The autoradiogram of the electrophoresis gel run at 5 °C (Figure 3B) shows formation of the three-way junction from the 14-mer oligonucleotides (1.07×10^{-5} M) in the presence of divalent Mg^{2+} ions (10 mM $MgCl_2$) (Figure 3B, lane 4). Magnesium dications are known to stabilise three-way junctions at low-to-intermediate ionic strengths.[9] In the absence of divalent ions the stability of the three-way junction was apparently not very high and the band corresponding to the three-way junction was weak (Figure 2B, lane 3). By contrast, this band became much stronger when the tetracationic cylinder $[Fe_2L_3]^{4+}$ was added (Figure 3B, lane 7). This observation is particularly remarkable, because the cylinder was present in the sample at the concentration three orders of magnitude lower than that of $MgCl_2$ (the ratio single strand/cylinder was 3:1). Also, interestingly, besides stabilising the three-way junction structure, the cylinder also stabilised the dimer (Figure 3B, lane 6) and reduced its migration in the gel in comparison with the migration of the duplex formed in absence of the helicate. The dimer represents a Y-shaped junction (for example, a replication fork). At the branch point of such a Y-shaped junction there are four bases, rather than the six found at the three-way junction branch-point. Nevertheless these four bases may form a binding site similar to that of the three-way junction and it may be hypothesised that the cylinder can bind at the centre of the dimer at this Y-shaped junction. While binding of the cylinder to the three-way junction has little effect on the DNA structure,[6] binding to this Y-shaped structure will significantly alter the shape and flexibility of the dimer. Such binding would be expected to enlarge the projection area of the complex in the direction of its migration in the gel and lead to a slowing of the migration of the complex through the gel pores; this is what is observed. Interestingly, the binding of the cylinder to the three-way junction caused a small acceleration in its electrophoretic mobility relative to the three-way junction induced by Mg^{2+} (Figure 3B, lane 7). This may be related to the ability of the cylinder to stabilise the three-way junction solely by binding in the centre of the junction. The high concentrations of magnesium may imply binding both at the branch point and in the arms; thus the difference in migratory ability may relate to differences in

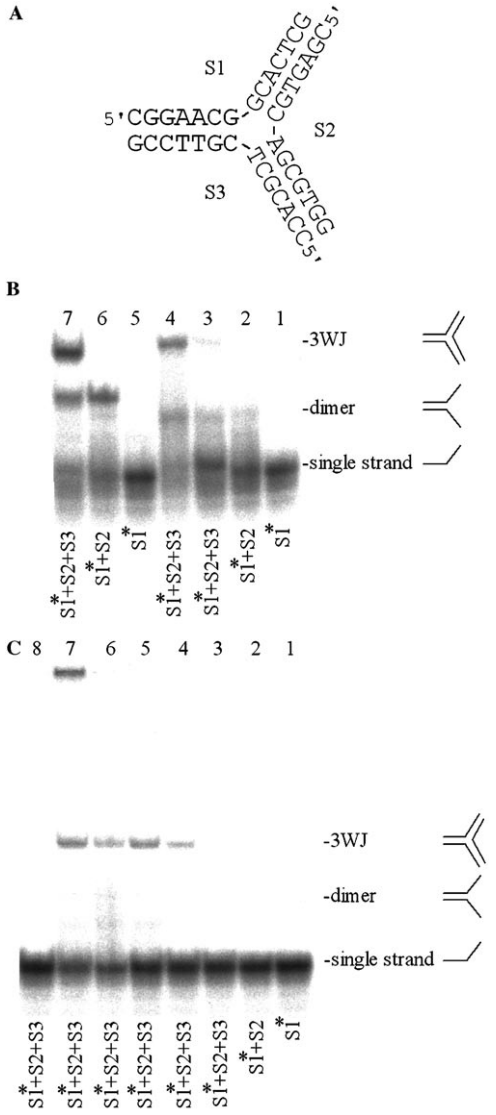

Figure 3. Recognition and stabilisation of a three-way junction assembled from nonpalindromic oligonucleotides by the helicate $[Fe_2L_3]^{4+}$ (racemic mixture). A) The sequences and numbering of the oligonucleotides used to assembly a three-way junction with seven bps per arm. Sequences were taken from reference [7]. B) Autoradiogram of the gel run at 5 °C showing assembly of the three-way junction. Lanes 1–3: Controls containing one, two or all three strands in the buffer, respectively. Lane 4: Strands S1, S2, and S3 in the buffer and 10 mM $MgCl_2$. Lanes 5–7: One, two and three strands, respectively, in the buffer mixed with $[Fe_2L_3]^{4+}$ at 3:1 ratio (strand/cylinder). The asterisk indicates the (^{32}P) labelled strand used in each lane. C) Autoradiogram of the gel run at 25 °C. Lanes 1–3: Controls containing one, two or all three strands, respectively, in the buffer. Lanes 4–7: Strands S1, S2 and S3, respectively, in the buffer mixed with $[Fe_2L_3]^{4+}$ at 6:1, 3:1, 3:2 and 3:10 (strand/cylinder) ratios, respectively. Lane 8: Strands S1, S2 and S3, respectively, in the buffer and 10 mM $MgCl_2$. The asterisk indicates the (^{32}P) labelled strand used in each lane.

the flexibility of the arms of the structure under the different conditions. An alternative explanation is that a larger amount of bound Mg^{2+} is required to stabilise the junction and this leads to a lower overall charge on the migrating species that is reflected in slightly slower migration of the complex through the gel pores.

The crystallographic study[6] revealed that the crystals were only obtained for the complexes of $[Fe_2L_3]^{4+}$ with the oligonucleotides that made it possible to form the three-way junctions with the central thymidines or deoxyriboadenosines. Hence, it has been suggested[6] that $[Fe_2L_3]^{4+}$ may bind selectively to three-way junctions with a central TA sequence. The results of this work (Figure 3) show that $[Fe_2L_3]^{4+}$ may also bind the three-way junctions, with central sequences in which the GC sequence prevails.

The same experiments undertaken at 5 °C (Figure 3B) were also performed at 25 °C. The results confirmed that the three-way junctions with only seven bps per arm were not stable in the gel at 25 °C (Figure 3C, lane 3) even if the sample contained a very high concentration of $MgCl_2$ (Figure 3C, lane 8). Nevertheless, the cylinder was still capable of stabilizing three-way junctions during electrophoresis (Figure 3C, lanes 4–7), which serves to further highlight the extraordinary recognition ability and stabilising influence of these agents on three-way junctions. Higher concentrations of the cylinder did not result in a further enhancement of the formation of the three-way junction (Figure 3C, lanes 6, 7); rather, at the single strand/cylinder ratio of 3:10, DNA started to precipitate (Figure 3C, lane 7).

The crystal structure of the cylinder in complex with a hexanucleotide revealed a close match between the size and shape of the central cavity in the three-way junction and the dimensions of the cylinder.[6] To further explore the importance of the precise size and shape of the cylinder and how it affects the recognition and stabilisation of the three-way junctions, a bulkier cylinder $[Fe_2(L\text{-}CF_3)_3]^{4+}$ was employed. This cylinder is derived from the parent cylinder $[Fe_2L_3]^{4+}$, but has additional CF_3 groups attached to the carbon in the middle of the ligand. This addition results in a dramatic enlargement of the diameter of the cylinder so that it is unlikely that such a cylinder would fit into the central cavity of the three-way junction. Consistent with this, the results shown in Figure 4 (lanes 5,6) demonstrate that $[Fe_2(L\text{-}CF_3)_3]^{4+}$ exhibits no stabilizing effect on the three-way junctions and just precipitates the DNA as other tetracations; tests with other tetracationic compounds, a dinuclear ruthenium complex with 4+ charge, $[(tpy)Ru(dtdeg)Ru(tpy)]Cl_4$ (Figure 4, lanes 7,8) and spermidine (data not shown), also demonstrated no stabilisation effect on the three-way junction. These results are consistent with the view and support the hypothesis that the size and shape of the cylinder are crucial for recognition and stabilisation of three-way junctions.

The supramolecular cylinders, such as $[Fe_2L_3]^{4+}$, are helical and exist in two enantiomeric forms, *M* and *P*. It has been previously shown[1,2] that there are differences in DNA binding of the $[Fe_2L_3]^{4+}$ enantiomers. The *M* enantiomer exhibits higher affinity for double-stranded DNA than *P* enantiomer. Moreover, although the crystal structure of the cylinder in complex with the three-way junction was obtained from a solution containing racemic cylinder, the structure

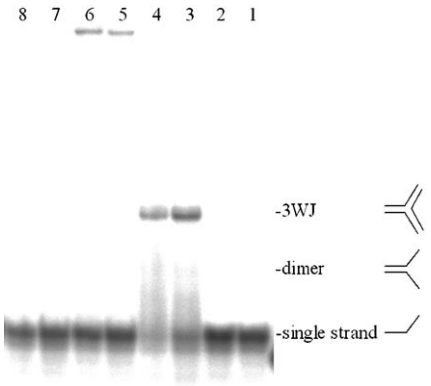

Figure 4. Recognition and stabilisation of a three-way junction assembled from nonpalindromic oligonucleotides by the helicate $[Fe_2(L\text{-}CF_3)_3]^{4+}$ and [(tpy)Ru(dtdeg)Ru(tpy)]Cl_4. Autoradiogram of the gel run at 25°C. Lanes: 1, control containing all three strands in the buffer; 2, strands S1, S2 and S3 in the buffer and 10 mM $MgCl_2$; 3, 4, strands S1, S2 and S3 in the buffer mixed with $[Fe_2L_3]^{4+}$ at 3:1 and 3:2 (strand/cylinder) ratios, respectively; 5, 6: strands S1, S2 and S3 in the buffer and with $[Fe_2(L\text{-}CF_3)_3]^{4+}$ at 3:1 and 3:2 (strand/cylinder) ratios, respectively; 7, 8, strands S1, S2 and S3 in the buffer and with [(tpy)Ru(dtdeg)Ru(tpy)]Cl_4 at 3:1 and 3:2 ratios, respectively. See Figure 3 for the sequences and numbering of the oligonucleotides used to assembly a three-way junction.

contained only the *M* cylinder bound in the DNA junction. To explore this further, the interactions of pure enantiomers with three-way junctions assembled from three 14-mer nonpalindromic oligonucleotides were examined by PAGE at room temperature (Figure 5). The results revealed that *M* enantiomer was more efficient in stabilizing three-way junctions than *P* enantiomer. The radioactivity associated with each band in the autoradiogram of the gel shown in Figure 5A was quantified and the fraction of the three-way junction plotted as a function of the cylinder concentration was plotted (Figure 5B). These results indicate that although the *M* enantiomer is more effective than the *P* enantiomer at stabilising the three-way junction structure, both enantiomers are capable of stabilising this structure.

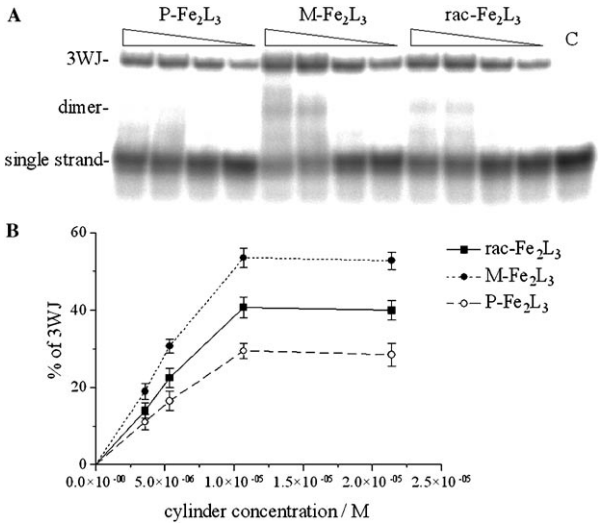

Figure 5. Recognition and stabilisation of a three-way junction assembled from nonpalindromic oligonucleotides by enantiomeric forms, *M* and *P* of the helicate $[Fe_2L_3]^{4+}$. A) Autoradiogram of the gel run at 25°C. Strands S1, S2 and S3 in the buffer were mixed with racemate, *M* and *P* enantiomers of $[Fe_2L_3]^{4+}$ at 9:1, 6:1, 3:1 and 3:2 ratios (strand/cylinder), respectively. Lane c: Control containing all three strands in the buffer. B) Plot of the fraction of three-way junctions as a function of cylinder concentration.

The helicate $[Fe_2L_3]^{4+}$ ($L=C_{25}H_{20}N_4$) promotes formation of the three-way junction assembled from short palindromic oligonucleotides: Given the remarkable ability of the cylinders to stabilise three-way junctions even under conditions in which high Mg^{2+} concentration has no effect (Figure 4), we decided to explore whether three-way junctions from still shorter oligonucleotides might be stablised. For these studies we used 12-mer palindromic oligonucleotide of sequence 5′-TATGGTACCATA-3′, which has the potential to form a three-way junction with arms six bps long (Figure 6A). In the past three-way junctions with arms shorter than seven bps have been shown[8] to be unstable on the gel even at low temperature, when they were stabilised by divalent ions. Using this palindromic oligonucleotide, we found that both enantiomers of the parent cylinder $[Fe_2L_3]^{4+}$ were

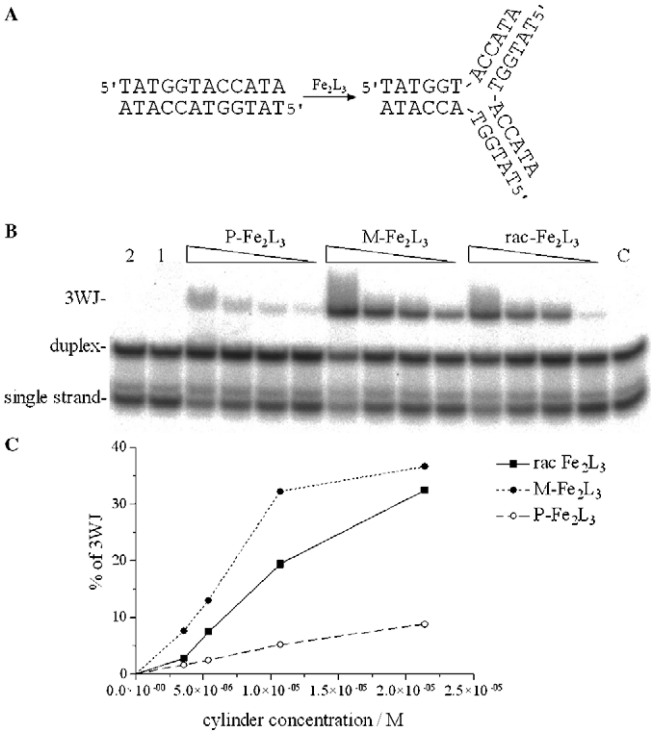

Figure 6. Recognition and stabilisation of a three-way junction assembled from short palindromic oligonucleotides by the helicate $[Fe_2L_3]^{4+}$. A) Schematic diagram showing formation of the three-way junction from the palindromic duplex promoted by $[Fe_2L_3]^{4+}$. B) Autoradiogram of the gel run at 5°C. Palindromic duplex in the buffer was mixed with racemate, *M* and *P* enantiomers of $[Fe_2L_3]^{4+}$ at 9:1, 6:1, 3:1 and 3:2 ratios (strand/cylinder), respectively. Lane c: Control containing duplex in the buffer. Lanes 1, 2: Palindromic duplex in the buffer mixed with bulky cylinders $[Fe_2(L\text{-}CF_3)_3]^{4+}$ and $[Fe_2(L\text{-}Ph)_3]^{4+}$, respectively. C) Plot of the fraction of three-way junction as a function of cylinder concentration.

able to promote formation of the three-way junction and stabilise this structure during electrophoresis (Figure 6B). In contrast, bulky helicates $[Fe_2(L\text{-}CF_3)_3]^{4+}$ and $[Fe_2(L\text{-}Ph)_3]^{4+}$ (Figure 6B, lanes 1,2), spermidine (data not shown) or high concentrations of Mg^{2+} (data not shown) did not facilitate formation of this three-way junction. The *M* enantiomer was several times more efficient (Figure 6B) in promoting and stabilizing this three-way junction than its counterpart. The same experiment was repeated with a 14-mer with an identical central sequence and very similar results were obtained (not shown).

The helicate $[Fe_2L_3]^{4+}$ ($L=C_{25}H_{20}N_4$) binds to three-way junctions with unpaired nucleotides: The initial crystallographic study described recognition of a DNA Y-shaped three-way junction with perfect pairing (since it was formed from a palindromic hexamer).[6] Such three-way junctions are known to be fairly rigid and to have an open cavity at their core. However this is not the only structure possible for three-way junctions. For example, a common feature of naturally occurring three-way junctions, and in particular of RNA three-way junctions, is the presence of unpaired nucleotides at the branch point of the junction.[10] It has been shown[8] that unpaired bases stabilise the formation of three-way junctions by allowing the three-way junctions to fold to form asymmetric structures in which coaxial stacking exists between two helical arms. Evidently, such a three-way junction structure differs significantly from that of Y-shaped three-way junctions with no unpaired nucleotides. Most importantly, in their relaxed state, three-way junctions with unpaired nucleotides do not contain open central cavities that could be occupied by the cylinders, such as $[Fe_2L_3]^{4+}$.[11]

The three-way junctions containing six bps per arm were designed as illustrated in Figure 7A; the strands S1–12 and S2–12 are shown. The strands S1-AA-12 and S2-TT-12 included two adenines or thymines at the positions marked in Figure 7A with "#" and "*", respectively. Three different three-way junctions were prepared, mixed with cylinders (racemic mixture, *M* and *P* enantiomers of $[Fe_2L_3]^{4+}$) and tested for stability in the PAA gel at 5 °C. One three-way junction had no unpaired bases, while two others contained an AA or TT unpaired sequence at the branch point of the junction. The parent cylinder stabilised the Y-shaped three-way junction as well as both three-way junctions with unpaired nucleotides (Figure 7B–D). As it is also described above, the bulky cylinders had no effect on the stability (data shown only for the three-way junction with TT unpaired sequence in Figure 7D, lanes 2,3) and the *M* enantiomer was more efficient in stabilizing three-way junctions than the *P* enantiomer. The molecular-level detail

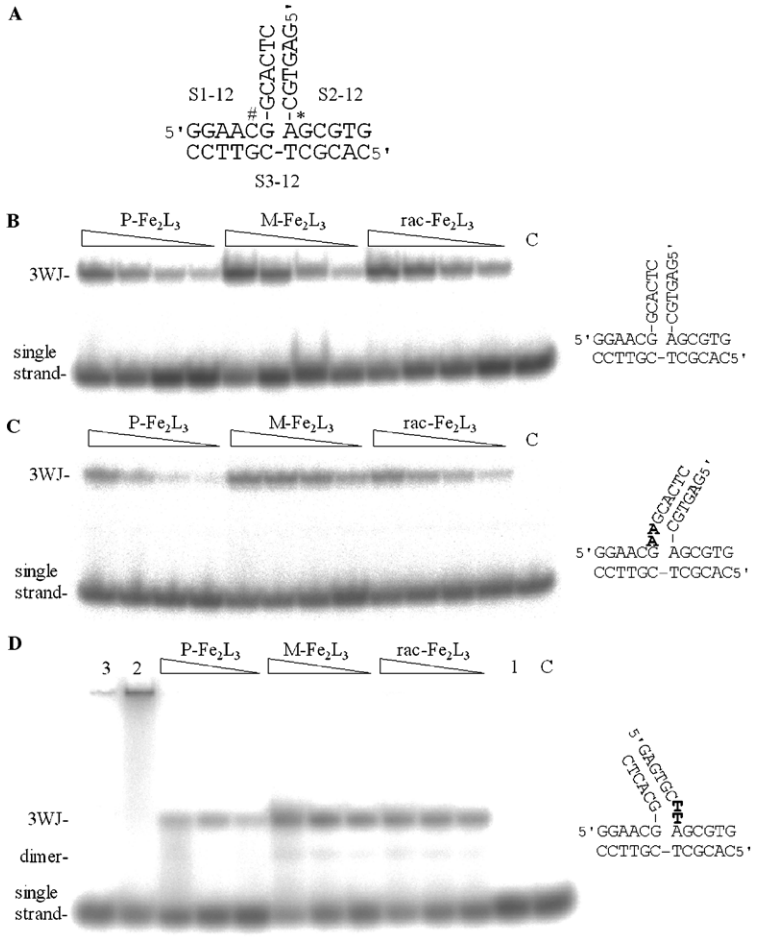

Figure 7. Recognition and stabilisation of a three-way junction with unpaired nucleotides by the helicate $[Fe_2L_3]^{4+}$. A) schematic diagram of the oligonucleotides used to form three-way junctions with unpaired bases. The points where unpaired bases AA and TT were inserted are indicated with # and *, respectively. B) Autoradiogram of the gel run at 5 °C. Strands S1–12, S2–12 and S3–12 in the buffer were mixed with racemate, *M* and *P* enantiomers of $[Fe_2L_3]^{4+}$ at 9:1, 6:1, 3:1 and 3:2 ratios (strand/cylinder), respectively. Lane c: Control containing all three strands in the buffer. C) Autoradiogram of the gel run at 5 °C. Strands S1-AA-12, S2–12 and S3–12 in the buffer were mixed with racemate, *M* and *P* enantiomers of $[Fe_2L_3]^{4+}$ at 9:1, 6:1, 3:1 and 3:2 ratios (strand/cylinder), respectively. Lane c: Control containing all three strands in the buffer. D) Autoradiogram of the gel run at 5 °C. Strands S1–12, S2-TT-12 and S3–12 in the buffer were mixed with racemate, *M* and *P* enantiomers of $[Fe_2L_3]^{4+}$ at 6:1, 3:1 and 3:2 ratios (strand/cylinder), respectively. Lane c: Control containing all three strands in the buffer. Lane 1: All three strands in the buffer and 10 mM $MgCl_2$. Lanes 2, 3: All three strands in the buffer mixed with bulky cylinders $[Fe_2(L\text{-}CF_3)_3]^{4+}$ and $[Fe_2(L\text{-}Ph)_3]^{4+}$, respectively.

of this stabilisation is the subject of ongoing study; however, given the failure of the bulky cylinders to stabilise these structures, it seems reasonable to hypothesise that just as the cylinders induce the three-way junction with a palindromic sequence, with these unpaired three-way junctions the cylinders can open up the junction point and insert, perhaps in a similar fashion to the manner in which they stabilise the Y-shaped dimer (vide supra).

Conclusion

The results herein show that the parent cylinder $[Fe_2L_3]^{4+}$ recognises three-way junctions and binds to them and that recognition of the three-way junction structure by the cylinder is not an artifact of crystallisation, but a real and potentially very powerful new mode of DNA recognition. They also indicate how effective these agents are in stabilizing the three-way junction structure, with a considerable enhancement of stabilisation over that by divalent cations. While the high positive charge (4+) is expected to contribute to the stabilisation of the three-way junctions, the precise size and shape of the cylinder plays a key role; bulky cylinders and other tetracations do not afford the same stabilisation to three-way junctions. The results confirm that both *M* and *P* enantiomers can stabilise the three-way junction structure, but that *M* is more effective than *P*. Given the perfect fit of the *M* enantiomer in the junction, presumably binding the *P* enantiomer in the centre of the junction would cause the helical sense of the twist at the junction point to be inverted; thus we would expect the *P* enantiomer to be less stabilizing. X-ray quality crystals of oligonucleotides in combination with the cylinder were only obtained for sequences containing a flexible AT sequence at the junction point.[6] The studies herein demonstrate that this is not an inherent requirement for recognition and that the cylinder can also stabilise junctions with GC pairs at the junction. Finally, our studies herein demonstrate that recognition is not restricted to "perfect" three-way junctions created by palindromic DNA, but that the cylinder can stabilise other Y-shaped junctions, such as that formed at a fraying point in duplex DNA (as at a replication fork) and other DNA three-way junction structures, such as those containing unpaired nucleotides (perhaps by opening up this structure to access the central cavity). Thus the three-way junction recognition by this class of supramolecular cylinders is revealed to be an exciting new binding mode that has much wider scope than the simple recognition of a palindromic "perfect" three-way junction formed from sequences containing a central AT step. While the scope of junction recognition is clearly greater than hitherto reported, the studies herein do focus on and provide information about sequences that have been specifically investigated because they are able to support different three-way junction and Y-shaped structures; the cylinder has previously[1,2] been shown also to bind and coil natural DNA that contains long tracts of polymeric duplex and may not readily afford such structures. Studies are continuing in our laboratories to probe further the recognition of various Y-shaped oligonucleotide junctions.

Experimental Section

Chemicals: The synthesis of the cylinder $[Fe_2L_3]Cl_4$ ($L = C_{25}H_{20}N_4$; Figure 1a) has been described previously.[1–3,12] The cylinders $[Fe_2(L\text{-}CF_3)_3]Cl_4$ (Figure 1b) and $[Fe_2(L\text{-}Ph)_3]Cl_4$ (Figure 1c) also followed these previously outlined procedures. The dinuclear ruthenium complex [(tpy)Ru(dtdeg)Ru(tpy)]Cl_4 (dtdeg = bis[4′-(2,2′:6′,2″-terpyridyl)]-diethyleneglycolether, tpy = 2,2′:6′,2″-terpyridine; Figure 1d) was provided for this study by Dr. Karlijn van der Schilden and Prof. Jan Reedijk from Leiden University, the Netherlands.[13] The synthetic oligodeoxyribonucleotides used in this work were purchased from VBC-genomics (Vienna, Austria). T4 polynucleotide kinase was purchased from New England Biolabs (Beverly, MA). [γ-^{32}P]-ATP was from MP Biomedicals, LLC (Irvine, CA). Acrylamide and bis(acrylamide) were from Merck KgaA (Darmstadt, Germany).

Methods: Oligonucleotides were purified as described previously;[14,15] in the present work the quoted molar concentrations are related to the single strands. Purification of oligonucleotides with the aid of HPLC was carried out on a Waters HPLC system consisting of Waters 262 Pump, Waters 2487 UV detector and Waters 600S Controller with MonoQ HR 5/5 column. The samples were analysed by PAGE (15% polyacrylamide) in buffered solutions consisting of tris(hydroxymethyl)amino methane (89 mM), borate, pH 8.3 (89 mM) and NaCl (10 mM). Stoichiometric amounts of oligonucleotides (1.07×10^{-5} M, related to strand) were mixed in the buffer to form a three-way junction. One strand was 5′-end-labelled by using T4 polynucleotide kinase and [γ-^{32}P]ATP. Absorption spectra were measured with a Beckmann DU-7400 spectrophotometer. Gels were visualised by using the BAS 2500 FUJIFILM bioimaging analyser, and the radioactivities associated with bands were quantitated with AIDA image analyser software (Raytest, Germany).

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