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Oligonucleotides Containing a Lysine Residue as 3′–3′ Junction for Alternate Strand Triple Helix Formation

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Abstract—Oligonucleotides with a 3′–3′ inversion of polarity site assured by one lysine residue have been synthesized, characterized and used as third strands in alternate strand triple helix formation. UV melting studies and molecular mechanics calculations have been carried out to investigate the stability and the geometry of these new triplexes.

Introduction

Oligonucleotide (ODN) directed triple helix formation[1–4] can interfere with gene expression at the transcription level, as many in vitro and in vivo experiments recently demonstrated.[5–7] The strongest limitation to the application of this approach in therapeutics and biotechnology is related to the relatively low stability of triple helical complexes under physiological conditions; sufficiently stable triplexes can be achieved only targeting quite long (at least 16–17 bases) tracts of homopurines, which are rarely found in biologically relevant DNA sequences. Attempts to extend the repertory of potential DNA targets include the recognition of short homopurine sequences alternating on the two strands of duplex DNA, in an approach called *alternate strand triple helix formation*:[8] $(\text{purine})_m(\text{pyrimidine})_n$ sequences can be selectively recognized by third strands simultaneously and cooperatively binding to the adjacent purine blocks on alternate strands, by switching strand at the junction between the oligopurine and the oligopyrimidine domain. Polypyrimidine third strands, via Hoogsteen triads formation, can be efficient triplex forming oligonucleotides (TFOs) provided that they contain a convenient linker introducing the appropriate inversion of polarity (i.e., a 3′–3′ or a 5′–5′ internucleoside junction) so that both the 5′–3′ domains of the TFO have the required orientation with respect to the target duplex.

Several ODN analogues containing a 3′–3′ inversion of polarity motif have been described in the literature as TFOs in an alternate strand approach; among others, 1,2-dideoxy-D-ribose,[8] oligomers of 1,3-propanediol,[9] an *o*-xylenyl unit[10] and an interbase linkage[11] were reported to efficiently tether the 3′-ends of the two 3′–5′ domains of the third strand. Less promising proved to be 5′–5′ tethered ODNs as third strands in alternate strand triplex formation, as the resulting triplexes always resulted to be dramatically unstable.[9]

We recently studied ODNs containing one 3′–3′ phosphodiester bond as TFOs in an alternate strand recognition, synthesizing several 15-, 16- and 24-mers through an efficient solid-phase method.[12,13] Thermal denaturation experiments, gel retardation assays, CD and DSC spectra have been carried out to investigate triplex formation and stability.[12,14,15] To get a deeper insight into sequence effects–stability relationships of such triplexes, we also undertook molecular mechanics

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calculations, particularly aiming at clarifying the structural details at the inversion site level.[15,16]

Since molecular models indicated that not all the nucleotides present at, or in the proximity of, the 3′–3′ inversion of polarity site would participate in the Hoogsteen hydrogen bonding with the target duplex, appropriate replacements for one nucleotide unit at the 3′–3′ junction have been also looked for. Particularly a 1,2,3-propanetriol and a *cis*,*cis*-1,3,5-cyclohexanetriol residue have been inserted in the 3′–3′ region, leading to modified ODNs whose hybridization and physico-chemical properties have been studied.[17,18]

In this frame, we here report the synthesis, characterization and UV melting behaviour of two new alternate strand triplexes (**c** and **t**, Fig. 1) with TFOs containing a lysine residue as the 3′–3′ junction (**6** and **7**). Molecular mechanics calculations were performed to interpret the different stability of the modified triplexes.

Results and Discussion

In the search for optimal linkers as 3′–3′ junction in polypyrimidine TFOs, we have explored the potential of L-lysine, a flexible, three-functional, naturally occurring molecule, where the COOH group can be used for the anchorage to the solid support, and the α and ε amino groups be exploited to grow the two 3′–5′ ODN domains forming the TFO, by formation of two phosphoramidate bonds with the two 3′-phosphate ends of the ODN tracts. We therefore used a solid support

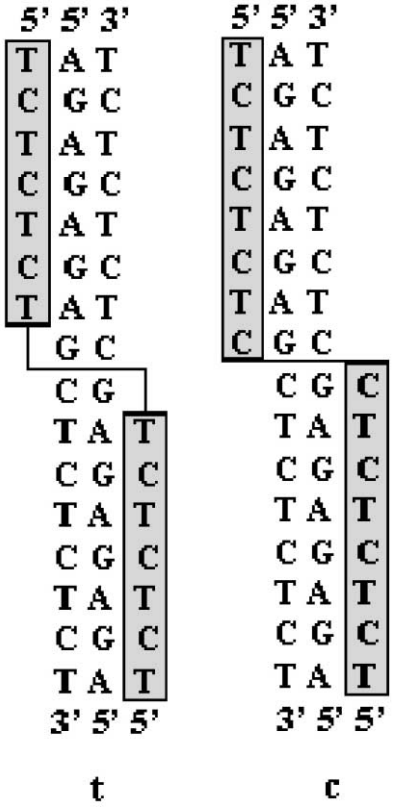
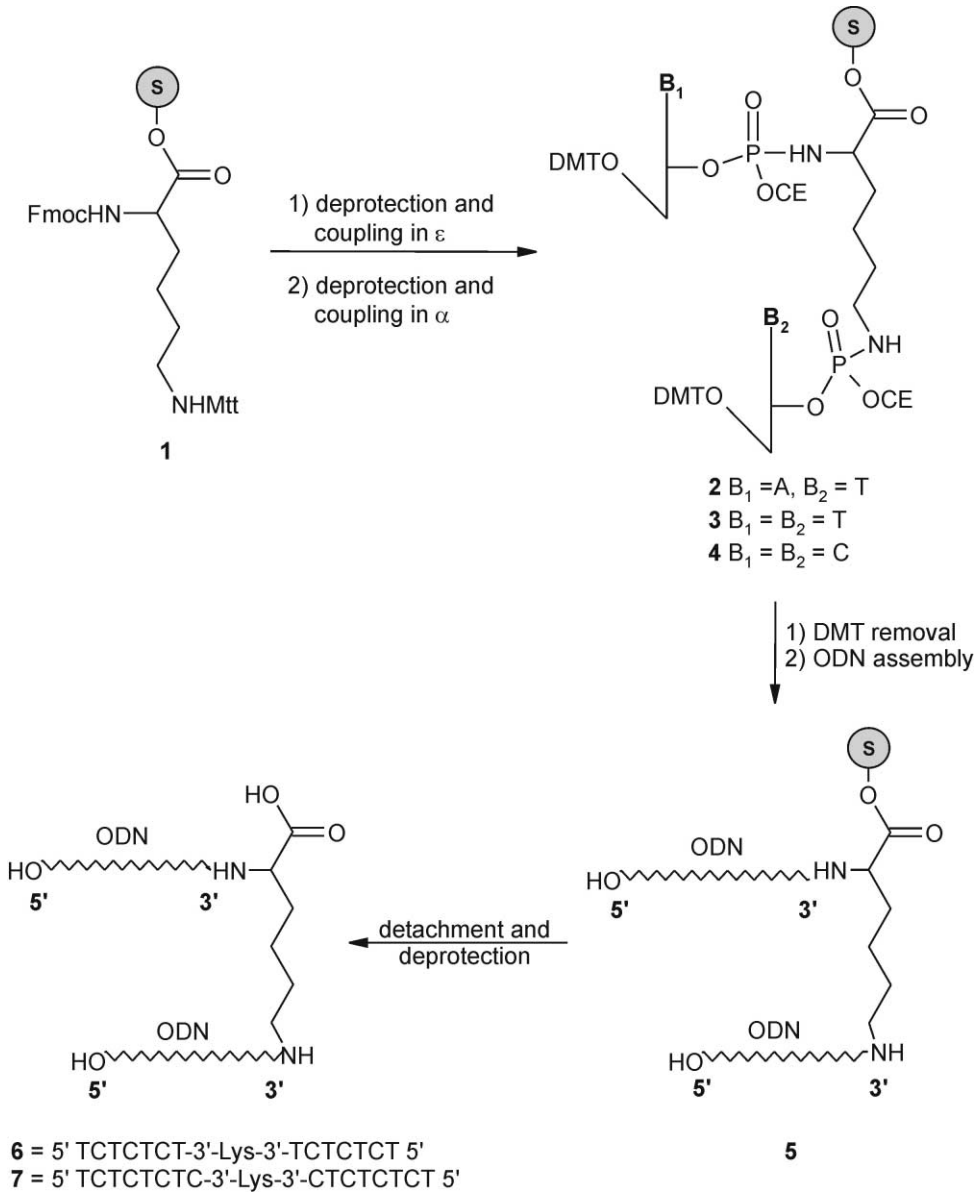


Figure 1.

(Tentagel, a copolymer polystyrene–polyethylene glycol), functionalized with one lysine residue having orthogonal protecting groups (Fmoc and Mtt) for the α- and the ε-amino functions, respectively (**1**, Scheme 1), which can be exploited for the automated preparation of 3′–3′ linked oligonucleotides, having symmetrical or non symmetrical sequences with respect to the inversion of polarity motif. By virtue of the different reactivity of the two amino groups (α and ε) in the lysine residue, the simultaneous addition of one nucleotide unit at both the deprotected NH_2 groups in the solid phase did not lead to satisfactory yields, in any case affording a complex mixture of the expected lysine-dinucleotide together with both the possible nucleotidyl-lysine monomers as the predominant components. Optimization of the yields (>94% for the α-amino group and >98% for the ε-amino group) in the first nucleotide attachments could be achieved performing the coupling step on the two amino groups separately by the following procedure: (i) removal of the ε-Mtt group by treatment with 1% TFA in CH_2Cl_2; (ii) washings with DIEA/CH_3CN (1:1, v/v); (iii) coupling cycle with the chosen 2′-deoxyribonucleoside phosphoramidite unit, including the oxidation step; (iv) removal of the α-Fmoc group by treatment with piperidine/DMF (1:4, v/v); (v) coupling cycle with the chosen 2′-deoxyribonucleoside phosphoramidite unit, including the oxidation step. Submitting **2** (or **3** or **4**) to the standard deprotection and detachment treatment with aq ammonia (overnight, 50 °C) at this level, led to a complex mixture, which, analyzed by HPLC, showed, among others, the presence of 2′-deoxyadenosine and/or thymidine or 2′-deoxycytidine in the eluates. The formation of nucleosides at this stage could be ascribed to the alkaline hydrolysis of the 3′-phosphoester bonds in **2** (or **3** or **4**), occurring to a not negligible extent, which anyway would prove to be extremely detrimental to the final yields of the assembled 3′–3′ TFO. A possible explanation of such behaviour could be the lower rate at which the deprotection of the 2-cyanoethyl groups—via β-elimination—occurs, with respect to a standard *O*-2-cyanoethyl-phosphotriester group, so rendering the cleavage of the 3′-phosphomonoester bond a competitive reaction. This hypothesis was confirmed by testing an alternative deprotection procedure, involving a previous pyridine/Et_3N (1:1, v/v) treatment, to remove the 2-cyanoethyl protecting groups, before the aq ammonia addition. Following this method, supports **2**, **3** and **4** released only the expected A-Lys-T, T-Lys-T and C-Lys-C, whose structures have been confirmed by ^1H, ^{31}P NMR and FAB mass data. For the synthesis of symmetrical TFOs **6** and **7**, starting from **3** or **4**, respectively, the ODN chain was completed by simultaneous growing on both the 5′-ends following an automated phosphoramidite procedure,[19] using longer coupling cycles (8 min) and the 3′-phosphoramidites building blocks in higher concentration (45 mg/mL) than in standard oligonucleotide assembly protocols. In all the coupling steps, yields were always superior to 98%, as checked by DMT tests in the DCA treatments. After deprotection and detachment from the solid support, symmetrical sequences **6** and **7**[20] have been purified by HPLC and characterized by MALDI-TOF mass spectrometry, and their binding properties to the same



Scheme 1.

16-mer target duplex have been investigated by UV melting analysis. These TFOs have alternating C,T residues, which among the previously studied sequences formed the most stable triplexes, and differ from each other in length, **6** being a 14-mer and **7** a 16-mer. In addition to proving the feasibility of the proposed synthetic strategy to obtain oligonucleotides containing a lysine residue as 3′–3′ junction, these experiments were aimed at investigating if the two nucleotides flanking the 3′–3′ inversion of polarity site, crossing the major groove at the level of the GC/CG dinucleotide box in the target duplex, could be involved in some sort of recognition process. As deduced comparing the T_m data reported in Table 1, at acidic pH the triple helical complex **c** is more stable than **t**, while at pH = 6.0 the difference in stability is less striking; at pH = 6.6 and 7.0 no triplex formation could be found in both cases. The larger pH dependence of triple helix **c** with respect to **t** is consistent with the greater number of cytosine residues present in 16-mer **7**.[21]

The lysine junction is a linker with a high degree of conformational freedom and contains a sufficient number of covalent bonds between the two NH_2 arms to link the 3′-ends of the two DNA domains of the third strand (both in **6** and in **7**) without presumably causing energetically demanding distortions or bending of the triplex structures. In order to confirm this hypothesis, UV melting data were integrated into molecular models of the two triple helices. The results of molecular mechanics calculations are shown in Figure 2. Inspection of the reported structures reveals that in triplex structure **t** the target double helix contains the GC/CG

Table 1. T_m values (°C)/pH for triplexes **t** and **c**

pH	t	c
5.5	30.3	35.4
6.0	20.9	21.6
6.6	< 15	< 15

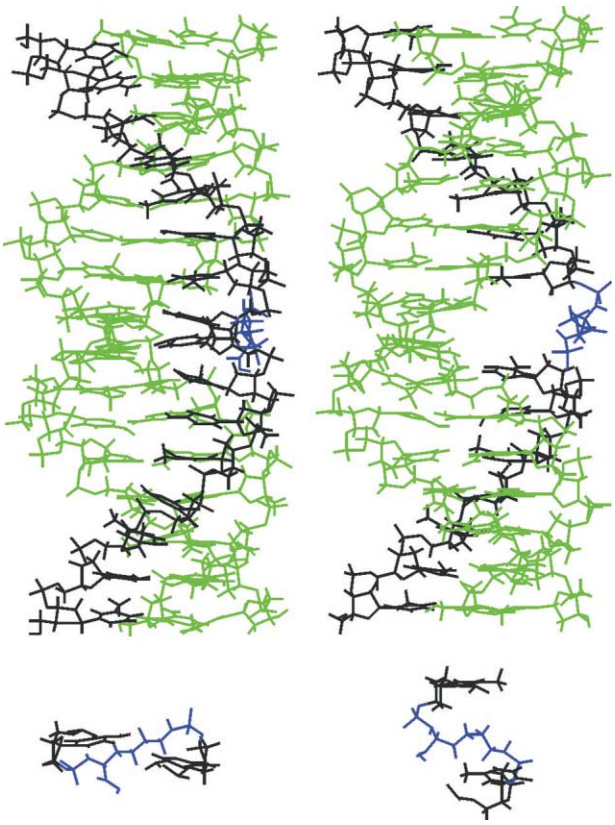


Figure 2. Triple helical structures of triplex **c** (on the left) and triplex **t** generated by molecular mechanics. In bottom the corresponding junction regions are also reported. The Watson–Crick strands are green, the third strands are black. The linker is shown in blue.

dinucleotide box which does not participate in Hoogsteen base pair interactions, the linker spanning these base pairs. The initial distances between 3′-oxygens of the nucleotides involved in the junction were 9.9 Å for triplex **t** and 15.9 Å for triplex **c** and became 8.7 and 9.2 Å, respectively, after minimization. The linking distances appear to be suitable to accommodate the lysine residue whose backbone is oriented parallel to the helical axis in triplex **t** and perpendicularly in triplex **c**. The most striking result is that in both cases the lysine junction is able to cross the major groove of the duplex without causing distortions and all the nucleotides of the third strands are involved in the hydrogen bonds with the target duplex. Consequently, structure **c** is stabilized by a higher number of interactions when compared to structure **t**, justifying the difference in thermal stability.

Conclusions

Tentagel solid support **1**, incorporating a lysine residue orthogonally protected with Fmoc and Mtt groups on the α- and ε-amino functions, has been efficiently prepared and exploited in the fully automated synthesis of two new C,T-containing oligonucleotides with the aminoacid residue as 3′–3′ linker. Starting from supports **3** and **4**, where the first two nucleotide units are attached to the lysine through stable phosphoramidate bonds, a simultaneous growing of the two 3′–5′ domains of the ODN chains was efficiently carried out, synthesizing symmetrical 3′–3′ oligomers **6** and **7**, used as triple helix forming oligonucleotides targeting duplexes of the type 5′-(purine)$_m$(pyrimidine)$_n$-3′ in an alternate strand recognition approach. Thermal denaturation analysis confirmed their ability to cooperatively hybridize to duplex DNA at acidic pH, while no triplex formation was observed at neutral pH. 16-mer **7** showed a higher affinity than 14-mer **6** towards the same target duplex, thus suggesting that also the GC/CG dinucleotide box on the duplex at the level of the inversion site can be targeted by the third strand. The modeling studies reveal that the lysine junction can be easily accomodated within the major groove of the duplex preserving all the Hoogsteen hydrogen bonds in the oligonucleotide third strand. In conclusion, the lysine residue has proved to be an efficient and flexible aminoacidic linker for 3′–3′ oligonucleotides to be used as triplex forming oligonucleotides in an alternate strand recognition approach.

Experimental

General methods

Tentagel resin (0.27 meq OH groups/g) was purchased from Rapp Polymere, Tubingen, Germany. The solid support functionalization with Fmoc-(Mtt)-Lys–OH (purchased from NovaBiochem) was carried out in a short glass column (5 cm length, 1 cm i.d.) equipped with a sintered glass filter, a stopcock and a cap following a

reported procedure.[22] In the case of the synthetic oligomer forming the duplex, the oligonucleotides were assembled on a Millipore Cyclone Plus DNA synthesizer, using commercially available 3′-*O*-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite 2′-deoxyribonucleosides as building blocks following a standard synthetic protocol.[19] HPLC analyses and purifications were performed on a Beckman System Gold instrument equipped with a UV detector module 166 and a Shimadzu Chromatopac C-R6A integrator. Thermal denaturation experiments were carried out on a Cary 1E Varian spectrophotometer equipped with a Haake PG20 thermoprogrammer with detection at $\lambda = 260$ nm.

Preparation of supports 2, 3 and 4

Support **1** (0.27 meq/g, by Mtt spectroscopic quantitation) was prepared according to a previously reported procedure[22] by reaction of Tentagel resin (0.27 meq/g of OH groups) with Fmoc-Lys-(Mtt)–OH in the presence of DCCI/HOBt in CH_2Cl_2/DMF. In a typical experiment, 50 mg (0.0135 mg) of support **1** were first treated with 1% TFA in CH_2Cl_2 till decoloration of the eluates, then subjected to washings first with DIEA/CH_3CN (1:1, v/v) and then exhaustively with CH_3CN. One standard coupling cycle with the chosen 3′-*O*-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite-2′-deoxyribonucleoside was then carried out, including the oxidation step. DMT test performed on a weighed sample of the resin showed a quantitative insertion of the first nucleotide unit on the ε–NH_2 group of the lysine residue. The resin was then treated with piperidine/DMF (1:4, v/v) for three cycles of 15 min each, exhaustively washed with CH_3CN and then subjected to one coupling cycle with the chosen 3′-*O*-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite-2′-deoxyribonucleoside, including the oxidation step. Incorporation of the nucleotide unit at the α-NH_2 group of the lysine residue, calculated by the increase in DMT groups, was 94%. Supports **2**, **3** and **4** were then submitted to standard DMT removal, and successively to deprotection and detachment procedure by reaction with pyridine/Et_3N (1:1, v/v) for 30 min at rt, followed by an overnight treatment with concd aq ammonia at 50 °C. The supernatants were filtered and the supports washed with water. The combined filtrates and washings were concentrated in vacuo, redissolved in water, analyzed and purified by HPLC on a Nucleosil 100-5 C-18 column (4.6×250 mm, 7 μm) eluted with linear gradients of CH_3CN in TEAB 0.1 M, pH = 7.0, from 0 to 30% in 30 min. In the representative case of A-Lys-T, the peak with retention time 23.80 min was collected and lyophilized.

A-Lys-T. ^{1}H NMR (CD_3OD, 400 MHz), δ 8.73 (H-2 adenine, s, 1H); 8.58 (H-8 adenine, s, 1H), 7.62 (H-6 thymine, s, 1H); 6.61 and 6.24 (2 H-1′, 2 dd, 1H each); 4.80 and 4.37 (2 H-3′, 2 m, 1H each); 4.39 and 4.13 (2 H-4′, 2 m, 1H each); 3.90–3.77 (2 H_2-5′ and H-α, m, 5H); 3.00 (1 H-2′, m, 1H), 2.90 (CH_2-ε, m, 2H); 2.79, 2.45 and 2.17 (3 H-2′, m, 3H); 1.94 (CH_3 thymine, s, 3H); 1.64 (CH_2-β, m, 2H); 1.48 (CH_2-δ, m, 2H); 1.42 (CH_2-γ, m, 2H). ^{31}P NMR (CD_3OD, 161.98 MHz), δ 10.8 and 8.77. FAB MS (negative ions): m/z 762 $(M-H)^-$.

Solid-phase synthesis of the oligonucleotides

3′–3′ linked oligonucleotides **6** and **7** were synthesized exploiting functionalized Tentagel solid supports **3** and **4**. After a standard capping procedure, the ODN chain was completed by simultaneous assembly on both the 5′-ends following a phosphoramidite protocol[19] including final DMT removal, using coupling cycles of 8 min and the 3′-phosphoramidites building blocks in 45 mg/mL concentration. In all the coupling steps, yields were always superior to 98%, as checked by DMT tests in the DCA treatments.

Deprotection, purification and characterization of the lysine-containing oligonucleotides

Deprotection and detachment of oligomers **6** and **7** from solid support **5** were achieved first by treatment with pyridine/Et_3N (1:1, v/v) for 30 min at rt, then by an overnight treatment with concd aq ammonia at 50 °C. The supernatant was filtered and the support washed with water. The combined filtrate and washings were concentrated in vacuo, redissolved in water, analyzed and purified by HPLC on a Nucleogen SAX column (4.6×250 mm, 7 μm) eluted with linear gradients of KH_2PO_4 (20% CH_3CN, pH = 7.0) from 1 to 350 mM in 30 min for **6** and in 50 min for **7**, flow 0.8 mL/min. The isolated oligomers, having the following retention times: **6** = 20.90 min; **7** = 18.24 min, were collected and successively desalted by gel filtration on a Sephadex G25 column eluted with H_2O. By HPLC analysis on a Partisphere Whatman RP18 analytical column (125×4.0 mm, 5 μm), the isolated 3′–3′ oligomers resulted to be more than 98% pure.

The synthesized compounds were characterized by MALDI-TOF MS: oligonucleotide **6**, negative ions, mass calculated $[M]^- = 4890$; mass observed 4890; positive ions, mass calculated $[M+H]^+ = 4891$; mass observed 4891; oligonucleotide **7**, positive ions, mass calculated $[M+H]^+ = 4913$; mass observed 4915.

Thermal denaturation experiments

The concentration of the synthesized ODNs was determined spectrophotometrically at $\lambda = 260$ nm and at 85 °C, using the following molar extinction coefficients for each base:[23] 15,400 (A); 11,700 (G); 7300 (C); 8800 (T) $cm^{-1}\,M^{-1}$.

A 140 mM KCl, 5 mM NaH_2PO_4, 5 mM $MgCl_2$ solution was used for the melting experiments, carried out at different pH values (5.5, 6.0 and 6.6). Melting curves were recorded realizing a concentration of approximately 1 μM for each strand in 1 mL of the tested solution in Teflon stoppered quartz cuvettes of 1 cm optical path length. The resulting solutions were then allowed to heat at 80 °C for 15 min, then slowly cooled and kept at 5 °C for 20 min. After thermal equilibration at 10 °C, UV absorption at $\lambda = 260$ nm was monitored as a function of the temperature, increased at a rate of 0.5 °C/min, typically in the range 10–80 °C. When performing the same experiments at a rate of 0.2 °C/min, no

significant difference was detected in the melting profiles, which in any case were found to be essentially superimposable to the renaturation profiles, thus showing the triplex formation as an equilibrium binding process. The melting temperatures, reported in Table 1, were determined as the maxima of the first derivative of absorbance versus temperature plots.

Molecular mechanics calculations

The initial structures of the two triplexes were generated from the helical co-ordinates given by Liu,[24] who reported a B-like conformation for the duplex portion in a triplex. The nitrogen atoms of the lysine NH_2 group (α and ε) were connected with the two oxygen atoms of the 3′-phosphate ends at the junction site of the third strand. The charges on the atoms of protonated cytosines were reassessed in order to take into account the protonation. New parameters for the phosphoramidate bonds were added to the Amber force field[25] for molecular mechanics calculations involving the NP group. The stretch, bend, and torsion force constants were assigned to the NP group by analogy with phosphate constants in Amber force field.[25] The bond stretch force constants for the NQ–P and CT–NQ bonds (NQ is atom type of nitrogen in phosphoramidate linkage) were set slightly higher than in the unmodified backbone in order to model the possible conjugation in the NP group utilizing the parameters reported by Ding et al.[26] The water molecules that were <2.3 Å from any solute atoms were removed. The two triplexes were made electrically neutral by addition of sodium counterions and then, any triplex individually was placed in a box $40.1\times40.1\times56.1$ Å of Monte Carlo TIP3P[27] water, with periodic boundary conditions. For both the triple helices the same minimization procedure was used. Cut-off of 12 Å was applied to the non-bonded potential. Energy minimization was performed using 1500 steps of the steepest descent method followed by the conjugate gradient method until convergence to a rms gradient of 0.1 kcal mol^{-1} $Å^{-1}$.

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