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PAPER

Cationic modified nucleic acids for use in DNA hairpins and parallel triplexes†

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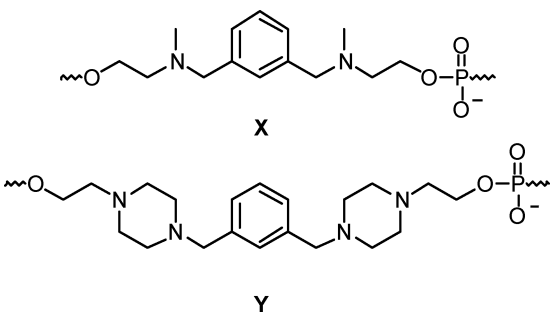
Non-nucleosidic DNA monomers comprising partially protonated amines at low pH have been designed and synthesized. The modifications were incorporated into DNA oligonucleotides *via* standard DNA phosphoramidite synthesis. The ability of cationic modifications to stabilize palindromic DNA hairpins and parallel triplexes were evaluated using gel electrophoresis, circular dichroism and thermal denaturation measurements. The non-nucleosidic modifications were found to increase the thermal stability of palindromic hairpins at pH 8.0 as compared with a nucleosidic tetraloop (TCTC). Incorporation of modifications at the 5′-end of a triplex forming oligonucleotide resulted in a significant increase in thermal stability at low pH when the modifications were placed as the 5′-dangling end.

Introduction

DNA and RNA hairpins are essential secondary structures involved in protein interactions and regulation of gene expression.[1–4] DNA hairpins are normally less thermally stable and not as common as their corresponding RNA hairpins. However, the DNA hairpin structure has been widely studied.[5–9] Replacement of the nucleosidic hairpin loop by non-nucleosidic linkages such as hexaethylene glycol,[10] stilbene derivates,[11–13] and a variety of aromatic linkages,[14–17] have resulted in stabilization of the DNA hairpin structure. Furthermore, structural shifts between the intramolecular hairpin and the intermolecular self-complementary double stranded DNA (dsDNA) have been reported with loops comprising modified nucleic acids capable of forming metal-mediated base pairs upon addition of transition-metal ions.[18–20]

Modified oligonucleotides comprising amines prone to protonation under physiological conditions have been investigated to some extent.[21–29] However, these studies have mainly been focused on stabilizing parallel triplexes due to the close proximity of the triplex forming oligonucleotide (TFO) and the target purine strand allowing interstrand electrostatic and ionic interactions.[30–31] In addition, cationic oligonucleotides have been shown to improve nuclease resistance and cellular uptake and therefore represent an interesting class of modifications in DNA technology.[32]

To our knowledge, the effect on the DNA hairpin comprising a cationic charged end-cap has not been investigated. The optimal linkages should comprise amines prone to protonation at relative high pH and with a terminal oxygen distance close to the optimal 16–17 Å between the 3′- and 5′-phosphate-oxygen of the B-type DNA duplex.[13] We designed two simple modified nucleic acids, **X** and **Y** (Fig. 1), with a maximal terminal oxygen distance of ~14.5 Å to ~19.5 Å, respectively. The choice of 1,3-bis(aminomethyl)benzene as the core structure resulted in a fairly rigid structure with an intramolecular distance of ~7.3 Å between the two amines, close to the intrastrand distance between two phosphate groups (7 Å),[33] allowing the modified nucleic acids to be used in parallel triplex studies on electrostatic interaction with backbone phosphates.[34] Tertiary amines were chosen to avoid protection groups during automated DNA synthesis. Moreover, based on pK_a values of related tertiary amines[35–39] (see ESI†), expected pK_a values of monomer **X** are pK_a^1 ≈ 8–9 and pK_a^2 ≈ 5, whereas pK_a^1 ≈ 7–8 and pK_a^2 ≈ 4 are expected for monomer **Y**.



X

Y

Fig. 1 Modified monomers **X** and **Y**.

Herein we report the effect of the incorporation of the cationic modified nucleic acids into end capped dsDNA hairpins and

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† Electronic supplementary information (ESI) available: pK_a-values of related tertiary amines in H_2O; Gel electrophoresis with **ON2** and increasing NaCl concentration and **ON1–ON3** with Mg^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}. Melting curves of thermal denaturation experiments with **ON1**, **ON6** and **D1** with and without Cu^{2+} at pH 5.0 and pH 8.0; ON-concentration dependence at pH 8.0 with **ON1–2**, **ON6** and **D1**. T_m (°C) data for thermal denaturation study with **X** and **Y** as bulge insertion in parallel triplex and with **X** and **Y** as replacement of a thymidine monomer in parallel triplex. NMR and IR spectra of compound **1–6**. See DOI: 10.1039/c1ob05085k

parallel triplexes with the modification attached to the 5′-end of the TFO as the dangling end, or for targeting the phosphate backbone of 5′-overhang dsDNA (Fig. 2).

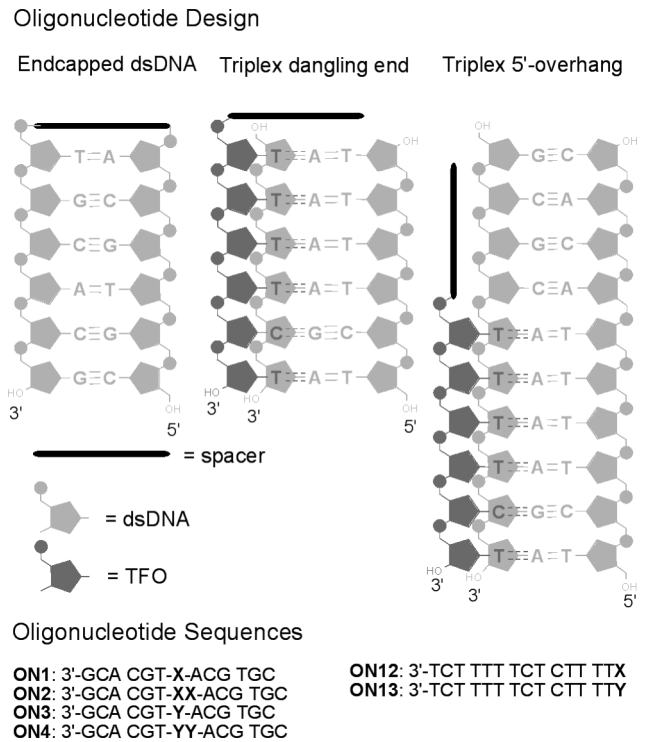


Fig. 2 Oligonucleotide design and modified sequences.

Results and Discussion

Synthesis

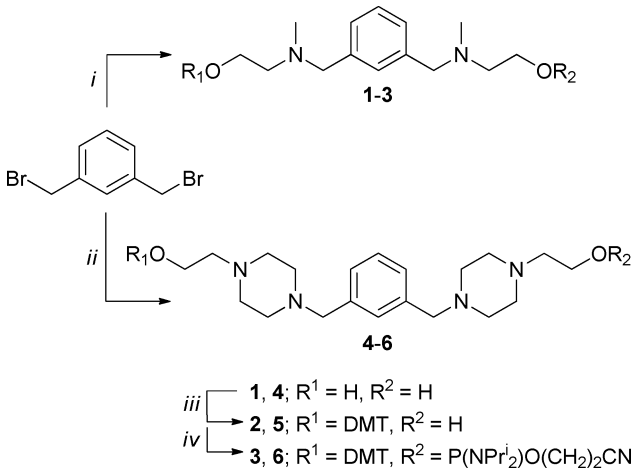
Modified nucleic acids **X** and **Y** were synthesized from commercially available 1,3-bis(bromomethyl)benzene by treatment with 2-(methylamino)ethanol or *N*-(2-hydroxyethyl)piperazine yielding diols **1** and **4**, respectively (Scheme 1). Subsequent mono-protection with DMT-chloride afforded the alcohols **2** and **5**, which were converted to the phosphoramidites **3** and **6** using 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite and diisopropylammonium tetrazolide. Phosphoramidites of **X** and **Y** were incorporated into oligonucleotides using standard automated DNA synthesis.

Palindromic DNA Hairpin Study

For the study on the influence of cationic end-capped dsDNA hairpin we chose a 12-mer palindromic oligonucleotide comprising the modified nucleic acids in the middle (Fig. 2). The palindromic hairpin design allowed the study of possible transformation from intramolecular into intermolecular dsDNA under different pH values and in the presence of metal ions.

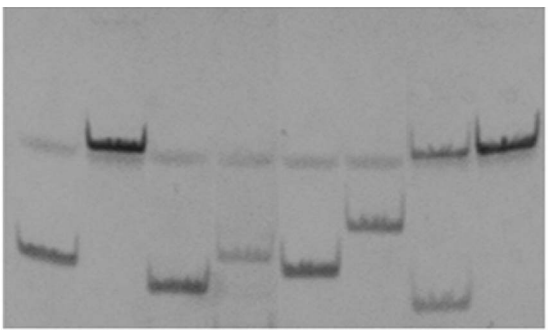
Gel Electrophoresis and Circular Dichroism (CD) Study

Since palindromic DNA oligonucleotides can form intramolecular DNA hairpin structures and intermolecular dsDNA depending on the sequence and ionic conditions,[40–41] native PAGE was used



Scheme 1 Synthesis of modified nucleic acids **X** and **Y**. Reagents and conditions (i) 2-(methylamino)ethanol, Na_2CO_3, MeCN reflux, overnight, 74%; (ii) *N*-(2-hydroxyethyl)-piperazine, Na_2CO_3, MeCN reflux, overnight, 70%; (iii) DMT-Cl, pyridine, 0 °C to RT, overnight, 57% (2) and 53% (5); (iv) diisopropylammonium tetrazolide, 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite, CH_2Cl_2, 0 °C to RT, overnight, 36% (3) and 100% (6).

to examine the secondary structures of the oligonucleotides (Fig. 3). The gel electrophoresis was carried out at 4 °C to slow the equilibration between intermolecular dsDNA and hairpin DNA.



	A	B	C	D	E	F	G	H
A	ON-ref	3′-TGT CAG ACC GGC						
B	D1	3′-TGT CAG ACC GGC 5′-ACA GTC TGG CCG						
C	ON1	3′-GCA CGT-**X**-ACG TGC						
D	ON2	3′-GCA CGT-**XX**-ACG TGC						
E	ON3	3′-GCA CGT-**Y**-ACG TGC						
F	ON4	3′-GCA CGT-**YY**-ACG TGC						
G	ON5	3′-GCA CGT ACG TGC						
H	D1	3′-TGT CAG ACC GGC 5′-ACA GTC TGG CCG						

Fig. 3 Non-denaturing 20% PAGE; 25 μM of **ON-ref**, **D1** and **ON1–5** in TB-buffer, 100 mM NaCl, pH 8.0, 4 °C.

As references for the gel mobility of ssDNA and dsDNA, a non-palindromic 12-mer oligonucleotide (**ON-ref**) and the duplex thereof (**D1**) comprising the same composition of nucleobases as the palindromic sequences (**ON1–5**) were used. It was found that the unmodified **ON5** formed both a hairpin and an intermolecular duplex (*Lane G*) as a band with the same mobility as reference

Table 1 T_m (°C) data for hairpin melting, evaluated from UV melting curves (λ = 260 nm)[a]

Entry	Sequence	T_m, °C pH 5.0	Δ T_m, °C	pH 8.0	Δ T_m, °C	$\Delta T_{m\,(pH\,8.0–pH\,5.0)}$
ON1	3′-GCA CGT-**X**-ACG TGC	71.2	+4.3	77.6	+7.8	+6.4
ON2	3′-GCA CGT-**XX**-ACG TGC	67.8	+0.9	75.4	+5.6	+7.6
ON3	3′-GCA CGT-**Y**-ACG TGC	74.0	+7.1	75.5	+5.7	+1.5
ON4	3′-GCA CGT-**YY**-ACG TGC	63.0	–3.9	70.5	+0.7	+7.5
ON5	3′-GCA CGT ACG TGC	63.9 [38.6][b]	—	67.0 [48.0][b]	—	—
ON6	3′-GCA CGT-**TCTC**-ACG TGC	66.9	*ref.*	69.8	*ref.*	+2.9

[a] C = 5 μM ON in 10 mM Na_2HPO_4/NaH_2PO_4, 100 mM NaCl, pH 5.0 and pH 8.0; [b] T_m of intermolecular dsDNA.

duplex **D1** (*Lane H*) and a second band with greater mobility than ssDNA (**ON-ref**; *Lane A*) were observed. For modified ONs comprising single or multiple insertions of **X** and **Y** (**ON1–4**, *Lane C–F*), only single bands were observed with a mobility greater than for duplex **D1** (*Lane H*). However, due to the increased molecular weight and the partially protonated **X** and **Y**, their motilities are lowered as compared to the hairpin of **ON5** (*Lane G*).[42–43] Therefore hairpin formation was not conclusive. It is worth mentioning that Nakano *et al.*[41] have shown that increasing Na^+ concentration stabilizes the intermolecular DNA duplexes. In our case, gel electrophoresis performed using increasing concentrations of NaCl did not result in the formation of modified intermolecular DNA duplex (see ESI†). Furthermore, addition of metals and transitions metals such as Mg^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} did not result in any change in gel mobility (see ESI†).

To ensure that observed bands in the gel electrophoresis study were DNA hairpins, CD spectra of **D1**, **ON1–5** and **ON6** comprising a TCTC-loop (see Table 1) as reference for of the DNA hairpin were investigated (Fig. 4). At pH 5.0 and pH 8.0, all palindromic ONs (**ON1–6**) formed complexes exhibiting similar CD-profiles. Thus a positive band at 285 nm and two negative bands at 257 nm and 210 nm were observed for **ON1–4** at both pHs, as similarly reported for B-type DNA hairpins.[16,44] CD spectra of **ON5** and **ON6** comprising "no loop" and a TCTC-loop, respectively, differed only marginally from CD spectra of **ON1–4**. Moreover, **D1**, forming a 12-mer B-type dsDNA, resulted in a significantly different CD profile with an additional shoulder at 266 nm and only one negative band at 244 nm. This confirms the intramolecular hairpin formation for **ON1–6** under the given conditions.

Thermal Denaturation Study

All palindromic sequences were examined by thermal denaturation at 260 nm using 5 μM concentrations due to the low hypochromicity for the 6-mer DNA hairpin (Table 1). The melting temperature (T_m, °C) was determined as the first derivative of the melting curves. Since the pK_a[2] values are in the range 4–5, the influence of the net positive charge of the hairpin loop was examined using pH 5.0 and pH 8.0.

As expected from the gel electrophoresis study, thermal denaturation of **ON5** resulted in two thermal transitions as the result of hairpin and duplex dissociation (67.0 °C and 48.0 °C at pH 8.0 respectively). At pH 8.0 **ON1–4** showed an increase in thermal stability as compared with **ON6**. A single incorporation of **X** (**ON1**) resulted in a highly stable DNA hairpin, $\Delta T_{m\,(pH\,8.0;ON1–ON6)}$ = +7.8 °C, whereas insertion of **Y** (**ON3**) resulted in a slightly less stable hairpin $\Delta T_{m\,(pH\,8.0;ON3–ON6)}$ = +5.7 °C. Increasing the loop-size (**ON2** and **ON4**) resulted in a decrease in thermal stability, which is also observed for increasing nucleosidic and non-nucleosidic loop sizes.[9,45] However, the maximal terminal oxygen distance of ~14.5 Å for **X** did not seem to affect the stability of the hairpin,

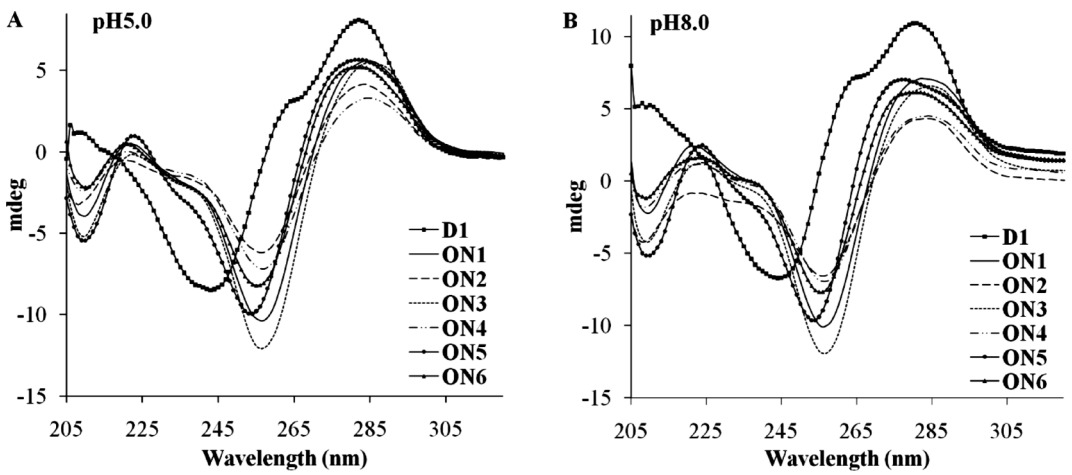


Fig. 4 CD spectra of duplex **D1** (3′-TGT CAG ACC GGC-5′/5′-ACA GTC TGG CCG-3′) and DNA hairpins **ON1–6** (see Table 1); 5 μM of each strand in 10 mM Na_2HPO_4/NaH_2PO_4, 100 mM NaCl, 20 °C at A) pH 5.0 and B) pH 8.0.

even through the optimal length is 16–17 Å.[13] Thermal stability decreased for all ONs at pH 5.0 relative to pH 8.0 as expected due to partial protonation of cytosine, destabilizing the Watson–Crick base pairing.[46] The surprisingly high thermal stability of **ON3** at pH 5.0 might be due to the ability of **Y** to comprise the two positive charges without affecting stabilizing π–π interactions and/or, due to its flexibility, formation of stabilizing hydrogen bonding between partially protonated amines and nucleobases. **ON1–2** and **ON4** showed larger pH sensitivity when compared with **ON6**, which might be the result of unfavourable ionic interactions, affecting the adjacent basepairing. It has been shown that transition-metal ions can destabilize DNA hairpins when the loop contained amines.[16] However, addition of Cu(II)-ions had no effect on thermal stability both at pH 5.0 and pH 8.0 for **ON1** (see ESI†). In addition, it is worth mentioning that T_m values were independent of ON-concentrations, confirming the hairpin structure (see ESI†).[7,47]

Parallel Triplex Study

Since the parallel triplex is pH sensitive, due to the requirement of cytosine N3 protonation for the formation of stable base triplet, a simple 14-mer TFO comprising only three cytosines to ensure specificity was chosen.[48–49] The TFO was modified at the 5′-end for convenience during automated DNA synthesis and two target dsDNA with and without a 5′-GCGC-overhang were used (Fig. 2).

Thermal Denaturation Study

UV thermal denaturing experiments with 5′-modified TFOs (**ON12–13**) targeting duplex **D2**, comprising a 5′-GCGC-overhang region, resulted in a slight increase in thermal stability as compared to the unmodified triplex **ON1/D2** at pH 5.1 and pH 6.0 with ΔT_m = 4.0–5.0 °C and ΔT_m = 2.0–3.5 °C, respectively (Table 2). These observations are in agreement with previously published results observed for TFOs comprising guanidinium moieties (ΔT_m = 3.5 °C per modification).[24,28] However, in our case **X** and **Y** were more pH-sensitive due to the lower pK_a values of the amines. Furthermore, the increased number of amino-groups in **Y** showed only a marginal impact on the thermal stability as compared with **X** at pH 5.1 and pH 6.0, indicating no further counter ion effect in spite of the amino-groups. Since the pK_a^1 values are above 6, monomers **X** and **Y** should be protonated under the applied conditions. Moreover, as the pK_a^2 values are in the range 4–5, some additional pH-dependence can be expected.

Unexpectedly, the effect of pH was remarkably high when 5′-modified TFOs were used to target duplex **D3**, placing **X** and **Y** as dangling ends. At pH 5.1, triplex and duplex melting overlaid (**ON12/D3** and **ON12/D3**) as result of a significantly large increase in thermal stability (ΔT_m = 12.5–13.5 °C). Furthermore, the partial protonation of the amines decreases when pH is increased which resulted in a dramatic destabilization of the modified triplexes at pH 6.0. Since the modifications are placed as dangling ends, interactions with adjacent phosphate-groups would be limited. The gain in thermal stability is comparable to the lid effect observed for large aromatic molecules capable of stabilizing DNA by π–π interactions.[50–51] However, since only weak π–π interactions between the sterically hindered benzene ring and adjacent nucleobases can be expected, we propose that the observed thermal stability at pH 5.1 arises from cationic-π interactions and hydrogen bond formation between terminal nucleobases and monomer **X** and **Y**, which due to the missing duplex overhang are believed to have a larger degree of freedom for conformational arrangements.

Similar thermodynamic stabilizations have been observed for double stranded DNA and RNA when a dangling nucleobase has been placed at either the 3′- or 5′-terminal ends.[52–56] The stabilizing effect has been assigned to an interplay of electrostatic interactions of the dangling residue and the closing basepair.[57] The dangling residue strengthens the hydrogen bonds of the terminal basepairs *via* favorable stacking and furthermore, removal or coordination of water molecules in the microenvironment enhances the thermal stability.[58] These assumptions are in agreement with the increased thermal stability observed for **ON12–13/D3** at lower pH due to protonation of amines. Furthermore, the lower thermal stability observed for triplexes with a 5′-GCGC-overhang region (**ON12–13/D2**) supports this theory since the observed stabilization is greater than what is expected from ionic interactions with the phosphate backbone.

Preliminary experiments with **X** and **Y** as bulge insertion, or replacing one nucleotide in a TFO, resulted in similar large decreases in thermal stability and was therefore not of interest in this study (see ESI†). Incorporation of flexible linkages into dsDNA has been shown to destabilize dsDNA due to the loss of interstrand nucleobase stacking.[59]

Table 2 T_m (°C) data for triplex melting, evaluated from UV melting curves (λ = 260 nm)[a]

		5′-GAAGCTCTTTTTCTCTTTTGCGC				5′-GAAGCTCTTTTTCTCTTTT			
		3′-CTTCGAGAAAAAGAGAAAACGCG				3′-CTTCGAGAAAAAGAGAAAA			
		D2 (ON7/ON8)				**D3 (ON9/ON10)**			
Entry	Sequence	pH 5.1[b]	ΔT_m	pH 6.0[b]	ΔT_m	pH 5.1[b]	ΔT_m	pH 6.0[b]	ΔT_m
ON11	3′-TCTTTTTCTCTTTT	37.0	*ref.*	24.0	*ref.*	37.0	*ref.*	23.5	*ref.*
ON12	3′-TCTTTTTCTCTTTTX	41.0	+4.0	26.0	+2.0	49.5[c]	+12.5	26.0	+2.5
ON13	3′-TCTTTTTCTCTTTTY	42.0	+5.0	27.5	+3.5	50.5[c]	+13.5	28.0	+4.5

[a] C = 1.5 μM of **ON11–13** and 1.0 μM of each strand of dsDNA (**D2–3**) 10 mM NaH_2PO_4, 100 mM NaCl, 0.1 mM EDTA, pH 5.1 and pH 6.0; duplex T_m = 63.5 °C (**D2**, pH 5.1), 63.0 °C (**D2**, pH 6.0), 54.0 °C (**D3**, pH 5.1), 52.0 °C (**D3**, pH 6.0); target regions are underlined for TFO hybridization. [b] The melting temperature (T_m, °C) was determined as the first derivative of the melting curves; [c] Triplex-duplex melting overlaid.

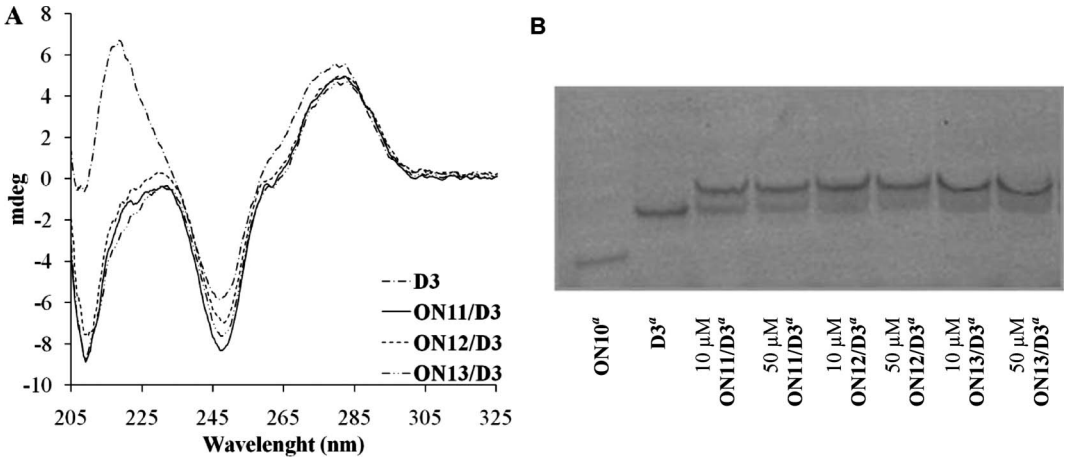


Fig. 5 A) CD spectra of duplex **D3** and triplexes **ON11–13/D3**; 2 μM of each strand in 10 mM NaH_2PO_4, 100 mM NaCl, 0.1 mM EDTA at pH 5.1, 20 °C. B) Non-denaturing 20% PAGE; 10 and 50 μM of **ON11–13** and 200 nM of **ON10** or **D3** in 50 mM HEPES-buffer, 100 mM NaCl, pH 5.0, 4 °C; a) purine strand comprising 3′-(6′-fluorescein) for visualization.

Gel Electrophoresis and Circular Dichroism (CD) Study

To examine the ability of modified TFOs to form parallel triplexes, circular dichroism and non-denaturing gel electrophoresis were used. CD spectra of duplex **D3** and triplexes **ON11–13/D3** are shown in Fig. 5A. The duplex and triplexes all formed a B-type conformation, as seen from the positive and negative band, with approximately the same magnitude at ~278 nm and ~248 nm, respectively.[44] For all triplexes a distinct negative band with a larger magnitude than for the duplex was observed at ~212 nm which is considered to be an indication of parallel triplex formation.[60–61] Non-denaturing 20% PAGE confirmed triplex formation for complexes **ON11–13/D3** at pH 5.0 (Fig. 5B. Unmodified TFO (**ON11**) and the modified TFOs (**ON12–13**), showed the same mobility for the triplex band. However, modified triplexes showed a smeared region between the duplex- and the triplex-bands, presumably as a result of a low kinetic stability of the parallel triplex.

Thermal Denaturation Study on Antiparallel and Parallel Duplex

For further investigation of the dangling end effect observed for the parallel triplex **ON12–13/D3**, the ability of monomers **X** and **Y** to stabilize antiparallel and parallel duplexes as the 5′-dangling end was examined. As can be seen from Table 3, no significant impact on the thermal stability of antiparallel duplexes (**ON12–13/ON14**) was observed and only a slight increase was observed for the parallel duplexes (**ON12–13/ON10**). Interestingly, it seems that the thermal stabilization observed for the 5′-dangling end parallel triplex is dependent on electrostatic interactions with the entire base triplet and only to a lesser extent the parallel basepair.

Conclusion

Herein we described the synthesis of monomers **X** and **Y** comprising tertiary amines, and their incorporation into oligonucleotides. Thermal denaturation studies of a 12-mer palindromic DNA comprising monomers **X** or **Y** as a loop resulted in a significant increase in thermal stability at pH 8.0 as compared with a tetraloop (TCTC), $\Delta T_m = 7.8$ °C and $\Delta T_m = 5.7$ °C for **X** and **Y**, respectively. Similar increases in thermal stability were observed at pH 5.0. However, greater pH sensitivity was observed for hairpins comprising **X** and **Y**-loops as compared with a TCTC-loop. This effect is considered to be the result of intramolecular loop repulsion affecting the adjacent basepairing. It is worth mentioning that no intermolecular dsDNA duplexes were observed under the applied conditions and that T_m values were not affected by addition of Cu(II) ions. Gel electrophoresis and CD studies confirmed the formation of a B-type hairpin DNA structure.

Thermal denaturation studies using 5′-modified TFOs targeting dsDNA with a 5′- GCGC-overhang resulted in a slight increase in thermal triplex stability and only at relatively low pH ($\Delta T_{m(pH5.1)}$ = 4.0–5.0 °C). However, significant gain in thermal stability at pH

Table 3 T_m (°C) data for parallel and antiparallel duplex melting, evaluated from UV melting curves (λ = 260 nm)[a]

		5′-CTTCGAGAAAAAGAGAAAA				3′-CTTCGAGAAAAAGAGAAAA			
		ON14				**ON10**			
Entry	Sequence	pH 5.1[b]	ΔT_m	pH 6.0[b]	ΔT_m	pH 5.1[b]	ΔT_m	pH 6.0[b]	ΔT_m
ON11	3′-TCTTTTTCTCTTTT	39.0	*ref.*	37.5	*ref.*	30.5	*ref.*	24.0	*ref.*
ON12	3′-TCTTTTTCTCTTTTX	38.0	–1.0	37.0	–0.5	34.0	+3.5	25.0	+1.0
ON13	3′-TCTTTTTCTCTTTTY	38.0	–1.0	37.5	0.0	34.0	+3.5	26.5	+2.5

[a] C = 1.0 μM of each strand in 10 mM NaH_2PO_4, 100 mM NaCl, 0.1 mM EDTA, pH 5.1 and pH 6.0; target regions are underlined for duplex; [b] The melting temperatures (T_m, °C) were determined as the first derivative of the melting curves.

5.1 (ΔT_m = 12.5–13.5 °C) was observed when monomer **X** and **Y** were placed as dangling ends adjacent to the base triplet. No significant increase in thermal stability was observed at pH 6.0 (ΔT_m = 2.0–3.5 °C), presumably due to the partial deprotonation of amines.

These pH-sensitive monomers represent a new class of cationic modified nucleic acids for the use in DNA hairpins and parallel triplexes.

Experimental Procedures

General Information

NMR spectra were recorded on a Varian Gemini 2000 spectrometer at 300 MHz for ^{1}H, 75 MHz for ^{13}C and 121.5 MHz for ^{31}P. Internal standard used in ^{1}H NMR was TMS (δ: 0.00) for $CDCl_3$; in ^{13}C NMR was $CDCl_3$ (δ: 77.16); in ^{31}P NMR was H_3PO_4 (δ: 0.00) used as external standard. Accurate ion mass determinations were performed using the Ionspec 4.7 T HiResMALDI Ultima Fourier transform (FT) mass spectrometer (Ion Spec, Irvine, CA) and the 4.7 T HiResESI Ultima (FT) mass spectrometer. The [M+ H/Na]$^+$ ions were peak matched using ions derived from the 2,5-dihydroxybenzoic acid matrix. Thin layer chromatography (TLC) analyses were carried out with use of TLC plates 60 F_{254} purchased from Merck and visualized in an UV light (254 nm). The silica gel (0.040–0.063 mm) used for column chromatography was purchased from Merck. Solvents used for column chromatography and reagents were used as purchased without further purification.

Synthesis

2 - ((3 - (((2 - Hydroxyethyl)methylamino)methyl)benzyl)methylamino)ethanol (1). 1,3-Bis(bromomethyl)-benzene (3.00 g, 11.4 mmol), Na_2CO_3 (4.52 g, 42.6 mmol) and 2-(methylamino)ethanol (1.75 g, 23.3 mmol) were refluxed in dry MeCN (100 mL) for 22 h. At room temperature the reaction mixture was filtered and concentrated under reduced pressure. The residue was purified by column chromatography with silica gel; $CHCl_3$: MeOH (5%) : NEt_3 (1%). **1** was isolated as thick yellow oil. Yield 2.12 g, 74%. ^{1}H NMR ($CDCl_3$) δ 2.26 (s, 6H, 2 × NCH_3), 2.59 (t, J = 5.4 Hz, 4H, 2 × CH_2CH$_2$OH), 3.26 (br. s, 2H, 2 × CH$_2$OH), 3.58 (s, 4H, 2 × PhCH_2N), 3.62 (t, J = 5.4 Hz, 4H, 2 × CH$_2$CH_2OH), 7.18–7.29 (m, 4H, Ph). ^{13}C NMR ($CDCl_3$) δ 41.9 (2 × NCH$_3$), 58.4, 58.5 (2 × CH$_2$CH$_2$OH, CH$_2$$CH_2$OH), 62.3 (2 × Ph$CH_2$N), 128.2, 128.4, 129.8, 138.6 (Ph). HR-MALDI-MS: m/z calcd for $C_{14}H_{24}N_2O_2Na^+$ [M$^+$ Na]$^+$ 275.1730, found 275.1718.

2-(4-(3-((4-(2-Hydroxyethyl)piperazin-1-yl)methyl)benzyl)piperazin-1-yl)ethanol (4). 1,3-Bis(bromomethyl)benzene (2.00 g, 7.6 mmol), Na_2CO_3 (3.01 g, 28.4 mmol) and N-(2-hydroxyethyl)piperazine (2.12 g, 16.3 mmol) were refluxed in dry MeCN (50 mL) for 26 h. The reaction mixture was filtered at room temperature and concentrated under reduced pressure. The residue was crystallized from MeCN yielding **4** as a white solid. Yield 1.93 g (70%). ^{1}H NMR (DMSO) δ 2.33–2.38 (m, 20H, 2 × CH_2CH$_2$OH, 4 × NCH_2CH_2N), 3.42 (s, 4H, 2 × PhCH_2N) 3.46 (t, J = 5.4 Hz, 4H, 2 × CH$_2$CH_2OH), 4.34 (br. s, 2H, 2 × CH$_2$OH), 7.13–7.24 (m, 4H, Ph). ^{13}C NMR (DMSO) δ 52.6, 53.2 (4 × NCH$_2$$CH_2$N) 58.5 (2 × CH$_2CH_2$OH), 60.3 (2 × CH$_2$$CH_2$OH), 62.1 (2 × Ph$CH_2$N), 127.4, 127.9, 129.1, 138.1 (Ph). HR-MALDI-MS: m/z calcd for $C_{20}H_{34}N_4O_2H^+$ [M$^+$ H]$^+$ 363.2755, found 363.2744.

2 - ((3 - (((2 - (4,4′ - Dimethoxytrityloxy)ethyl)methylamino)methyl)benzyl)methylamino)ethanol (2). The diol (**1**; 1.33 g, 5.3 mmol) was dissolved in dry pyridine (50 mL). DMTCl (1.79 g, 5.3 mmol) was added at 0 °C and under Ar. After 30 min the reaction mixture was allowed to reach room temperature and left overnight. The pyridine was removed under reduced pressure before the residue was purified by column chromatography with silica gel; EtOAc : Et_3N (1%) followed by $CHCl_3$: NEt_3 (1%) : MeOH (5–10%). **2** was isolated as a yellow oil. Yield 1.66 g, 57%. ^{1}H NMR ($CDCl_3$) δ 2.21 (s, 6H, 2 × NCH_3), 2.57 (t, J = 5.4 Hz, 2H, CH_2CH$_2$OH), 2.66 (t, J = 5.7 Hz, 2H, CH_2CH$_2$ODMT), 3.20 (t, J = 5.7 Hz, 2H, CH$_2$CH_2ODMT), 3.52, 3.54 (2 × s, 2H, 2 × PhCH_2N), 3.60 (t, J = 5.4 Hz, 2H, CH$_2$CH_2OH), 3.78 (s, 6H, 2 × OCH_3), 6.81 (d, J = 9.0 Hz, 4H, Ar), 7.19–7.46 (m, 13H, Ar). ^{13}C NMR ($CDCl_3$) δ 41.7, 43.1 (2 × NCH$_3$), 55.30, 55.33 (2 × OCH$_3$), 57.2 (CH$_2$CH$_2$ODMT), 58.5 (NCH$_2$$CH_2$) 62.2, 62.3, 62.8 (CH$_2$$CH_2$ODMT, 2 × Ph$CH_2$N), 86.1 ($CAr_3$), 113.1, 126.7, 127.77, 127.84, 128.1, 128.3, 129.8, 130.2, 136.6, 138.4, 139.4, 145.3, 158.5 (Ar). HR-MALDI-MS: m/z calcd for $C_{35}H_{42}N_2O_4H^+$ [M$^+$ H]$^+$ 555.3218, found 555.3216.

2-(4-(3-((4-(4,4′-Dimethoxytrityloxyethyl)piperazin-1-yl)methyl)benzyl)piperazin-1-yl)ethanol (5). The diol (**4**; 1.90 g, 5.2 mmol) was dissolved in dry pyridine (50 mL). DMTCl (1.95 g, 5.8 mmol) was added at 0 °C. After 30 min the reaction mixture was allowed to reach room temperature and left overnight. The pyridine was removed under reduced pressure before the residue was purified by column chromatography. With silica gel; $CHCl_3$: sat. $NH_{3(MeOH)}$ (1%) : MeOH (1–10%). **5** was isolated as yellow foam. Yield 1.85 g, 53%. ^{1}H NMR ($CDCl_3$) δ 2.51–2.65 (m, 20H, CH_2CH$_2$OH, CH_2CH$_2$ODMT, 4 × NCH_2CH_2N), 3.20 (t, J = 6.0 Hz, 2H, CH$_2$CH_2ODMT), 3.48, 3.50 (2 × s, 4H, 2 × PhCH_2N), 3.59 (t, J = 5.4 Hz, 2H, CH$_2$CH_2OH) 3.77 (s, 6H, 2 × OCH_3), 6.81 (d, J = 9.6 Hz, 4H, Ar) 7.18–7.45 (m, 13H, Ar). ^{13}C NMR ($CDCl_3$) δ 53.0, 53.3, 53.9 (4 × NCH$_2$$CH_2$N), 55.3 (2 × O$CH_3$), 57.8, 58.3 (CH$_2CH_2OH, CH_2CH_2$ODMT), 59.3, 61.9 (CH$_2$$CH_2OH, CH_2$$CH_2$ODMT), 63.08, 63.15 (2 × PhCH$_2$N), 86.2 (CAr$_3$), 113.1, 126.7, 127.8, 128.0, 128.1, 128.2, 128.3, 130.1, 136.6, 138.08, 138.14, 145.3, 158.5 (Ar). HR-MALDI-MS: m/z calcd for $C_{41}H_{52}N_4O_4H^+$ [M$^+$ H]$^+$ 665.4061, found 665.4059.

(3 - (((2 - ((2 - Cyanoethyl)(diisopropylamino)phosphinoxy)ethyl)-methylamino)methyl)benzyl) - (2 - (4,4′ - dimethoxytrityloxy)ethyl)methylamine (3). The alcohol (**2**; 1.50 g, 2.7 mmol) and diisopropylammonium tetrazolide (2.32 g, 13.6 mmol) were dissolved in dry CH_2Cl_2 (30 mL) under Ar and cooled to 0 °C. 2-Cyanoethyl N,N′,N′-tetraisopropylphosphordiamidite 1.71 g, 5.7 mmol) was added dropwise before the reaction mixture was allowed to reach room temperature and left overnight. The reaction mixture was filtered through celite which was washed with cyclohexane: EtOAc (20%): NEt_3 (5%), the residue was concentrated under reduced pressure before purified by vacuum column chromatography with silica gel; cyclohexane : EtOAc (20–30%) : Et_3N (4–10%). **3** was isolated as yellow oil. Yield 800 mg (36%). ^{1}H NMR ($CDCl_3$) δ 1.14–1.19 (m, 12H, 2 × CH(CH_3)$_2$), 2.20, 2.24 (2 × s, 6H, 2 × NCH_3), 2.57–2.68 (m, 6H, CH_2CH$_2$OP, CH_2CH$_2$ODMT, CH$_2$CH_2CN), 3.20 (t, J = 6.0 Hz, 2H, CH$_2$CH_2ODMT), 3.51, 3.52

(s, 4H, 2 × PhCH_2N), 3.54–3.75, 3.80–3.86 (m, 6H, CH_2CH_2OP, 2 × CH(CH_3)$_2$, CH_2CH_2CN) 3.78 (s, 6H, 2 × OCH_3), (6.80 (d, J = 9.0 Hz, 4H, Ph) 7.18–7.45 (m, 13H, Ph). ^{13}C NMR ($CDCl_3$) δ 20.4, 20.5 (CH_2CN) 24.6, 24.7, 24.8 (2 × CH(CH_3)$_2$) 42.9, 43.06, 43.13, 43.2 (2 × CH(CH_3)$_2$, 2 × NCH_3), 55.30, 55.32 (2 × OCH_3), 57.2 (CH_2CH_2CN), 57.9, 58.0 (CH_2CH_2ODMT), 58.4, 58.7 (CH_2CH_2OP), 61.7, 61.9 (CH_2CH_2ODMT), 62.2 (CH_2CH_2OP) 62.8, 62.9 (2 × PhCH_2N), 86.1 (CAr_3), 113.1, 126.7, 127.7, 127.8, 128.2, 128.3, 129.6, 130.1, 136.6, 139.0, 139.3, 145.3, 158.4 (Ar). ^{31}P NMR ($CDCl_3$) δ 148.7. HR-ESI-MS: m/z calcd for $C_{44}H_{59}N_4O_5PNa^+$ [M$^+$ Na]$^+$ 777.4115, found 777.4093.

4-((3-((4-((2-Cyanoethyl)(diisopropylamino)phosphinoxy)ethyl)piperazin-1-yl)methyl)benzyl)-(4,4′-dimethoxytrityloxyethyl)piperazine (6). The alcohol (**5**; 0.46 g, 0.7 mmol) and diisopropylammonium tetrazolide (0.19 g, 1.1 mmol) were dissolved in dry CH_2Cl_2 (20 mL) under Ar and cooled to 0 °C. 2-Cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite was added dropwise (0.66 g, 2.2 mmol) before the reaction mixture was allowed to reach room temperature and left overnight. The reaction mixture was concentrated under reduced pressure before the residue was purified by column chromatography with silica gel; cyclohexane : EtOAc(50%) : Et_3N (0.5%). **6** was isolated as a yellow oil. Yield 599 mg (100%). ^{1}H NMR ($CDCl_3$) δ 1.15–1.19 (m, 12H, 2 × CH(CH_3)$_2$), 2.49–2.64 (m, 22H, CH_2CH_2OP, CH_2CH_2ODMT, 4 × NCH_2CH_2N, CH_2CH_2CN), 3.19 (t, J = 6.3 Hz, 2H, CH_2CH_2ODMT), 3.48, 3.49 (2 × s, 4H, 2 × PhCH_2N), 3.52–3.75, 3.80–3.89 (2 × m, 6H, CH_2CH_2OP, 2 × CH(CH_3)$_2$, CH_2CH_2CN) 3.78 (s, 6H, 2 × OCH_3), (6.80 (d, J = 9.0 Hz, 4H, Ph) 7.18–7.45 (m, 13H, Ph). ^{13}C NMR ($CDCl_3$) δ 20.4, 20.5 (CH_2CH_2CN) 24.62, 24.67, 24.71, 24.76, 24.81 (2 × CH(CH_3)$_2$), 43.1, 43.2 (2 × CH(CH_3)$_2$), 53.2, 53.3, 53.7, 53.9 (4 × NCH_2CH_2N), 55.3 (2 × OCH_3), 58.3 (CH_2CH_2CN), 58.5, 58.7 (CH_2CH_2OP, CH_2CH_2ODMT), 61.9 (CH_2CH_2OP, CH_2CH_2ODMT), 63.2 (2 × PhCH_2N), 86.1 (CAr_3), 113.1, 126.7, 127.8, 128.0, 128.07, 128.13, 128.3, 130.1, 136.6, 138.1, 145.3, 158.5 (Ar). ^{31}P NMR ($CDCl_3$) δ 149.0. HR-ESI-MS: m/z calcd for $C_{50}H_{69}N_6O_5PH^+$ [M$^+$ H]$^+$ 865.5145, found 865.5128.

Synthesis and Purification of Modified Oligonucleotides

Modified oligonucleotides (**ON1–4** and **ON12–13**) were synthesized in 0.2 µmol on 500 Å CPG supports using Expedite Nucleic Acid Synthesis System Model 8909 (Applied Biosystems). Commercial and modified phosphoramidites (**X** and **Y**) were coupled using standard procedures with an extended coupling time (10 min) for the latter. The CPG-supports were treated with 32% aqueous ammonia (1 mL) and deprotected at 55 °C overnight. ONs were purified on reverse-phase semipreparative HPLC on a Waters Xterra MS C18 column (10 µm, 7.8 × 150 mm). DMT was cleaved with 80% aq. AcOH (100 µL) for 20 min and ONs were precipitated by addition of 1 M aq. NaOAc (150 µL) and EtOH (550 µL) at –18 °C. ON-purity was checked by ion-exchange chromatography on a LaChrom system (Merck Hitachi) using a GenPak-Fax column (Waters). MALDI-TOF mass spectrometry analyzes was performed on a Voyager Elite Bio spectrometry Research Station (Perspective Biosystems). m/z calcd. for **ON1** [M]$^+$ 3960.7 found 3958.5, m/z calcd. for **ON2** [M]$^+$ 4275.0 found 4276.5, m/z calcd. for **ON3** [M]$^+$ 4069.9 found 4066.4, m/z calcd. for **ON4** [M]$^+$ 4493.4 found 4492.7, m/z calcd. for **ON12** [M]$^+$ 4467.2 found 4467.1, m/z calcd. for **ON13** [M]$^+$ 4575.3 found 4572.9.

3′-(6′-Fluorescein)-labeled **ON10** was synthesized in a 0.2 µmol scale on a 3′-(6′-fluorescein) CPG support, using MerMade 4 Automated DNA Synthesizer from BioAutomation Corporation, using standard procedures. Purification and DMT-cleavage was accomplished using C_{18} cartridges (0.2 µmol puri-pak cartridges from ChemGenes corporation) using standard procedures. MALDI-TOF mass spectrometry analysis was performed on a Bruker Daltonics Autoflex MALDI TOF in the negative mode using 3-hydroxypicolinic acid as a matrix and dibasic ammonium citrate as a co-matrix, m/z calcd. for **ON10** [M]$^+$ 6454.4 found 6450.7. Oligonucleotides were desalted using C18 ziptips (Millipore) prior to loading on the MALDI plate.

Thermal Denaturation Measurements

Hairpin study. T_m measurements were performed on a CARY 100Bio UV-Vis spectrophotometer using a 2 × 6 Multicell block with Peltier temperature controller. T_m solutions containing 5.0 µM of ON(s) in the corresponding buffer were heated to 90 °C and afterward cooled to 15 °C. Absorbance was measured at 260 nm from 15 °C to 95 °C with a heating rate of 1.0 °C min^{-1}. The melting temperatures (T_m, °C) were determined as the maximum of the first derivative plots of the melting curves.

Triplex study. T_m measurements were preformed on a PerkinElmer Lambda 35 UV/VIS spectrometer with a PTP 6 thermostat and PerkinElmer Templab 2.00 Software. Triplexes were formed by mixing 1.0 µM of each ssDNA and 1.5 µM of the TFO in the corresponding buffer solution. The solutions were heated to 80 °C for 5 min and afterward cooled to 5 °C and kept at this temperature for 30 min. The absorbance of triplexes was measured at 260 nm from 5 °C to 80 °C with a heating rate of 1.0 °C min^{-1} or 0.5 °C min^{-1}. The melting temperatures (T_m, °C) were determined as the maximum of the first derivative plots of the melting curves. All melting temperatures are within the uncertainty ± 0.5 °C as determined by repetitive experiments.

CD Measurements

Hairpin study. CD measurements were performed on an Applied Photophysics Chirascan CD spectrometer (150 W Xe arc) with a Quantum Northwest TC125 temperature controller using quartz optical cells with a path length of 1 cm. The spectra were recorded at 20 °C in identical solutions as used for T_m measurements. CD spectra were obtained in the range from 205 to 340 nm with a data pitch of 1 nm. The spectra were taken as an average of five scans and corrected for solvent background.

Triplex study. CD measurements were performed on a Jasco J-815 CD spectrometer fitted with a Jasco CDF-426S/15 temperature controller using quartz optical cells with a path length of 0.5 cm. The spectra were recorded in the same buffer that was used for T_m studies using 2.0 µM concentration of each ON at 20 °C. CD spectra were obtained with 100 mdeg sensitivity in the range from 200 to 350 nm with a data pitch of 0.1 nm. The spectra were taken as an average of five scans, corrected for solvent background.

Gel Electrophoresis

Hairpin study. Non-denaturing 20% PAGE was performed using 1×TB-buffer, 100mM NaCl, pH 8.0 and at 4 °C using 10W. Samples of 25 μM of **ON-ref**, **D1**, **ON1–5** were prepared in 1×TB-buffer with a dye/glycerol mix (20%), heated to 90 °C and cooled before loaded on the gel. The gel was visualized using Stains-All® and destained in H_2O.

Triplex study. Non-denaturing 20% PAGE was performed using 50mM HEPES-buffer adjusted to pH 5.0 using HCl, 100 mM NaCl and at 4 °C using 9W.[62] Samples of 10 mM or 50 mM of **ON11–13** and 200 nM of **ON10** or **D1** (purine strand comprising 3′-(6′-fluorescein)) were prepared in the buffer with a dye/glycerol mix, heated to 90 °C and cooled down before being loaded onto the gel. The gel was visualized using fluorescence scanner FLA-5000 from Fujifilm with filter LPB/Y510/#220-000/01 and excitation laser at 473 nm.

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