Triplex formation at physiological pH: comparative studies on DNA triplexes containing 5-Me-dC tethered at N^4 with spermine and tetraethylenoxyamine

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**ABSTRACT**

Oligodeoxynucleotides with spermine conjugation at C4 of 5-Me-dC (sp-ODN) exhibit triple helix formation with complementary Watson–Crick duplexes, and were optimally stable at physiological pH 7.3 and low salt concentration. This was attributed to a favored reassociation of the polycationic third strand with the anionic DNA duplex. To gain further insights into the factors that contribute to the enhancement of triplex stability and for engineering improved triplex systems, the spermine appendage at C4 of 5-Me-dC was replaced with 1,11-diamino-3,6,9-trioxaundecane to create teg-ODNs. From the triple helix forming abilities of these modified ODNs studied by hysteresis behaviour and the effect of salts on triplex stability, it is demonstrated here that teg-ODNs stabilise triplexes through hydrophobic desolvation while sp-ODNs stabilise triplexes by charge effects. The results imply that factors in addition to base stacking effects and interstrand hydrogen bonds are significantly involved in modulation of triplex stability by base modified oligonucleotides.

**INTRODUCTION**

Oligodeoxynucleotide (ODN) directed triplex formation is increasingly attracting attention due to its therapeutic potential (1–7). Triple helix formation depends on Hoogsteen hydrogen bonds between thymine (T) with an A:T base pair (T*A:T triplet) and protonated cytosine (C^+) with a G:C base pair (C^+*G:C triplet) (8–12). This arrangement requires a polypurine central strand for constituting triplexes which are optimally stable at non-physiological pH 5.6–6.0. Several approaches have recently appeared in the literature to overcome these limitations through the use of chemically modified nucleobases (13–21) in the third strand to increase its affinity to duplex DNA at intracellular conditions and employing pyrimidine analogs endowed with bidirectional hydrogen bonding when present in the central strand (22–25). Spermine is known to promote triplex stabilization, both upon external addition (26–28) as well as upon conjugation to ODN at the 5′-terminus (29,30), 2′O (31) and C5 of dU (32). The polycationic effect of spermine on triplex stabilization has also been realised through linking of basic peptides (33). We have recently demonstrated that ODNs with spermine conjugation at C4 of 5-Me-dC (sp-ODN) (34) exhibit triple helix formation with complementary Watson–Crick duplexes, with optimal stability at low salt concentration and physiological pH 7.3; the corresponding unmodified ODNs do not form triplexes in these conditions (35,36). Further, N3 protonation of C (conjugated with spermine) in the third strand was not observed in sp-ODN triplexes and the loss in stability thereby due to the absence of the (C)N3-H--N7(G) Hoogsteen bond is compensated by favorable electrostatic interactions of the sperminyl side chain with DNA, leading to an enhanced association with the duplex. Towards understanding the role of the tetraprotonated spermine side chain in causing triplex stability, this appendage at C4 of 5-Me-dC was replaced with the tetraethylenoxyglycolamine that has only a single protonation site, to obtain teg-ODNs for constituting triplexes. We present here comparative biophysical studies of triplexes derived from sp-ODN and teg-ODN in the third strand employing measurements of thermal stability, hysteresis and the effect of salts on thermal transitions of the corresponding triplexes. It is demonstrated that teg-ODNs when present as a third strand, form triplexes with increased stability even at neutral pH, in contrast to the unmodified controls that form triplexes only at a lower pH. The origin of the teg-ODN triplex stability is shown to be due to hydrophobic desolvation in contrast to sp-ODNs which stabilise the triplex by charge effects.

**MATERIALS AND METHODS**

All chemicals used were of reagent quality or better grade. Base protected standard nucleoside phosphoramidites and 5′-DMT-nucleoside derived controlled pore glass supports (CPG) were purchased from Cruachem UK. T4 polynucleotide kinase and Klenow polymerase from United States Biochemicals and [γ-^32^P]ATP, [α-^32^P]CTP from Bhabha Atomic Research Center, Bombay were used for radiolabelling of oligonucleotides.

**Oligonucleotide synthesis, purification and primer extension**

All oligonucleotides were synthesized on 1.3 µmol scale on a Pharmacia GA plus DNA synthesizer using CPG and nucleobase (A, G, C and T) protected 5′-O-(4,4′-dimethoxytrityl)

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deoxyribonucleoside-3′-O-[(N,N-diisopropylamino)-β-cyanoethyl phosphoramidite] monomers, followed by deprotection with aqueous NH₃. The teg-monomer 3 was synthesised by the procedure described below. All oligonucleotides were purified by reversed phase FPLC on a C18 column and the purity was rechecked on reverse phase HPLC using buffer systems A: 5% CH₃CN in 0.1 M triethylammoniumacetate (TEAA) and B: 30% CH₃CN in 0.1 M TEAA with a gradient A to B of 1.5%/min at a flow rate of 1.5 ml/min. Retention time for teg-ODNs: 12 (11.86 min), 13 (11.65 min), 14 (11.84 min), 15 (12.08 min) and 16 (12.22 min). To check the migration behavior of teg-ODNs on PAGE the purified oligonucleotides were labeled at the 5′-end using 5′-[γ-32P]ATP by T4 polynucleotide kinase according to standard procedures (37). The radiolabeled oligonucleotide samples were run on a 20% polyacrylamide gel containing 7 M urea with 90 mM Tris-borate-EDTA (pH 8.3) as buffer. Samples were mixed in formamide, heated to 70°C for 5 min and then cooled in an ice bath before loading on the gel. Autoradiograms were developed after 1 h exposure using an intensifying screen.

The primer extension reactions were carried out in a total volume of 20 µl containing 50 mM Tris–HCl (pH 7.6), 10 mM MgCl₂, 10 µM each of dATP, dGTP, TTP, [α-32P]CTP and 2 µM of appropriate annealed duplex. The reactions were initiated by addition of 2 U Klenow Polymerase I at 25°C for 1 h. The reactions were terminated by freeze-drying, dissolved in formamide and then analysed by denaturing gel electrophoresis as described above.

**Scheme 1.**

![Scheme 1](image)

\[
X = 5′-\text{Me-DC-(N⁴-spermine)} \\
Y = 5′-\text{Me-DC-(N⁴-o-deoxyaninotetraethyleneglycol)}
\]

4. (1.0 g, 5.2 mmol, 7.5 eq.) were stirred in dry pyridine (2 ml) at 70°C for 48 h. Pyridine was removed from the reaction mixture under vacuum and the residue was taken in ethylacetate (20 ml), washed with water (10 ml), dried over anhydrous sodium sulfate and evaporated to dryness. The residue was treated with ethyltrifluoroacetate in absolute ethanol at ambient temperature under basic conditions to get the N-trifluoroacetamide derivative 2. Compound 2 was purified by silica gel column chromatography using an eluent chloroform/methanol/triethylamine 9:0:9:0:1, yield: 0.40 g (71%). ¹H NMR (CDCl₃): 7.68(s, 1H), 7.60(bs, 1H, exchangeable with D₂O), 7.44–7.39(m, 2H), 7.33–7.21(m, 7H), 6.84–6.80(d, 4H), 6.48–6.42(t, 1H, J = 6.5 Hz), 5.50(bs, 1H, exchangeable with D₂O), 4.56(m, 1H), 4.10(m, 1H), 3.79(s, 6H), 3.75–3.31(m, 1H), 2.64–2.53(m, 1H), 2.26–2.20(m, 1H) and 1.48(s, 3H). ¹³C NMR (CDCl₃): 163.7, 158.7, 156.6, 144.7, 137.4, 135.8, 130.2, 128.3, 128.0, 117.2, 103.2, 104.6, 86.7, 86.1, 85.9, 72.0, 70.5, 70.3, 70.1, 69.5, 68.8, 63.8, 55.3, 42.0, 40.7, 39.7 and 12.4. FAB MS: 815 (M⁺ + 1).

The phosphoramidite monomer 3 was prepared from compound 2 by following the procedure as described in ref. 38. ³¹P NMR (CDCl₃): 149.8 and 149.3 p.p.m.

**Melting experiments**

Duplex and triplex melting experiments were carried out in 25 mM Tris, pH 7.0–7.3 buffer, containing varying amounts of salts NaCl, Na₂SO₄, NaClO₄ as mentioned in each case. Appropriate oligonucleotides, each at a strand concentration of 1 µM based on a UV absorbance of 260 nm calculated using molar extinction coefficients of dA = 15.4, dC = 7.3, dG = 11.7, T = 8.8 cm²/mmol, were mixed and heated at 70°C for 3 min, allowed to cool to room temperature followed by overnight storage at 4°C. The A₂₆₀ nm at various temperatures were
recorded using a Perkin Elmer Lambda 15 UV/VIS spectrophotometer, fitted with a water jacketed 5-cell holder and a Julabo temperature programmer with a heating rate of 0.5°C/min over 5–75°C. Dry nitrogen gas was flushed in the spectrophotometer chamber to prevent moisture condensation at temperatures <15°C. The triplex dissociation temperature \((t_m)\) was determined from the midpoint of the first transition in the plots of fraction absorbance change versus temperature and were further confirmed by differential \((dA/dT)\) versus \(T\) curves. The \(t_m\) values are accurate to ±0.5°C over the reported values. The reassociation of third strand with complementary duplex was studied by hysteresis experiments (36) in which the samples were heated (0.5°C/min) and maintained above triplex–duplex transition temperature for 10 min to achieve constant absorbance, followed by cooling (0.5°C/min).

RESULTS AND DISCUSSION

Synthesis and characterization of teg-ODNs

The 5-Me-dC-N4-(teg) monomer 3 required for site-specific incorporation into oligonucleotide sequences was synthesised (Scheme 1) from displacement-coupling reaction (39,40) of 5′O-DMT-O4-(2,5-dimethylphenyl)-5-methyl dC 1 with 1,11-diamino-3,6,9-trioxanadecane 4, followed by protection of 6′-amino group as trifluorooacetate and subsequent 3′O-phosphoramidation by standard procedures (38). The attachment of diaminopolyoxyethylene at C4 of pyrimidine by following a similar strategy and its incorporation into ODNs has been used for synthesis of oligonucleotides bearing reporter functional groups (41,42). The diamine 4 was obtained from tetraethyleneglycol in three steps (43), with purification and complete characterization of products at all stages by chromatographic and spectroscopic methods. The modified dC-amidite monomer 3 was purified to homogeneity by column chromatography and characterised through 31P NMR and mass spectroscopy. This was incorporated into the oligonucleotide sequences 12–16 at the indicated positions, followed by aqueous NH3 treatment for complete deprotection and FPLC purification. The ODNs were 5′end radiolabelled and their purity was rechecked by denaturing polyacrylamide gel electrophoresis. Spermine conjugated ODNs (sp-ODNs) 7–11 were synthesised as reported earlier (35,36).

Thermal stability of teg-ODN duplexes and triplexes

Figure 1 and Table 1 document UV \(t_m\) results on duplexes and triplexes derived from teg-ODNs. The duplexes from teg-ODN containing different degrees of substitutions (5:12, 5:13, 5:15 and 5:16) exhibited lower \(t_m\)s compared with the control unmodified duplex 5:6, similar to the behaviour of sp-ODN duplexes (35); the destabilising order being control 5:6 = (3′) 5:12 < (M) 5:13 < (3′,5′) 5:15 < (3′,5′,M) 5:16. The terminally modified teg-ODNs 12 and 15 with the complementary 24mer duplex 17:18 showed triplex transitions even in the absence of Mg++, in contrast to the control 6*17:18 where triplex was not observed. Under these conditions, the triplex transitions with teg-ODNs 13 and 16 were not detectable perhaps due to the destabilising nature of the centrally modified oligonucleotides and/or a low hypochromism. From an application perspective the terminal modifications are more advantageous than the internal one as they are less destabilising.

In the presence of Mg++, all teg-ODNs formed triplexes and the \(t_m\)s of terminally modified triplexes 12*17:18 and 15*17:18 were enhanced by 13 and 18°C respectively compared with the triplex formed in the absence of Mg++. The trisubstituted teg-ODN 16*17:18 was less stable than the control 6*17:18. The triplex \(t_m\) of teg-ODNs decreased upon lowering of pH (12*17:18, 50 mM sodium acetate, pH 5.5, \(t_m\) 41°C, 20 mM Tris, pH 7.3, \(t_m\) 47°C, in presence of 100 mM NaCl) and 20 mM MgCl2, \(\Delta t_m\)pH 7.3–5.5 = 6°C) and was qualitatively similar to that seen with the

![Figure 1](image-url)
corresponding \( sp \)-ODN triplexes (\( \Delta T_m \) pH 7.3–5.5 = 11°C) (36) under identical conditions, but with a lower magnitude. In \( sp \)-ODN, such a behaviour was attributed to a protonated spermine side chain at C4 causing a lower pK\(_a\) of N3 due to electrostatic repulsion, as compared with that of dC in unmodified ODN. Unlike \( sp \)-ODNs, \( teg \)-ODNs lack multiple protonation sites in the side chain; however, the presence of a single protonated amino function at terminus may slightly disfavour N3 protonation due to electrostatic repulsion and hence destabilise the triplexes but to a lesser extent compared with \( sp \)-ODNs. No change in \( t_m \) of unmodified triplex \( 6^{*17}:18 \) was observed upon external addition of 1 mM 1,11-diamino-3,6,9-trioxaundecane, unlike that seen from external addition of spermine which enhances triplex stability (26–28). To understand the role of conjugated polyoxyethyleneamino chain in \( teg \)-ODN in inducing stable triplexes, the differential behaviour of heating and cooling curves (hysteresis) were examined.

**Hysteresis in \( teg \)-ODN triplexes**

We have shown earlier that the stability of \( sp \)-ODN triplexes arises from favoured reassociation with complementary duplex mediated by polycationic spermine chain (36). To examine corresponding effects in \( teg \)-ODN triplexes, heating and cooling curves were recorded for triplex transitions, both in the presence and absence of \( Mg^{2+} \) and the results are shown in Figure 2. No significant hysteresis for triplex ⇔ duplex transition was observed with \( teg \)-ODN triplexes in presence of \( Mg^{2+} \) upon cooling (Fig. 2a). However, in the absence of \( Mg^{2+} \), \( teg \)-ODN significantly exhibited a strong hysteresis (Fig. 2b) which was absent in the corresponding \( sp \)-ODN triplex, both with and without \( Mg^{2+} \) (Fig. 2c and d). In the case of \( sp \)-ODN triplexes, the electrostatic interaction of the protonated side chain with the phosphate backbone and/or hydrogen bonding with nucleobases, causes enhancement of the reassociation rate. In case of \( teg \)-ODN having polyether functions that are non-protonated under the experimental conditions, the observed triplex stability must arise from factors other than charge effects, for example, hydrophobic desolvation of the major groove of the duplex by the appended polyethylene glycol side chain of the third strand. The polyether side chain may alter the hydration network in its vicinity in the major groove, thereby improving the third strand association via hydrophobic and hydrogen bonding interactions with neighbouring DNA strands.

It has been previously reported (44) that external addition of polyethylene glycols in the range PEG 400–3400 in molar concentrations, induces a dramatic change in the melting temperature of duplex and triplex with increasing concentration and molecular weight of polymer. The observed effect of PEGs was attributed to a combination of multiple factors including salt concentration, water activity and solution crowding (45–47). In light of this, comparative effects of salts on \( sp \)- and \( teg \)-ODN triplex transitions were studied.

**Effect of salts on stability of \( sp \)-ODN and \( teg \)-ODN triplexes**

The triplex stabilities are strongly influenced by the presence of salt and are more cation specific as compared with duplex stability (48). In addition to electrostatic interaction, salts can also influence/affect hydrophobic interaction in condensation and precipitation of biopolymers (49,50). The stability of proteins is influenced by the hydrating ability of anions which follows the order \( SO_4^{2–} > Cl^– > ClO_4^{–} > CNS^{–} \). This has also been recently employed to establish the hydrophobic nature of the calicheamycin–DNA interaction which is enhanced in the presence of a strong antichaotropic agent such as \( Na_2SO_4 \) but decreases in the presence of a weakly hydrated chaotropic agent such as \( LiClO_4 \) (51). A comparative study of the effects of these salts on \( sp \) and \( teg \)-ODN triplexes should therefore help in defining the nature of interaction (charge/hydrophobic) of the side chains conjugated to the third strand with the duplex.

Both \( sp \)-ODN and \( teg \)-ODN triplexes showed enhancement of \( t_m \) upon increasing NaCl concentration from 100 to 900 mM (Fig. 3B). The melting of \( teg \)-ODN triplexes were more salt dependent than those of \( sp \)-ODN triplexes, as evident from a higher slope for \( teg \)-ODN triplex in salt concentration–\( t_m \) plot (data not shown). Comparative UV–\( t_m \) data for triplexes containing either \( sp \)-ODN or \( teg \)-ODN recorded in Tris buffer containing salts of different compositions are indicated in Figure 3B. Addition of 100 mM \( Na_2SO_4 \) to the buffer instead of 100 mM NaCl had no major consequence on the \( t_m \) of \( sp \)-ODN triplexes irrespective of the position and degree of substitution (A, B, C) except in the case of trisubstituted \( sp \)-ODN triplex which was slightly destabilised. On the other hand, 100 mM \( Na_2SO_4 \) induced a significant rise in \( t_m \) of \( teg \)-ODN triplexes, whose magnitude increased with the degree of substitution (D, E, F).

In the case of trisubstituted \( teg \)-ODN (Fig. 3A), triplex formation which was undetectable with 100 mM NaCl, showed up only in the presence of 100 mM \( Na_2SO_4 \). This rise in \( teg \)-ODN triplexes in the presence of 100 mM \( Na_2SO_4 \), could arise from increased concentrations of either cation (Na\(^+\)) or anion (SO\(_4^{2–}\)). Hence, a set of UV–\( t_m \) experiments on 3′-monosubstituted triplexes \( 7^{*17}:18 \) (\( sp \)-ODN) and \( 12^{*19}:20 \) (\( teg \)-ODN) were carried out at different compositions of NaCl and \( Na_2SO_4 \), keeping the total cation concentration constant and the results are depicted in Figure 3B. Increasing Na\(^+\) concentration from 500 mM [Fig. 3B(a)] to 900 mM [Fig. 3B(c)] enhanced the \( t_m \) of both \( sp \)-ODN and \( teg \)-ODN triplexes. In the presence of 100 mM NaCl with 400 mM \( Na_2SO_4 \) (total [Na\(^+\)] = 900 mM) [Fig. 3B(b)], \( sp \)-ODN showed a lower \( t_m \) compared with that at 900 mM NaCl alone [Fig. 3B(c)]. In contrast, the \( teg \)-ODN triplex was equally stable in both salt compositions. Similarly, addition of 400 mM LiClO\(_4\) [Fig. 3B(d)] induced a slight destabilization of \( sp \)-ODN triplex \( t_m \) as compared with that at 500 mM NaCl [Fig. 3B(a)], while no such effect was seen with \( teg \)-ODN triplex. Thus, salts have differential effects on \( teg \) and \( sp \)-ODN triplexes with more stabilization effect on \( teg \)-ODN triplexes than for \( sp \)-ODN. The observed order of induced stability by the anions \( SO_4^{2–} > Cl^– > ClO_4^{–} \) which is the same as their hydrating abilities suggests that hydrophobic effects from the polyethylenoxy side chain could contribute significantly to the stability of the \( teg \)-ODN triplex (51).

**Gel retardation and primer extension with \( sp \)-ODN and \( teg \)-ODN**

The relative cationic charge contributions to the properties of \( sp \)-ODN and \( teg \)-ODN can also be probed by their electrophoretic behaviour. We had previously observed that \( sp \)-ODNs were considerably retarded in gel mobility on PAGE, compared with the unmodified ODNs and the retardation increases with the degree of substitution (36). The mobility retardation in electrophoresis results
Figure 2. Hysteresis curves of teg-ODN triplex 15*17:18 (a and b) and sp-ODN triplex 10*17:18 (c and d) at pH 7.3 in Tris buffer containing NaCl (100 mM). (a) and (c) in the presence of MgCl₂ (20 mM); (b) and (d) in the absence of MgCl₂; solid lines show heating curves while dashed lines show cooling curves.

from the positive charges of conjugated spermine chain and we sought to examine this effect in teg-ODNs which have only a single terminal amino group in side chain. Similar to sp-ODNs, the 5’-end ³²P-labelled teg-ODNs showed retardation on gel compared with unmodified ODNs, but lower compared with that of sp-ODN. The retardation (Table 2) increased with the degree of substitution and interestingly exhibited a dependence on the position of the modification in the sequence. The terminally modified monosubstituted ODNs (3’/5’) in each class had not only closer Rᵅ values, but retarded slightly more than the centrally modified ODNs. Since the molecular weight differences between the two types of modifications is <10, the observed retardation differences among sp- and teg-ODNs arise mostly from charge effects, the higher positive charge in sp-ODN leading to a relatively greater retardation.

Table 2. Gel retardation data on sp-ODNs and teg-ODNs

<table>
<thead>
<tr>
<th>Modified side</th>
<th>Rᵅ value</th>
<th>sp-ODN</th>
<th>teg-ODN</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’</td>
<td>0.76 (9)</td>
<td>0.83 (14)</td>
<td></td>
</tr>
<tr>
<td>3’</td>
<td>0.76 (7)</td>
<td>0.84 (12)</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>0.84 (8)</td>
<td>0.89 (13)</td>
<td></td>
</tr>
<tr>
<td>5’, 3’</td>
<td>0.56 (10)</td>
<td>0.70 (15)</td>
<td></td>
</tr>
<tr>
<td>5’, 3’, M</td>
<td>0.34 (11)</td>
<td>0.60 (16)</td>
<td></td>
</tr>
</tbody>
</table>

³²P value for unsubstituted ODN, 1.0

It would be interesting to see the effect of conjugation of polyamine and polyether functions to nucleobases in oligonucleotides on their ability to act as substrates in biological reactions. The chain extension reactions were individually carried out by DNA polymerase Klenow Pol I (52) using a common 25mer ODN 19 as the template and sp-ODNs 7–11 and teg-ODNs 12–16 as primers, all in unlabeled form. The presence of [α-³²P]dCTP as one of the dNTPs in the reaction mixture enabled unambiguous detection of only the primer extended products incorporating the radiolabel, as analysed by denaturing gel electrophoresis followed by autoradiography shown in Figure 4. The 5’-end ³²P-labeled 19 (lane 1) used as a marker, confirmed that both classes of modified oligomers, regardless of the position and degree of modification, act as efficient primers to yield 25mer extended products. The characteristic gel mobility retardation seen for the modified primers (Table 2) was also evident in their corresponding extended chains, with teg-ODN products showing a lower retardation than sp-ODN products.

Origin of triplex stability: sp-ODN versus teg-ODN

The experimental data presented in this and an earlier paper (36) clearly demonstrate that oligonucleotides with a spermine or a tetraethylenoxyamine side chain appended at C4 of 5-Me-dC form stable triplexes under low salt conditions. UV difference spectra and pKᵢ measurements indicate that N3 of sp-ODN is predominantly non-protonated at pH 7.0 and hence handicapped by the loss of one HG hydrogen bond with N7 of dG in central...
strand. The stability of the sp-ODN triplex under low salt conditions (100 mM NaCl) is due to enhanced reassociation of the duplex and third strand, caused by favored interaction of conditions (100 mM NaCl) is due to enhanced reassociation of the primer extended products obtained from sp-ODNs 9, 7, 8, 10 and 11 respectively while lanes 7, 8, 9, 10 and 11 correspond to the primer extended products from teg-ODNs 12, 13, 15, 16 and 14 respectively. Lane 1 shows 5’-end labeled 19 (25mer) as marker for size comparison.

CONCLUSIONS

Our studies on triple helices employing spermine and polyether conjugated ODNs as third strand have shown remarkable stabilization at physiological pH, conditions under which ODNs containing dC and 5-Me-dC fail to show triplex formation. The stability of sp-ODN triplexes at low salt conditions can be attributed to an accelerated reassociation of third strand with duplex, while teg-ODNs stabilize triplexes through hydrophobic desolvation. In contrast to the ionic interactions of sp-ODN, hydrophobic binding in teg-ODN is weaker. These results suggest that triplex stabilities can be modulated via covalent conjugates through factors other than base stacking and interstrand H-bonding. Since polyamines and polyoxyethylenes are well known to interact with membranes, studies on sp and teg-ODNs presented here may have significance in evolving newer strategies to improve cell permeability and in the development of ligand conjugated oligonucleotide analogues as non-cytotoxic candidates for antisense/antigene therapeutics.

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Figure 3. (A) Bar diagram shows anion dependence of sp-ODN and teg-ODN triplex $t_{in}$ in Tris buffer at pH 7.3. L stands for buffer containing NaCl (100 mM) and R stands for buffer containing Na$_2$SO$_4$ (100 mM). A (7*17:18), B (10*17:18) and C (11*17:18) represent sp-ODN triplexes with single, double and triple modifications respectively. D (12*17:18), E (15*17:18) and F (16*17:18) represent teg-ODN triplexes with single, double and triple modifications respectively. (B) Bar diagram shows salt dependence of sp-ODN 7*17:18 and teg-ODN 12*17:18 triplex $t_{in}$ in Tris buffer at pH 7.3 having different salt composition. (a) 500 mM NaCl (b) 100 mM NaCl + 400 mM Na$_2$SO$_4$ (c) 900 mM NaCl and (d) 100 mM NaCl + 400 mM LiClO$_4$.

Figure 4. Autoradiogram of denaturing PAGE of chain extended products obtained using 19 (25mer) as template and sp-ODNs and teg-ODNs as primers (18mer). As both primer and template are unlabelled, only the primer extended products are seen due to the incorporation of [α-32P]dCTP during extension reaction. Lanes 2, 3, 4, 5 and 6 correspond to the primer extended products obtained from sp-ODNs 9, 7, 8, 10 and 11 respectively while lanes 7, 8, 9, 10 and 11 correspond to the primer extended products from teg-ODNs 12, 13, 15, 16 and 14 respectively. Lane 1 shows 5’-end labeled 19 (25mer) as marker for size comparison.