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

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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 tetraethyleneoxyglycolamine 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 phopsphoramidites and 5′*O*-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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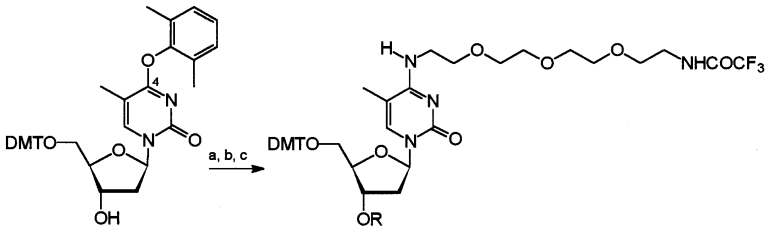

1

2 R = H
3 R = P(N-iPr$_2$)O(CH$_2$)$_2$CN

a. $H_2N(CH_2)_2O(CH_2)_2O(CH_2)_2O(CH_2)_2NH_2$ (**4**), b. ethyltrifluoroacetate and c. P(N-iPr$_2$)$_2$O(CH$_2$)$_2$CN/tetrazole

5						A	A	G	A	A	A	A	A	A	G	A	A	A	A	A	A	G	A	-d		
6			d-	T	T	**C**	T	T	T	T	T	T	**C**	T	T	T	T	T	T	**C**	T					
7						C							C							X						
8						C							X							C						
9						X							C							C						
10						X							C							X						
11						X							X							X						
12						C							C							Y						
13						C							Y							C						
14						Y							C							C						
15						Y							C							Y						
16						Y							Y							Y						
17	*d-*	G	C	C	A	A	G	A	A	A	A	A	G	A	A	A	A	A	A	G	A	C	G	C			
18		C	G	G	T	T	C	T	T	T	T	T	C	T	T	T	T	T	T	C	T	G	C	G	*-d*		
19	*d-*	C	C	A	G	C	T	A	A	A	G	A	A	A	A	A	A	G	A	A	A	A	A	G	A		

X = 5-Me-dC-(N^4-spermine)
Y = 5-Me-dC-(N^4-ω-deoxyaminotetraethyleneglycol)

Scheme 1.

deoxyribonucleoside-3′-*O*-[(*N*,*N*-diisopropylamino)-β-cyanoethyl phosphoramidite] monomers, followed by deprotection with aqueous NH_3. 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% CH_3CN in 0.1 M triethylammoniumacetate (TEAA) and B: 30% CH_3CN 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-^{32}P]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 $MgCl_2$, 10 µM each of dATP, dGTP, TTP, [α-^{32}P]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.

3′-*O*-(2-Cyanoethyl-*N*,*N*-diisopropylphosphoramido)-5′-*O*-(4,4′-dimethoxytrityl)-5-methyl-4-*N*-(11-*N*-trifluoroacetamido-3,6,9-trioxaundecyl)-2′-deoxycytidine (3)

5′-*O*-DMT-C4-(2,6-dimethyl)phenyl-5-methyluridine (34), **1**, (0.45 g, 0.7 mmol) and 1,11-diamino-3,6,9-trioxaundecane **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 as an eluent chloroform/methanol/triethylamine 9:0.9:0.1, yield: 0.40 g (71%). ^{1}H NMR δ($CDCl_3$): 7.68(s, 1H), 7.60(bs, 1H, exchangeable with D_2O), 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 D_2O), 4.56(m, 1H), 4.10(m, 1H), 3.79(s, 6H), 3.75–3.31(m, 18H), 2.64–2.53(m, 1H), 2.26–2.20(m, 1H) and 1.48(s, 3H). ^{13}C NMR δ($CDCl_3$): 163.7, 158.7, 156.6, 144.7, 137.4, 135.8, 130.2, 128.3, 128.0, 127.0, 113.3, 102.4, 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. ^{31}P NMR δ($CDCl_3$): 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, Na_2SO_4, $NaClO_4$ 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 cm^2/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 $A_{260\ nm}$ 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-N^4-(*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-O^4-(2,5-dimethylphenyl)-5-methyl dC **1** with 1,11-diamino-3,6,9-trioxaundecane **4**, followed by protection of ω′-amino group as trifluoroacetate and subsequent 3′*O*-phosphoramidation by standard procedures (38). The attachment of diaminopolyoxyethylenes 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 ^{31}P NMR and mass spectroscopy. This was incorporated into the oligonucleotide sequences **12–16** at the indicated positions, followed by aqueous NH_3 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_ms 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.

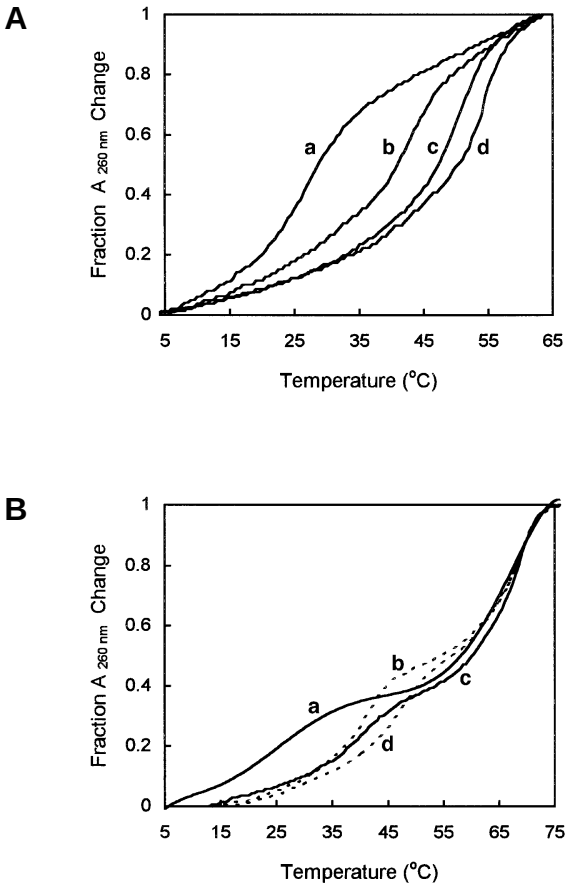

Figure 1. (**A**) UV-melting profiles of duplexes constituted from *teg*-ODNs in Tris buffer (pH 7.3) containing NaCl (100 mM) and $MgCl_2$ (20 mM). (a) **5:16**, (b) **5:15**, (c) **5:13** and (d) **5:6**. (**B**) UV melting profiles of triplexes constituted from *teg*-ODNs in Tris buffer (pH 7.3) containing NaCl (100 mM) and $MgCl_2$ (20 mM). (a) **16*17:18**, (b) **15*17:18**, (c) **13*17:18** and (d) **12*17:18.**

Table 1. Melting data for *teg*-ODN duplexes and triplexes

Duplex	t_m °C	Triplex	t_m °C –Mg^{++}	+Mg^{++}
5:6	50	6*17:18	nd	30
5:12	50	12*17:18	34	47
5:13	47	13*17:18	nd	42
5:15	42	15*17:18	23	41
5:16	29	16*17:18	nd	24

nd, not detected.

In the presence of Mg^{++} all *teg*-ODNs formed triplexes and the t_ms 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 $MgCl_2$, Δt_m pH 7.3–5.5 = 6°C) and was qualitatively similar to that seen with the

corresponding *sp*-ODN triplexes (Δt_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^{++} 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^{++} upon cooling (Fig. 2a). However, in the absence of Mg^{++}, *teg*-ODN significantly exhibited a strong hysteresis (Fig. 2b) which was absent in the corresponding *sp*-ODN triplex, both with and without Mg^{++} (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 $SO_4^{2-} > Cl^- \approx Br^- > ClO^{4-} > CNS^-$. This has also been recently employed to establish the hydrophobic nature of the calicheamicin–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 (F in 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 t_m of *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 stablility by the anions $SO_4^{2-} > Cl^- > ClO_4^-$ which is the same as their hydrating abilities suggests that hydrophobic effects from the polyethyleneoxy 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

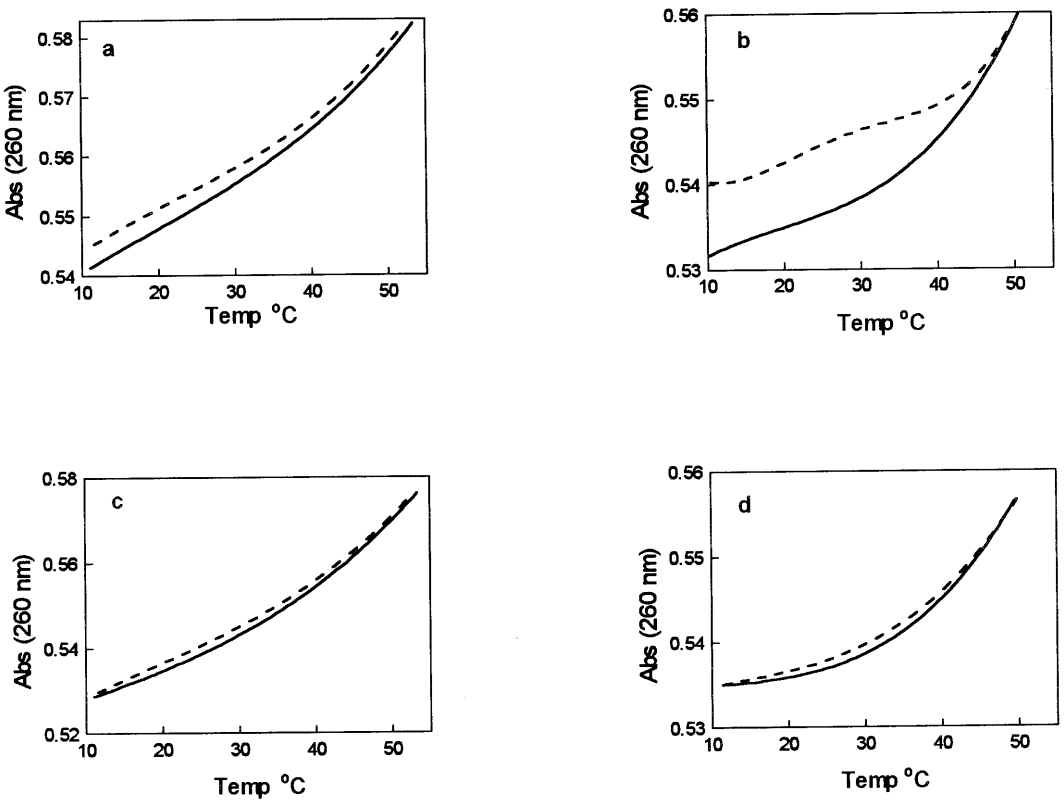

Figure 2. Hysteresis curves of *teg*-ODN triplex **15*17:18** (**a** and **b**) and *sp*-ODN triplex **10*17:18** (**c** and **d**) at pH 7.3 in Tris buffer containing NaCl (100 mM). (a) and (c) in the presence of $MgCl_2$ (20 mM); (b) and (d) in the absence of $MgCl_2$; solid lines show heating curves 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 ^{32}P-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 R_f 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[a]

Modified side	R_f value	
	sp-ODN	*teg*-ODN
5′	0.76 (**9**)	0.83 (**14**)
3′	0.76 (**7**)	0.84 (**12**)
M	0.84 (**8**)	0.89 (**13**)
5′, 3′	0.56 (**10**)	0.70 (**15**)
5′, 3′, M	0.34 (**11**)	0.60 (**16**)

[a]R_f 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-^{32}P]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 ^{32}P-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 tetraethyleneoxyamine side chain appended at C4 of 5-Me-dC form stable triplexes under low salt conditions. UV difference spectra and pK_a 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

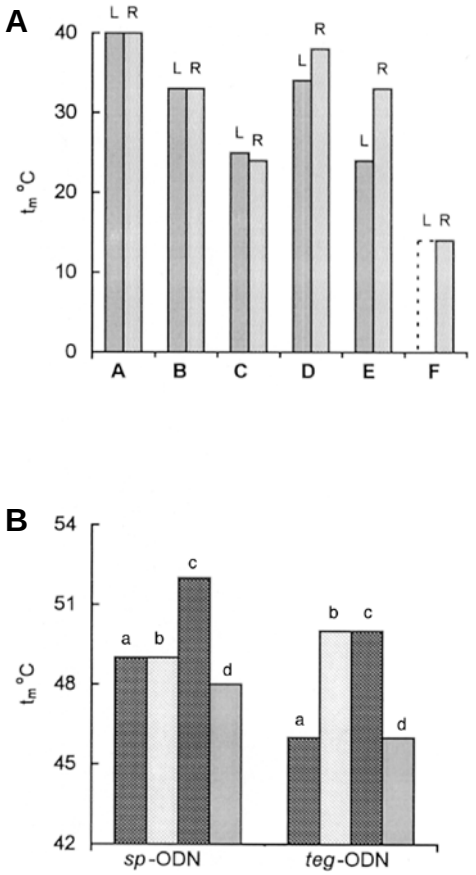

Figure 3. (**A**) Bar diagram shows anion dependence of *sp*-ODN and *teg*-ODN triplex t_m in Tris buffer at pH 7.3. L stands for buffer containing NaCl (100 mM) and R stands for buffer containing Na_2SO_4 (100 mM). A (**7*17:18**), B (**10*17:18**) and C (**11*17:18**) represent *sp*-ODN triplexes with 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_m in Tris buffer at pH 7.3 having different salt composition. (a) 500 mM NaCl (b) 100 mM NaCl + 400 mM Na_2SO_4 (c) 900 mM NaCl and (d) 100 mM NaCl + 400 mM $LiClO_4$.

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 conjugated cationic spermine electrostatically with the anionic phosphate backbone and through possible hydrogen bonding interaction with bases on the duplex. In the case of *teg*-ODN triplexes, which lack a polycationic side chain, the triplex stability arises from a fine tuning of the microenvironment by the polyether side chain in terms of desolvation, facilitating a hydrophobic interaction. This is based on the effect of strongly hydrated, antichaotropic agent Na_2SO_4 which enhanced the t_m of *teg*-ODN triplexes in contrast to the weakly hydrated chaotropic agent $NaClO_4$ which destabilised the *teg*-ODN triplex. This is consistent with a hydrophobic nature of the interaction of the *teg* side chain with DNA duplex. In contrast to the ionic interaction of *sp*-ODN, hydrophobic binding in *teg*-ODN is weaker as shown by a lower stabilizing and a greater hysteresis effect, but nevertheless significant, since *teg*-ODNs form triplexes with a

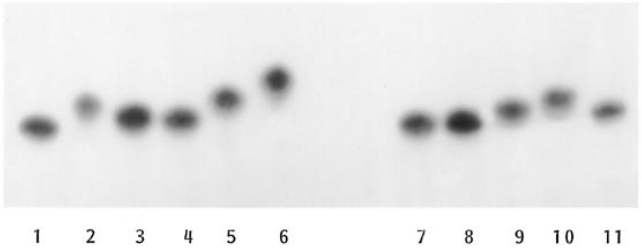

Figure 4. Autoradiogram of denaturing PAGE of chain extended products obtained using **19** (25mer) as template and *sp*-ODNs and *teg*-ODNs as primers (18mer). As both primer and template are unlabelled, only the primer extended products are seen due to the incorporation of [α-^{32}P]dCTP during extension 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.

better stability compared with unmodified controls. In both *sp*- and *teg*-ODNs, substitutions in the interior are less stabilizing than substitutions towards the 3′/5′ termini. This is apparent from the nucleation-zipping model for association–dissociation of duplex (53), also valid for triplexes (54) where nucleation involving terminal 3–4 base triplets governs the rate determining step for association of the third strand with duplex.

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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