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Triplex formation at physiological pH by 5-Me-dC-N^4-(spermine) [X] oligodeoxynucleotides: non protonation of N3 in X of X*G:C triad and effect of base mismatch/ionic strength on triplex stabilities

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ABSTRACT

Oligodeoxynucleotide (ODN) directed triplex formation has therapeutic importance and depends on Hoogsteen hydrogen bonds between a duplex DNA and a third DNA strand. T*A:T triplets are formed at neutral pH and C$^+$*G:C are favoured at acidic pH. It is demonstrated that spermine conjugation at N^4 of 5-Me-dC in ODNs 1–5 (sp-ODNs) imparts zwitterionic character, thus reducing the net negative charge of ODNs 1–5. sp-ODNs form triplexes with complementary 24mer duplex 8:9 show foremost stability at neutral pH 7.3 and decrease in stability towards lower pH, unlike the normal ODNs where optimal stability is found at an acidic pH 5.5. At pH 7.3, control ODNs 6 and 7 carrying dC or 5-Me-dC, respectively, do not show any triple helix formation. The stability order of triplex containing 5-Me-dC-N^4-(spermine) with normal and mismatched duplex was found to be X*G:C ~ X*A:T > X*C:G > X*T:A. The hysteresis curve of sp-ODN triplex 3*8:9 indicated a better association with complementary duplex 8:9 as compared to unmodified ODN 6 in triplex 6*8:9. pH-dependent UV difference spectra suggest that N3 protonation is not a requirement for triplex formation by sp-ODN and interstrand interaction of conjugated spermine more than compensates for loss in stability due to absence of a single Hoogsteen hydrogen bond. These results may have importance in designing oligonucleotides for antigene applications.

INTRODUCTION

The design of oligonucleotides capable of inhibiting either translation via duplex formation with mRNA through Watson–Crick hybridization (antisense strategy) or transcription via a triple helix formation with a natural DNA duplex through Hoogsteen base pairing (antigene strategy) is currently a very active research area (1,2). The criteria for the analogues of ODN to be useful for the above therapeutic purposes are that these should be cell permeable and more nuclease resistant than the natural ODNs without compromising their binding ability to the target single strand or duplex nucleic acids. Since the negative charge on nucleic acid is a major deterrent for membrane permeation, ODNs which are non-ionic or those with diminished net charge have acquired importance for synthesis and study of biophysical properties. Much of the efforts towards this aim have centered on modification of sugar–phosphate backbone or replacement with dephosphono chemical moieties (2b). Nucleobase modification provides an alternative approach and of late has received much attention (3–7). It was shown that zwitterionic ODNs carrying a lipophilic cation at each nucleotide base form duplexes at physiological pH and low salt conditions (8). Here, the base tethered ammonium cations replace metal ions which normally neutralize the negative phosphate charges, thus improving ODNs membrane permeability and nuclease resistance due to attendant changes in charge and size of monomer units.

Triple helix formation depends on Hoogsteen hydrogen bonds between thymine and A:T base pairs (T*A:T triplet) and between protonated cytosine and G:C base pairs (CH$^+$*G:C triplet) (1c,9). Due to the molecular necessity of protonated C (CH$^+$) in the third strand (10), triplex formation in natural/unmodified nucleic acids is pH dependent with an optimum stability at non-physiological pH 5.6–6.0. This specificity and pH dependence of triad base pairing in triple helix formation have led to the design of chemical modifications that increase the affinity of third strand at intracellular pH 7.1–7.6 (3,4,5a,7). Substitution of 5-Me-dC for dC in third strand has extended the compatible pH range for triplex formation to 6.5 (11).

Polyamines and their bio-conjugates have potential as biological effector molecules with bright prospects for medicinal developments (12). The naturally occurring polyamines, spermine and spermidine are largely protonated at physiological pH and exhibit net positive charge close to +4 and +3, respectively. Their inherent polycationic nature and conformational mobility of chain, encourage electrostatic interaction with the anionic phosphates of polynucleotides resulting in specific molecular

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interaction in duplex DNA. Spermine is also known to favor triple helix formation when present in millimolar concentrations (13) and it was recently shown (14) that spermine conjugation to the 5′-end of oligonucleotides leads to improved triple helix stability at pH 6.5. In view of the positive attributes of both 5-Me-dC (11) and spermine (13,14) in promoting triple helix formation, we envisioned (15) that oligonucleotides carrying both moieties as in 5-Me-dC-N^4-(spermine), may have a constitutive effect on triplex formation at physiological pH. It was indeed found that incorporation of 5-Me-dC-N^4-(spermine) into the Hoogsteen strand of a DNA triplex remarkably stabilized the triplexes at desirable physiological pH, even without Mg^{2+}, as compared to non-formation of triplexes with unmodified third strand. In this paper, we examine the effect of mismatches and the possible role of conjugated spermine in enhancing the stability of triplex, in particular on the N3 protonation and association/dissociation equilibria. Conjugation of spermine to a nucleobase also permits multiple incorporation at internal sites leading to an increased local concentration of spermine for enhanced triplex stability. From a therapeutic perspective, this is an attractive idea since intracellular environment cannot be manipulated to achieve conditions favorable for triplex existence.

MATERIALS AND METHODS

Base-protected nucleoside phopsphoramidites and nucleoside derivatised controlled pore glass supports (CPG) were purchased from Cruachem UK. All chemicals used were of reagent quality or better grade. Modified nucleoside monomers for pK_a measurements were synthesized according to an earlier report (15). T4 polynucleotide kinase, snake-venom phosphodiesterase and alkaline phohphatase were purchased from Boehringer Mannheim. 5′-[γ-^{32}P]ATP was purchased from Bhabha Atomic Research Center, Bombay.

Oligonucleotide synthesis, purification and labelling

All oligonucleotides were synthesized on 1.3 µmol scale on a Pharmacia GA plus DNA synthesizer using CPG support and base protected 5′-O-(4,4′-dimethoxytrityl)deoxyribonucleoside-3′-O-[(diisopropylamino)-β-cyanoethylphopsphoramidite] monomers, followed by deprotection with aqueous NH_3. The protected 5-Me-dC-N^4-(spermine) phosphoramidite was prepared according to the earlier reported procedure (15a) for incorporation at the desired positions in oligonucleotide sequences (**1–5**) (Fig. 1). All oligonucleotides were purified by reversed phase HPLC on Novapak C18 column using the buffer systems A: 5% CH_3CN in 0.1 M triethylammoniumacetate (TEAA) and B: 30% CH_3CN in 0.1 M TEAA using a gradient A→B of 1.5%/min at a flow rate of 1.5 ml/min. Retention time for ODNs: **1**, 10.87 min; **2**, 10.73 min; **3**, 10.82 min; **4**, 11.38 min; **5**, 11.57 min; and **6**, 10.31 min. The purified oligonucleotides were labeled at the 5′-end with T4 polynucleotide kinase and 5′-[γ-^{32}P]ATP according to standard procedures (16). The radiolabeled oligonucleotide samples were run on a 20% polyacrylamide gel containing 7M urea and with Tris-borate–EDTA (pH 8.3) as buffer. Samples were mixed in formamide, heated to 70°C and cooled on an ice bath before loading on the gel. Autoradiograms were developed after 1 h exposure using an intensifying screen.

Base composition analysis

The base composition of modified oligonucleotides were confirmed by enzymatic hydrolysis (17). Oligonucleotides **1–5**, (0.5 OD_{254} U) were dissolved in 10 mM KH_2PO_4 (100 µl, pH 7.0) containing $MgCl_2$ (10 mM) and treated with snake venom phosphodiesterase (10 µl, 1 mg/0.5 ml) and alkaline phosphatase (10 µl, 1 U/µl) at 37°C for 12 h. This hydrolysate (2 µl) was analyzed on analytical C18 RP-column and eluted with 0.1 M triethylammonium acetate, at pH 6.5 with flow rate of 1 ml/min and the peaks were detected using a photodiode array detector. The retention times (Rt) for (i) standard nucleosides were: dC, 1.2 min; dG, 2.3 min; dT, 2.7 min; and dA, 4.4 min and (ii) for the enzymatic hydrolysates were: dC, 1.2 min, λ_{max} 271 nm; 5-Me-dC-N^4-(spermine), 1.4 min, λ_{max} 280 nm; and dT, 2.7 min, λ_{max} 269 nm. The enzymic hydrolysate of **5** showed complete absence of dC and presence of 5-Me-dC-N^4-(spermine) and dT.

pH titrations

The pK_a of N3 in modified nucleoside monomers dC, 5-Me-dC, 5-Me-dC-N^4-(*n*-propyl), 5-Me-dC-N^4-(*n*-butylamino) and 5-Me-dC-N^4-(spermine) were determined by titration of their individual aqueous solutions (1 mg/ml) with aqueous NaOH (2 mM) by incremental addition of aliquots of 40 µl, at 25°C under constant stirring. After each addition the solution was allowed to equilibriate for 5 min followed by pH measurement using standard pH electrode. pK_a were determined by following reported literature procedure (5c). The pK_a of nucleosides dC and 5-Me-dC-N^4-(spermine) incorporated into the oligonucleotides (**6** and **5**) were determined by UV titration of their aqueous solutions (1 µM) with aq NaOH at 45°C and the titration was monitored at 260 nm (11d,18). The pH stability curves of triple helices (**6*8**:**9**) and (**3*8**:**9**) were obtained at 4°C by similar UV titration monitored at 260 nm (11d). All oligonucleotide samples for titration were prepared in aq solutions containing 100 mM NaCl and 20 mM $MgCl_2$.

Melting experiments

Duplex and triplex melting experiments were carried out in the following buffers containing 100 mM NaCl with or without 20 mM $MgCl_2$: 50 mM NaOAc, pH 5.5; 10 mM PIPES [piperazine-N-N′-bis(2-ethanesulphonic acid)], pH 6.0; and 25 mM Tris, pH 7.0–7.3. Appropriate oligonucleotides, each at a strand concentration of 1 µM based on 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/µmol were mixed and heated at 70°C for 3 min, cooled to room temperature followed by overnight storage at 4°C. The A_{260} at various temperatures were recorded using Perkin Elmer Lambda 15 UV/VIS spectrophotometer, fitted with a peltier heating controller and temperature programmer with heating rate of 0.5°C/min over the range of 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 T_m of triplexes containing mismatches were similarly determined under identical conditions.

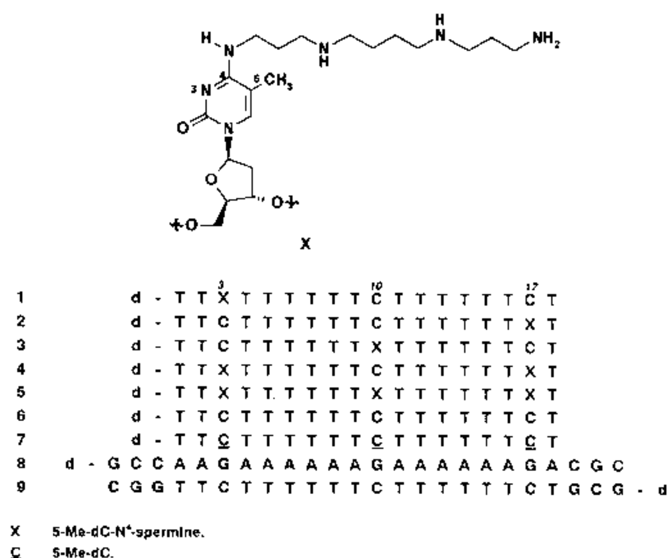

Figure 1. X = 5-Me-dC-(N^4-spermine).

The association T_m of triplexes were studied by hysteresis experiments in which the samples were heated (0.5°C/min) and maintained at triplex–duplex transition temperature (45°C for **6*8**:**9** and 57°C for **3*8**:**9**) for 10 min to achieve constant absorbance, followed by cooling (0.5°C/min). All hysteresis experiments were done in Tris buffer (20 mM, pH 7.2), containing either NaCl (100, 400 and 800 mM) or NaCl (100 mM) with $MgCl_2$ (20 mM).

The UV-difference spectra were obtained using graphic software of Perkin Elmer Lambda 15 UV/VIS spectrophotometer by subtracting individual spectra of corresponding triplexes at pH 2.3, 5.3 and 7.3. All spectra were recorded at constant temperature (5°C) in buffer containing Tris (25 mM) and NaCl (100 mM) with or without $MgCl_2$ (20 mM).

RESULTS AND DISCUSSION

Synthesis and characterization of spermine-oligonucleotide (sp-ODN) conjugates

Oligonucleotides containing 5-Me-dC-N^4-(spermine) (Fig. 1) and 5-Me-dC were synthesized according to earlier reported procedures (15). The 5-Me-dC-N^4-(spermine) monomer was incorporated at specific positions into oligonucleotide sequences **1–5**. After completion of synthesis, final on-column detritylation was followed by standard aqueous NH_3 treatment to yield the fully deprotected oligonucleotides **1–5**. The laser desorption mass spectral measurement of the HPLC purified oligonucleotide **1** gave the expected mass 5561.1 (M-H) and 5603.1 (M-2H+K) confirming the presence of modified moiety.

To further ensure that polyamine conjugated nucleobases have survived the synthetic chemistry of oligonucleotide assembly by phosphoramidite approach and the subsequent ammoniacal deprotection, enzymatic hydrolyses of **1–5** were done using snake venom phosphodiesterase and alkaline phosphatase (17). RP-HPLC analysis of the hydrolysate indicated the presence of the modified nucleoside (**X**) in addition to the normal deoxynucleosides. The modified nucleoside base **X** could also be unambiguously assigned by its different uv spectral shift (280 nm) as compared with standard dC (271 nm). The spermine conjugated oligonucleotides **1–5** used presently consisted of 18 nucleotide (nt) oligomers with a single modification towards the 5′- side (**1**, **X**3), the 3′-side (**2**, **X**17), and at the center (**3**, **X**10) and those with double (**4**, **X**3 and **X**17) and triple (**5**, **X**3, **X**10 and **X**17) insertions of 5-Me-dC-N^4-(spermine). ODNs having either dC **6** or 5-Me-dC **7** were also synthesized as above for control experiments.

Altered electrophoretic mobility of sp-ODN

The homogeneity of synthesized oligonucleotides is apparent from single bands obtained on denaturing PAGE, in which the ODNs exhibited different migratory aptitudes as a function of degree of spermine substitution for the same length of oligonucleotides. This behavior on gel electrophoresis revealed the expected zwitterionic character due to covalent presence of positively charged spermine in the negatively charged oligonucleotide. The relative electrophoretic migration of various oligomers with respect to unmodified oligo **6** (1.00) are **1** (0.87), **4** (0.68), **5** (0.55), and **9** (0.90). Thus the oligomers **1**, **4** and **5** containing one, two and three spermine molecules, respectively, per DNA strand, exhibited considerable retardation in mobility on the denaturing polyacrylamide gel as compared to unmodified 18mer **6** and 24mer **9**, suggesting a lowering of net negative charge on sp-ODN by the conjugation of spermine moiety. The oligomer **1** with mono spermine substitution showed mobility lower than unmodified 18mer **6** but similar to that of unmodified 24mer **9**. This indicates that retardation in gel mobility arises from neutralization of the charge rather than an increase in size. The sp-ODNs **4** and **5** with higher substitutions show further retardation in their mobility compared with the 24mer **9** suggesting the additive nature of the neutralization effect upon increasing the number of spermine molecules per DNA strand (8).

Triplex formation by sp-ODN

The triplexes bearing spermine on the third strand were generated by hybridization of sp-ODNs **1–5** with duplex **8**:**9** constituted from unmodified 24mer oligonucleotides containing polypurine and polypyrimidine stretches. The 5′ and 3′ ends in duplex **8**:**9** were designed to avoid concatenation and to resolve triplex–duplex transition from that of duplex–single strand. The thermal denaturation profiles of hybrids constituted from sp-ODNs with the control duplex **8**:**9** in Tris buffer (pH 7.3) containing NaCl (100 mM), showed a biphasic dissociation (Fig. 2). The transition in the lower temperature range 25–40°C corresponds to melting of third strand and that around 67°C corresponds to duplex denaturation. The formation of triplexes is also supported by UV-mixing curves (11b), generated by addition of third strand to preformed duplex by continuous variation method which indicated a 1:1 stoichiometry for sp-ODN:duplex hybrids. Among the sp-ODNs **1–5** which differ in the number and sequence-position of modifications, triplexes derived from the third strands containing spermine conjugation towards 5′/3′-termini (**1*8**:**9** and **2*8**:**9**) gave better thermal stability compared to modification in the center (**3*8**:**9**) (Table 1). Although the triplex T_m slightly decreased with the increasing number of modifications, triplex formation was still observable with the trisubstituted sp-ODN **5** at physiological pH and temperature. In all cases, the underlying duplex melting temperature remained the same (67°C). In order to rule out formation of any secondary structures involving

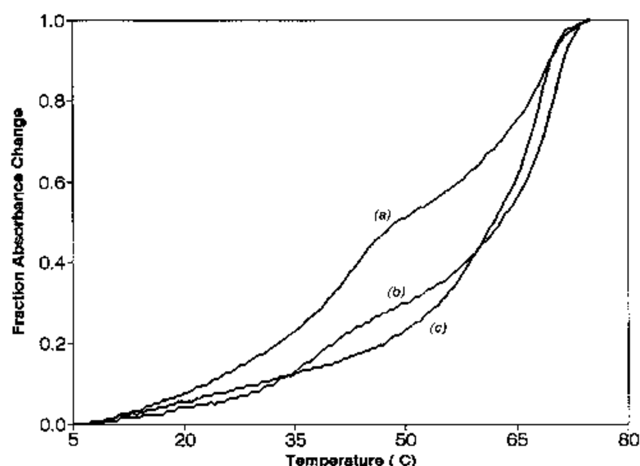

Figure 2. Melting profiles at pH 7.3 for (**a**) **3*G**:**C**, (**b**) **3*C**:**G** and (**c**) **3*T**:**A** in buffer containing 25 mM Tris, 100 mM NaCl, 20 mM $MgCl_2$.

sp-ODNs, control thermal denaturation experiments were performed in the absence of duplex. Non-existence of any ordered structure in sp-ODNs alone was indicated by uneventful T_m curves.

Table 1. Triplex–duplex transition temperatures[a] (T_m °C) of sp-ODN triplexes in the absence of $MgCl_2$

Third strand	No. of spermine/strand	pH 7.3	6.5	6.0
1	1	40	36	34
2	1	40	36	35
3	1	33	31	30
4	2	33	32	31
5	3	25	24	23
6	–	nd	nd	28
7	–	nd	nd	38
7[b]	–	33	–	–

[a]All T_m were obtained from melting experiments of the corresponding triplexes. nd, not detected.
[b]ODN **7** with 1 mM added spermine.

The ODNs **6** and **7** containing dC or 5-Me-dC, respectively, which are devoid of conjugated spermine showed no triple helix formation under the above conditions. The triplexes from these were obtained at pH 7.3 in the presence of either 100 mM NaCl containing 20 mM $MgCl_2$ or high NaCl (800 mM) concentration. The stabilities of triplexes from sp-ODNs **1–5** measured with added $MgCl_2$ (20 mM) showed an enhancement of T_m by 6–7°C. Significantly, triplexes from mono sp-ODNs (**1*8**:**9**, 47°C and **2*8**:**9**, 46°C) were as stable as that from ODN with three 5-Me-dC (**7*8**:**9**, 46°C) in the presence of $MgCl_2$. The formation of the triplex (**6*8**:**9**) was also examined in presence of externally added spermine (1 mM). A T_m of 33°C obtained with 1 mM extraneous spermine is similar to that from sp-ODN **5**, in which the concentration of appended spermine (equivalent to [ODN]) is only ~1 µM.

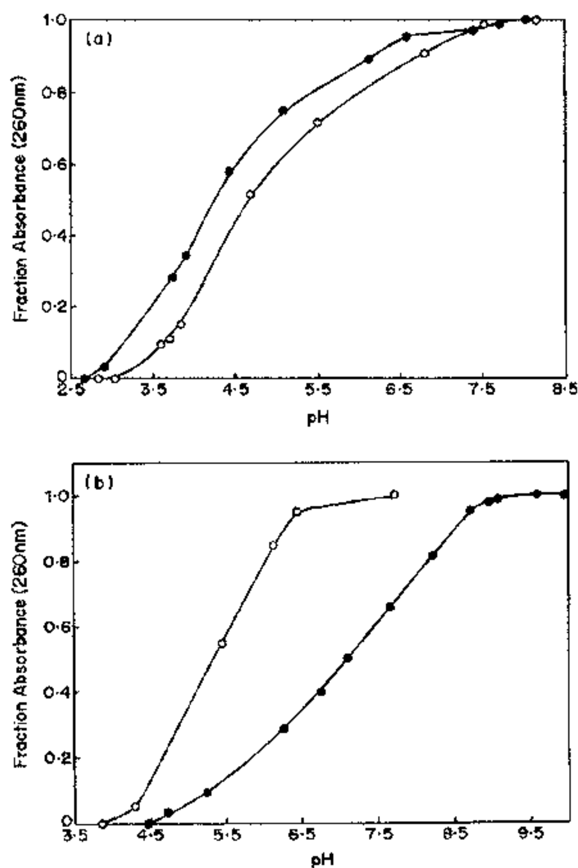

Figure 3. (**a**) Protonation curves for **5** (●) and **6** (○) as a function of pH at 45°C. (**b**) Stability of triplex **6*8**:**9** (○) and **3*8**:**9** (●) as a function of pH.

pH dependence of sp-ODN triplexes

The pK_a of N3 in dC was considered to be one of the critical factors in determining the stability of triplexes (10,11b). To analyze the effect of N^4 substitution, pK_as of various N^4-substituted nucleosides 5-Me-dC-N^4-(*n*-propyl), 5-Me-dC-N^4-(*n*-butylamino) and 5-Me-dC-N^4-(spermine) were measured by pH titration and found to be 4.5, 3.8 and 3.7, respectively, as compared with 4.3 and 4.4 for dC and 5-Me-dC, respectively (5c,11b). N^4 substitution with a simple alkyl chain (e.g *n*-propyl) does not seem to affect the pK_a of N3 while the presence of even a single amino group in the side chain lowers the pK_a by 0.5–0.6 U as seen for 5-Me-dC-N^4-(*n*-butylamino) nucleoside. Increasing the number of amino groups on the side chain as in 5-Me-dC-N^4-(spermine) did not have any further perceptible effect on pK_a of N3. In dC nucleosides with N^4 substituents carrying amino groups, the lower pK_a of N3 may arise due to the fact that prior existence of a positive charge on the side chain alkyl amino group (having higher pK_a) may disfavor a second protonation of N3. The four pK_a values for spermine (10.97, 10.27, 9.04 and 8.03) as reported in the literature (19) are distinctly higher than N3 pK_a of dC and its analogs, and hence no influence persists from the spermine side chain in the N3 pK_a measurements. Upon incorporation into oligonucleotide, pK_a values for N3 of dC 4.6 (11d,18) and 5-Me-dC-N^4-(spermine) 4.2 were enhanced relative to the monomer by 0.3–0.5 U (Fig. 3a).

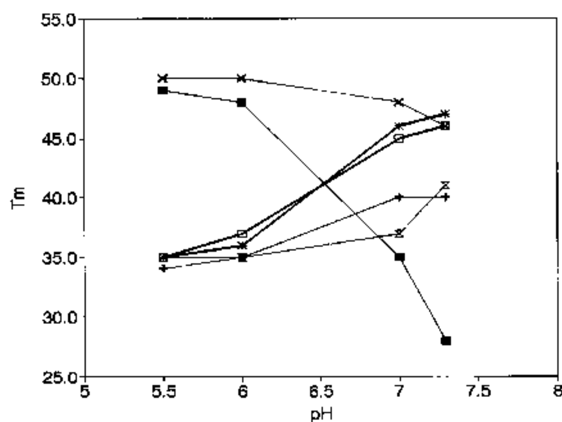

Figure 4. pH dependent T_m of triplexes constituted from the duplex **8**:**9** and **1** (*), **2** (□), **3** ( ), **4** (+) **6** (■) and **7** (×) as third strand in presence of Mg^{2+}.

The pH dependence of triplex stability is critical for *in vivo* applications and hence we examined the effect of pH on triplex formation from ODNs **1–5** in the presence of Mg^{2+}. The unmodified triplex (**6*8**:**9**) exhibited a large increase in T_m towards the acidic pH with a plateau around pH 5.6–5.8, while 5-Me-dC containing triplex (**7*8**:**9**) showed only a marginal increase in T_m at lower pH (Fig. 4). The pH dependence of T_m for sp-ODN containing triplexes (**1–5*8**:**9**) showed an opposite pattern; T_m was maximum in the pH range 7.1–7.4 and displayed moderate destabilization at acidic pH. Even in the absence of Mg^{2+} this stability pattern was retained in sp-ODN triplexes (Table 1). The most significant and useful result is that sp-ODNs have optimum triplex stability at physiological conditions. The pH dependent stabilities of triplexes **6*8**:**9** and **3*8**:**9** were also determined by UV titration (11d) at 4°C and monitored at 260 nm. The titration curves (Fig. 3b) indicated a better stability of sp-ODN triplex at neutral pH as compared with the unmodified triplex.

Protonation status of N3: UV-spectral study

Among the four nucleobases, only dC shows significant near UV spectral changes which are pH dependent (20). This property has been used to investigate the N3 protonation state of base dC in triplex (21) and tetraplexes (22). The spectral shift in UV of dC as a function of pH, although characteristic, is small in magnitude and can be unambiguously identified only by difference UV-spectroscopy. The predominant protonation of N3 in dC at pH (3.0) lower than pK_a is indicated by the appearance of a characteristic negative band at 288 nm in the difference spectra Δ_{UV} pH(7.3–2.3) and Δ_{UV} pH(5.3–2.3) while not so significant in the difference spectrum Δ_{UV} pH(7.3–5.3) (Fig. 5a). This behavior persisted even upon incorporation of dC into the oligonucleotide since N3 pK_a does not change appreciably. The effect of spermine conjugation at N^4 of dC on N3 protonation can be similarly characterized by UV-difference spectral data (Fig. 5b). N3 protonation of 5-Me-dC-N^4-(spermine) is also accompanied by a negative band in difference spectra Δ_{UV} pH(7.3–2.3) and Δ_{UV} pH(5.3–2.3) but interestingly, the wavelength is shifted to 298 nm in nucleoside and to 294 nm in sp-ODN. These results are in agreement with pK_a values of dC (4.3) and 5-Me-dC-N^4-(spermine) (3.7) reported above. In view of the above characteristic observation, we examined the UV-difference spectra of triplexes from sp-ODN constituted at different pHs, to determine the protonation status of N3. The validity of this approach is substantiated by a recent report (21) which demonstrated that in unmodified triplexes third strand dC residues are protonated slightly even at pH 7.0, far above its intrinsic pK_a. In unmodified triplex **6*8**:**9**, Δ_{UV} pH(7.3–5.3) spectral pattern with negative band at 292 nm confirmed that N3 of dC is appreciably protonated even above pH 5.3 and much more below pH 5.3 (Fig. 5c). In contrast to this, in sp-ODN triplex **5*8**:**9**, N3 of 5-Me-dC-N^4-(spermine) residues are negligibly protonated in the pH range 7.3–5.3 as seen by the lack of a negative band at 294 nm (Fig. 5d). The origin of a negative band in the difference spectrum of triplex **6*8**:**9** [(Δ_{UV} pH(7.3–5.3)] is from dC protonation in the third strand rather than those in duplex. This was confirmed by lack of any negative band characteristic of protonation in UV-difference spectrum of duplex **8**:**9** in the pH range 7.3–5.3. The existence of stable triplexes from sp-ODN in this pH range was shown by T_m data and pH stability curve. N3 protonation of 5-Me-dC-N^4-(spermine) occurred at pHs <5.3 as observed in the difference spectrum Δ_{UV} pH(5.3–2.3) in which the characteristic negative band appeared at 294 nm. The sp-ODN triplexes in this pH range have lower stability than at pH 7.0. Thus, conditions under which most stable sp-ODN triplexes are seen by T_m (pH 7.3, without Mg^{2+}), no N3 protonation was detected by UV-difference spectral data. Similar UV-difference spectra were also obtained for unmodified and sp-ODN triplexes at different temperatures (not shown), with and without $MgCl_2$ and the result was in agreement with the above observations.

Sequence specificity

To study the stringency of triplex formation by sp-ODNs (**1–5**), triplexes were constituted with duplexes containing all four Watson–Crick base pairs at positions complementary to 5-Me-dC-N^4-(spermine) of the third strand. Apart from **X*G**:**C**, the other triplexes (**X*C**:**G**, **X*A**:**T** and **X*T**:**A**) contain mismatched Hoogsteen base pairing of **X** with duplexes. Amongst them, a decreased stability was observed in the following order: **X*G**:**C** ~ **X*A**:**T** > **X*C**:**G** > **X*T**:**A** (Table 2), which is similar to that reported for unmodified base triad by the affinity cleavage method (23). However, this order differed slightly from that obtained from thermal denaturation studies by Helene *et al.* (24).

Table 2. Mismatch specificity

Triplex	T_m with $MgCl_2$		T_m without $MgCl_2$	
X*Y:**Z**	first	second	first	second
C*G:**C**	28	67	nd	–
X*G:**C**	41	67	32	62
X*C:**G**	36	69	29	62
X*A:**T**	40	67	27	60
X*T:**A**	nd	–	nd	–

[a]All T_m were obtained from melting experiments of the corresponding triplexes.

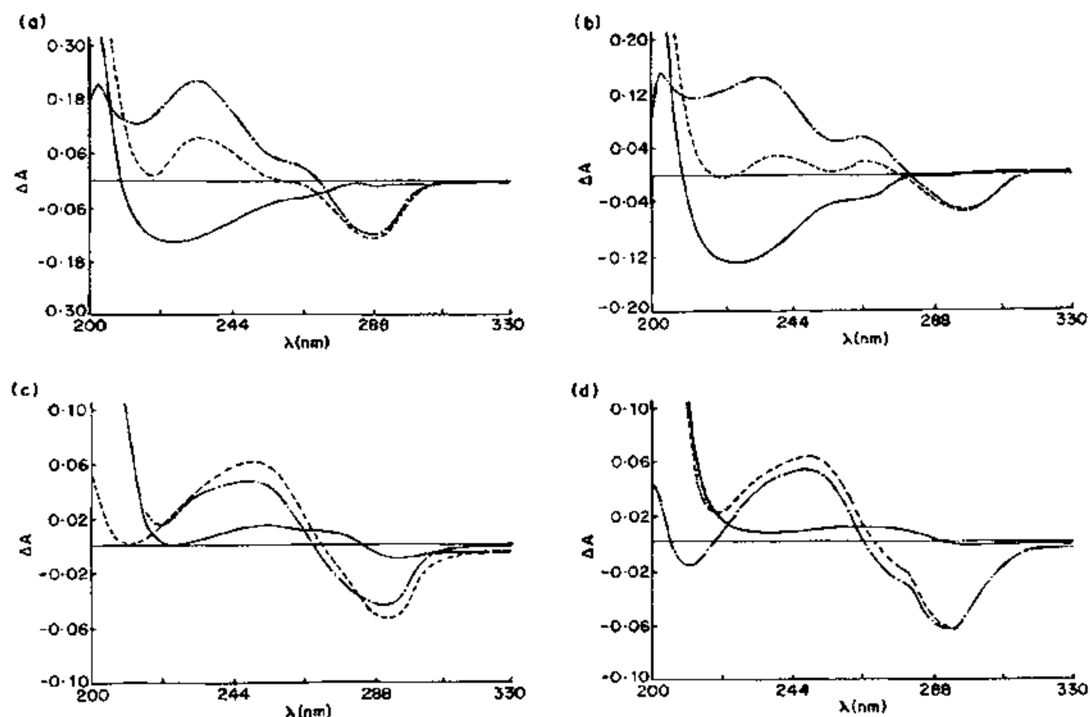

Figure 5. UV-difference spectra at 5°C of dC (**a**), 5-Me-dC-(N^4-spermine) (**b**), **6*8**:**9** (**c**) and **5*8**:**9** (**d**). Different line styles represent Δ_{UV} pH(7.3–5.3) [—], Δ_{UV} pH(7.3–2.3) [- - -] and Δ_{UV} pH(5.3–2.3) [– · – · –]

Hysteresis in triplex transitions and effect of ionic strength

It is known from the literature (25) that association constant K_a for triplex formation is ~1000 times lower than that for duplex and the rate of third strand hybridization with the duplex is ~100 times slower than association of duplex from single strands. As a consequence, hysteresis is observed for normal triplex association/dissociation which is dependent on both pH and ionic strength (26). At neutral pH and 100 mM NaCl, the association rates are much slower than the dissociation while at lower pH (5.8) where triplex stability is higher, the heating and cooling curves tend to merge. To understand the role of appended spermine in triplex stabilization through strand association and dissociation events, relative behaviors of heating and cooling curves were examined for both control (**6*8**:**9**) and modified (**3*8**:**9**) triplexes under identical conditions. This is also necessary in view of the gel retardation results that indicated an intramolecular charge neutralization which may significantly influence the triplex association/dissociation equilibria.

The unmodified hybrid (**6*8**:**9**) formed triplex at neutral pH only in the presence of 800 mM NaCl and upon cooling from 46 to 5°C, no detectable duplex–triplex transition was noticed (Fig. 6a), indicating an incomplete association of triplex even at 5°C in the cooling experiment. The heating/cooling rates were slow enough (0.5°C/min) to ensure an equilibrium attainment. A similar hysteresis was observed when unmodified triplex was constituted in 100 mM NaCl in the presence of either divalent cation (20 mM Mg^{2+}) or externally added polycation (1 mM spermine). In contrast to this, under identical conditions of pH and ionic strength (100 mM NaCl with 20 mM $MgCl_2$ or 800 mM NaCl alone, pH 7.2), triplex from sp-ODN exhibited nearly superimposable heating-cooling curves with well defined triplex-duplex transitions (Fig. 6b). This implies that appended spermine favours the association process in triplex formation by increasing the third strand affinity to duplex.

Since ionic strength has critical effects on association/dissociation rates, the hysteresis experiments on sp-ODN containing triplexes were carried out at varying salt concentrations. As expected, both heating and cooling T_ms shifted to higher values with increase in ionic strength and showed a linear dependence in the range 100–800 mM NaCl (Fig. 7). The magnitude of hysteresis (ΔT_m 1–3°C) although small, was enhanced at higher salt concentration suggesting a slightly retarded association of sp-ODN with complementary duplex at higher ionic strength.

Origin of stabilization in sp-ODN triplex

Our rationale for covalent conjugation of spermine to 5-Me-dC present in Hoogsteen strand of triplex to constitutively enhance the triplex stability is well supported by the UV T_m results. The higher stability of sp-ODN containing triplex is perhaps due to the extra binding energy resulting from interaction of duplex with the polyamine appended to the third strand. While triplexes from unmodified analogs (**6*8**:**9**) forms only in the presence of $MgCl_2$ (20 mM), sp-ODN (**1**–**5**) triplexes are seen even in the absence of $MgCl_2$. Addition of $MgCl_2$ to sp-ODN triplexes results in further stabilization with higher T_m for triplex to duplex transition as expected for the effect of increased ionic strength. In all cases, the underlying duplex T_m in the triplex was unaffected implicating a direct role of appended spermine in triplex stabilization. The stability of sp-ODN triplex (**3*8**:**9**) containing a single spermine chain in micromolar concentrations (~ 1 μM) was equivalent to that of the unmodified triplex (**6*8**:**9**), but in the presence of millimolar concentrations of externally added spermine. This

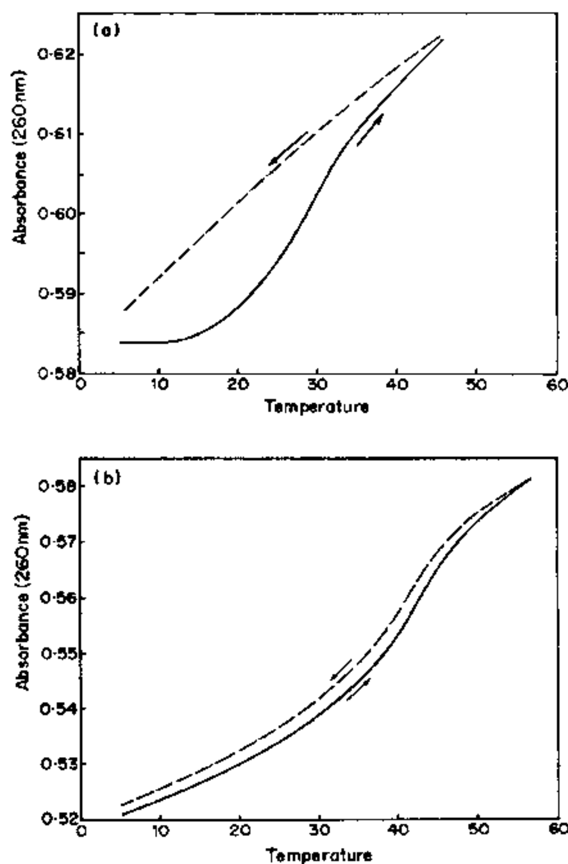

Figure 6. Hysteresis curve for triplex **6*8**:**9** (**a**) and **3*8**:**9** (**b**) at pH 7.3 in buffer containing 25 mM Tris and 800 mM NaCl, solid line shows heating curve while dashed line shows cooling curve at a heating or cooling rate of 0.5°C/min.

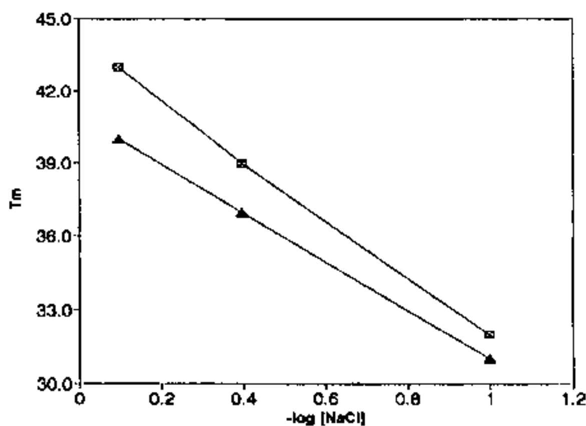

Figure 7. Heating (□) and cooling (▲) T_m of triplex **3*8**:**9** as a function of salt concentration.

amounts to a 1000-fold higher 'local' or 'effective' concentration effect offered by the conjugated spermine over free spermine (14). The mono sp-ODNs **1** and **2** are as effective in triplex formation at physiological pH as ODN containing three 5-Me-dC residues. Either increasing the number of spermine modification per ODN or decreasing the pH to acidic range destabilized the triplex. While the pH effect is opposite to that seen for unmodified or 5-Me-dC containing triplex, even at the level of three modifications, the sp-ODN triplexes had better stability at pH 7.0 compared with the unmodified triplex. In contrast to stabilization seen for triplexes, spermine conjugation significantly destabilized the duplexes where the dC-N^4-(spermine) unit is involved in Watson–Crick hydrogen bonding mode (15). This suggests different electronic/steric requirements for Hoogsteen base pairing in triplexes as compared to Watson–Crick base pair of duplex.

The factors leading to triplex stability from the appended polyamine may arise as a combined consequence of several effects: (i) altered pK_a of N3 of 5-Me-dC by N^4-(spermine) substitution; (ii) stabilization by additional hydrogen bonding interactions of spermine with complementary or adjacent base pair (27); (iii) intra/inter strand electrostatic phosphate neutralization imparted by polycationic appendage (8,14); (iv) entropic changes from counter-ion effects during melting as predicted from polyelectrolyte theory (28); and (v) favoured association/dissociation equilibria.

pK_a effects. From a study of triplexes containing 5-substituted dC, pK_a of N3 was postulated to be a major determinant of triplex stability, with a higher pK_a leading to more stable triplexes at physiological pH (11b). In this context, it is interesting to see from present results that, although pK_a of N3 in 5-Me-dC-N^4-(spermine) monomer is lower than that of dC by 0.7 U, its incorporation leads to stable triplex formation under conditions where corresponding unmodified ODN analogue fails to form triplex. On the basis of monomer pK_a, the optimal stability of sp-ODN triplexes should shift to lower pH (acidic) while the experimentally observed results indicate a shift to higher pH (neutral). The N3 pK_a factor is therefore highly unlikely to be the cause for improved stability of sp-ODN containing triplexes. A pK_a shift of 0.3–0.5 U to higher values upon incorporation of monomer into oligonucleotide is consistent with earlier observations (11d).

The pH dependent UV-difference spectral studies show that N3 protonation is not necessary for triplex formation by sp-ODN at physiological pH. N3 protonation which occurs at acidic pH (<5.3) as seen from UV-difference spectra actually destabilizes the sp-ODN triplex (Fig. 5d). This is a remarkable result which suggests that at neutral pH where sp-ODN triplex stability is maximal, in the triad **X*G**:**C**, where **X** is held by only one Hoogsteen hydrogen bond possible between N^4-H of **X** and O6 of **G**. Thus the loss in triplex stability arising from absence of the second Hoogsteen hydrogen bond due to non-protonation of N3 is more than compensated for by interaction of appended spermine chain with the duplex.

Interestingly, in the present study on sp-ODNs **1–5**, a decrease of triplex stability was noticed upon lowering the pH, in reverse trend to that seen with unmodified oligonucleotides (Fig. 4). This may arise perhaps as a consequence of pH induced change in conformation of appended spermine chain, which at low pH, may assume orientations unfavorable for specific interactions with the backbone or the base pairs. The orientation change may also occur from the imino tautomer of 5-Me-dC-N^4-(spermine) due to partial double bond character of exocyclic C4-N bond (7a). This is less likely to occur at physiological pH since the N3 pK_a for 5-Me-dC-N^4-(spermine) is very low (3.70) but may become reasonable at acidic pH (5.5). The fact that increasing the degree of spermine substitution also lowers triplex stability suggests electrostatic repulsion, either among the spermine chains or

between spermine and protonated C^+, as a major causant of low pH destabilization of sp-ODN triplexes.

Effect of mismatches. Thermal denaturation studies of triplexes containing a single Hoogsteen mismatched triad of 5-Me-dC-N^4-(spermine) with Watson–Crick doublets CG, TA and AT showed that the inverted doublet CG also formed triplex, although less effective than the original GC (Fig. 2; Table 2). Of the other pairs, AT and TA, only the former exhibited any detectable triplex transition. With both CG and AT doublets, a carbonyl group (O6 of G and O4 of T) is available in the first base of the triad as a hydrogen bond acceptor in major groove (29). It is possible that the N^4-spermine side chain from the third strand can span across the major groove to form additional hydrogen bonds from its imino/amino group with the O6/O4 carbonyls of G and T. Thus C and A are well tolerated in the central purine position of the triad, with protonation requirement of C in the third strand assuming less significance and hence favouring triplex even at neutral pH. This may have utility for employing 5-Me-dC-N^4-(spermine) as a Hoogsteen mismatch tolerator, without loss of stability. Spermine chain may also participate in hydrogen bonding with WC base pair of adjacent triads and this possibility should lead to considerable sequence dependence of triplex stabilization and may be understood better by a study of nearest neighbor effect in sp-ODN triplex.

Counter-ion effects. From a recent study of electrostatic effects in triplexes in intramolecular triplexes (30), it was observed that electrostatic interactions between protonated C (C^+) of third strand and phosphate backbone offer considerable stabilization in a global, sequence independent manner. However, repulsion between adjacent C^+ can cause successful negation of the above electrostatic advantage, thereby imparting local sequence dependent effects. Application of polyelectrolyte theory to DNA suggests that DNA melting is accompanied by release of a part of counter-ions from DNA bound state to bulk as the condensed charge density is reduced in the melting process. In zwitterionic DNA, such as that from sp-ODNs **1–5** described here, or 5-hexylamino-2′-dC incorporated DNA reported earlier (8b), the high charge density in triplex/duplex form is partially balanced by the covalently bound, non-diffusible cations, offering a unique stabilization effect.

Electrostatic neutrality. The intramolecular phosphate neutralization by polycationic spermine leading to a net diminished charge for sp-ODNs **1–5** is clearly apparent from mobility retardation seen for them in gel electrophoresis and better association of sp-ODN in triplex formation. Mono spermine 18mer ODN **1** migrates equal to an unsubstituted 24mer **9** and the migration is systematically retarded as a function of degree of spermine substitution. Such a correspondence illustrates the zwitterionic character of sp-ODNs **1–5** similar to those reported by Switzer *et al*. (8b). An analogous effect of interstrand phosphate charge neutralization is possible in triplex. Such charge neutralization effects by externally added cations are known to lead to electrophoretic retardation of high molecular weight (1–5 kb) linear/plasmid DNA due to counter-ion condensation effects (31). The third strand of DNA asymmetrically partitions the major groove (32) and the N^4-linked spermine chain may position itself across the major groove accessing the phosphate backbone of either of WC duplex strands.

Effect of spermine on dissociation/association equilibrium. The nature of the observed hysteresis (Fig. 6) and its salt dependence (Fig. 7) indicated that the appended spermine may assist third strand association with complementary duplex. Normally, hysteresis in triplex melting as seen with **6*8**:**9** arises from a slower association of third strand with duplex during cooling as compared to dissociation rates during heating (26). The insignificant hysteresis phenomena seen in sp-ODN triplex (100 mM NaCl) suggests that the association rate is comparable to that of dissociation rate, i.e., the association is enhanced relative to that seen in unmodified triplex. In presence of higher salt concentration (800 or 100 mM NaCl with 20 mM $MgCl_2$), the hystereis is marginally increased (ΔT_m ~3°C) due to a slower association. This perhaps arises from accumulated salt cations on duplex surface which electrostatically disfavors the approach of cationic spermine chain. Thus, stable triplex formation by sp-ODNs at physiological pH may be a combined consequence of intermolecular hydrogen bonding and electrostatic stabilization leading to a favored association event.

The overall experimental results presented here suggest that when spermine molecule is covalently attached to the third strand, it may still be accommodated in the deep major groove of the duplex DNA along with the third strand, yielding a stable triple helix. However, it is difficult at this juncture to delineate the extent of contributions by each of the above factors for enhanced stability of sp-ODN triplexes.

Conclusions

This paper reports studies on stability of triple helices employing spermine conjugated ODNs as third strand. Triplexes are observed in the absence of $MgCl_2$ at physiological pH, conditions under which ODNs containing dC **6** or 5-Me-dC **7** fail to show triplex hybridization. The simple and site-specific spermine conjugation permits introduction of polycations at desired, multiple internal sites in oligonucleotide sequences, thereby imparting significant zwitterionic character to DNA. N3 protonation is not a requirement for triplex formation by sp-ODNs since the corresponding loss in stability is more than compensated for by spermine's favorable interactions with duplex DNA. The increased stability of sp-ODN triplexes is also a consequence of favoured association of third strand with the duplex. The observed nature of mismatch tolerances may enlarge the scope of sp-ODN applications in triplexes. A lower net negative charge arising from multiple substitution, may assist the cellular uptake of ODNs. This conjecture is strengthened by the recent findings that coadministration of ODNs with cationic lipids significantly enhances the membrane permeability (33,34). The synthetic DNA–ligand complexes have also found utility in receptor mediated gene transfer (35) and the presence of spermine binding sites on the cell surface (36) may aid uptake of sp-ODNs. Since polyamines can also form metal complexes, the compounds such as those synthesized here, may in addition have potential utility in site-directed cleavage of DNA.

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