

between spermine and protonated  $C^{+}$ , as a major cause of low pK destabilization of sp-ODNs 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 1). Of the other pairs, AT and TA, only the former exhibited any detectable triplex formation. 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 [20]. 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 pronounced 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 tolerant, 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 neighbour effect in sp-ODN triplex.

**Cationic ion effects.** From a recent study of electrostatic effects in triplexes in intermolecular triplexes [46], 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 8-8 described here, or 2-thiacytosine-2'-dC incorporated DNA reported earlier [18], the high charge density in triplex/duplex form is partially balanced by the covalently bound, non-diffusible cations, offering a unique stabilization effect.

**Electrostatic neutralization.** The intermolecular phosphate neutralization by polycations spermine leading to a net diminished charge for sp-ODNs 8-8 is clearly apparent from melting retardation seen for them in gel electrophoresis and better association of sp-ODN in triplex formation. Also spermine (from ODN 8 migrates equal to an unsubstituted 24mer B and this migration is systematically retarded as a function of degree of spermine substitution. Such a correspondence illustrates the zwitterionic character of sp-ODNs 8-8 similar to those reported by Switzer et al. [16]. 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 (1-3 kb) linear/plasmid DNA due to counter-ion condensation effects [11]. The third strand of DNA asymmetrically partitions the major groove [37] and the  $N^4$ -linked spermine chain may position itself across the major groove accessing the phosphate backbone of both 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 d $N^4$ B $N^9$  arises from a slower association of third strand with duplex during cooling as compared to dissociation rate during heating [28]. 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 unsubstituted triplex. In presence of highest salt concentration (500 or 100 mM NaCl with 20 mM MgCl<sub>2</sub>), the hysteresis is marginally increased (LJ<sub>50</sub> -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 accumulated in the deep major groove of the duplex DNA along with the third strand, yielding a stable triplex 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 triplex helices employing spermine conjugated ODNs as third strand. Triplexes are observed in the absence of MgCl<sub>2</sub> at physiological pH, conditions under which ODNs containing dC $N^4$  or 5-Me-dC $N^4$  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. No 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 tolerance 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 neutralization of ODNs with cationic lipids significantly enhances the membrane permeability [33,34]. The synthetic DNA-lipid complexes have also found utility in receptor mediated gene transfer [37] and the presence of spermine binding sites on the cell surface [35] may aid uptake of sp-ODNs. Since polycations 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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