PNA C–C$^+$ *i*-motif: superior stability of PNA TC_8 tetraplexes compared to DNA TC_8 tetraplexes at low pH†

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Study of self-assembly of PNA TC_8 monitored by UV thermal transition at 295 nm indicates formation of a C–C$^+$ tetraplex (*i*-motif) in acidic pH, with higher stability than the analogous dTC_8.

Telomeric DNA has guanine (G) and cytosine (C) rich DNA sequence regimes. G-rich DNA oligomers are well known to form G_4-tetrads *via* WC and HG hydrogen bond mediated cyclic structures.[1] The complementary C-rich sequences form tetramers *via* the semiprotonated C–C$^+$ base pairs held by three hydrogen bonds to form parallel double strands.[2] Two such double strands interdigitating through C–C$^+$ base pairs lead to a four-stranded *i*-motif structure (Fig. 1(a)). The opposed dipoles of exocyclic C2-carbonyl and N4-amino groups favour interaction of consecutive base pairs by alternate stacking of the amino and carbonyl groups.[3]

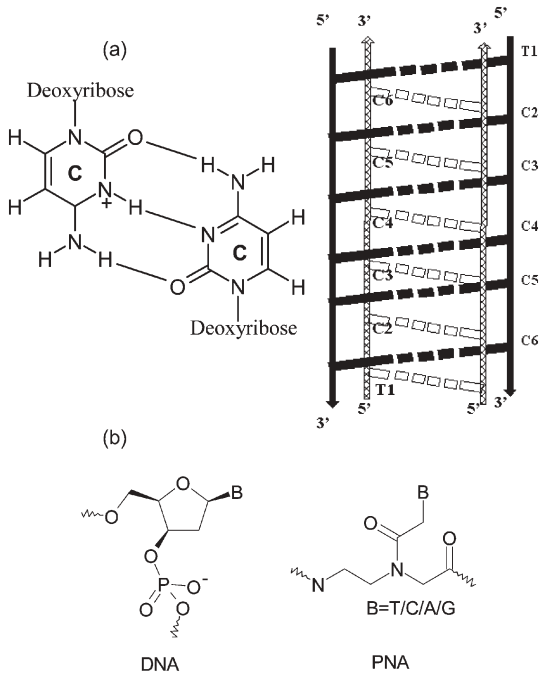

Fig. 1 (a) Schematic diagram of the *i*-motif in DNA. (b) Chemical structures of DNA and PNA.

NMR spectroscopy[3,4] has been extensively used to characterise the solution structure of the *i*-motif in oligonucleotides $d(TC_5)$, $d(T_2C_8T_2)$, dA_2C_4 and dC_4A_2. Rich and co-workers[5] have the solved the *i*-motif structure in several C-rich oligonucleotides by X-ray crystallography, while Raman spectroscopy[6] was used to characterise the *i*-motif in DNAs dTC_3 and dTC_8. In UV spectra, C and protonated C$^+$ show a large absorption difference at 295 nm.[7] Hence UV-thermal transitions monitored at 295 nm, show a reverse sigmoidal pattern, which is characteristic of C–C$^+$ tetraplex formation.[7] The thermodynamics and kinetics of *i*-motif formation in modified oligonucleotides has also been studied by UV at 295 nm.[8]

DNA and RNA have very versatile auto-association properties, the range of which extends from formation of duplexes to triplexes and tetraplexes.[9] RNA has been shown to lack the ability to form *i*-motif structures.[10] Considerable interest is now growing in the study of tetraplexing properties of mimics of natural oligonucleotides such as phosphorothioates,[8a] LNA[11] and PNA.[12] While G_4 tetraplex formation was successfully demonstrated recently in PNA,[13,14] it was reported that the PNA H–$C_4A_4C_4$–Lys–NH_2 did not form C–C$^+$ tetraplexes at pH 7.0.[14] Owing to favourable steric factors, it was shown that a PNA analogue gly-ala-PNA forms C–C$^+$ complexes in a C_4-tetramer, but not in a C_8-octamer.[15] Thus, *no* reports exist so far on successful C–C$^+$ tetraplexing properties of unmodified *aeg*-PNA. We herein present the first observation on C–C$^+$ tetraplexing properties of unmodified PNA sequences TC_4 and TC_8, analogous to the isosequential DNA, but with higher thermal stability in the acidic pH range.

To study the *i*-motif in PNAs, we synthesised PNAs TC_n corresponding to different lengths (Table 1). TC_2 (PNA **1**), TC_3 (PNA **2**), TC_4 (PNA **3**) and TC_8 (PNA **4**) were synthesized by standard procedures on solid phase method using Boc-chemistry (for details see ESI†). For comparative study, the DNA sequences $d(TC_8)$ and $d(TC_8)$ were synthesized on an *ABI*-DNA synthesizer. All sequences were purified by HPLC to homogeneity and characterized by mass spectrometry.

Table 1 Oligomers for the study of the *i-motif* of PNA

	Sequences of PNA/DNA
1	H_2N–T–C–C–βala–COOH
2	H_2N–T–C–C–C–βala–COOH
3	H_2N–T–C–C–C–C–βala–COOH
4	AcHN–Lys–T–C–C–C–C–C–C–C–C–$CONH_2$
5	d(TCCCC)
6	d(TCCCCCCCC)

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† Electronic supplementary information (ESI) available: Experimental procedures, HPLC and mass spectra of PNAs **1–4**, pH dependent UV spectra and UV–T_m measurements. See http://dx.doi.org/10.1039/b506870c

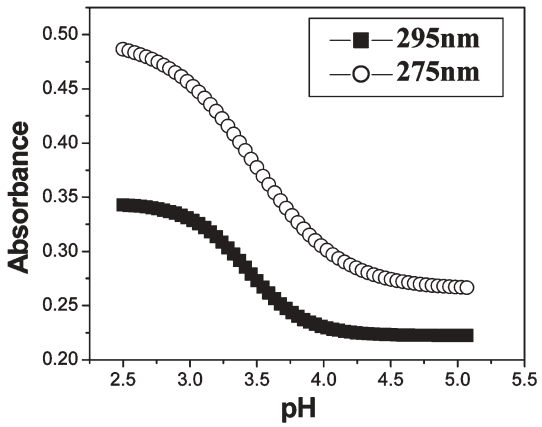

Fig. 2 UV absorbance at 275 and 295 nm of PNA **4** as a function of pH.

The UV spectra of PNA **4** were recorded at different pH values in the range 2.8–5.1 at 25 °C. The band at 275 nm found at acidic pH 2.8 slowly decreased in intensity and shifted to lower wavelength at 260 nm, with increase in pH (see ESI†). Earlier, it had been observed that the difference in absorbance spectra of protonated and non-protonated cytosine in DNA/RNA is maximum in the region 290–295 nm.[10] Fig. 2 shows a plot of UV absorbance at 275 and 295 nm in PNA **4** as a function of pH and the absorbance differences between protonated and non-protonated C in PNA are greater at 275 nm. From these data, the pK_a for N3 of C in PNA is obtained as 3.45, which is significantly lower than the pK_a of 4.8 reported for N3 of C in DNA/RNA.[10]

The formation of C–C$^+$ tetraplexes from PNAs **3**, **4** and d(TC)$_8$ **6** at pH 3.0, 4.5, 5.0, 6.5 and 7.0 were monitored at 295 nm, for a true comparison with the tetraplex formation in d(TC)$_n$ as per the reported procedures.[7,10] The temperature dependent UV-absorbance results obtained are shown in Fig. 3.

Table 2 pH Dependent T_m of TC$_8$ in PNA and DNA[a]

	T_m (°C) at varying pH					
pH	3.0	4.5	5.0	6.0	6.5	7.0
PNA **4**	67.4	55.0	46.0	nf	nf	nf
DNA **6**	58.4	58.7	55.7	50.4	52.0	nf

[a] nf indicates not formed.

The melting experiments were done in 100 mM sodium acetate buffer for the pH range 3.0–5.0 and 10 mM phosphate buffer for the pH range 6.0–7.0. The successful formation of tetraplexes in different sequences was indicated by observance of negative sigmoidal transitions (Fig. 3). Accurate T_m values were obtained from the first derivative curves and the T_m data for PNA **4** and DNA **6** are shown in Table 2. The PNAs **1** (TC$_2$) and **2** (TC$_3$) failed to show tetraplex formation at any of the pH conditions. PNAs **3** and **4** showed formation of strong C–C$^+$ tetraplexes at pH 3 and 4.5, respectively. Significantly, these PNA C–C$^+$ tetraplexes were much more stabilised (by 10–20 °C) compared to the analogous DNA C–C$^+$ tetraplexes. The stability of PNA C–C$^+$ tetraplexes were also dependent on pH. A comparison of pH dependent T_m of different PNA and DNA C-oligomers (Table 2) reveals that PNAs **3** and **4** form tetraplexes only in the acidic regime, up to pH 5.5. At pH 5.0, the PNA C-oligomers **3** and **4** form tetraplexes while at pH 6.0, no tetraplex formation is observed for these oligomers. This is seen from the reversal of melting curves for PNA oligomers at pH higher than 6 (Fig. 3). In comparison, the isosequential DNA C-oligomers **5** and **6** show tetraplex formation up to pH 6.5. Both PNA and DNA C-oligomers fail to form tetraplexes at pH 7.0. The pH effect on tetraplex stability is more drastic for PNA C-oligomers with $\partial T_m/\partial$pH being 10, while that for DNA is only about 3. The difference in the cut-off pH for tetraplex formation in PNA (5.5)

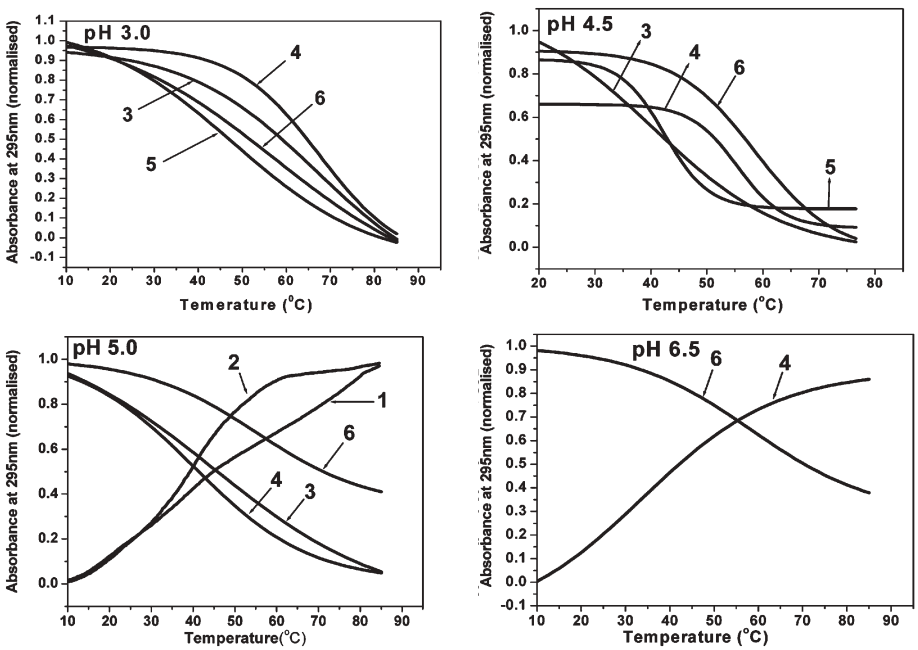

Fig. 3 UV–T_m of PNA and DNA at different pH values.

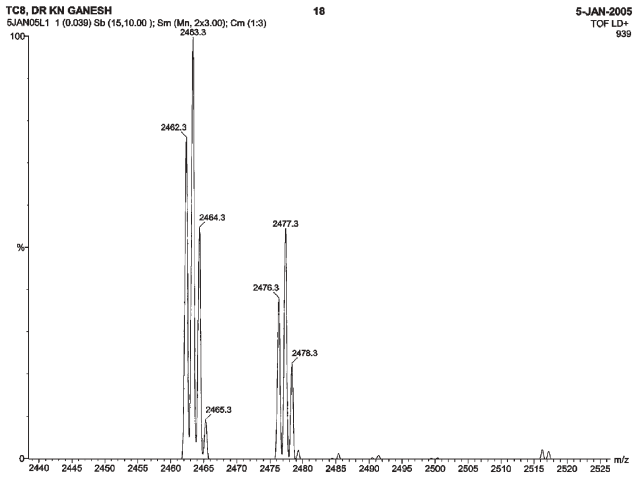

Fig. 4 MALDI-TOF mass spectrum of PNA **4**.

and DNA (6.5) is perhaps a reflection of the lower pK_a of N3-C in PNA (Fig. 2) compared to that in d(TC)$_n$.[10]

The identity of PNA C-oligomers, is supported by mass spectral data (Fig. 4). The MALDI-TOF mass spectra of PNA **4** TC_8 oligomer exhibited two sets of peaks separated by 14 mass units. While the cluster at *m*/*z* 2463 corresponds to the calculated ($C_{99}H_{137}N_{47}O_{30}$) mass of M^+, the cluster at *m*/*z* 2477 corresponds to $(4M + 2H^+ + 2Na^+)^{4+}$.

In summary, this communication demonstrates the hitherto unknown formation of the C–C$^+$ tetraplex in unmodified C-oligomeric PNAs. It is shown that in the acidic pH 3.0–5.0 range, PNA C–C$^+$ tetraplexes possess significantly higher stability compared to analogous DNA C–C$^+$ tetraplexes. Recently, it was reported[14] that the PNA $C_4A_4C_4$–Lys–NH_2 did not show formation of C–C$^+$ tetraplexes at pH 7.0. Up to now, no modified DNAs or their analogues have been known to form a more stable *i*-motif than natural DNA.[2,7] In light of this and the current interest in modified peptide nucleic acid analogues,[16] the first observation and characterization of C–C$^+$ tetraplexes reported here, holds promise to further examine the role of the PNA backbone structure in tetraplex formation.‡ The effect of modified backbones and sequences on influencing the self-assembling properties of nucleic acids has current importance in the development of practical applications for therapeutics and diagnostics.[11,12]

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Notes and references

‡ Though the present results do not give a direct evidence for interdigitation of base pairs, the UV characteristics similar to that of DNA C–C$^+$ tetraplexes, suggest that PNA TC_8 may have a similar arrangement. A similar structure is proposed for gly-ala PNA[15]

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