CHAPTER 5A

PNA TETRAPLEXES: BIOPHYSICAL STUDIES OF G-TETRAD OF aep-PNA
## CHAPTER 5A: PNA TETRAPLEXES: BIOPHYSICAL STUDIES OF G-TETRAD OF aep-PNA

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5A.1 INTRODUCTION

Nucleic acids are versatile in forming self-assembled structures. One of the most important class of these are the quadruplexes, specifically derived from self association of G-bases observed in DNA/RNA telomeres (Figure 1a). Telomere is a repeating structure in DNA sequences associated with proteins at the termini of eukaryotic chromosomes (Figure 1b) and has an important role in replication of the end regions of the chromosomes within the cell nucleus. The basic structure of telomeres is formed by repeated occurrence of G nucleotides of DNA. The guanine-rich sequences of DNA are known to have an ability to form G-tetrads leading to four-stranded secondary structure. The inventory of sequences forming telomeric structures correspond to d(TTTTGGGG), d(TTGGGG) and d(TTAGGG) present in sequences from *Oxytricha*, *Tetrahymena*, and *humans* respectively. The regular short repeat sequences leading to G-quadruplexes of DNA fall into the general motif d(T\textsubscript{3}-(T/A)-G\textsubscript{3}).

![Figure 1: (a) Chemical structure of DNA and RNA. (b) Typical structure of cell nucleus.](image-url)
The secondary structural inventory of DNA related to or derived from Watson-Crick base-pairing include structures such as hairpins and cruciforms. Higher order DNA structures such as triplexes and quadruplexes are stabilized by cyclic Hoogsteen (HG) hydrogen bonding arising from association of four guanines (Figure 2). It is reported that the G-rich sequences in DNA may adopt non-B folded, quadruplex structures shown in Figure 2 in the presence of monovalent ions (K\(^+\), Na\(^+\) etc) at physiological conditions. The crystal structure of DNA sequence 5'-TGGGGT-3', corresponding to telomere of *Tetrahymena*, was shown to form parallel stranded G-tetrads in presence of Na\(^+\), Ca\(^+\), K\(^+\) or Tl\(^+\) in crystals and in solution.

![Figure 2: above: Hydrogen pattern in G-tetrad and a monovalent cation (Na\(^+\)/K\(^+\)) occupies the central position. below: (A) Diagonal loops; (B) Two parallel edgewise loops. (C) Two antiparallel edgewise loops; (D) Adjacent parallel strands with edgewise loops; (E) Alternating antiparallel strands with edgewise loops.](image-url)
Like DNA, the elements of G-tetrad formation are also evident in RNA. For example, RNA G-tetrad structure is observed in filamentous *bacteriophage fd*\(^{13}\). Recently, the fragile X mental retardation protein (FMRP) has been shown to bind RNA structures formed by G-tetrads.\(^{14}\) The RNA tetrads of r(UGGGU)\(_4\) also form parallel stranded structures like DNA in presence of Sr\(^{2+}\).\(^{15}\) The G-repetitive units in long sequences of DNA give different types of folded tetraplex structures. Folding topology of G-tetrads in solution critically depends on the number of G-rich repetitive units, the nature of sequence and the concentration of metal ions. The well-established folding topology in DNA G-quadruplexes are given in Figure 2 as (A) diagonal loops protruding on either side of the guanine tetrad core, (B) two parallel edgewise loops protruding on the same side, (C) two antiparallel edgewise loops protruding on the same side, (D) adjacent parallel strands with edgewise loops protruding on opposite sides and (E) alternating antiparallel strands with edgewise loops protruding on opposite sides.\(^{15}\)

5A.1.1 Characterization of G-tetrad formation in DNA

The G-tetrad structures of DNA are very much important in biology. A number of techniques and established methodologies have been applied to characterize their structures. These include chemical probing, NMR, crystallography, circular dichroism, Raman spectroscopy, gel electrophoresis, ultraviolet absorption and ESI-mass spectroscopy and are widely used to characterize the G-tetrad formed structures in nucleic acids.

5A.1.1a Chemical and Enzymatic Probes

Chemical and enzymatic probes are useful tools for the study of telomeric DNAs. The guanine base in a G-quartet is nearly saturated with hydrogen bonds, and therefore the G-quartet structure is particularly sensitive to these types of perturbations. In the absence
of detailed NMR or crystallographic data, chemical probing methods offer the best diagnostic evidence for G-quartet formation.

(1) **Nuclease Sensitivity.** The G-quartet structures are resistant to hydrolysis by both endo- and exo-nucleases.\(^{16}\)

(2) **UV Crosslinking.** The ultraviolet light (UV)-induced covalent crosslinking of thymines is characteristic of the folded G-quartet form. Thymidine residues from different repeats brought into proximity by folded structures may be detected via photo crosslinking to form T-T dimers by 2+2 cycloaddition.\(^{17}\)

(3) **Chemical Protection.** The most characteristic signature of G-quartet formation is the strong steric protection conferred by the structure to methylation of the guanine N7 by dimethyl sulfate (DMS). This is caused by their involvement in hydrogen bonds. Thus DMS can used to distinguish telomeric and non-telomeric G-rich DNA sequences. N7 of guanines in G-quartet are also resistant to chemical modification by diethylpyrocarbonate (DEPC).\(^{18}\)

**5A.1.1b Nuclear Magnetic Resonance**

Nuclear magnetic resonance (NMR) is an invaluable tool for the study of nucleic acid structures and enables elucidation of folded conformations. Initial NMR studies of telomeric DNA sequences did not provide detailed structural information except indicating the presence of unusual structures. The NMR study of d(TTGGGG)\(_4\) revealed the presence of nonstandard G-G base pairs and the existence of guanines in the unusual syn conformation.\(^{19}\) The first detailed NMR study of a G-quartet structure was done on the oligomer d(GGTTTTTGG), which was shown to form a tetramolecular complex by calorimetric analysis.\(^{20}\) The significant finding of these studies was that the glycosidic torsion angle alternated between syn and anti for each adjacent pair of Gs along the strand (Figure 3). Such alternation of glycosidic torsion angles was also observed in the similar
sequence d(GGTTTTCGG). The second significant result from NMR studies of d(GGTTTTTGG) was the existence of multiple conformers that were in slow exchange on the NMR timescale. Qualitative NMR studies also revealed that the nature of the tetraplexes formed by telomeric DNAs differed according to the presence of Na⁺ or K⁺ as the counterion and that the stability of the structures depended on the monovalent cation present. Both d(TTGGGG)₄ and d(TTAGGG)₄ were studied in Na⁺-phosphate or K⁺-phosphate buffers and showed a complexity of the imino proton spectrum indicating presence of multiple species at the high substrate concentrations required for NMR studies. The imino proton spectrum was markedly different for both oligomers in Na⁺ and K⁺ buffers. Temperature dependent imino proton spectra also revealed that the K⁺ quadruplex were more stable than the Na⁺ tetraplexes with imino protons still observable for the K⁺ quadruplex of d(TTGGGG)₄ even at 90 °C.

![Figure 3: Syn and anti conformation of nucleoside](image)

**5A.1.1c X-Ray Crystallography**

X-ray crystallographic analysis of d(GGGGTTTGGGG) supported the general features postulated for the G-quartet core structure. The glycosidic torsion angles alternate syn-anti-syn-anti along the strand and around in any given G-quartet. The thymidine loops are located on opposite ends of the G-quartet core in head-to-tail fashion,
unlike the structure suggested by NMR. The crystal structure exposes the detail structural features in G-quartets, which are difficult to observe with NMR. It was seen that the base geometry is often distorted from the ideal square planar arrangement of guanines that are somewhat buckled out of the plane of the G-quartet. The crystal structure of DNA sequence 5'-TGGGT-3', corresponding to telomere of Tetrahymena was shown to form parallel stranded G-tetrads in presence of Na⁺, Ca⁺, K⁺ or Tl⁺ in crystals and in solution (Figure 4). The central channel of this entities have also shown the considerable charge density resulting from bound potassium ion located between the second and third G-quartets at the center of the core structure.

![Figure 4: Crystal structure of d(TG₄T) in presence of monovalent ion.](image)

5A.1.2 Polymorphism in DNA G-quartet

The contrast between the structures derived from X-ray crystallography and NMR spectroscopy for the same oligomeric sequence adequately illustrated polymorphic nature of telomeric DNAs. The numerous structures possible for telomeric DNAs make it difficult to determine a priori, as to which of these structures are formed under a given set of conditions. The interesting aspect of telomeric DNA structures is that the same bases can be assembled in many different ways with similar basic hydrogen-bonding structures.
and this flexibility lead to polymorphism in telomeric DNA. The following classes of polymorphism are observed in DNA (Figure 5a-d).

![Figure 5: Geometry arrangement of polymorphism; (a) Parallel vs Antiparallel Strands; (b) Strand Stoichiometry; (c) Glycosidic Conformation; (d) Loop Geometry; (e) Ion-Binding.](image)

(1) **Parallel vs Antiparallel Strands:** One fundamental source of structural variation is the possibility of forming different G-quartets in which the strands have different polarities. Four strands can come together in a tetrameric complex in at least three different ways as shown in Figure 5a. There are two permutations: all four strands can be parallel or two parallel-two antiparallel strands. In principle, three parallel strands and one anti-parallel strand could form a tetraplex, but this type of structure has not yet been observed. G-
quartet structures can be formed with the relative arrangement of adjacent backbones that are all parallel, alternate with antiparallel, or adjacent antiparallel combination.\(^\text{27}\)

(2) **Strand Stoichiometry:** The same oligomer can form different types of structures by association of one or more molecules. For example, a telomeric DNA sequence containing four repeats can form an intramolecular quadruplex, a dimer, or a telomeric quadruplex, as shown in Figure 5b.\(^\text{27}\) This type of polymorphism depends on the concentration of the DNA. Representative members for each of these three classes have been characterized structurally and are illustrated in Figure 5b. Thus, the association of one, two or four strands can form G-quartet structures.

(3) **Glycosidic Conformation:** Guanines in G-quartets are observed in both syn and anti conformation. In principle, a string of four G residues can adopt different combinations of glycosidic conformations. The only observed patterns thus far are all-anti and alternating syn-anti. Guanines involved in the same quartet that are on parallel strands have the same glycosidic torsion, while guanines on antiparallel strands have opposite glycosidic torsions, as illustrated in Figure 5c.\(^\text{28}\) The glycosidic conformation changes the relative orientations of the bases on adjacent G-quartets, and thus can affect the stacking energy between G-quartets. Thus adjacent Gs in the same G-quartet can have the same or the opposite glycosidic torsion angle depending on whether their constituent strands are parallel or antiparallel.

(4) **Loop Geometry:** Depending on whether the G-quartet formation is unimolecular or bimolecular, the G-strings can be connected by a variety of combinations of loop crossings. For example, in dimeric species, loops can join adjacent or diagonal strands, with the two loops oriented in a head-to-tail or head-to-head fashion. Figure 5d\(^\text{29}\) illustrates these types of polymorphism.
(5) **Ion-Binding Geometry:** The metal ions may interact with the G-quartet structures in different ways and with different stoichiometries. The ion binding geometry is known from its crystal structure with certainty only for d(GGGGTTTTGGGG) in the potassium (K⁺) form. The electron density for the positive ion is found in between the second and third G-quartet levels. Other ion stoichiometries are possible, with ions binding to every G-quartet, or to every other G-quartet, as shown in Figure 5e.^[30]

### 5A.1.3 Nondenaturing electrophoresis

*Nondenaturing polyacrylamide gel* is used for the separation and purification of fragments of double-stranded DNA while *denaturing polyacrylamide gel* is used for the separation and purification of single stranded fragments of DNA.[^31] Nondenaturing (native) gel electrophoresis is a simple but powerful method for the analysis of telomeric DNA structures. Electrophoretic mobility is dependent on the size, shape, and charge of the molecule as it passes through the gel matrix. In denaturing gels, DNA fragments exist primarily as single strands that migrate according to their molecular weights. In nondenaturing gels, DNA fragments that adopt particular structures migrate differently from single stranded DNAs of the same length. This technique is therefore useful for distinguishing different topological structures adopted by oligonucleotides such as multiple or oligomeric structures and ion dependent structures and these are also temperature dependent.

**Assay for Structure formation:** Unusual gel mobilities were obtained for telomeric DNA structure. Multimerization of telomeric DNA sequence occurs by interaction of the terminal fragments, and the stability of the oligomers formed depends strongly on whether Na⁺ or K⁺ is present. Different telomeric oligonucleotides assume compact high mobility forms on native gels[^22] at low temperatures (5 °C). In contrast, oligonucleotides from telomeric C-strands, or oligonucleotides without G-strings, did not form such high
mobility species. This simple assay is routinely used for analysing structures in telomeric DNA sequences.

Assay for multiple or oligomeric structures: Native gels are particularly useful for detection of multiple species in an oligonucleotide, due to alternate conformers or higher-order structures. Telomeric DNAs are polymorphic in that they form both multiple conformers and oligomeric species. Alternate conformers typically have similar, yet distinct, mobilities, while dimers and other structures exhibit quite different mobilities. G-quartet structures, frequently detected as thermodynamic mixtures of forms by native gel electrophoresis can be formed by dimerization or tetramerization of oligonucleotides. Also, higher-order structures formed by the end-to-end association of quadruplexes has been studied by using native gels.

Strand stoichiometry: A simple and elegant method for determining the stoichiometry of a complex involves mixing oligonucleotides that have tails of different lengths that do not perturb the ability to form G-quartet structures. If equimolar amounts of an oligonucleotide “A” are mixed with oligonucleotide “B”, then the stoichiometry of a given complex can be determined from the number of mixed species (dimers “AA”, “AB” and “BB” and tetramers “AAAA”, “AAAB”, “AABB”, “ABBb”, and “BBBB”) that are produced. This simple technique has been used to demonstrate dimer formation by telomeric oligonucleotides and tetramer formation in nontelomeric and telomeric oligonucleotides.

Ion-dependent structure formation: Telomeric DNAs exhibit a strong preference for binding certain cations. Consequently, the counterion used in a native gel experiment can change the stability of structured forms. Dimerization of macronuclear DNA from Oxytricha is preferentially stabilized by $K^+$ in native gels. Oxytricha telomeric oligonucleotides do not form a structure in the absence of added counterions or in the
presence of Li⁺ but readily form a structure in the presence of Na⁺ or K⁺ added to the gel running buffer. K⁺ stabilized the quadruplex formed by r(UGGGGU) better than Na⁺, and Sr₂⁺ also stabilizes quadruplex formation.³⁶

**Temperature dependent structure formation:** Because the dissociation of G-quartet structures is often slow, native gels can be used to monitor the temperature dependence of structure formation. *Oxytricha* telomeric DNA dimers are stable in K⁺ up to 70 °C. Gels run at different temperatures have been used to compare the relative stabilities of a set of related sequences or simply to monitor structure formation.²⁷

### 5A.1.4 Thermal denaturation by UV and CD spectroscopy

#### 5A.1.4a UV-Spectra

The UV spectra of telomeric DNAs typically exhibit two overlapping peaks in the 260 to 280 nm range. Characteristic absorption changes are observed upon folding of telomeric DNAs. The absorbance at 275 nm typically decreases by ~10%, and the absorbance at 295 nm increases by ~100%.³⁸ Consequently, these wavelengths can be used to monitor folding or unfolding processes.

Several studies have used thermal denaturation monitored by changes in UV or CD to obtain thermodynamic information on telomeric DNAs. In general, telomeric sequences are very stable, and their stability depends strongly on the monovalent cation present and the nature of sequence.³⁹ Telomeric DNA sequences also undergo slow folding and unfolding kinetics and this presents experimental difficulties in optimizing conditions and comparing results. The parallel quadruplexes formed by d(TTTTGGG) in solution are extremely stable, with a ΔG° of -47 kcal/mol at 25 °C. In contrast, the quadruplex formed by d(TGGGT) exhibits a ΔG° of - 7 kcal/mol.⁴⁰ The main difference between these two
sequences is the presence of a 3' terminal T residue that can greatly affect the stability of the quadruplex structure. The difference in stability of a parallel and antiparallel hairpin dimer structure adopted by same sequences has been determined using thermal denaturation. The antiparallel structure formed by d(GGGGTGGGGG) is -5 kcal/mol less stable than the parallel structure adopted by d(3'-GGGGTT-5'-5'-TTGGGG-3'). This difference represents the net change between forcing glycosidic torsion angles to the syn conformation, the differential stacking and ionic interaction energies in the two structures.

5A.1.4c CD-Spectra

The circular dichroism (CD) spectra of telomeric DNAs are very much dependent on the conformation and sensitive to base stacking geometry. Two basic forms of CD spectra are typically observed for telomeric DNAs: type I with a positive CD band at 265 nm and a negative band at 240 nm and type II showing a positive band at 295 nm and a negative band at 260 nm. The two types of CD spectra are strongly correlated to the conformation of the G-quartet core. The parallel quadruplex structure formed by d(3'-GGGGTT-5') where all guanines are in the anti conformation exhibits a type I CD spectrum. The antiparallel quadruplex formed by d(GGGGTGGGGG) where the guanines have alternate syn-anti conformation exhibits a type II CD spectrum. Presence of multiple conformations and mixture of parallel and antiparallel quadruplexes often make it difficult to assign structures by CD data alone.

5A.1.5 Application of quadruplexes

Quadruplex structural motif is adopted by the chromosome telomeres, immunoglobulin switch region and regulatory region of oncogenes. Thus the G-quadruplex is seen as a promising target for anticancer drug design with ever-increasing
discoveries of G-quadruplex structure binding proteins, such as human DNA topoisomerase I, BLM (Bloom's syndrome protein), WRN (Werner's syndrome protein), in the Recq family of helicases, SV40 large tumor antigen helicase and so forth. The novel supramolecular architecture of G-quartets has also led to the development of interesting and functional non-covalent assemblies such as G-wire, ion-channels and self-assembled ionophores. In recent years, considerable efforts have been directed towards the synthesis and investigation of new DNA analogs with improved binding properties with nucleic acids other than the natural canonical counterparts. Search for more stable quadruplexes from modified analogs compared to natural DNA may also help in understanding the complex mechanism of quadruplex formation.

5A.2 RATIONALE AND OBJECTIVE OF PRESENT WORK

The quadruplex formation by many modified oligonucleotides, for examples LNA (Locked Nucleic acid) and PNA have been well studied. Since, PNA was developed to mimic Watson-Crick and Hoogsteen base pairing, they also ideally participate in G-quartet formation in mainly two-ways: (1) G-rich PNA forming hybrid quadruplexes alone or in presence of DNA templates and (2) Self-assembly of G-rich sequences of PNA-DNA chimeras. Armitage, et. al have discussed the formation of PNA-DNA hybrid quadruplexes by strand invasion and overhang effect. The quadruplex formation by PNA-DNA chimeras – ^5'TGGG^3'-t, ^5'TGG^3'-gt, t-^5'GGGT^3' and tg-^5'GGT3' where lower and upper case letters indicate PNA and DNA residues respectively, have been reported. Recently, TG homo oligomer PNA was also shown to form quadruplex at pH 7.4 in presence of cations by electrospray ionization mass spectrometry (ESI-MS) and confirmed by ^1'H NMR and thermal stability measurements by UV absorbance change at 305 nm with temperature experiment.
During previous studies of PNA properties by chemical modification in this laboratory, the aminoethyl prolyl (aep) PNA emerged as one of the useful analogues (Figure 6). This PNA analog is positively charged and has constrained chiral backbone as a ring, instead of the linear achiral backbone of aeg-PNA (Figure 6). This part of the Chapter describes the tetraplexing properties in G-rich sequences of aeg-aep mixed backbone and aep-PNA oligomers to understand the effect of chirality and conformational rigidity on the tetraplexing stability of PNA.

The specific objectives of this chapter are

(i) Synthesis of T and G monomers of L-cis-(2S,4S)-aep-PNA and aeg-PNA.

(ii) Synthesis of G-rich sequences of aep- and aeg-PNA oligomers

(iii) Comparative study of G-tetrad forming properties in aep-PNA, aeg-PNA and DNA (figure 6).

5A.3 PRESENT WORK

5A.3.1 Synthesis of aep-PNA-(T/G) monomers

1-(N-Boc-aminoethyl)-4S-(N3-benzoylthymine-1-yl)-2S-proline methyl ester was synthesized from 4R-hydroxy compound 6 by N1-alkylation of N3-Benzoyl protected thymine at C-4 of prolyl ring under Mitsunobu reaction conditions as described before.
(Scheme 1). The reaction was accompanied by inversion at C4 lead to the 4S isomer. The ester compound 8 upon treatment with NaOH in aqueous methanol for 24h got hydrolysed with the cleavage of the N3-benzoyl group. Neutralization of the excess alkali with Dowex 50 H+ and work-up gave 1-N-(Boc-aminoethyl)-4S-(N3-benzoylthymine-1-yl)-2S-proline carboxylic acid 9 in quantitative yield.

1-N-(Boc-aminoethyl)-4S-(2-amino-6-chloropurine-9-yl)-2S-proline methyl ester 11 was prepared by N9-alkylation of 2-amino-6-chloropurine with 1-N-(Boc-aminoethyl)-4R-(O-mesityl)-2S-proline methyl ester compound 10 in presence of base (K₂CO₃) and catalytic amount 18-crown-6 in DMF by as reported procedure.⁵⁷

The aminoethyprolyl (aep) guanine monomer 12 was obtained by simultaneous hydrolysis and oxidation of 1-N-(Boc-aminoethyl)-4S-(2-amino-6-chloropurine-9-yl)-2S-proline methyl ester 11 using NaOH in aqueous methanol. The initial ester hydrolysis was

**Scheme 1**: Synthesis of aep-PNA-(T/G) monomers

[Diagram of the synthesis process]

**Reagents**: (i) N3-Bz-Thymine, DIAD, PPh₃, dry THF, 55%; (ii) 1N NaOH, CH₂OH·H₂O (1:1) overnight, 90%; (iii) MeSO₂Cl, dry Et₃N, dry DCM, 0 °C, 3 hr, 80%; (iv) 2-Amino-6-chloropurine, K₂CO₃, 18-crown-6, DMF, 70°C, overnight, 65%. 
completed within 10 minutes, followed by complete conversion of the 6-chloro to the 6-oxo-function after 75h (Scheme 1).

The \(N\)-(Boc-aminoethyl)-(thymin-1-yl)-glycine (T) and \(N\)-(Boc-aminoethyl)-(guanin-9-yl)-glycine (G) monomer (Figure 7) were synthesized from reported procedures (see chapter 2).

\[ \text{Figure 7: Chemical structure of aeg-PNA} \]

5A.3.2 Synthesis of aep-PNA and aeg-PNA oligomers

The syntheisis of following PNA oligomers was carried out by solid phase synthesis method following Boc-Chemisty of peptide synthesis.

1. \(H_2N-T-G-G-G-T-\beta\text{-ala-MF}\)
2. \(H_2N-T-G-G-G-\beta\text{-ala-MF}\)
3. \(H_2N-T-G-g-G-T-\beta\text{-ala-MF}\)
4. \(H_2N-T-G-G-G-t-\beta\text{-ala-MF}\)
5. \(H_2N-t-g-g-t-\beta\text{-ala-MF}\)
6. \(H_2N-t-g-g-g-\beta\text{-ala-MF}\)
7. \(H_2N-t-g-g-g-\beta\text{-ala-MF}\)
8. \(H_2N-t-g-g-\beta\text{-ala-MF}\)
9. \(H_2N-t-g-\beta\text{-ala-MF}\)

\(G, T = \text{aeg-PNA}; g, t = \text{L-cis-(2S,4S)-aep-PNA} \) and MF=Merrifield resin.

The aep-PNA oligomers were cleaved from the solid support using TFMSA to yeild oligomers with ‘C’ terminal carboxylic acids. The cleaved oligomers were initially desalted by size exclusion chromatography over G25 sephadex and subsequently purified by FPLC on a reverse phase C column. The purity of the oligomers was re-checked by reverse phase analytical HPLC on C18 column and confirmed by MALDI-TOF and ESI
mass spectrometry. Some representative HPLC profiles and mass spectra are shown in appendix.

5A.3.3 Characterization of PNA

The pure oligomers PNA 1-9 were characterized by MALDI-TOF and ESI mass spectroscopy and their spectral data is given Table 1.

5A.4 G-Quartet formation by aep-G / aeg-G-PNA and DNA: Comparative Study

The efficacy of the aep-G quartet formation under physiological conditions by aeg-G-PNA and DNA was studied using UV and CD spectroscopies, isothermal titration calorimetry and mass spectroscopy techniques.

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<th>Observed Mass</th>
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<td>9 H₂N-g-t-β-ala</td>
<td></td>
<td></td>
<td>643</td>
</tr>
<tr>
<td>10 d(3'-T-G-G-G-G-T-5')</td>
<td>Synthesized on ABI DNA synthesizer</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a G, T = aep-PNA and g, t = L-cis-(2S,4S)-aep-PNA.
5A.4 RESULTS

5A.4.1 UV-Tm Studies

The UV-spectrum of aeg- and aep-PNAs recorded in 10mmol potassium phosphate buffer and 100mmol of KCl is shown in Figure 8. Two broad peaks in the range at 253-273 nm were seen in spectra of both aeg- and aep-PNA. This suggests the formation of G-tetrad in aeg-PNA well as in aep-PNA. The UV spectrum of control DNA TG4T (10) in same buffer condition exhibited broad overlapping of two peaks in this range.5

The stabilities of G-tetrad complexes of aeg-, aep-PNA and DNA were studied by temperature dependent UV absorbance changes. The UV-thermal denaturation experiments were performed by following absorbance at 295 nm where the characteristic change occur due to disruption in tetrad structure.58 It is reported that the UV hypochromicity at 285-300 nm decreases with increase in temperature for DNA G-quadruplexes. The negative sigmoidal transition at 295 nm resulting from melting of quadruplexes suggests a co-operative effect. Tetraplex stability is dependent on the nature and concentration of metal ion (Na+/K+) and pH 7.0-7.4, and hence thermal stability of G-tetrads of aep-, aeg-PNA and DNA under different physiological conditions were studied at 295 nm. The results are discussed in following paragraphs.

Figure 8: A. UV-spectra of aeg-PNA 1-2, aeg-aep-PNA 3-4; B. aep-PNA (5-8) and DNA 10 at 10 °C
5A.4.1a Salt concentration dependent stability of G-tetrad s in PNA

Figure 9 shows the UV-melting profiles of PNAs 1 and 3-5 at 295 nm in sodium phosphate buffer (10 mM) and NaCl (100 mM) at pH 7.4. These profiles show inverse-sigmoidal curves at 295 nm, characteristic of UV melting of G-quartets. The tetrplex melting temperature of aeg- and aep-PNAs extracted from first derivative curves (see appendix) of the melting profiles are given in Table 2. Figure 9A, shows the melting of PNAs 1-5 in 10 mM sodium phosphate and 100 mM NaCl ([Na^+] ). The Tm values indicate that single aep-PNA modification (g) at C-terminus or in the middle enhance Tm by 1 to 1.5 °C, while all modified aep-(g) PNA 5 only slightly destabilized (∆Tm = -0.8 °C) the tetrplexes compared to control. Interestingly, the aep modification led to enhancements in hypochromicity, with maximum effect (20%) seen with all-modified aep-PNA-5.

To see the effect of different metal ions in tetrplexing nature of PNA, UV-melting experiments with PNA 1, PNA 3, PNA 6 and DNA 10 were performed in potassium phosphate (10 mM) buffer devoid of any Na^+ ion at same pH (7.4). The melting profiles of these PNA are shown in Figure 9B and their Tm values are given in Table 2. The Tm values indicate that K^+ induces significant stabilization of aeg-aep-PNA tetrplex (ΔTm = +5.8 °C) compared to Na^+, while show that either no effect of salt or slight stabilized.

The Tm values (Figure 9B) suggest that 10 mM K^+ in phosphate buffer significantly stabilizes the control aeg-PNA (Tm = +5.8 °C) while destabilizing the aep modified PNAs and DNA. However increasing [K^+] at higher buffer concentration (100 mM) enhanced the Tm of both aeg and aep-PNAs by 13 °C and 9 °C respectively. Addition of 100 mM KCl further enhanced the Tm by 1-2 °C. Thus G-quartet formation in aeg-PNA 1, aep-PNA 5 and PNA 3 (chimerae of aeg- and aep-PNA) is more preferable in presence of K^+ than Na^+. The overall hypochromicity at 295 nm also generally increased
with all PNA s (8-20%). The overall results of salt effects indicate that K\(^+\) stabilized PNA tetraplexes much more than Na\(^+\).

**5A.4.1b pH-Dependent studies**

Since aep-PNAs are protonatable at ring nitrogen at physiological pH, tetraplexing properties in aep-PNAs were examined at different pHs. The melting profiles of PNAs in 10mM potassium phosphate buffer having 100 mM KCl at pH's 5.8, 6.4 and 7.8 were determined (Figure 10) and results are shown in Table 3. At higher pH of 7.8, the melting profiles of PNA 2, PNA 5 and PNA 6 (Figure 10A) monitored at same wavelength are similar to the characteristic melting profile of tetraplexes of DNA. The Tm values of all modified aep-G PNAs 5 and 6 were slightly destabilized compared to unmodified aeg-PNA by ΔTm of 2-4 °C.

At slightly lower of pHs 7.0, (Figure 10B and Table 3) and pH 6.4 (Figure 10C and Table 3) the stability of quadruplexes marginally increased to that at pH 7.8 and for the different PNAs, the stabilities were similar. All the different PNAs were more stable than the corresponding DNA sequence 10 by 6-7 °C.
Table 2: UV-melting temperature (Tm) of PNAs at different concentration of metal ion*

<table>
<thead>
<tr>
<th>Figure of UV-Melting curve</th>
<th>PNA/DNA</th>
<th>List of PNA and DNA</th>
<th>Buffer Condition</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>at λ_{max} = 295nm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9A</td>
<td>1</td>
<td>H₂N-T-G-G-G-G-T-β-ala</td>
<td>10mmol. Sodium</td>
<td>46.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>H₂N-T-G-G-G-G-T-β-ala</td>
<td>Phosphate with 100</td>
<td>48.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>H₂N-T-G-G-G-G-T-β-ala</td>
<td>mmol NaCl</td>
<td>47.6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>H₂N-t-g-g-g-t-β-ala</td>
<td></td>
<td>45.7</td>
</tr>
<tr>
<td>9B</td>
<td>1</td>
<td>H₂N-T-G-G-G-G-T-β-ala</td>
<td>10mmol. Potassium-</td>
<td>52.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>H₂N-T-G-G-G-G-T-β-ala</td>
<td>Phosphate</td>
<td>48.0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>H₂N-t-g-g-g-t-β-ala</td>
<td></td>
<td>46.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>d(3'-T-G-G-G-G-T-5')</td>
<td></td>
<td>44.5</td>
</tr>
<tr>
<td>9C</td>
<td>1</td>
<td>H₂N-T-G-G-G-G-T-β-ala</td>
<td>100mmol. Potassium-</td>
<td>65.9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>H₂N-T-G-G-g-T-β-ala</td>
<td>Phosphate</td>
<td>57.2</td>
</tr>
<tr>
<td>9D</td>
<td>1</td>
<td>H₂N-T-G-G-G-G-T-β-ala</td>
<td>100mmol. Potassium-</td>
<td>67.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>H₂N-T-G-G-g-T-β-ala</td>
<td>Phosphate with 100</td>
<td>58.2</td>
</tr>
</tbody>
</table>

*All Tm's average 2/3 measurement and accurate with in ±0.5 °C. All experiments aer done at pH 7.4.

Figure 9: UV-melting profile of aeg-PNA and aep-PNAs A. PNA 1, 3-5.; B. PNA 1, 3, 5 and 10.; C. PNA 1 and PNA 3.; D. PNA 1 and PNA 3 at different concentration of metal ion. Derivatives curves are given in appendix.

At slightly acidic pH 6.4 (Figure 10C), aeg- and aep-PNA 2, 5 and 6 generally show the signature of G-tetrad formation, with the melting profiles showing complicated
pattern perhaps due to multiple transitions. At acidic pH 5.4 (Figure 10D), the negative sigmoidal signature of G-quadruplex disappeared completely and interestingly was replaced by patterns of typical double helix transitions.

<table>
<thead>
<tr>
<th>Figure of UV-Melting curve</th>
<th>PNA/DNA</th>
<th>List of PNA and DNA Sequence</th>
<th>pH of Buffer</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>at λ_{max} = 295nm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10A</td>
<td>2</td>
<td>H₂N-T-G-G-G-G-β-ala</td>
<td>7.8</td>
<td>63.1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>H₂N-t-g-g-g-g-tβ-ala</td>
<td>61.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>H₂N-g-g-g-g-tβ-ala</td>
<td>59.2</td>
<td></td>
</tr>
<tr>
<td>10B</td>
<td>1</td>
<td>H₂N-T-G-G-G-G-Tβ-ala</td>
<td>7.0</td>
<td>64.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>H₂N-T-G-G-G-G-β-ala</td>
<td>63.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>H₂N-g-g-g-g-tβ-ala</td>
<td>64.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>d(3'⁻T-G-G-G-G-T-5')</td>
<td>57.2</td>
<td></td>
</tr>
<tr>
<td>10C</td>
<td>2</td>
<td>H₂N-T-G-G-G-G-β-ala</td>
<td>6.4 Multiple</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>H₂N-t-g-g-g-g-tβ-ala</td>
<td>63.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>H₂N-g-g-g-g-tβ-ala</td>
<td>64.8</td>
<td></td>
</tr>
<tr>
<td>10D</td>
<td>2</td>
<td>H₂N-T-G-G-G-G-β-ala</td>
<td>5.8 Non-characteristic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>H₂N-g-g-g-g-tβ-ala</td>
<td>57.2</td>
<td></td>
</tr>
</tbody>
</table>

All Tm's average ±0.5°C measurement and accurate with in ±0.5 °C. All experiments aer done in 10 mM Potassium Phosphate containing 100 mM KCl.

Figure 10: UV-melting profile of PNAs at different pH: A. PNA 2, 6 and 5.; B. PNA 1-2 and 5.; C. PNA 2, 5 and 6.; D. PNA 2, 3 and 6 in same concentration of K⁺ metal ions 110mM. Derivatives curves are given appendix.
5A.4.1c Length Dependent study of G-quartet in aep-PNA

The UV melting curves of all modified aep-PNAs 5-9 corresponding to different lengths were recorded in potassium phosphate (100mM) containing 100 mM of KCl. Monitored at 295 nm, only PNAs 5, 6 and 7 exhibited characteristic tetraplex melting profiles (Figure 11A). The Tm values (Table 4) of all PNAs 5 and 6 were higher than DNA 10 while that of PNA 7 was slightly lower than DNA 10. The shorter aep-PNAs 8 and 9 did not form any tetraplexes under these conditions. Although PNA 8 exhibited duplex type transition, PNA 9 failed to show any transition (Figure 11B). The Tm of aep-PNA 1 in these conditions is 61.0 (Table 4).

Table 4: UV-melting temperature (Tm) of at different length of aep-PNAs

<table>
<thead>
<tr>
<th>Figure</th>
<th>PNA/DNA</th>
<th>List of PNA Sequence</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11A</td>
<td>5</td>
<td>H$_2$N-t-g-g-g-t-$\beta$-ala</td>
<td>59.3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>H$_2$N-g-g-g-t-$\beta$-ala</td>
<td>56.4</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>H$_2$N-g-g-g-t-$\beta$-ala</td>
<td>50.1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>d (3'-T-G-G-G-T-5')</td>
<td>54.0</td>
</tr>
<tr>
<td>11B</td>
<td>1</td>
<td>H$_2$N-T-G-G-G-T-$\beta$-ala</td>
<td>61.0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>H$_2$N-g-t-$\beta$-ala</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>H$_2$N-g-$\beta$-ala</td>
<td>nd</td>
</tr>
</tbody>
</table>

* All Tm’s average 2/3 measurement and accurate with in ±0.5 °C. All experiments are done at pH 7.4.

Figure 11. UV-Melting profile of aep-PNA A. PNA 5, 6, 7 and DNA 10; B. aep-PNA 1, 8 and 9. Derivatives curves are given appendix.
5A.4.2 Additional results on G-tetraplexes of PNA

5A.4.2a Hysteresis: Intramolecular vs Intermolecular folding

Two types of G-quartets have been observed in DNA: *intermolecular* and *intramolecular*. These can often be distinguished by hysteresis experiments: dissociation upon heating leads to a decrease in absorbance resulting in an inverse-sigmoidal curve, characteristics of tetraplexes\(^9\) and upon cooling at same rate, re-association should lead to an increase of absorbance, characteristic of intermolecular quadruplexes. The heating and cooling curves of UV-profile of *aep*-PNA 5 and PNA 6 are given in Figure 12, which suggest that PNA 5 and PNA 6 perhaps form intermolecular quartet.

![Figure 12: UV-melting profile of A. PNA 5 and B. PNA 6 in 10mM Potassium Phosphate and 100mM KCl at pH 7.4 and wavelength 295nm.](image)

5A.4.2b Circular Dichroism spectroscopy

The CD-spectra of *aep*-PNAs of different lengths and DNA 10 are shown in Figure 13. The CD spectrum of DNA 10 has one maxima at 262 nm and one minima at 238.5 nm, which is characteristic of G-quadruplex. The CD-spectra of \(L\)-cis-(2S,4S)-*aep*-PNAs 5-8 also show one maxima (271 nm) and one minima (223.5 nm), but location of both maxima and minima in these PNAs are different from DNA.
5A.4.2c Mass Spectral Data

Recently Ghosh, et. al.\textsuperscript{53} has reported the characterization of a PNA\textsubscript{4} quadruplex by electrospray ionization mass spectrometry (ESI-MS) to study the molecularity of the PNA species and seek evidence for Q-PNA. ESI-MS has been used to observe noncovalent intermolecular complexes of DNA and PNA-DNA hybrids.\textsuperscript{69} G-rich sequences of PNA as in TG3 has been analyzed by positive ion nano electrospray ionization mass spectrometry (nano-ESI-MS).\textsuperscript{73} Analysis at a cone voltage of 60 V and source temperature of 30 °C showed peaks corresponding to a triply charged species at $m/z$ 1716.9 and a doubly charged species at $m/z$ 2575.2.10 The associated molecular weight (MW) for these peaks was 5148, consistent with tetramer formation by PNA TG3 (MW of PNA TG3) 1287 (0.5 Da). Peaks corresponding to (M4 + 2H + Na)$^{3+}$, (M4 + 2H + K)$^{3+}$, and (M4 + H + 2K)$^{3+}$ were also seen, which supports that TG3 forms tetramers by ESI-MS.\textsuperscript{59}
### Table 5: aeg-PNA and L-cis-(2S,4S)-aep-PNA sequences*

<table>
<thead>
<tr>
<th>PNA</th>
<th>Calculated Molecular Mass</th>
<th>ESI mass spectra</th>
<th>MALDI-TOF mass spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1786.70</td>
<td>3664.03, 2469.36, 2096, 1299.36</td>
<td>M⁺ = 1787.3</td>
</tr>
<tr>
<td>2</td>
<td>1518.58</td>
<td>-</td>
<td>M⁺ = 1518 (4M+3K+Na+H)⁺ = 1242; (4M+Na+K+3H)⁺ = 1227.6;</td>
</tr>
<tr>
<td>3</td>
<td>1784.73</td>
<td>1787.70</td>
<td>1782, (4M+3Na+6K+H)⁺ = 743.1 and (4M+3Na+K+3H)⁺ = 1034.10</td>
</tr>
<tr>
<td>4</td>
<td>1784.73</td>
<td>1786.2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1774.86</td>
<td>M⁺ = 1774.86; (4M+8K+H)⁺ = 741.4, (4M+7K)⁺ = 1056.49, (4M+K+8H)⁺ = 794.04 (4M+6K+Na+2H)⁺ = 817.59.</td>
<td>M⁺ = 1774.86; (M⁺ + 2Na⁺ + 2K⁺)⁺ = 7223.44</td>
</tr>
<tr>
<td>6</td>
<td>1510.58</td>
<td>1593, 1555.48, 1510,</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1221.28</td>
<td>1263</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>931.98</td>
<td>1011.86, 995.88, 973.88, 931.9</td>
<td>973.88-</td>
</tr>
<tr>
<td>9</td>
<td>643</td>
<td>643</td>
<td>643</td>
</tr>
</tbody>
</table>

*G, T = aeg-PNA and g, t = L-cis-(2S,4S)-aep-PNA.

The MALDI-TOF PNA 1-9, aeg-PNA 2, single modified aep-PNA 3 and homooligomer aep-PNAs 5-6 are listed in Table 5. The expected mass peaks exhibited along with additional peaks derived from addition of Na⁺ and K⁺ ions. The observed M⁺ from MALDI-TOF could be fitted into mass compositions as shown in Table 5. (4M+3K+Na+H)⁺ for PNA 1, (4M+3Na+6K+H)⁺ for PNA 2, (M⁺ + 2Na⁺ + 2K⁺)⁺ for PNA 3 and (4M+K+3H)⁺ are evidence for tetraplex.

#### 5A.4.2d Isothermal titration calorimetry

The stability of G-tetrad depends both upon the concentration of metal ion and the concentration of DNA oligomer. The structure of tetraplexes will be disrupted by dilution, which was studied by “Isothermal titration calorimetry” (ITC) experiment. In this experiment the annealed and the unannealed PNA tetraplexes were separately titrated (diluted) with buffer and the rate of change in heat of dilution (dq/dt) was recorded at 20 °C. The ITC plot of dq/dt vs volume of titrant for PNA 1, PNA 3 and PNA 5 given in
Figure 14. It is seen that the plots of annealed samples of \textit{aeg-PNA 1}, \textit{aeg-aep-PNA 3} and \textit{aep-PNA 5} show characteristic titration curves indicating heat evolved during the dilution of tetraplexed PNA, while ITC plots of PNA 1 and PNA 5 without annealing do not show the any ordered curves, expected for tetraplex formation. The data shown have been corrected for buffer dilution effects. These experiments, gave further proof for tetraplex formation by PNA, though the data are insufficient to obtain any thermodynamic information.

\textbf{Figure 14: ITC Graph of PNAs in Buffer Solution unannealed and annealed}
5A.5 DISCUSSION

The effect of PNA backbone modification as in aep-PNA is expected to significantly affect the stability of the G₄-quadruplexes. The UV, UV-Tm, CD and Mass spectral data presented in last section suggests that formation G₄-quadruplexes by aep-PNA is significantly modulated according to conditions.

G-rich aeg-PNA sequences are known to form G₄-tetraplexes in the presence of monovalent metal cation. The UV-spectra of aeg-PNAs (1 and 2), aeg-aepPNAs (3 and 4) and aep-PNAs (5-9) are quite similar having two broad peaks in 260-280 nm range, suggesting tetraplex formation by these PNAs. The UV-absorbance vs temperature plot derived from UV-melting shows negative sigmoidal curves, characteristic of tetraplex formation. The stability of G₄-tetraplexes of aeg- and aep-PNA units is almost same in presence of Na⁺ at pH 7.4. G₄-tetraplexes formed by the aeg-PNA 1 in presence of K⁺ at pH 7.4 are more stable than aep-PNA 6 (ΔTm = 2-6 °C). On further, increasing the concentration of [K⁺] upto 100 mM, the stability of tetraplexes is significantly changed. Since the prolyl ring nitrogen of aep-PNA is protonated even at neutral pH, the thermal stability of aep-PNA were measured at different pHs in the range (7.8-5.8). The formation of tetraplexes is restricted to the pH range 7.8-6.4 and the stability of aeg and aep-PNAs in phosphate buffer is almost similar at pH 7.8, 7.4 and 7.0. The Tm values of these PNAs at pH 6.4 could not be determined due to appearance of multiple transitions. The formation of tetraplexes in aeg- and aep-PNA at the acidic pH 5.8 is seriously disfavored and their melting profiles correspond to typical duplex transition in agreement with previous literature.

To find the required minimum length in aep-PNA for formation of stable tetraplexes, length dependent study in aep-PNAs (5-9) was done in the presence of [K⁺] = 110 mM at pH 7.4. The results indicated that only tetramer or longer PNAs t₄, t₄ and t₅
formed stable G-quartets. The shorter sequences like tg2 and tg did not show any signature of G4-tetraplex in UV-thermal experiments.

The study of *intrastrand* vs *interstrand* G-quadruplex formation of PNA was carried out by heating-cooling experiments. Interstrand association is kinetically slower than intrastrand association. The heating and cooling melting profiles of PNAs 4 and 5 indicated slower re-association than dissociation, which suggested formation *inter*-molecular rather than *intra*-molecular quartets.

The CD-spectra of *aep*-PNAs (5-9) are slightly different from that of known quadruplex forming DNA 10. In *aep*-PNAs (5-9) the maxima is observed at wavelength 265-270 nm, similar to that of DNA 10. The minima at 220nm in PNAs is slightly different than that of DNA 10 at 238-243 nm. The difference in minima of CD-spectra of *aep*-PNAs may be due to unusual conformation of its prolyl ring. The ring nitrogen being protonatable even at neutral pH, may alter the puckering of the prolyl ring, affecting the secondary structure of *aep*-PNA.

The formation of G-tetraplexes was also observed in mass spectra (MALDI-TOF and ESI) of *aeg-* and *aep*-PNA. The presence of ion peaks at expected tetrameric molecular mass composition of PNA 1-6 suggests the formation of G-quadruplexes.

From the above results of UV-thermal denaturation experiments, it is seen that the stability of the G4-quartets is highly dependent upon the concentration of monovalent metal ions. To examine the role of metal ion ([M+], the tetraplexes of PNA in buffer solution contains salt was diluted with same buffer and measuring the evolved heat of dilution with substraction of values (buffer dilution) by ITC experiment. These ITC results for tetraplexed PNAs qualitatively showing that dilution disrupts the tetraplexes.
5A.6 CONCLUSIONS

The synthesis and characterization G-rich sequences of aeg-PNA and aep-PNA are reported. The formation of G-tetrad structures in aep- and aeg-PNA is observed under different conditions of pH, salt and metal ions K⁺ and Na⁺. The stability of G-tetrad structure in aep- and aeg-PNAs are almost similar and both of them stabilize the G-tetrad structures better than in DNA. The formation of quadruplexes is also supported by mass spectroscopy, CD-spectra and ITC.

5A.7 EXPERIMENTAL

5A.7.1 Synthesis of aeg- and aep-PNA monomer and oligomer

The synthesis of thymine and guanine monomers of aeg- and aep-PNA was achieved by following the same procedures as reported in Chapter 1. These monomers were used to synthesize the corresponding PNA oligomers by solid phase synthesis procedure reported in Chapter 4.

5A.7.2 UV-Melting Experiment

The concentrations of the synthesized PNA s were determined spectrophotometrically at λ 260 nm at 80 °C, by use of the molar extinction coefficient calculated for unstacked oligonucleotides [11700 cm⁻¹ M⁻¹ (G); 8800 cm⁻¹ M⁻¹ (T)] in buffer solution, used for the melting experiments. UV melting experiments were performed on Lambda-35 UV Spectrometer (Perkin-Elmer) equipped with a thermal melt system, PTP-6 Peltier Temperature Programmer with water circulator Thermohake K20.

Melting curves were recorded with a concentration of approximately 10 μM of single strand PNA s in 2 mL of the buffer solution in Teflon-sealed quartz cuvettes of 1 cm optical path length. The resulting solutions were then heated at 80 °C for 15 min, then slowly cooled and kept at 20 °C for 20 min. After thermal equilibration at 20 °C, the UV
absorption at \( \lambda \) 295 was monitored as a function of the temperature, increasing at a rate of 0.5 °C/min. UV-thermal denaturation method was used to study the change in UV-absorbance, which was recorded with respect to increase in temperature at constant wavelength. The results were normalized and the data was analysed using Origin 5.0 (Microsoft Corp.).

5A.7.3 Circular Dichroism spectral studies

5-10 \( \mu \)M stock solution of each PNA (5-8) and DNA 10 strands taken in 2 mL of 100mM potassium phosphate buffer with salt 100mM KCl was annealed by heating at 90 °C for 5min, followed by slow cooling to room temperature and kept at room temperature for 30 min then, refrigerate in for 72 h. The CD spectra of the refrigerated samples of all PNA were recorded on a Jasco J-715 spectropolarimeter at temperature 10 °C by accumulation 5 scans and a scan speed of 200nm/min.

5A.7.4 ITC-Measurements

The VP-ITC micro Calorimeter was used to study dilution effect on G-quartet structure of PNA. In this experiment, annealed sample was diluted with the same buffer in which sample was prepared. The dilution was done by successive injection of 10 \( \mu \)L PNA samples to 1.5 mL of buffer in cell at interval of 20 seconds at 15 °C.

5A.7.5 Mass Spectroscopy

Electron spray ionization mass spectrometer (QSTAR MultiView1.5.0 TOF-MS-IN) was used for characterization of G-quartet formation. The 2 \( \mu \)M solution of PNA in 1mL of methanol was used for recording mass spectra. ABI-MALDI-TOF spectrometer was used to record mass of PNA by using Voyager spec electronic software. The mass of neat PNA was recorded in presence of matrices CHCA (\( \alpha \)-cyano-4-hydroxycinnamic acid).
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1. HPLC of PNA 1-6

PNA 1

PNA 2

PNA 3

PNA 4

PNA 5

PNA 6

PNA 7

PNA 8
2. Derivative curves of UV-Melting profiles aeg-/aep-PNA 1-6 and DNA 10
3. MALDI-TOF of PNA 1-2 and 4

PNA 1
\[ M^+ = 1786.70 \]
\[ (M+3Na)^+ = 1802.75 \]
\[ (M+2K+Na+H)^+ = 1814.25 \]
\[ (M+K+2Na+H)^+ = 1817.75 \]

PNA 2
\[ M^+ = 1518 \]
\[ (4M+3K+Na+H)^+ = 1242 \]
\[ (4M+Na+K+3H)^+ = 1227.6 \]

PNA 4
\[ M^+ = 1782 \]
\[ (4M+3Na+6K+H)^{10+} = 743.1 \]
\[ (4M+3Na+K+3H)^{10+} = 1034.10 \]
4. ESI mass of PNA 3 and 5

PNA 3: M' = 1782.70
(4M)^+ = 793.25
(4M)^+ +2K = 871.33

PNA 5
M' = 1774.86
(4M+8K+H)^+ = 741.4
(4M+7K)^+ = 1056.49
(4M+K+8H)^+ = 794.04
(4M+7K)^+ =
(4M+6K+Na+2H)^+ = 817.59

PNA 5: M' = 1774.86
(4M+2K+Na+3H)^+ = 1200.50
(4M+7K)^+ = 1056.49
(4M+7K)^+ =
(4M+6K+Na+2H)^+ = 817.59
5. MALDI-TOF of PNA 5 (with salt Na⁺)

PNA 5

\[ M^- = 1774.86 \]
\[ (M^- + 2H)^+ = 1776.86 \]

PNA 6 (H₂N-g-g-g-g-T-β-ala)

\[ (M^+ + K^+ + 4H)^+ = 1553.58 \]
\[ (4M^+ + K^+ + 3H)^+ = 1564.08 \]
\[ (4M^+ + 2Na^+ + K^+ + H)^+ = 1575.08 \]
\[ (4M^+ + Na^+ + 3K)^+ = 1588.58 \]
\[ (M^+ + 2K^+ + Na^+ + H)^+ = 1547.08 \]
6. ESI mass of PNA 5-6

PNA 5; M⁺ = 1774.86
(4M+2K+Na+3H)= 1029.06
(4M+2K+Na+3H)= 1050.92
(4M+4K+1H)" = 1451.2

PNA 6
M⁺ = 1510
(M+3K+Na+H)⁺ = 1651.3
7. ESI mass spectra of PNA 5

- ESI mass spectra of PNA 5
- Mass range: 800.0 to 2000.0 amu
- Acquisition Time: Thu, Sep 23, 2004 at 3:39:26 PM

Mass spectra showing peaks at:

- \( M^+ = 1774.86 \)
- \( (M^+ + 2Na^+ + 2K^+) = 723.44 \)
- \( (M^+ + 2Na + 3K^+) = 759.25 \)

- ESI mass spectra of PNA 5
- Mass range: 800.0 to 2000.0 amu
- Acquisition Time: Thu, Sep 23, 2004 at 3:39:26 PM

Mass spectra showing peaks at:

- \( M^+ = 1774.86 \)
- \( (4M^+ + 2Na^+ + 2K^+) = 723.44 \)
- \( (M^+ + 2Na + 3K^+) = 759.25 \)
8. MALDI-TOF of PNA 1

PNA 1

$M^+ = 1786.70$

$(2M + 2Na^+ + K^+)^+ = 3660.4$
9. MALDI-TOF of PNA 7-9

PNA 7
$M^+ = 1221.28$
$(M+K+3H)^+ = 1263.28$

PNA 8
$M^+ = 930.98$
$(M+K+3H) = 973.98$
$(M+K+Na+2H) = 995.98$

PNA 9
$M^+ = 643$
$(M+K'+2H)^+ = 684$
$(M+K'+Na'+H)^+ = 706$
10. MALDI-TOF of PNA 3

PNA 3
M⁺ = 1785
(4M)⁹⁺ = 794.33
CHAPTER 5B
PNA TETRAPLEXES: BIOPHYSICAL STUDIES OF i-MOTIF OF aeg-PNA
CHAPTER 5B: PNA TETRAPLEXES: BIOPHYSICAL STUDIES OF i-MOTIF OF aeg-PNA

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5B.1 INTRODUCTION

In continuation of the previous section, the study of other class of tetraplexes namely the i-motif in nucleic acids is described in this section. The telomeric DNA has guanine (G) and cytosine (C) rich DNA sequence regime and G-rich DNA oligomers are well known to form G₄-tetrads via combination of Watson-Crick and Hoogsteen hydrogen bond mediated cyclic structures, as elaborated in the earlier Section. In comparison, the C-rich sequences form tetramers via the semi protonated C-C⁺ base pairs held by three hydrogen bonds to form parallel double strands. Two such double strands interdigitating through C:C⁺ base pairs lead to the four-stranded i-motif structure (Figure 1). The opposed dipoles of exocyclic C2-carbonyl and N4-amino groups favour such an interaction of consecutive base pairs by alternate stacking of the amino and carbonyl groups.

![Figure 1: (a) Chemical structures of DNA; (b) Schematic diagram of i-motif in DNA.](image)

Recently, the discovery of i-motif structures and investigation of their properties in context to possible biological relevance is extensively described within the framework on quadruplexes of DNA/RNA. The structure of the DNA oligomer with continuous C-stretch (5'-TC₆-3') at acidic pH formed a four-stranded complex, in which two base-paired parallel-stranded duplexes are intimately associated via their base pairs, which are fully intercalated. The relative orientation of the duplex pairs is anti-parallel so that each base
pair is face-to-face with its neighbour. The possibility of forming i-motif structure with C-rich ribonucleic acids (RNA) has been investigated. The C-rich sequence of RNAs as r(UC₅), r(C₅), r(C₃U) and r(UC₃) associate into multiple intercalated structures at acidic pH. The conformational differences between RNA i-motif and the DNA i-motif arise from the C3'-endo pucker of the RNA sugars. The orientations of the intercalated C:C⁺ pairs in RNA result in a slight widening of the narrow grooves at the steps where the hydroxyl groups come in close contact. Two types of i-motif structures are postulated in RNA sequence r(UC₅). The major one is the i-motif similar to DNA and the minor one is different with respect to stacking topology due to 2'-OH/2'-OH, repulsive contacts in the fully intercalated structure. The free energy of the RNA i-motif (on average -4 kJ mol⁻¹ per C·C⁺ pair) is half the value of DNA i-motif structures.

5B.2 RATIONALE OF PRESENT WORK

5B.2.1 Structural characterization of the i-motif structure in DNA/RNA

The structure of i-motif in nucleic acid is characterized by many known techniques like NMR-spectroscopy, X-ray crystallography, Raman spectroscopy, CD and UV-spectroscopies.

5B.2.1a NMR-Spectroscopy

A tetrameric DNA structure with protonated C:C⁺ base pairs has been studied by NMR. Oligomers containing continuous tracts of cytidine form hemiprotonated base pairs at acidic pH and are double-stranded. The structure of the DNA oligomer 5'-d(TCCCCC) at acidic pH is found to be a four-stranded complex in which two base-paired parallel-stranded duplexes are intimately associated, with their base pairs fully intercalated. The NMR spectrum indicates the structure to be highly symmetrical with the
four strands being equivalent. A model derived by energy minimization with constrained molecular dynamics shows excellent compatibility with the observed nuclear Overhauser effects (NOEs) such as inter-residue sugar-sugar NOEs H1'-H1', H1'-H2" and H1'-H4', which are diagnostic for such tetrameric structures. Proton exchange transfer occurs easily from a donor hydrogen (DH) to an acceptor (A) via hydrogen-bonded complex (DH-A). Exchange requires the disruption of the base pair, followed by chemical exchange from the open pair and it occurs even at neutral pH in the absence of added catalyst. The formation of i-motif in d(TC₈), d(TC₃) and d(T₂C₂T₂) has also been characterized by NMR.

5B. 2.1.b Characterization of i-motif Structure by Crystallography

i-Motif structure in C-rich sequences of DNA has also been characterized by single crystal X-ray diffraction. The i-motif structure assigned by NMR and X-ray are not quite similar, but show some gross similarities. The X-ray structure reveals much more microheterogeneity that can be visualized in the NMR structure. Differences are found in phosphate orientations and the relative positioning of the adjacent chains. There is a great similarity in the interactions of the bases and the overall packing of the sugar phosphate backbones. Both analyses show a large number of C4'-exo puckers, with minor differences in the helical twist. This organization, termed as the intercalation motif (i-motif), is distinctly different from either the DNA duplexes or the G₄-DNA quadruplexes in which four planar guanine residues are found in cyclic hydrogen bonding. Gehring et. al. attribute the stability to van der Waals stabilization between the sugar phosphate backbones across the narrow groove and the opposite dipole orientations of the carbonyl and amino groups. The closer stacking of the bases of 3.1 Å, instead of the more familiar 3.4 Å arising from the exocyclic residue overlapping with the local n-electron clouds, is
also known to impart additional contribution to stability. The crystal structure of a four intercalated DNA sequence d(C4) is also reported (Figure 2).16

![Figure 2: Crystal structure of d(C4)](image)

**5B.2.1c Raman spectroscopy**

Raman spectroscopy is an effective probe of nucleic acid secondary structure determination in both solution and crystalline samples.17 Raman spectra of solutions of 5′-dCMP, d(CCCT), and d(C8) were excited at 514.5 nm. Raman frequencies of well-resolved bands are accurate to within (1.5 cm⁻¹). For d(CCCT) and d(C8), spectral intensities were normalized to the phosphodioxy stretching band at 1092 cm⁻¹, which is essentially invariant to pH change in the range of present interest. Raman spectra of single crystals of d(CCCT) were obtained at 514.5 nm excitation. The nucleic acid i-motif, which results from antiparallel intercalation of two parallel-stranded duplexes containing hemiprotonated cytosine base pairs [(C:C)⁺], is characterized by a unique Raman signature. Both thermostability (Tm) and the extent of cytosine protonation (pKC) in i motif quadruplexes of d(CCCT) and d(C8) have been monitored. The crystal structure is conserved in aqueous solution, despite the fact that C3′-endo conformation is rarely seen in deoxynucleosides. Stabilization of the i motif by cytosine base pairing and stacking, estimated here as <0.17 kcal/mol of (C:C)⁺, is apparently sufficient to compensate for the
incorporation of C3'-endo sugars in the backbone of the solution quadruplex. In the
d(CCCCT) crystal, the four phosphodiester strands are not conformationally identical,
leading to an asymmetric quadruplex. The Raman signature of hemiprotonated cytosine
base pairs is distinct from the signatures of unprotonated and protonated cytosines, making
it useful as marker for protonated cytosines. This should be of value in assessing the extent
of cytosine protonation/hemiprotonation in duplex, triplex, and quadruplex structures of
DNA.

5B.2.1d Characterization of i-motif structure by CD

The structural characterization of i-motif in DNA by CD of d(C3TA2)3C3 indicated
little dependence on the cation species.\(^{18}\) In CD profiles with either Na\(^+\) or K\(^+\), a positive
band around 275 nm and a negative one near 250 nm existed at neutral pH, whereas a peak
near 288 nm and a trough near 256 nm appeared at pH 5.5.\(^{19}\) The red-shift in the CD
bands as the pH is lowered to 5.5, which are attributed to the i-motif structure.

5B.2.1e Thermal denaturation study by UV

In UV spectra, protonated cytosines show characteristic absorption at 295 nm.\(^{20}\)
Hence UV-thermal transitions monitored at 295 nm show a reverse sigmoidal pattern
characteristic of formation of C-C\(^+\) tetraplexes like G-quartet structure.\(^{21}\) The kinetic and
thermodynamic aspects of i-motif formation in modified oligonucleotides has been studied
by UV at 295 nm.\(^{22}\)

DNA and RNA have very versatile auto-association properties,\(^{8}\) the range of which
extends from formation of duplexes to triplexes and tetraplexes. RNA lacks ability to form
i-motif structures in some sequences.\(^{23}\) Considerable interest is now growing in the study
of tetraplexing properties of mimics of natural oligonucleotides such as
phosphorothioates,\(^{24}\) LNA,\(^{25}\) and PNA.\(^{26,27}\) While G\(_4\) tetraplex formation was successfully
demonstrated recently in aeg-PNA,\textsuperscript{28} it was reported that the PNA H-C<sub>4</sub>A<sub>4</sub>C<sub>4</sub>-Lys-NH<sub>2</sub> did not form C-C<sup>+</sup> tetraplexes at pH 7.0. However, The i-motif formation in alanine-PNA was observed by Diedersen, \textit{et. al.}\textsuperscript{29} Based on steric factors, it was shown that ala-PNA forms C-C<sup>+</sup> complexes in C<sub>4</sub> tetramer, but not in C<sub>8</sub>-octamer (Figure 3). The successful confirmation of i-motif structure by UV and NMR in TC<sub>4</sub> sequences of ala-PNA encourages us to trace tetraplexing properties in 1-N-aminoethylglycine peptide nucleic acid (aeg-PNA). As described in the preceding Chapters, PNA is one of most promising mimic of nucleic acid with remarkable thermal stability over DNA as both in duplex and G-quadruplexes. However, no report exists so far on successful tetraplexing properties of unmodified PNA.

![Figure 3: Chemical structure of alanine PNA](image)

\section*{5B.2.2 Objective}

Recently, a large number of nucleic acid mimics have been developed, for antisense therapeutics, but few reports exist on i-motif formation by them. Due to the interesting result of ala-PNA, we chose to examine aeg-PNAs with C-rich sequences TC<sub>n</sub> (n = 2, 3, 4, 8) to study the pH dependent tetraplex formation by temperature dependent UV-spectroscopy (Figure 4).
5B.3 PRESENT WORK

5B.3.1 Synthesis and characterization of aeg-PNA oligomers

The cytosine (C) and thymine (T) monomer (Figure 5) of aminoethylglycine-(aeg)-PNA monomers were synthesized by using synthetic procedures described earlier. For \(i\)-motif study in PNA, the following oligomers of PNA TC\(n\) were synthesized on solid support Merrifield resin, using \(t\)-Boc chemistry using similar procedure as described in Chapter 4.
1. H$_2$N-T-C-C-β-ala-MF; (TC$_2$)
2. H$_2$N-T-C-C-C-β-ala-MF; (TC$_3$)
3. H$_2$N-T-C-C-C-β-ala-MF; (TC$_4$)
4. AcHN-Lys-T-C-C-C-C-C-C-C-C-CONH-MBHA; (TC$_s$)

All these PNAs sequences were cleaved from resin using TFA-TFMSA and they were purified to homogeneity by HPLC and characterized by MALDI-TOF mass spectroscopy. MALDI-TOF mass spectra of TC$_2$ (PNA 1), TC$_3$ (PNA 2), TC$_4$ (PNA 3) and TC$_s$ (PNA 4) pure PNA 1-4 (Table 1) are shown in Table 1. The HPLC chromatogram and mass spectra of PNA 1-4 are given in an appendix to this Chapter. For comparative study, the DNA sequences d(TC$_g$) and d(TC$_g$) were synthesized on ABI-DNA synthesizer. A typical mass spectra of TC$_4$ (PNA 3) and TC$_s$ (PNA 4) are shown in Figure 6.

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

<table>
<thead>
<tr>
<th>PNA</th>
<th>Sequences of PNA</th>
<th>Molecular Formula</th>
<th>Molecular weight (Calculated)</th>
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<tbody>
<tr>
<td>1</td>
<td>H$_2$N-T-C-C-β-ala</td>
<td>C$<em>{34}$H$</em>{69}$N$<em>{15}$O$</em>{13}$</td>
<td>859.86</td>
</tr>
<tr>
<td>2</td>
<td>H$_2$N-T-C-C-C-β-ala</td>
<td>C$<em>{44}$H$</em>{62}$N$<em>{20}$O$</em>{15}$</td>
<td>1111.11</td>
</tr>
<tr>
<td>3</td>
<td>H$_2$N-T-C-C-C-C-β-ala</td>
<td>C$<em>{54}$H$</em>{75}$N$<em>{25}$O$</em>{18}$</td>
<td>1361.36</td>
</tr>
<tr>
<td>4</td>
<td>AcHN-Lys-T-C-C-C-C-C-C-C-C-CONH$_2$</td>
<td>C$<em>{99}$H$</em>{137}$N$<em>{47}$O$</em>{30}$</td>
<td>2465.49</td>
</tr>
</tbody>
</table>
PNA 4

$M' = 2463.48$

$(4M+2Na+2H)^{+} = 2477.33$

Figure 6: MALDI-TOF mass spectrum of PNA 3 and PNA4

PNA 3

$M' = 1359.36$ (Calculated)

$(4M+2K+2H)^{+} = 1379.40$

(Observed)
5B.4 RESULTS

5B.4.1 Biophysical study of i-motif in PNA by UV spectrophotometer

In the following section, the structures of G-tetrad in aeg-PNA and aep-PNA have been studied by use of temperature dependent UV-spectroscopy.

5B.4.1a Determination the pKₐ for N3 of cytosine

Determination of the pKₐ for N3 of cytosine (C)⁵,¹⁷,²³ in monomer and oligomer PNA ⁴ is determined by their UV-spectra. The UV spectra of PNA ⁴ were recorded at different pHs 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 the lower wavelength 260 nm as the pH is increased. Earlier, it has 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.³⁴ Figure 7 shows a plot of UV absorbance at 275 and 295 nm in PNA ⁴ and in

![Figure 7: Left. The UV-spectra of cytosine monomer and PNA ⁴. Right. The plot of UV-absorbance vs. pH at 275 and 295 nm.](image-url)
cytosine monomer as a function of pH and it is seen that the spectral differences between protonated and non-protonated C in PNA are more at 275 nm. From these data, the pK_a for N3 of C in PNA is obtained as 3.42 for PNA 4 and 3.72 for PNA C-monomer, which is significantly lower than that seen for N3 of C in DNA/RNA, which is about 4.8. The formation of C-C" tetraplexes in PNA 3, 4 and d(TC)_8 at pHs 3.0, 4.5, 5.0, 6.5 and 7.0 were monitored at 295 nm, for a true comparison with the established tetraplex formation in d(TC)_8 by following absorption at 295 nm.

5B.4.2 i-motif formation in aeg-PNA at different pHs

At pH 3.0: The UV melting profiles (absorbance vs. temperature) of PNAs 3, 4 and DNA 5, 6 followed at 295 nm are given in Figure 8A. The observed inverse sigmoidal plots of melting profiles are characteristics for i-motif and G-quartets. This preliminary observation of tetraplexes in these sequences suggested the formation of i-motif in PNA at low pH 3.0. The Tm values of PNA 3, PNA 4 and DNA 5 were extracted from the first derivative of their respective melting curves and given in Table 2.

At pH 4.5: The UV melting profile (absorbance vs. temperature) of PNA 3, 4 and DNA 5, 6 monitored at 295 nm are given in Figure 8B and these are also show similar type of negative sigmoidal curves. Hence, the PNA 3, 4 and DNA 5, 6 are also forming i-motif structure at this acidic pH 4.5 and the Tm values extracted from first derivatives plot are given in Table 2.

At pH 5.0: The UV-melting profiles of PNA 1-4 and DNA 6 at pH 5.0 are shown in Figure 8C, which suggests successful formation of i-motif in PNA 3, 4 and DNA 6 295 nm. The Tm value of PNA 3, PNA 4 and DNA 6 shows presence of i-motif at this pH. PNAs 1-2 do not have negative sigmoidal transition characteristic of i-motif under these conditions.
At pH 6.0 and 6.5: The melting profiles of PNA 4 and DNA 6 at pH 6.0 are shown in Figure 8D and at pH 6.5 in Figure 8E. The results indicate absence of i-motif structure in PNA 4 and retention of i-motif in DNA 6.

Table 2: Tm of PNA and DNA i-motif at different pH

<table>
<thead>
<tr>
<th>PNA/ DNA</th>
<th>Sequences of PNA</th>
<th>Tm at different pH (°C)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H2N-T-C-C-β-ala</td>
<td>47.5 43.0 ns - -</td>
</tr>
<tr>
<td>2</td>
<td>H2N-T-C-C-C-β-ala</td>
<td>67.3 40.0 40.5 - -</td>
</tr>
<tr>
<td>3</td>
<td>H2N-lysT-C-C-C-C-C-C-C-CONH2</td>
<td>67.4 55.0 46.0 nf nf</td>
</tr>
<tr>
<td>4</td>
<td>d(5'-T-C-C-3')DNA</td>
<td>47.5 43.0 ns - -</td>
</tr>
<tr>
<td>5</td>
<td>d(5'-T-C-C-C-C-C-C-C-C-3')DNA</td>
<td>58.4 58.7 55.7 50.4 52.0</td>
</tr>
</tbody>
</table>

nf: i-motif is not formed; ns: not studied

* 3.0 4.5 5.0 6.0 6.5

Figure 8: UV melting profile of aeg-PNA TCn series
At neutral pH 7.0: In Figure 8F, the melting profiles of both DNA 6 and PNA 4 suggest complete absence of i-motif in both cases. On the other hand, the profile indicates duplex formation. Tms of these duplex transitions are obtained 28.8 °C for PNA 4 and 39.9 °C for PNA 6.

5B.4.3 Isothermal Titration Calorimetry (ITC)

ITC was also used to study the dissociation of tetraplex of PNAs 3 and 4 and DNA 5 and 6 (see in appendix). In ITC, the enthalpic change associated with dissociation was measured incrementally as a function of dilution by buffer. In both cases, the titration

![Figure 9: A. ITC profile of DNA 5, B. DNA 6, C. PNA 3 and PNA 4.](image-url)
profile corresponded to classical saturation isotherms, with the liberated heat slowly leveling off after the dissociation event. The data were corrected for solvent dilution effects by subtraction of the blank buffer titration data. The total enthalpy computed from the integrated area of the isotherms indicated that the enthalpy change in dissociation of TC₈ was about twice that of TC₄ (Figures 8A-8D)

5B.5 DISCUSSION

The temperature dependent UV-absorbance results on C-rich PNA and DNA sequences are shown in Figure 8. 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 PNA/DNA sequences was indicated by observance of negative sigmoidal transitions (Figure 8). The accurate Tm values obtained from the first derivative curves and the Tm data for PNA 4 and DNA 6 are shown in Table 2. The PNAs 1 (TC₂) and 2 (TC₃) failed to show tetraplex formation at any of the pH conditions. The PNA 3 and PNA 4 showed formation of strong C-C⁺ tetraplexes at pHs 3 and 4. Significantly, these PNA C-C⁺ tetraplexes were much more stabilized compared to the analogous DNA C-C⁺ tetraplexes by 10-20°C at low pH. The stability of PNA C-C⁺ tetraplexes was also dependent on pH. A comparison of pH dependent Tm of different PNA and DNA C-oligomers (Table 2) reveals that PNAs 3 and 4 form tetraplexes only in the acidic regime, upto 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 higher pH (Figure 3). In comparison, the isosequential DNA C-oligomers 5 and 6 shows tetraplex formation upto 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 ∂Tm/∂pH being 10,
while that for DNA is only about 3. The difference in the cut-off pH in PNA (6.0) and DNA (6.5) for tetraplex formation is perhaps a reflection of the lower pKₐ of N3-C in PNA (Figure 2) compared to that in d (TC)ₙ. The lower pKₐ of N3-C in PNA compared to that in DNA is due to electronic effects: C in DNA is linked to glycosidic carbon while not so in PNA. These cause alterations of pKₐ of N-3 C in PNA compared to DNA. Such differences in nucleobase properties due to electronic effects may partly explain other important biophysical effects displayed by Pans, different from those of DNA.

5B.6 CONCLUSIONS

While i-motif in C-rich sequences of DNA is well established, a similar motif in PNA was hitherto unknown. The synthesis and characterization of PNA TCₙ (n = 2, 3, 4 and 8) were done to study i-motif in these PNA by UV-spectroscopy. The results clearly demonstrate formation of i-motif in PNA via C-C⁺ base pairing in acidic pH 3.0-5.0 range. PNA C-C⁺ tetraplexes possess significantly higher stability compared to analogous DNA C-C⁺-tetraplexes. The absence of i-motif at higher pH in PNA in contrast to DNA arises from the lower pKa of N-3 of C in PNA compared to DNA.
5B.7 EXPERIMENTAL

5B.7.1 Synthesis of aeg-PNA monomers and oligomer

The synthesis of thymine (T) and cytosine (C) monomer of aeg-PNA was done by following the same procedure as in Chapter 1. These monomers were used to synthesize the PNA oligomers by solid phase synthesis procedure described in Chapter 4.

5B.7.2 UV-Melting Experiments

UV melting experiments were performed on Lambda-35 UV Spectrometer (Perkin-Elmer) equipped with a thermal melt system, PTP-6 Peltier Temperature Programmer with water circulator Thermohake K20. The samples 2 mL were transferred to quartz cell and sealed with Teflon stopper after degassing with nitrogen gas for 15 min and equilibrated at the starting temperature for at least 30 min. The OD at 260 nm was recorded in steps from 10-85 °C with temperature increment of 0.2 °C/min. The results were normalized and analysis of the data was performed on using Origin 5.0 (Microsoft Corp.).

The concentrations of the synthesized PNAs were determined spectrophotometrically at λ 260 nm at 80 °C, by use of the molar extinction coefficients calculated for unstacked oligonucleotides [6700 (C); 8800 (T) cm^{-1} M^{-1}] in buffer solution. Melting curves were recorded with a concentration of approximately 10 μM of single strand in 2 mL of the tested solution in Teflon-sealed quartz cuvettes of 1 cm optical path length. The resulting solutions were then heated at 80 °C for 15 min, then slowly cooled and kept at 20 °C for 20 min. After thermal equilibration at 20 °C, the UV absorption at λ 295 nm was monitored as a function of the temperature, increasing at a rate of 0.5 °C/min. Temperature dependent UV-absorbance was recorded at constant wavelength 290 nm. A plot of absorbance vs. temperature was obtained to determine the thermal melting point. In
this case shape of melting curve is negative sigmoidal due to decreased hypochroism at 295 nm.

5B.7.3 ITC Experiments

The VP-ITC micro Calorimeter was used to study dilution effect on G-quartet structure of PNA. In this experiment, annealed sample was diluted with same buffer in which sample was prepared. The dilution was done by successive injection of 10μL PNA sample in 1.5mL of buffer in cell at interval of 20 seconds at 15°C.

5B.7.4 Mass Spectroscopy

5B.7.4a ESI- Mass spectroscopy

Electron spray ionization mass spectrometer (QSTARMultiView1.5.0 TOF-MS-IN) was used for characterization of G-quartet formation. The 2 μM solution of PNA in 1mL of methanol was used for recording mass spectra.

5B.7.4b MALDI-TOF- Mass spectroscopy

ABI-MALDI-TOF spectrometer was used to record mass of PNA by using Voyager spec electronic software. The mass of neat PNA was recorded in presence of matrices CHCA (α-cyano-4-hydroxycinnamic acid).
5B.8 REFERENCES


30. Dueholm, K. L.; Egholm, M.; Behrens, C.; Christensen, L.; Hansen, H. F.; Vulpius, T.; Petersen, K. H.; Berg. R. H.; Nielsen, P. E.; Buchardt, O. Synthesis of peptide nucleic acid monomers containing...
the four natural nucleobases: Thymine, Cytosine, Adenine, and Guanine and their oligomerization. 


### 5B.9 APPENDIX

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1. HPLC of PNA

PNA 1

PNA 3

mV Volts

PNA 4

mV Volts
2. MALDI-TOF spectra of PNA 1 and PNA 2

Voyager Spec #1 MC=>AdvBC(32.0.5.0.1) => NR(2.0.0) => AdvB

PNA 1

M⁺ = 857.85 (Calculated)
(4M+K+3H)²⁺ = 867.5 (Observed)

M⁺ = 857.85 (Calculated)
(4M+3K+Na)²⁺ = 893.0 (Observed)

Voyager Spec #1 MC=>AdvBC(32.0.5.0.1) => NR(2.0.0) => AdvB

PNA 2

(4M+2K+Na+H)⁴⁺ = 1134.5
4. MALDI-TOF spectra of PNA 1 and PNA 2

**PNA 1**

- \( M^+ = 1359.36 \) (Calculated)
- \( (4M+2K+2H)^+ = 1379.40 \) (Observed)

**PNA 3**

\( M^+ = 2463.48 \)

**PNA 4**

- \( (4M+2N+2H)^+ = 2475.48 \)
5. UV-Spectra of PNA 4 and cytosine monomer of aeg-PNA

Figure 10: First derivative curve of UV-melting profile profile of TCₙ sequences of aeg-PNA