CHAPTER 1

INTRODUCTION
1.1 Nucleic Acids: Chemical Structure

Nucleic acids are long, thread-like biopolymers, dominating the modern molecular science after the Watson-Crick discovery of the double helical structure of DNA.¹ Their vital roles are fundamental for the storage and transmission of genetic information within cells and contain all information required for transmission and execution of steps necessary to make proteins which are another important class of biopolymers, important for cellular function. Nucleic acids are made up of a linear array of monomers called nucleotides.

![Figure 1: Model of DNA](image)

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Self-recognition by nucleic acids through complementary base pairing is one of the fundamental processes in biological systems. DNA is the basic genetic material, consisting of two complementary strands held together by Watson-Crick hydrogen bonds through the donor-acceptor sites of the four nucleobases A, T, G and C (Figure 2). All biological functions of DNA take place via molecular recognition. The structure of the double-stranded DNA allows it to have a number of molecular interactions through electrostatic, intercalation and groove binding mechanisms (Figure 3) with other molecules such as proteins, drugs, metal ions etc. Such molecular recognitions mediated by weak non-covalent interactions are important in regulating the biological functions.

**Figure 2:** Base pair recognition by Watson-Crick Hydrogen bonding

**Figure 3** The three primary binding modes seen in DNA
1.2 Oligonucleotide as therapeutic agents

The concept of antisense oligonucleotides as potential therapeutic agents introduced by Zamecnik and Stephenson has aroused much interest in search of potent DNA mimics. Antisense oligonucleotides (Figure 4) recognize a complementary sequence on target mRNA through Watson-Crick base pairing and form a duplex (RNA-DNA hybrid) that is not processed by the protein synthesis machinery and hence would retard the expression of the corresponding protein. When target proteins are disease related, this will have a therapeutic value.

![Diagram of normal cell, antisense inhibition, and antigen inhibition](image)

**Figure 4:** Mechanism of action of antisense and antigen oligonucleotides

In another approach, the 'antigene strategy', (Figure 4) obstruction of gene expression can be achieved by binding of oligonucleotides to duplex DNA through Hoogsteen hydrogen bonds (Figure 5) leading to the formation of a triple helix. Thus the double stranded DNA itself can act as a target for the third strand oligonucleotides or analogs and the limitation for triplex formation is that it is possible only at homopurine stretches of DNA.¹⁰,¹¹
Figure 5 Triplexes involving Hoogsteen and Watson-Crick base pairing

In order for the triplex strategy to be effective, it requires oligonucleotides that effectively compete with ligands bound to DNA. Hence one of the major research goals is to achieve better triplex forming abilities with modified oligonucleotides. Triplex-forming oligonucleotides (TFOs) that bind DNA in a sequence-specific manner may provide an effective way to modulate selectively gene expression via transcriptional repression, mutagenesis and recombination.\textsuperscript{12-14} Binding of a TFO requires the presence of a relatively long and uninterrupted homopurine:homopyrimidine tract in DNA to ensure optimal stability and specificity of the triple helical complex.\textsuperscript{12-14}

The prime requisites for oligonucleotides to be effective as antisense oligonucleotides are (a) they should have high specificity to the RNA template, the sense strand, (b) improved cellular uptake and (c) resistance to cellular enzymes eg. Nucleases and proteases. The latter attribute (b and c) are also necessary for the access of antigen drugs.

Natural oligonucleotides have been shown to exhibit both antisense and antigenic properties in vitro.\textsuperscript{15} However, a serious drawback that limits the use of oligonucleotides as therapeutic agents is that they are rapidly degraded by nucleases in vivo.\textsuperscript{15a}
This has lead to several chemical modifications\textsuperscript{15} of the oligonucleotide structure to impart them the resistance towards cellular enzymes.\textsuperscript{16} The various possible sites of modifications in a nucleotide are shown in Figure 6.

1.3 Chemical Modifications of DNA

1.3.1 Phosphate modified linkages

The first generation backbone modifications and the most widely studied and effective analogue is the phosphorothioate. Although first introduced into DNA enzymatically by Eckstein and co-workers the phosphoramidite method followed by thiolation has greatly facilitated the synthesis of these ODNs.

In the first generation ‘antisense oligonucleotides’ (Figure 7) the phosphodiester backbone has been replaced by phosphorothioates 1\textsubscript{a}, phosphorodithioates 1\textsubscript{b}, methyl phosphonates 1\textsubscript{c}, hydroxymethyl phosphonates 2, phosphotriesters 3, and phosphoramidates 4 as shown in Figure 7. The backbone modifications displayed a greatly improved resistance towards nuclease\textsuperscript{17} and a therapeutic agent based on phosphorothioates already has been approved as a drug by US FDA.\textsuperscript{18} The chemical
modifications also modulate the binding ability of analogs to complementary sequences.

![Phosphate modifications](image)

**Figure 7** Phosphate modifications

### 1.3.2 Oligonucleotides with backbones not containing phosphorous

To increase the nuclease resistance and binding affinity, several modifications have been introduced. In which the four atom chain \( W, X, Y \) and \( Z \) in DNA phosphodiester backbone of 5 has been replaced by other combination of atoms (Figure 8). Only a few of these phosphodiester mimics have shown good binding but none showed the potential to be a good drug.

The second generation modifications comprises of backbone replacements involving elimination of phosphorous atom from the phosphodiester backbone (Figure 8). A common problem for all anionic analogs is the ineffective permeation of cellular membranes. Anionic ODNs are taken up by endosomes, but are unable to cross the endosomal membrane in the absence of cationic lipids.\(^{19}\) Based on this observation, neutral isosters of the phosphodiester linkage (Figure 8) have been designed.\(^{20}\) An
increasing number of neutral ODN analogs have been developed that do not contain stereogenic phosphorous centers.\textsuperscript{21} Substitution of the PO linkage with neutral SO\textsubscript{2} group resulted in a series of sulfanyl containing linkages. Sulfones \textsuperscript{1}\textsuperscript{22a}, sulfonamides \textsuperscript{2}\textsuperscript{22b} and sulfamates \textsuperscript{3}\textsuperscript{22c} (Figure 8) have been prepared as ODN analogs, but very little in vitro and cellular data are available.

![Phosphodiester linkage modifications](image)

**Figure 8:** Phosphodiester linkage modifications

The formacetal linkage \textsuperscript{4} exhibits somewhat inferior sequence specific binding affinities.\textsuperscript{23} Slightly increased RNA binding properties were observed with the 3'-thioformacetal \textsuperscript{5}\textsuperscript{24} and N-methylhydroxylamine \textsuperscript{6}\textsuperscript{25} linkages. 5'-thioformacetal containing ODNs do not hybridize as well as the unmodified ODNs to either RNA or DNA. Replacement of the PO backbone by amide groups 7-11 resulted in neutral and achiral linkages that were tested for RNA binding and nuclease stability. Analogs 9 and 11 were identified as having good binding affinity to the RNA targets and high stability towards
cellular nucleases. Modifications of the 2'-position with a methoxy provided ODNs with even greater binding affinity and nuclease stability. Carbonate 12 and carbamate 13 linkages have also been reported as replacements for the PO backbone. The carbonate linkage has been prepared as dimer, but no biochemical data is available. The 5'-N-carbamate linkage 13 is chemically stable, and cytidine hexamers were found to bind complementary DNA and RNA with high affinity. However the thymidine hexamer carbamate ODN bound nucleic acid targets with relatively low affinity. Replacement of the PO linkage with an acetylenic bond 14 resulted in decreased RNA affinity.

1.3.3 Boranophosphate DNA

These are designed by replacing one of the non-bridging oxygen atoms in the phosphodiester group of DNA with borane (BH₃) (Sood et al., 1990; Spielvogel et al., 1991; Sergueev et al., 1998) (Figure 9). The boranophosphate diester is isoelectronic with phosphodiesters, isosteric with the methylphosphonate group and is chiral. These negatively charged oligos are highly water soluble, but more lipophilic than DNA.

![Figure 9: Structure of boranophosphate DNA](image-url)
1.4 Sugar modifications

Sugar modifications have also been used to enhance stability and affinity. The $\alpha$-anomer of a 2'-deoxyribose sugar has the base inverted with respect to the natural anomer (Figure 10). ODNs containing (\(-\)anomer sugars are resistant to nuclease degradation and bind in a parallel mode to the RNA target.\(^30\)

\[\text{Figure 10} \text{ 2’ modified oligonucleotides}\]

1.4.1 Sugar skeleton modifications

Apart from the 2’modifications of sugar moiety the other modifications related to sugar skeleton are hexitol nucleic acids (HNA), D-Altrio nucleic acids (ANA), pentapyranosyl, and threofuranosyl (TNA).

\[\text{Figure 12} \text{ The family of four (4’→3’) penta-pyranosyl and threio fuanosyl oligonucleotides}\]
The first two modifications were discovered by Herdewijn et al.,\textsuperscript{31a} and the later two modifications were introduced by Eschenmoser group. The (l)-\(\alpha\)-lyxopyranosyl-(4’to3’)-oligonucleotide system a member of a pentopyranosyl oligonucleotide family containing a shortened backbone is capable of cooperative base-pairing and of cross-pairing with DNA and RNA. In contrast, the corresponding (d)-D-ribopyranosyl-(4’(3’))-oligonucleotides do not show base-pairing under similar conditions.\textsuperscript{31b}

1.5 Locked nucleic acids (LNA)

LNA is a bicyclic nucleic acid where a ribonucleoside is linked between the 2’-oxygen and the 4’-carbon atoms with a methylene unit (Figure 13). Locked Nucleic Acid (LNA) was first described by Wengel\textsuperscript{32} and Imani\textsuperscript{33a} et al in as a novel class of conformationally restricted oligonucleotide analogues.

![Locked nucleic acid (LNA)](image)

**Figure 13** Locked nucleic acid (LNA)

The design and ability of oligos containing locked nucleic acids (LNAs) to bind super coiled, double-stranded plasmid DNA in a sequence-specific manner has been described by Hertoghs et al.\textsuperscript{33b} for the first time. The main mechanism for LNA oligos binding to plasmid DNA is demonstrated to be by strand displacement. LNA oligos are
more stably bound to plasmid DNA than similar peptide nucleic acid (PNA) 'clamps' for procedures such as particle mediated DNA delivery (gene gun).

1.6 Carbocyclic nucleic acid

In carbocyclic nucleic acids, the furanose ring is completely replaced by saturated cycloalkane or cycloalkene rings. The replacement of the furanose moiety of DNA by a cyclohexene ring gives cyclohexene nucleic acids (CeNA).\textsuperscript{34}

![Figure 14 Carbocyclic analogues](image)

Incorporation of cyclohexenyl nucleosides in a DNA chain increases the stability of a DNA/RNA hybrid. CeNA is stable against degradation in serum and a CeNA/RNA hybrid is able to activate E. Coli RNase H, resulting in cleavage of the RNA strand. In case of carbocyclic pyranosyl analogues, cyclohexanyl-nucleic acid (CNA, Figure 14) was prepared in both enantiomeric (D/L) forms and D-CNA hybridizes to complementary RNA as compared to DNA with reduced affinity.
1.7 Morpholino nucleosides

So far, only a few attempts to replace the entire (deoxy) ribose phosphate backbone have been successful. One of this is the morpholino oligomer (Figure 15) wherein the monomers are linked through a carbamate linkage.\textsuperscript{35a} The second generation of morpholino DNA with a phosphoramide\textsuperscript{35b} linkage exhibited better stability \textit{in vitro} assay. To avoid the loss of bioactivity through major structural modifications and impart only the nuclease resistance, oligonucleotides having only 5' or 3' terminal modifications have been studied.

![Morpholino nucleoside](image)

\textbf{Figure 15} Morpholino nucleoside

These have a central core of unmodified DNA structure, but at the ends have either phosphorothioates or O2'-derivatized nucleosides that are resistant to 5'/3' exonuclease susceptibility. Although these exhibited favorable properties for antisense activity, many phosphorothioates showed non-antisense effects as well as leading to adverse clinical side reactions.
Table 1 Activities of various DNA analogues towards Rnase H and nuclease

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<tr>
<td>Peptide Nucleic acids</td>
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<td>++</td>
<td>NO</td>
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</tbody>
</table>

-Rapidly degraded by Nuclease, + Resistant to Nuclease, ++ NO nuclease degradation

1.8 Base modifications

Modification of the heterocyclic bases may enhance binding affinity with the complementary RNA fragment via WC bonding or with the duplex DNA via Hoogsteen bonding and may impart nuclease resistance. These modifications may have an impact on stacking interaction, hydrogen-bonding, donor/acceptor properties, pKa, steric or electrostatic effects. Selected modified bases are shown in the Figure 16.

![Modified Nucleobases](image)

Figure 16 Modified Nucleobases
1.9 Spectroscopic methods for studying the DNA duplexes, triplexes and DNA-PNA complexes.

A number of physicochemical techniques have been used to study the properties of duplexes and triplexes of nucleic acids. In the spectroscopic methods, UV absorbance and circular dichroism are very sensitive to the interactions of nearby bases which are stacked in the helical complexes. The principles are outlined in the following sections.

1.9.1 UV Spectroscopy

The two strands of a DNA helix readily come apart when hydrogen bonds between its paired bases are disrupted. This can be accomplished by heating a solution of DNA (called DNA-melting) or by adding acid or alkali to ionize its bases. The melting temperature (Tm) is defined as the temperature at which the duplex and the single strands exist in equal proportion (50% each). DNA double helix, in which the two strands are held together by several reinforcing bonds, is a highly cooperative structure. The double helix is stabilized by the stacking of bases as well as by base pairing. Hydrogen bonding contributes in the order of 5-15 kcal/mol/base pair to the stability of the nucleic acid helix (electronic or intrinsic energy). This contribution is selective, i.e., there is a preferential stability of the Watson-Crick guanosine-cytosine (G-C) pair relative to all other pairs. Stacking interactions contribute approximately the same amount as H bonding. The DNA melting is readily monitored by measuring its absorbance at a wavelength of 260 nm. A plot of absorbance against the temperature of measurement gives a sigmoidal curve in the case of duplexes and the midpoint of the transition gives the Tm. In case of triplexes, the first dissociation leads to the duplex (Watson-Crick duplex) and the third strand (Hoogsteen strand), followed by the duplex dissociation at higher temperature into two
single strands. The DNA triplex melting shows a characteristic double sigmoid transition with separate melting temperature (Tm) for each transition. The lower melting temperature corresponds to the triplex (duplex transition while the second transition gives the Tm of the duplex single strand (Figure 17).

**Duplex melting**

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Annealing
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Hypsochromic shift  Hyperchromic shift

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**Triplex melting**

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Annealing
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Figure 17 Schematic representation of thermal dissociation of DNA double and triple helices. Mid point of the transition corresponds to the melting temperature.
1.9.2 Circular dichroism

Circular dichroism\textsuperscript{37-38} is a technique to study the chiral molecules that have chromophores. In case of nucleic acids, the sugar units of the backbone provide chirality and the bases attached to the sugars are the chromophores. In the CD spectrum of a polynucleotide with stacked bases, the magnitude of CD signals is larger in the 260-280 nm region and significantly higher at 200 nm than that of individual bases. The base stacking in a chiral fashion induces the coupling of CD transitions leading to characteristic patterns. Single stranded ODNs are structurally less well defined than duplex ODNs and their CD signal is smaller. The CD pattern of the nucleic acid reflects the polymorphic forms of DNA such as A-, B-\textsubscript{\textast}, and Z- forms. The CD signature of B-form DNA as seen from longer to shorter wavelength is a positive band centered at 275 nm, a negative band at 240 nm, with a crossover at 258 nm (Figure 18). A-DNA is characterized by a positive CD band at 260 nm that is larger than the corresponding B-DNA band and a fairly intense negative band at 210 nm. Naturally occurring RNAs and RNA-DNA hybrids adopt this polymorphic form. The left handed Z-form DNA shows a negative CD band at 290 nm and a positive band at 260 nm.

![Figure 18 CD profiles of ssDNA and RNA](image)

Figure 18 CD profiles of ssDNA and RNA
1.10 Peptide Nucleic Acids:

PNA is peptide nucleic acid and was invented by Neilsen et. al 1991\textsuperscript{39} with physical properties similar to DNA or RNA but differing in the composition of its "backbone." PNA does not occur naturally and is synthesized for use in biological research and diagnostics.

![Figure 19 Chemical structure of DNA and PNA](image)

DNA and RNA have backbone containing deoxyribose and ribose sugars respectively, whereas the backbone of PNAs is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds (Figure 19). The various purine and pyrimidine bases are linked to the backbone by methylene carbonyl bonds. PNAs are depicted like peptides, with the N-terminus at the first (left) position and the C-terminus at the right. Since the backbone of PNA contains no charged phosphate groups, the binding between
PNA/DNA strands is stronger than between DNA/DNA strands due to the lack of electrostatic repulsion. Early experiments with homopyrimidine strands (strands consisting of only one repeated pyrimidine base) have shown that the Tm ("melting" temperature) of a 6-base thymine PNA/adenine DNA double helix was 31°C in comparison to an equivalent 6-base DNA/DNA duplex that denatures at a temperature less than 10 °C. Mixed base PNA molecules are true mimics of DNA molecules in terms of base-pair recognition. PNA/PNA binding is stronger than PNA/DNA binding.

Synthetic peptide nucleic acid oligomers have been used in recent years in molecular biology procedures, diagnostic assays and for potential therapeutics. Due to their higher binding strength it is not necessary to design long PNA oligomers for use in these roles, which usually require oligonucleotide probes of 20-25 bases. The main concern of the length of the PNA-oligomers is to guarantee the specificity. PNA oligomers also show greater specificity in binding to complementary DNAs, with a PNA/DNA base mismatch being more destabilizing than a similar mismatch in a DNA/DNA duplex. This binding strength and specificity also applies to PNA/RNA duplexes. PNs are not easily recognized by either nucleases or proteases, making them resistant to enzyme degradation. PNs are also stable over a wide pH range. Finally, their uncharged nature should make crossing through cell membranes easier, which may improve their therapeutic value.
1.10.1 Triplex formation with complementary DNA and RNA

Homopyrimidine peptide nucleic acids are known to form highly stable and sequence specific triplexes upon binding to complementary homopurine sites of ss and dsDNA.\textsuperscript{42} Displacement of the second, homopyrimidine strand takes place upon binding of PNA to dsDNA, forming a so-called P-loop. The extremely high stability of PNA\textsubscript{2}/DNA triplexes\textsuperscript{43,44} is at least partly due to the charge neutrality of the PNA backbone, that excludes electrostatic repulsion from the DNA molecule, and the presence of additional hydrogen bonds between the Hoogsteen strand of the PNA and the oxygen atoms of the DNA backbone. A unique property of PNAs is their ability to displace one strand of a DNA double helix to form strand invasion complexes, which is an additional attribute for their application as antisense/antigene agents. Such strand invasion process is inefficient or absent in DNA or any other DNA analogues studied so far. The strand invasion by PNA (Figure 20) is dictated by the formation of triple helical structures via Watson-Crick and Hoogsteen binding modes and is by far confined to the polypyrimidine PNA oligomers, which form PNA\textsubscript{2}:DNA triplexes.

1.10.2 Duplex formation with complementary DNA and RNA

PNAs obey Watson- Crick rules of hybridization with complementary DNA (Figure 20) and RNA. Antiparallel PNA-DNA hybrids are considerably more stable than the corresponding DNA-DNA complexes.\textsuperscript{40} The increased stability results in an increase in Tm of approximately 1 °C/base. Antiparallel PNA-RNA duplexes are even more stable compared to DNA-RNA hybrids, and PNA-DNA duplexes.\textsuperscript{41} The stability of parallel PNA-DNA and PNA-RNA duplexes is almost exactly the same as that of (antiparallel)
DNA-DNA and DNA-RNA duplexes respectively. An interesting aspect of PNA-DNA duplex formation is, that the Tm decreases with increase in salt concentration (ionic strength) which is contrast to that of DNA-DNA duplex, for which increase in Tm with salt concentration is observed. Base pair mismatches result in reduction of the Tm value by 8-20 °C. This discrimination is, in some cases, approximately double that observed for DNA-DNA duplexes.

![Diagram showing different types of PNA-DNA interactions](image)

**Figure 20** Schematic representation of PNA binding for targeting ds DNA, PNA (Thick line)

1.11 Structure of PNA complexes

Till date the three-dimensional structures of four PNA complexes have been established. The PNA-RNA and PNA-DNA duplex structures were determined by NMR methods, while the structures of a PNA₂-DNA triplex, PNA-PNA duplex and PNA-RNA duplexes were solved by X-ray crystallography (Figure 21).
Figure 21 Structures of PNA complexes shown in (a) side view (b) top view. The complexes from left to right PNA-RNA, PNA-DNA duplex, PNA-DNA-PNA and PNA-PNA (ref Erickson, M. Nielsen, P. E. Quart. Rev. Biophysics 1996, 29, 369.)

1.11.1 Structure of PNA-DNA duplexes

Almost complete structural information has been deduced from the NMR spectroscopic study of two antiparallel PNA-DNA duplexes (Figure 21). The DNA strand is in a conformation similar to the B-form, with a glycosidic anti-conformation, and the deoxyribose in C2'-endo form. A more recent NMR study showed that an octameric antiparallel PNA-DNA duplex contained elements of both A-form and B-form. The primary amide bonds of the backbone are in trans conformation and the carbonyl oxygen atoms of the backbone-nucleobase linker point towards the carboxy-terminus of the PNA strand. The CD spectra of antiparallel PNA-DNA complexes are similar to DNA-DNA spectra and indicate the formation of right handed helix.
1.11.2 Structure of PNA-RNA duplexes

The first report elucidating the structure of a nucleic acid-PNA hydrogen-bonded complex was reported by Brown et al.\textsuperscript{45} from solvent NMR solution structure study of hexameric PNA, GAACCTC, with complementary RNA. The study revealed that in PNA all bases form Watson-Crick base pairs, the glycosidic torsion angle in the RNA strand indicates an \textit{anti}-conformation, and the ribose sugars are in the 3'-\textit{endo} form. The RNA strand thus resembles an A-form structure. The tertiary amide bonds all are in the \textit{cis}-conformation. The carbonyl group of the tertiary amide in PNA backbone is isosteric to the C2'-hydroxyl group, which increases the solvent contact of the carbonyl oxygen atom. The CD spectra of antiparallel PNA-RNA duplexes also indicate the formation of a right-handed helix with geometry similar to the A- or B-form.

1.11.3 Structure of PNA\textsubscript{2}-DNA triplexes

The structure of PNA\textsubscript{2}-DNA triple-helix was resolved by the X-ray crystal structure analysis of the complex formed by bis-(PNA) and its complementary antiparallel DNA (Figure 21).\textsuperscript{47} The nucleobases of the PNA strand bind to the DNA by Watson-Crick pairing and Hoogsteen hydrogen bonding. The structure exhibited, both A-form and B-form DNA, and forms a “P-helix” with 16 bases per turn. The DNA phosphate groups are hydrogen bonded to the PNA backbone amide protons of the Hoogsteen strand. These hydrogen bonds, together with additional Vander-waal’s contact and the lack of electrostatic repulsion are the main factors responsible for the enormous stability of the triplex. The deoxyribose sugar adopted C3'-\textit{endo} conformation like A-form and bases lie almost perpendicular to the helix axis, which is characteristic of B-form DNA. The crystal structure is in agreement with the CD spectra of PNA\textsubscript{2}-DNA.
triple-helices measured in solution.\textsuperscript{50} The X-ray structure of self-complementary PNA-PNA duplex bears a strong similarity to the P-form of PNA\textsubscript{2}:DNA triplex (Figure 21).\textsuperscript{48}

Several general conclusions can be drawn from these structural studies, because of its flexible backbone, PNA to a great extent is able to adapt its partner's conformation in the complexes. In the PNA-RNA and PNA-DNA duplexes the oligonucleotide adopts close to its natural A and B-conformations respectively in terms of sugar puckering, while the helix parameters have both A and B-form characteristics. The PNA however, prefers a unique, different helix form, the P-form, which is adapted to some extent in the PNA\textsubscript{2}:DNA triplex and completely in the PNA-PNA duplex. This P-helix is very wide (28 Å diameter) and has a very large pitch (18 base pairs). In terms of base pair conformations it is a very regular helix, and the base pairs are virtually perpendicular to the helix axis.

1.12 Chemical modifications of PNA

The limitations of PNA include low aqueous solubility, ambiguity in DNA binding orientation and poor membrane permeability. Structurally, the analogues can be derived from modifications of ethylenediamine or glycine sector of the monomer, linker to the nucleobase, the nucleobase itself or a combination of the above. The strategic rationale behind the modifications (Dueholm et al., 1997) are (i) introduction of chirality into the achiral PNA backbone to influence the orientation selectivity in complementary DNA binding, (ii) rigidification of PNA backbone via conformational constraint to preorganize the PNA structure and to entropically drive the duplex formation, (iii) introduction of cationic functional groups directly in the PNA backbone, in a side chain substitution or at the N or C terminus of PNA, (iv) to modulate nucleobase pairing either
by modification of the linker or the nucleobase itself and (v) conjugation with ‘transfer’ molecules for effective penetration into cells. In addition to improving the PNA structure for therapeutics, several modifications are directed towards their applications in diagnostics.

Figure 22 Chemical modifications of PNA

Solubility was improved by introducing positive charges in the PNA monomers or by introducing ether linkages in the backbone. Charges were integrated into the PNA by replacing the acetamide linker with a flexible ethylene linker\textsuperscript{53} (Figure 23a) or by the attachment of terminal lysine residues\textsuperscript{54} (Figure 23c).

Recently, a novel class of cationic PNA (Figure 23b) (DNG/PNA) analogs has been reported.\textsuperscript{55} In these alternating PNA /DNG chimeras, the O-(PO\textsubscript{2})-O- linkage of nucleotide was replaced by strongly cationic guanidino [N-C(\textequiv N+H)-N] function. These
analogs with neutral and positive linker showed high binding affinity with DNA/RNA targets.

![Chemical Structures]

**Figure 23** Positively charged PNAs a) Flexible ethylene linker, b) Guanidium linkages, C) Lysine residues

Introduction of negative charges in the PNA backbone (Figure 24) improved aqueous solubility and showed good binding with both DNA and RNA.

However, these modified complexes were found to be less stable compared to the unmodified PNA sequences. In a similar approach pPNA-Hyp chimeras were also synthesized. Ether linked PNAs (Figure 24) showed co-operative binding with complementary antiparallel RNA in a sequence specific manner. Oligomers with 29b showed significantly lower affinity than 24a due to the increased flexibility of the side chain homologation. The replacement of these monomers with a-methylated derivatives led to significant enhancement in RNA binding affinity in case of 2R stereoisomer.
Whereas, presence of 2S isomer resulted in substantial decrease in Tm indicating that the substitution in case of S configuration may sterically interfere with RNA binding.

![Chemical Structures]

**Figure 24** Anionic PNAs

To ensure sufficient water solubility for RNA binding experiments, lysine was attached to the N/C terminus of these oligomers (Figure 25). This was followed by another similar report using oxy-PNA oligomers bearing adenine as nucleobase. Binding studies of these oligomers with complementary DNA showed all-or-none type hybridization and with high sequence specificity useful for the detection of single base mismatch DNAs.
1.13 Construction of bridged structures

Conformationally preorganized DNA analogues such as locked nucleic acids\textsuperscript{32,33} (LNA) in which the prelocked 3\textsuperscript{'}-endo sugar conformation, like in DNA:RNA hybrids, favors its binding with complementary DNA/RNA sequences.\textsuperscript{32} Other examples include conformationally frozen six-membered cyclohexenyl, hexitol,\textsuperscript{31} and altrito\textsuperscript{31} nucleic acids.

The cis and trans rotamers arising from the tertiary amide linkage in each PNA monomer leads to different PNA:DNA/RNA hybridization kinetics in parallel and antiparallel hybrids due to the high rotational energy barrier between cis and trans rotameric populations. Any favorable structural preorganization of PNA may activate a shift in equilibrium towards the preferred complex formation because of the reduced entropy loss upon complex formation, provided that the enthalpic contributions suitably compensate. This may be achieved if the conformational freedom in aeg-PNA is reduced by bridging the aminoethyl/glycyl acetyl linker arms to give rise to cyclic analogs with preorganized structure. Such modifications also restrain the fluctuation domains of the
aeg-PNA (glycyl and ethylene diamine) along with introducing the chirality into PNA monomeric units with the possibility of further fine-tuning the structural features of PNA to mimic DNA.

1.13.1 PNA with 5-membered nitrogen heterocycles

The naturally occurring amino acid trans-4-hydroxy-proline, a five-membered nitrogen heterocycle with useful substitutions and well known and easily manipulated stereochemistry,\textsuperscript{51, 52} is a versatile, commercially available starting material amenable for creating structural diversity to mimic the DNA/PNA structures. Many researchers have exploited trans-4-hydroxy-L-proline for the synthesis of a wide range of chiral, constrained and structurally preorganized PNAs.

1.13.1a Aminoprololy PNA

The introduction of a methylene bridge between β-carbon atom of the aminoethyl segment and the α" carbon atom of the glycine segment of the aeg-PNA resulted in 4-aminoprololy PNA, achieved by creation of two chiral centers (Figure 26).\textsuperscript{51}

![Figure 26](image-url)
Incorporation of these monomers at N terminus of aeg-PNA enhanced the inherent binding and also showed significant discrimination in the orientation of binding. The stability of such complexes decreases with increasing number of chiral prolyl units and homooligomers derived from each of the diastereomers (L and D-hydroxy proline) completely failed to form duplexes. Incorporation of alternating 4-aminoprolyl and glycine units\textsuperscript{62} stabilize the complex suggesting that in the homo-oligomer, internucleoside distances are too low.

1.13.1b Gly-Pro-Peptide PNA

Lowe \textit{et al.}\textsuperscript{63} used 4-Hydroxyproline for the synthesis of a novel chiral prolylglycyl PNA. The methylene bridge was inserted between the \(\alpha''\)-carbon atom of the glycine unit and the \(\beta'\)-carbon atom of the nucleobase linker of aeg-PNA (Figure 27).\textsuperscript{64,65} Oligomers derived of Prolylglycyl derived PNAs did not bind to the target complementary sequences due to the high rigidity in the structure, which has the tertiary amide nitrogen as part of a cyclic ring system on the backbone.

\begin{center}
\includegraphics[width=\textwidth]{figure27.png}
\end{center}

\textbf{Figure 27}
1.13.1c Aminoethylprolyl PNA (*aep*-PNA)

Bridging of the α"'-carbon atom of the glycyl segment with the β'-carbon atom of the nucleobase linker by a methylene group, accompanied by replacement of the side chain carbonyl with a methylene group, leads to *aep*-PNA (Figure 28). The flexibility in the aminoethyl segment of *aeg*-PNA was retained, unlike that in the proline-glycine PNA. The oligomers comprising 4-((S)-2-((S/R) *aep*PNA thymine units showed very favorable binding properties towards the target sequences without compromising the specificity. The stereochemistry at the C-2 centre did not bring any significant effect on the binding ability of the homooligomeric sequences. The mixed pyrimidine hairpin sequences with cytosine and N-7 guanine *aep*PNA 66 units exhibited directional discrimination in binding to parallel/antiparallel DNA sequences.

![Diagram showing the structure of *aeg*-PNA and *aep*-PNA](image)

**Figure 28**

1.13.1d Pyrrolidinone PNA:

A methylene bridge was inserted between the α carbon atom of the aminoethyl segment and the β'-carbon atom of the acetyl linker to the nucleobase of *aeg*PNA (Figure 29). 67 The hybridization properties of PNA decamers containing this analogue with
complementary DNA, RNA and PNA strands were investigated. The oligomers incorporating the (3S,5R) isomer were shown to have the highest affinity towards RNA in comparison with DNA.\textsuperscript{68} The fully modified decamer bound to rU\textsubscript{10} with a small decrease in binding efficiency with compared to \textit{aeg}-PNA.

\[ \text{Figure 29} \]

In order to get the best characteristics from both the \textit{aeg}PNA and the \textit{aep}-PNA, monomer was synthesized restoring the amide character to the pyrroldine ring nitrogen \textit{via} selective C5 oxidation of \textit{aep}-proline derivatized intermediate\textsuperscript{69} (Figure 20). \textit{aepone}-PNA oligomer stabilizes the derived triplexes with DNA but destabilizes the complexes formed with poly (rA).

\[ \text{Figure 29a} \]
1.13.1e Prolyl-(β-amino acid) peptide PNA

The conformational strain in the alternating proline-glycine backbone was released by replacement of the α-amino acid residue by different β amino acid spacers with appropriate rigidity. Novel pyrrolidinyl PNAs comprising alternate units of nucleobases modified with D-proline, either D/L aminopyrrolidine 2 carboxylic acid, (1R,2S)-2-aminocyclopentanecarboxylic acid or β alanine were synthesized (Figure 30).

![Chemical structures](image)

*Figure 30*

1.13.1f Pyrrolidine PNA and pyrrolidine PNA-DNA chimera

Insertion of a methylene bridge in *deg*PNA, linking the α-carbon atom of the aminoethyl segment and the β'-carbon atom of the tertiary amide linker, afforded the pyrrolidine PNA Figure 31. A fully modified (2R,4S) pyrrolidine PNA decamer formed very stable complexes with both DNA and RNA targets. The incorporation of the (2S,4S) thymine monomer into oligomers and mixed pyrimidine oligomers resulted in a decreased binding efficiency with the target DNA/RNA sequences. The (2R,4R) isomer was incorporated into a PNA:DNA dimer amenable to the synthesis of PNA:DNA chimeras. The chimeric PNA:DNA bound to the target DNA with decreased efficiency relative to the native DNA.
Introduction of the α'-β methylene bridge led to another pyrrolidine-PNA in which, unlike the previous analogues, the base is away from the pyrrolidine ring by one carbon (Figure 31a).\textsuperscript{72d,e} The enantiomeric pairs (2S,4S) and (2R,4R) formed antiparallel complexes with RNA much stronger than that of aeg-PNA or other diastereomers. The (2R,4S) pyrrolidinyl PNA analogues of all four bases lacked the discrimination effects with respect to either parallel/antiparallel or DNA/RNA binding.

Figure 31a
1.13.1g A cyclopentane conformational restraint for a peptide nucleic

Based on molecular modeling studies (S,S) cyclopentadiamine ring was used for conformational restraint of the C2-C3 dihedral angle of the PNA backbone. The \textit{trans} cyclopentane modification improves the stability of PNA-DNA triplexes and PNA-RNA duplexes for a poly-T PNA.\textsuperscript{73} Recently cyclopenty1 PNAs\textsuperscript{74,75} having \textit{cis} and \textit{trans} isomers have been reported (Figure 32). The results suggest that these have a stereochemistry dependent stabilization effect on binding both DNA and RNA. The \textit{cp}PNAs have better selectivity for mismatch DNA sequence and a higher binding to complementary DNA sequence than the unmodified PNA.

![Diagram of aeg-PNA and cp-PNA](image)

\textbf{Figure 32}

1.14 Thiazane and thiazolidine PNA

Bregant, et al.\textsuperscript{76} introduced rigidity by induction of ring containing thiazane and thiazolidine in the backbone of PNA (Figure 33). With the presence of sulfur in ring, both PNAs showed improved solubility, but, the derived PNA/DNA and PNA/RNA triplexes were destabilized by large extent.
1.15 PNA with six membered ring structures

The six-membered ring systems are conformationally more rigid when compared to their five-membered counterparts due to high-energy barriers between chair-boat conformations and preferred low-energy equatorial dispositions of substituents. The binding abilities of hexose sugar phosphate containing oligonucleotide have been extensively studied by Eschenmoser et al. The ability of morpholino, hexitol, and cyclohexene oligonucleotides to bind to DNA/RNA is well established and is dictated by the conformational preferences of the six membered ring structures. The ability of six-membered ring to impart more rigidification into PNA backbone significantly effects the hybridization properties of PNAs.

1.15a Glucose amine nucleic acids (GNA)

Novel glucosamine based oligonucleotide analogs (GNA) derived from conformationally constrained sugar scaffold have been synthesized (Figure 34). GNA derived oligomers are highly water-soluble.
In contrast to the homo DNA and hexose oligonucleotides, these bind to RNA to form stable complexes with an overall affinity comparable to that of DNA to RNA and are sequence selective. Thermodynamic parameters suggested an entropy gain in GNA due to the pre-organized scaffold.

1.15b Aminopipecolyl PNA, pip-PNA

Insertion of an ethylene bridge between the α″-carbon atom of the glycyl segment and the β carbon of the aminoethyl segment afforded the isomeric analogue 5-aminopipecolyl PNA (Figure 35). The preliminary studies indicated that the homothymynyl mixed-aegPNA sequences consisting the 5-aminopipecolyl unit form stable complexes with target DNA oligomers.

Figure 34

Figure 35

37
1.15c Aminoethyl pypecoly PNA (aepi-PNA)

Bridging the α"-carbon of the glycy1 unit with the β'-carbon of the nucleobase linker by a two-carbon ethylene bridge leads to the homologous analogue of aep-PNA, namely, aepi-PNA (Figure 36).[^80a]

This chiral six-membered analogue with (2S,5R) stereochemistry upon incorporation into aeg-PNA-T8 homoooligomer or into a mixed T/C PNA oligomer at different positions stabilized the corresponding PNA₂:DNA triplexes. This is interesting since it was suggested earlier[^80c] that six-membered piperidines are unlikely to stabilize the PNA:DNA complexes.

![Figure 36 aepi-PNA](image)

1.15d Piperidinone PNA

Introduction of an ethylene bridge between the α carbon atom and β' carbon atom in the aminoethyl and acetyl linker resulted in a six-membered ring piperidinone PNA (Figure 37).[^80c] (3R,6R) and (3S,6R) Adenine monomers were synthesized and incorporated into aegPNA which resulted in a large decrease in the duplex stability.
1.15e Cyclohexyl PNA (ch-PNA)

One of the earliest PNA modifications was to constrain the flexibility in the aminoethyl segment by introducing a cyclohexyl ring (Figure 38).\textsuperscript{81} PNA oligomers that contain cyclohexyl rings in the aminoethyl part showed similar hybridization properties as unmodified aeg-PNA with complementary DNA where as the (R,R) cyclohexyl moiety lacked such a property. The complexes formed by the two isomers were of the opposite handedness, as evident from CD spectroscopy. The synthesis of ethyl \textit{cis}-(1\textit{S},4\textit{R}/1\textit{R},2\textit{S})-2-aminocyclohex-1-yl-N-(thymin-1-yl-acetyl) glycinate was reported \textit{via} enzymatic resolution of the \textit{trans}-2-azido cyclohexanols. The crystal structure of the intermediate showed an equatorial disposition of the tertiary amide group, with the torsion angle $\beta$ in the range 60$^\circ$-70$^\circ$. UV-Tm experiments showed that (1\textit{S},2\textit{R}) isomer preferred to bind RNA and (1\textit{R},2\textit{S}) isomer showed higher affinity towards DNA in homothymine sequences leading to stereo discrimination in recognition of DNA and RNA.\textsuperscript{82}
1.15f Aminoethyl-α-amino-cyclohexanoic acid:

Rigidity was introduced into the aeg-PNA by replacing the glycyl segment in the backbone by α-amino cyclohexanoic acid (Figure 39).\textsuperscript{83} Incorporation of these monomers into oligomers and their DNA/RNA binding properties has not yet been reported.

![Aminoethyl-α-amino-cyclohexanoic acid](image)

**Figure 39** Aminoethyl-α-amino-cyclohexanoic acid

1.15g Morpholino PNA

The set of morpholino analogues with phosphonate esters, amide or ester linkages between the morpholino nucleoside residues was synthesized (Figure 40).\textsuperscript{84} Preliminary results indicated that amide-linked morpholino PNA\textsubscript{s} were better accommodated in the complexes than the ester or the phosphonate linked oligomers.

![Amide-morpholine and Ester-morpholine](image)

**Figure 40**
1.16 Modified nucleobases

Incorporation of modified nucleobases (Figure 41) would facilitate in understanding the recognition process between natural nucleobases in terms of various factors such as hydrogen bonding and inter-nucleobase stacking. Further, new recognition motifs may also have potential applications in diagnostics. 2-aminopurine\(^{85}\) hydrogen bonds with U and T in reverse Watson-Crick mode and being inherently fluorescent, it permits the study of the kinetics of PNA-DNA hybridization process. 2,6-Diaminopurine has increased affinity and selectivity for thymine.\(^ {86} \)

\[
\begin{align*}
\text{2-aminopurine} & \quad \text{2,6-diaminopurine} & \quad \text{pseudocytosine} & \quad \text{E-base} \\
\text{xanthine} & \quad \text{thiouracil} & \quad \text{N4-benzoylcytosine} & \quad \text{6-thioguanine}
\end{align*}
\]

Figure 41 Modified Nucleobases

Pseudo-isocytosine\(^{87}\) is an efficient mimic of protonated cytosine for triplex formation. E-base\(^{88}\) was designed for the recognition of T:A base pair in major groove and forms a stable triad with T in central position.

Other modifications like hypoxanthine\(^{89}\), N4-benzoylcytosine and thiouracil\(^{90}\) have also been incorporated as modified nucleobases. Combination of thiouracil in PNA chain
and 2,6-diaminopurine in DNA has been used in the “double duplex invasion” for the first time. 6-Thioguanine\(^9\) was found to decrease PNA-DNA hybrid stability

1.17 PNA-Conjugates

The covalent linking of PNA hybrids to various other molecules like peptides, DNA, RNA etc. has been exploited to overcome the limitations of PNA such as aggregation, solubility, cellular uptake and resistance to cellular enzymes and to impart better therapeutic applications.

1.17.1 PNA-DNA Chimera

The successful applications of the remarkable DNA binding properties of PNAs are sometimes (sequence/length dependent) hampered by their tendency to self-aggregate and poor aqueous solubility. Overcoming these limitations and imparting other abilities

![Figure 42 PNA-DNA Chimera](image)

for therapeutic applications such as cellular uptake, RNase H activation properties, have been addressed by designing covalent hybrids or chimaeras of PNA with DNA,
functional peptides and other effect or molecules. Three types of PNA-DNA chimeras (Figure 42) are in place (i) 5'-DNA-linker X- PNA -pseudo-392 (ii) pseudo-5'-DNA-linker X- DNA-3' and (iii) pseudo -5'-PNA-linker X- DNA-3'. Synthetic protocols have been developed with protecting groups compatible for carrying out online synthesis of both PNA and DNA to generate the chimeras. Several interesting properties were noticed in such covalent hybrids such as co-operative stabilizing effects against proteases and nucleases, enhanced water solubility and duplex/triplex stabilities dependent on the structure of chimerae and the linker. The linker can also be a fragment of DNA to generate PNA-(5')-DNA-(3')- PNA chimera which formed stable duplexes with both DNA and RNA with a lower stability than corresponding DNA:DNA and DNA:RNA duplexes.

1.17.2 PNA-Oligopeptide chimera

The conjugation of PNA with peptides containing basic amino acids like lysine (Figure 43) or arginine resulted in an increase in solubility as well as formation of stable complexes with DNA93 due to the interaction of the positively charged amino acids of the peptide with the negative anionic phosphate groups of DNA. A PNA-peptide chimera involving linking a 10-mer oligopeptide containing serine, which is a substrate for protein kinase A was used to assay phosphorylation of serine by kinase.94 The 5-mer PNA

![Figure 43 PNA-Peptide conjugation](image)

43
sequence H$_2$N-TAGGGCOOH linked to the N-terminus of various homooligomeric peptides of cationic amino acids lysine, ornithine and arginine are shown to inhibit human telomerase.

1.17.3 PNA-Liposome chimera:

A liposome is a spherical vesicle with a membrane composed of a phospholipid bilayer used to deliver drugs or genetic material into a cell. Liposomes can be composed of naturally-derived phospholipids with mixed lipid chains.

![Structure of PNA-adamantyl conjugation](image)

**Figure 44** PNA-adamantyl conjugation

The use of liposomes for transformation or transfection of DNA into a host cell is known as lipofection. In an effort to equip PNAs with a lipophilic tail that would confer liposome affinity, PNA lipid (adamantyl) conjugates (Figure 44) have been prepared and studied their liposome mediated cellular uptake. Liposomal delivery that is often used for transfection with oligonucleotides has, not been successfully used for PNA transport.

In recent years, some peptides that translocate over the plasma membrane in an energy and endocytotic independent manner, have been designed and synthesized. An extensively studied sequence, derived from the third helix of the Antennapedia homeodomain, is called penetratin. Penetratin or penetratin analogs have been used to
transport, moreover penetratin is not the only transport peptide that can mediate PNA transport.\textsuperscript{98} Antisense PNAs targeted to Escherichia coli genes can specifically inhibit gene expression, and attachment of PNA to the cell-permeabilizing peptide KFKFFKFFK dramatically improves antisense potency. The improved potency observed earlier was suggested to be due to better cell uptake.

1.18 PNA-Applications

PNA has attracted major attention at the interface of chemistry and biology because of its interesting chemical, physical, and biological properties and potential to act as an active component for diagnostic, molecular biological and pharmaceutical applications. According increase application fields of PNA, the development of cost-effective method of PNA synthesis becomes more important.

![Figure 45 PNA-Lipid conjugate](image)

1.18.1 Diagnostic applications

PNA has received great attention due to its several favorable properties including chemical and thermal stability, resistance to the nuclease and protease, stronger and faster binding affinity to the complementary nucleic acid, hybridization under low salt
concentration, and higher specificity and sensitivity to the single mismatch. These properties make PNA a powerful tool for diagnostic applications.

1.18.1a PCR clamping

Inhibition of PCR amplification of a specific target by 'PCR clamping' by which a PNA oligomer is used to inhibit the amplification of a specific target, e.g., by direct competition with a PCR primer has been used very successfully to detect and screen for single base-pair gene variants.\textsuperscript{100,101} PNAs were shown to serve as primers\textsuperscript{102} for certain DNA polymerases eg. Klenow fragment of DNA polymerase I (E coli) and reverse transcriptases, even though they have no phosphate residues to interact with polymerase, which were presumed to be necessary for binding via highly conserved amino acid residues. When PNA carrying a 5'-amino- 5'deoxythymidine at the carboxyl terminal end was used as the primer, there was no elongation of PNA primer to yield a PNA-DNA chimera, in cases of phage T4, phage T7 exo (Sequenase 2.0), \textit{Thermus aquaticus} and Deep Vent exo DNA polymerases, as well as HIV-1 reverse transcriptase. It was also found that the elongation of PNA primer was less efficient for the Polymerase (\textit{Thermus thermophilus}) and the reverse transcriptases from avian myeloblastosis virus (AMV) and moloney murine leukemia virus (M-MuLV).\textsuperscript{103}

1.18.1b Lightup probes

The method involves the detection of specific nucleic acid sequences in homogeneous solution using a probe with such a dye covalently attached to PNA (peptide nucleic acid) via an aliphatic linker (Figure 46). This so called LightUp probe\textsuperscript{104} upon hybridisation with complementary DNA, results in affording a strong enhancement of the
fluorescence. Up to a fifty-fold increase in fluorescence intensity has been achieved with a Lightup probes. The common binding mode of asymmetric cyanine dyes is intercalation between the bases of DNA.

![Diagram of Lightup probes](image)

Figure 46 Schematic representation of lightup probes

1.18.1c Molecular beacons (MB)

These represent probes carrying a fluorophore and a quencher at their termini. These probes are ingeniously designed to exhibit a fluorescence signal on binding to complementary targets, thus allowing the real-time quantitative monitoring of hybridization (Figure 47).
MB and other fluorescent probes have become very useful tools for DNA diagnostics. To use them, however, DNA must be in a denatured single-stranded (ss) form to allow Watson-Crick pairing of the MB to the target site. This requirement limits applications of MB. To overcome this, Heiko Khun et al reported PNA beacons for duplex DNA.\textsuperscript{105}

\textbf{1.18.1d Q-PNA:}

It is a new fluorogenic method for sealed-tube PCR analysis using a quencher-labeled peptide nucleic acid (Q-PNA) probe. The Q-PNA hybridizes to a complementary tag sequence located at the 5' end of a 5' fluorophore-labeled oligonucleotide primer, quenching the primer's fluorescence. Incorporation of the primer into a double-stranded amplicon causes displacement of the Q-PNA such that the fluorescence of the sample is a direct indication of the amplicon concentration. The Q-PNA is able to quench multiple primers bearing distinct 5' fluorophores in a single reaction.\textsuperscript{106}

\textbf{1.18.1e Micro Array:}

An array is an orderly arrangement of samples. It provides a medium for matching known and unknown DNA samples based on base-pairing rules and automating the
process of identifying the unknowns. In PNA micro array technology PNA probe is synthesized either in-situ or by conventional synthesis followed by immobilization on-chip. The array is exposed to the labeled DNA, and the identity and the abundance of the complementary sequence are determined. This technology is use full in gene discovery, disease diagnosis, pharmacogenomics and toxicogenomics.

1.19 Biological applications

1.19.1a PNA as anticancer agent (Inhibition of human telomerase)

PNA-peptide duplexes, which can penetrate into cells, have been used in anticancer applications. In this manner, telomerase activity in human melanoma cells and tumour specimens was inhibited by PNA conjugated with Antennapedia derived peptide (Antp) at nm concentrations.

![Figure 48 Design of PNA-peptide conjugates for inhibition of human telomerase](image)

Telomerase is almost ubiquitously expressed in human tumors. Human telomerase is a ribonucleoprotein that adds repeated units of TTAGGG to the ends of chromosomes known as telomeres. Human telomerase consists of a catalytic protein subunit the telomerase- reverse transcriptase component (hTERT), one or more additional proteins
and an integral RNA component (hTR) that serves as a template for the synthesis of telomeric repeats.\textsuperscript{109} The high telomerase activity found in tumor cells has aroused interest in its use as a potential target for anticancer chemotherapy.\textsuperscript{110} Inhibition of telomerase activity by conventional DNA oligomers and phosphorothioates showed poor sequence selectivity of these compounds.\textsuperscript{111} \textit{In-vitro} studies by Corey et al\textsuperscript{112} using Telomere Repeat Amplification Protocol (TRAP) showed that PNA can inhibit the telomerase activity by binding to RNA component of enzyme in picomolar to nanomolar range and the inhibition is due to sequence-selective PNA-mediated inhibition of telomerase activity.

In another approach PNAs were introduced into the cells by transfection using cationic lipids\textsuperscript{113} (lipofection). These PNAs were directed to non-template regions of the telomerase RNA that can overcome RNA secondary structure and inhibit telomerase by intercepting the RNA component prior to the holoenzyme assembly. The presence of cationic peptides at the N-terminus of the PNA resulted in enhanced inhibition of telomerase activity when targeted to the RNA template.\textsuperscript{114} In addition to these applications, PNAs have been exploited for plasmid labeling\textsuperscript{115} and duplex DNA capture,\textsuperscript{116} PNAs composed of trans-4-hydroxy-L-proline based monomers and phosphono derivatives were used to isolate mRNA free of genomic DNA.

1.19.1b PNA as delivery agent.

A major limitation of non-viral gene therapy is the low efficiency of gene transfer into target cells. PNAs can be use as adapters to link peptides, drugs or molecular tracers to plasmid vectors. According to the binding site, the coupling of PNAs to plasmids has no effect either on the transcription of genes included in the plasmid or on the plasmid's
physiological activities. Thus, this approach allows circumventing such barriers to gene transfer and fixing drugs to plasmids in order to enhance the gene delivery or tissue specific targeting. Using a triplex forming PNA as linker, Braden et al. observed an 8 times higher nuclear localization of a coupled nuclear localization signal (NLS) than did the free oligonucleotide (Figure 49).

![Figure 49](image)

**Figure 49** Schematic representation of the target site in the antisense Cy-5 oligonucleotide hybridizing to the sense PNA-NLS peptide. The PNA-NLS/oligonucleotide complex binds to the karyopherin-α/β proteins. The complex is then transported into the nucleus.

1.20 Antisense and antigenic applications

Originally conceived as agents for double stranded DNA binding, the unique properties of PNAs as DNA mimics were first exploited for gene therapy drug design. PNAs can inhibit transcription (antigene) and translation (antisense) of genes by tight binding to DNA or mRNA. PNA-mediated inhibition of gene transcription is mainly due to the formation of strand invaded complexes or strand displacement in DNA target. Several in vitro studies have shown that the binding of PNA or bis-PNA to dsDNA can efficiently block transcriptional elongation and inhibit the binding of transcriptional factors and helicases. Thus, Boffa et al. reported that PNA invasion of the tandem CAG
repeat of the human androgen receptor and the TATA binding protein, inhibits the transcription of these genes. Application of PNA as antisense reagents was first demonstrated in 1992. The nuclear microinjection of a 15-mer PNA targeting the translation start region of SV40 large T antigen mRNA inhibited transcription in cell extracts. This inhibition was both

![Diagram](image)

Figure 50 Transcription initiation from PNA:DNA strand displacement loops

Schematic representation of in vitro transcription from purified DNA fragments containing a single (a) or a double [two sites in cis] (b) or two sites in trans (c) PNA target showing the displacement loops and the start and possible directions of RNA synthesis indicated by arrows (full line: observed).

sequence-specific and dose-dependent. More recently, Mologni et al\textsuperscript{120} reported the effect of 3 different types of antisense PNA on the in vitro expression of PML/RAR gene. The PNA used targeted various sites involving AUG sites, coding sequences and the 5'-untranslated region (UTR).

1.20.1 PNA as artificial transcription promoters

In genetics, a promoter is a DNA sequence that enables a gene to be transcribed. The promoter is recognized by RNA polymerase, which then initiates transcription. In RNA synthesis, promoters are a means to demarcate which genes should be used for
messenger RNA creation and, by extension, control which proteins the cell manufactures. Homopyrimidine peptide nucleic acids (PNAs) form loop structures when binding to complementary double-stranded DNA by strand displacement. RNA polymerase recognizes these and initiates RNA transcription from PNA/double-stranded DNA strand displacement complexes at an efficiency comparable to that of the strong Eschenchia coflac UV5 promoter.

1.20.2 PNA conjugates as artificial restriction enzymes

A restriction enzyme (or restriction endonuclease) is an enzyme that cuts double-stranded DNA. This method involves peptide nucleic acid (PNA)-directed design of a DNA-nicking system that enables selective and quantitative cleavage of one strand of duplex DNA at a designated site, thus mimicking natural nickases and significantly extending their potential (Figure 51). This system exploits the ability of pyrimidine PNAs to serve as openers for specific DNA sites by invading the DNA duplex and exposing one DNA strand for oligonucleotide hybridization. The resultant secondary duplex can act as a substrate for a restriction enzyme, which ultimately creates a nick in the parent DNA.\(^{121}\)

The efficiency of this cleavage is more than 10 fold enhanced when a tandem PNA site is targeted and the site is trans oriented. Thus, PNA targeting makes the single strand specific nuclease S1 behave like a pseudorestriction end nuclease. Tethering a metal binding ligand such as Gly-Gly-His tripeptide to bis-PNA has been used to probe the structure of the DNA. Gly-Gly-His tripeptide placed on either the Watson-Crick or Hoogsteen bis-PNA strand forms a nickel complex that mediates cleavage at specific sites on the proximal displaced and hybridized DNA strands.\(^{122}\)
1.21 Present work

The above sections describe the current literature on Peptide Nucleic Acids with reference to structural variations and biological applications. The strand invasion property along with its high affinity and specificity to complementary DNA/RNA has prompted it as a useful tool in therapeutics and biology. However, due to limitations like poor aqueous solubility, self-aggregation, poor cellular uptake and ambiguity in binding orientation has limited further exploitation of PNA in practical applications. In order to circumvent these problems further modifications and the synthesis of newer PNAs to improve their properties, continue to elicit interest. To overcome the problems associated with PNA, there is considerable interest in chemical modifications of PNA backbone to enhance the selectivity as well as the discrimination towards the DNA/RNA and cellular uptake. To induct these properties into PNA, certain degree of chirality and rigidity is needed in PNA backbone.
In this connection Chapter 2 deals with the synthesis and characterization of novel \textit{amp} monomers (Figure 52) and their site specific incorporation in to \textit{aeg} PNA oligomers. The rational behind the synthesis of \textit{amp} monomers was to induce rigidity and chirality in to the \textit{aeg} backbone. The synthesis of these \textit{amp}-monomers can be achieved by bridging the backbone atoms with the side chain as shown in (Figure 53) to bridge the \(\alpha\)-carbon of the ethylenediamine with \(\alpha'\)-carbon of the glycine unit.

Figure 52 \textit{amp} (aminomethylprolyl) monomers

This modification restricts movement in both the aminoethyl and the glycyl segments of the \textit{aeg}-PNA and restrain the fluctuation region of \(\gamma\) and \(\delta\) torsion angles.

Figure 53

55
Chapter 3 describes the biophysical studies various *amp* PNA oligomers (octamers) were synthesized, by incorporation of (2S,5R), (2S,5S), (2R,5S), (2R,5R) and (2S,4S,5R) *amp* PNA monomers at specific sites to investigate the binding efficiency and selectivity towards DNA/RNA. The UV-melting studies were carried out with all synthesized oligomers (Table-1) and the Tm data was compared with the control aeg-PNA-T₈. The CD spectra of *amp* PNA single strands and corresponding complexes with complementary DNA were recorded.

Chapter 4 is divided in to two sections, section I presents a brief account of various synthetic methodologies to achieve the synthesis of 2,5-disubstituted pyrrolidines.

Section II describes the chemical studies towards synthesis of (2S,5S) Pyrrolidine dicarboxylic acid and (2S,4S,5R) Bulgecinine (Figure 54) by employing electrochemical oxidation to functionalize the C5 carbon of the proline and 4-hydroxyl proline respectively. These disubstituted pyrrolidine based natural products with structure close to *amp* PNA monomers (Figure 52).

![Figure 54](image-url)
1.22 Reference


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