CHAPTER 1
INTRODUCTION
# CHAPTER 1: INTRODUCTION

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1.1 INTRODUCTION

1.1.1 Deoxyribose nucleic acid (DNA)

DNA is present in the nucleus of organisms and contains the genetic instructions specifying the biological development of all cellular forms of life and many viruses. It is 52 years since Watson and Crick proposed the double-helical structure for duplex DNA (Figure 1). The molecular architecture of DNA consists of a double-stranded helix of uniform diameter, a with right handed twist. The main chemical constituents of DNA are the sugar-phosphate unit present on the outside of the helix which constitutes the backbone of each strand and the nitrogenous bases adenine (A), thymine (T), guanine (G) and cytosine (C) which are pointed towards the center of the helix. Hydrogen bonds between complementary bases pairs (A:T; G:C) hold the two strands together (Figure 1).

![Figure 1: Left. DNA double helix; Right. Nucleotide and hydrogen bonding between nitrogenous bases.](image-url)
The double helix of DNA is nature’s simple and elegant solution to the problem of storing, retrieving, and communicating the genetic information of living organisms. The specificity and the reversibility of the hydrogen bond formation between the complementary nucleobases are one of the most important characteristic features, which allow the strands of the double helix to be unwound and then rewound in exactly the same configuration. The construction of DNA and design of its analogues for use in the recognition of specific DNA and RNA sequences has emerged as intellectual and practical assignment. The recognition of DNA and RNA sequences by complementary oligonucleotides is a central feature of biotechnology and is important for hybridization-based biological applications. The study of such complementary recognition is possible with the widely used experimental techniques and diagnostic protocols. This is vital to make antisense- or antigen-based inhibition as a practical approach to therapeutics. Zamecnik and Stephensen were the first to propose the use of synthetic antisense oligonucleotides for therapeutic purposes. The specific inhibition is based on the Watson-Crick base-pairing between the heterocyclic bases of the antisense oligonucleotide and of the target nucleic acid.

Various cellular processes can be inhibited depending on the site at which the antisense oligonucleotide hybridizes to the target nucleic acid (Figure 2). For an 'antisense' oligonucleotide to be able to inhibit translation, it must reach the interior of the cell unaltered. The requirements for this are the stability of the oligonucleotide towards extra- and intra-cellular enzymes and equally important is its ability to traverse the cell membrane. After reaching the cytoplasm, it must bind the target mRNA with sufficient affinity and high specificity. In addition, it must possess an adequate half-life inorder to elicit its action. The toxicity of the oligonucleotide should also be negligible to the cell. In the conceptually similar 'antigene' approach, the therapeutic oligonucleotide is targeted to
the complementary duplex DNA sequence to inhibit DNA replication. In order to meet all the requirements of a successful medicinal agent, it is necessary for normal oligonucleotides to be chemically modified in a suitable manner.\(^5\)\(^7\)

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**Figure 2:** Principle of action of antisense and antigene oligonucleotides.

1.1.2 DNA analogues

To address the combined task of improving the rate, affinity or specificity of oligonucleotide recognition, while the enhancing membrane permeability and resistance to nuclease digestion, several chemical modifications of DNA have been attempted (Figure 3).\(^8\) These principally include modification of the sugar-phosphate backbone and/or the nucleobases. The modifications of phosphate moiety resulting in phosphorothioates,\(^9\) phosphorodithioates,\(^10\) methylphosphonates,\(^11\) phosphoramidates\(^12\) and phosphotriesters\(^13\) have lead to the first generation ‘antisense’ oligonucleotides. These have shown promising results, with one drug Vitravene (ISIS)\(^14\) based on the phosphorothioates already approved by FDA for retinitis.
Most of the chemical modifications include replacement of the phosphodiester linkage\(^{11}\) by other atom W-X-Y-Z chain (Figure 4). Among these modifications, a few bind to complementary DNA/RNA fairly well, but none have exhibited the potency to be an effective drug. The replacement of the ribose sugar by hexose or carbocycles has not been very successful\(^{15}\) in terms of specificity of binding/hybridization. However,
morpholino oligomers, where the monomers are linked through neutral carbamate linkages\(^1\) or through phosphoramidate linkages (Figure 5a), have shown promising antisense activity as they have superior permeability properties.

![Figure 5: a. Morpholino oligomers and b. LNA](image)

The locked nucleic acids (LNAs) invented by Wengel et al.\(^1\) were found to exhibit unprecedented stability of their complexes with complementary DNA and RNA. They are also stable to 3'-exonucleolytic degradation and possess good water solubility. LNAs are oligonucleotides containing one or more 2'-O, 4'-C-methylene-\(\beta\)-D-ribofuranosyl nucleotides (Figure 5b).\(^1\) The conformational preorganization of LNA is thought to be instrumental in imparting the enhanced binding affinity to DNA.

1.1.3 Peptide nucleic acid (PNA)

During the course of research on nucleic acid analogues, the novel aminoethylglycyl peptide nucleic acid (aeg-PNA)\(^1\) (Figure 6) has emerged as one of the most successful DNA mimics for potential therapeutic and diagnostic applications. PNA was originally designed and developed as a mimic of a DNA-recognizing, major groove-binding, triplex forming oligonucleotide. PNA is neutral, achiral DNA mimics
that bind to complementary DNA/RNA sequences with high affinity and sequence specificity.\textsuperscript{22} In PNA, the natural nucleobases are attached via methylenecarbonyl linkers to an uncharged, pseudopeptide backbone composed of repeating \(N\)-(2-aminoethyl)glycyl units. PNA hybridizes to complementary DNA/RNA sequences via specific base complementation to form duplexes for mixed sequences and triplexes for homopyrimidine/homopurine sequences. It is perhaps the most successful outcome of the chemical modification approach in nucleic acid analogues. The complexes of PNA with DNA/RNA sequences generally show thermal stabilities higher than the corresponding DNA-DNA/RNA complexes, depending on the sequence. PNAs and their analogues are also resistant to proteases and nucleases.\textsuperscript{23}

PNAs bind to complementary DNA/RNA in either parallel or antiparallel modes, the antiparallel mode being slightly preferred over the parallel one. The antiparallel mode refers to the instance when the PNA 'N' terminus lies towards the 3' end and the 'C' terminus, towards the 5' end of the complementary DNA/RNA oligonucleotide. Likewise,
the parallel mode of binding is said to occur when the PNA 'N' terminus lies towards the 5'-end with the 'C' terminus towards the 3'-end of the complementary DNA/RNA oligonucleotide (Scheme 1).^24

**Scheme 1:** Parallel and antiparallel modes of PNA-DNA binding

![Scheme 1](image)

**1.2 PROPERTIES OF PNA**

**1.2.1 Physiochemical properties**

Peptide nucleic acids and DNA have no functional groups in common except for the nucleobases and the backbone linkages are quite different. Hence, the physicochemical property of PNA differs significantly from its DNA counterpart.^25

**1.2.1a Chemical Stability**

In contrast to DNA, which depurinates on treatment with strong acids, while PNAs are completely acid stable.^26 It is thus possible to synthesize PNAs by using standard protecting groups from peptide chemistry. However, under basic conditions, the N-terminal amino groups can initiate transamidation reactions.

**1.2.1b Solubility**

The PNA is a neutral molecule with a tendency for self-aggregation and limited water solubility. However, the introduction of charged groups, such as a C-terminal lysine, very much improves the properties.^24,27 PNA solubility drops with increasing length and
purine:pyrimidine ratio and homoadenine PNA polymer is highly water soluble. Positive charges can also be introduced by modification of the PNA backbone, for instance by replacement of the glycine by a lysine unit. The incorporation of only two such groups greatly increases the solubility of the oligomers. Alternatively, negative charges can be introduced, which show enhanced water solubility.\textsuperscript{28}

1.2.1c Binding affinity

PNAs complex efficiently to complementary DNA and RNA. The strongest binding affinity of PNA is to itself. As PNAs are uncharged, they appear to be predestined to form triple helical structures, in particular PNA$_2$:DNA, while PNA:(DNA)$_2$ triple helices are rarely observed for only certain sequences.\textsuperscript{29} The formation of (PNA)$_2$:DNA hybrids is favored, through strand displacement in double-stranded DNA.\textsuperscript{30} If the sequence is inappropriate for the formation of triple helices, then PNA:DNA, PNA:RNA\textsuperscript{31} or, if applicable, PNA:PNA duplexes are formed.\textsuperscript{32}

1.2.2 Structure of PNA:DNA complexes

The complexes formed by PNA with double stranded DNA targets are similar to hydrogen bonding pattern of nucleobases A/T/G/C in DNA. Homopyrimidine PNAs bind to double stranded DNA targets not by triplex formation as observed with the corresponding homomorphous DNA, but by an unusual and interesting mechanism of strand invasion (Figure 7).\textsuperscript{33} Homopyrimidine poly-T PNA binds to the complementary poly-A DNA of poly (A:T) duplex forming a PNA$_2$:DNA triplex, by displacing the poly-T DNA strand which forms a P- or a D-loop structure (Figure 7a).\textsuperscript{34} Homopurine PNA can invade a target DNA duplex (although with a lower efficiency) and form a PNA:DNA duplex, without any triplex formation, via displacement of one strand of the original DNA duplex (Figure 7b).\textsuperscript{35} Recently, pseudo-complementary PNAs have been demonstrated to
invade the target DNA duplex by double-duplex invasion, forming two PNA:DNA duplexes wherein, each PNA strand pairs with its complementary DNA strand (Figure 7c). In contrast to the strand displacement mode of binding, cytosine-rich homopyrimidine PNAs bind to target DNA duplexes as a third strand forming a PNA₂:DNA triplex (Figure 7d). The complexes formed by PNA with either DNA or RNA are in general, thermally more stable than the corresponding DNA:DNA or DNA:RNA complexes. PNA:DNA duplexes are more stable when purines are in the PNA strand rather than in the DNA strand. Among the duplexes involving PNA, the generally observed thermal stability is of the order PNA:PNA > PNA:RNA > PNA:DNA.

Figure 7: Strand invasion complexes of PNA-DNA: a) triplex invasion; b) duplex invasion; c) double duplex invasion; d) triplex formation.

The first report elucidating the structure of a nucleic acid-PNA hydrogen-bonded complex was put forth by Brown et al. The NMR solution structure of a hexameric PNA, GAACTC, with complementary RNA revealed a 1:1 complex that is an antiparallel, right-handed double helix with Watson-Crick pairing similar to the 'A' form structure of RNA.
duplexes. The achiral PNA backbone was found to assume a distinct conformation upon binding to the chiral RNA. This was followed by a crystal structure of a PNA_{2}:DNA triplex.\textsuperscript{40} The PNA hairpin used was discovered to give a 'P-type' helix\textsuperscript{41} that differed from previously reported nucleic acid structures. This helix was underwound, with a base tilt similar to B-form DNA. The bases were even more displaced from the helix than in A-form DNA. The deoxyribose sugars all have a C3'-endo conformation with an average inter-phosphate distance of 6.0 Å, similar to the A-form DNA. This conformation is consistent with the observation that PNAs, including hairpins, bind more tightly to RNA than DNA. The tilt of the base triplets is however, similar to that of B-form DNA. Another structure of a PNA:DNA duplex derived from NMR\textsuperscript{42} data and an X-ray crystal structure of a PNA:PNA duplex\textsuperscript{43} were also subsequently reported. The PNA:DNA duplex was found to be very similar to the B-conformation of DNA, but preferred a unique different helix form, the P-form. This helix is very wide (28 Å diameter) with a large pitch (18 base-pairs) and the base-pairs are almost perpendicular to the helix axis.

In general, from the crystal structure indicates that the PNA backbone seems to be flexible enough to adopt that conformation present in PNA:DNA/RNA hybrid (Figure 8).\textsuperscript{41} The oligonucleotide in the PNA:RNA and the PNA:DNA duplexes adopts a conformation close to its natural 'A' and 'B' form respectively, in terms of sugar puckering, while the helix parameters have both 'A' and 'B' form characteristics.
1.2.3 Antigene and antisense applications of PNA

Peptide nucleic acids are promising as candidates for designing gene therapeutic drugs. They require well identified targets and a well-characterized mechanism for their cellular delivery. In principle, two general strategies can be adapted to design gene therapeutic drugs. Oligonucleotides or their analogs are designed to recognize and hybridize to complementary sequences in a gene wherein they would interfere with the transcription of that particular gene (antigene strategy). Alternatively, nucleic acid analogs can be designed to recognize and hybridize to complementary sequences in mRNA and thereby inhibit its translation (antisense strategy). PNA's are chemically and biologically stable molecules and have significant effects on replication, transcription, and translation processes, as revealed from in vitro experiments. Moreover, no general toxicity of PNA has so far been observed. As we shall see, PNA can interfere with the translation
process, and PNA:dsDNA strand displacement complexes can inhibit protein binding and block RNA polymerase elongation.46b

1.2.3a Inhibition of transcription

Strand displacement complexes with DNA can create a structural hindrance to block the stable function of RNA polymerase and are thus capable of working as antigenic agents. Nielsen et al.48 have demonstrated that even an 8-mer PNA-Tₘ is capable of blocking phage T3 polymerase activity. The presence of a PNA target within the promoter region of IL-2Ra* gene has been used to understand the effect of PNA binding to its target on this gene expression. The PNA-DNA triplex arrests transcription in vitro and is capable of acting as an antigenic agent.44 But one of the major obstacles to applying PNA as an antigenic agent is that the strand invasion or the formation of strand displacement complex is rather slow at physiological salt concentrations. Several modifications of PNA have shown improvement in terms of binding.50

1.2.3b Inhibition of translation

The basic mechanism of the antisense effects by oligodeoxynucleotides is considered to be either a ribonuclease H (RNase H)-mediated cleavage of the RNA strand in oligonucleotide-RNA heteroduplex or a steric blockage in the oligonucleotide–RNA complex of the translation machinery (Figure 9).51 Oligodeoxynucleotide analogs such as phosphorothioates activate RNase H and thus hold promise of working as antisense agents. However, they also exhibit some nonspecificity in their action. PNA/RNA duplexes, on the other hand, cannot act as substrates for RNase H. The antisense effect of the peptide nucleic acid is based on the steric blocking of either RNA processing, or translation.52 Triplex-forming PNAs are able to hinder the translation machinery at targets in the coding region of mRNA. However, translation elongation arrest requires a
PNA:RNA triplex and thus needs a homopurine target of 10–15 bases. In contrast, duplex-forming PNAs are incapable of this. Triplex-forming PNAs can inhibit translation at initiation codon targets and ribosome elongation at codon region targets.

![Figure 9: Mechanisms of antisense activity. (A) RNase H cleavage induced by (chimeric) antisense-oligonucleotides. (B) Translational arrest by blocking the ribosome. See the text for details.](image)

1.2.3c Inhibition of replication

It is also possible for PNA to inhibit the elongation of DNA primers by DNA polymerase. Further, the inhibition of DNA replication is feasible when the DNA duplex is subjected to strand invasion by PNA under physiological conditions or when the DNA is single stranded during the replication process. Efficient inhibition of extrachromosomal mitochondrial DNA, which is largely single-stranded during replication, has been demonstrated by Taylor et al. The PNA-mediated inhibition of the replication of mutant human mitochondrial DNA is a novel (and also potential) approach towards the treatment of patients suffering from ailments related to the heteroplasmy of mitochondrial DNA. Here wild-type and mutated DNA are both present in the same cell. Experiments have shown that PNA is capable of inhibiting the replication of mutated
DNA under physiological conditions without affecting the wild-type DNA in mitochondria.

1.2.4 Interaction of PNA with enzymes

1.2.4a Rnase H

The activation of the intracellular enzyme Rnase H by oligonucleotides to cleave RNA bound to deoxyribonucleic acid oligomers depends on the chemical structure of Rnase H stimulating oligonucleotides. The antisense oligonucleotide with Rnase H activity (e.g., phosphorothioate oligos) is considered a better antisense inhibitor than only steric block activity (methylphosphonates and hexitol nucleic acids).\textsuperscript{55} Despite their remarkable nucleic acid binding properties, PNAs generally are not capable of stimulating Rnase H activity on duplex formation with RNA. However, recent studies have shown that DNA-PNA chimeras (see in next section) are capable of stimulating Rnase H activity.\textsuperscript{56} On formation of a chimeric RNA double strand, PNA-DNA chimera can activate the RNA cleavage activity of Rnase H.\textsuperscript{56} Cleavage occurs at the ribonucleotide parts base paired to the DNA part of the chimera. Moreover, this cleavage is sequence specific in such a way that certain sequences of DNA-PNA chimeras are preferred over others. They are also reported to be taken up by cells to a similar extent as the corresponding oligonucleotides. Thus, PNA/DNA chimeras appear by far the best potential candidates for antisense PNA constructs.

1.2.4b Polymerase and reverse transcriptase

In general, there is no direct interaction of PNA with either DNA polymerase or reverse transcriptase.\textsuperscript{57} However, different groups have shown indirect involvement of PNA in inhibiting these enzyme functions (activity) under \textit{in vitro} conditions. For example, PNA oligomers are capable of terminating the elongation of oligonucleotide
primers by either binding to the template strand or directly competing with the primer for binding to the template. Primer extension by MMLV reverse transcriptase was shown to be inhibited by introducing a PNA oligomer. In another experiment, Nielsen et al. demonstrated that the primer extension catalyzed by Taq-polymerase can be terminated by incorporating PNA-T_{10} oligomer into the system. The latter can bind to DNA dA_{10} sequence in the template and thereby terminate the primer extension. In addition, the reverse transcription can be completely inhibited by a pentadecameric antisense PNA, using a molar ratio of 10:1 (PNA/RNA), without any noticeable RNase H cleavage of the RNA.

1.2.4c Inhibition of human telomerase

Telomerase is a ribonucleoprotein and possesses an RNA component that can be targeted to effect inhibition of enzyme activity. The designed PNAs were introduced into cells by transfection using cationic lipids. These PNAs were directed to non-template regions of the telomerase RNA. The problems due to the RNA secondary structure were overcome by intercepting the RNA component prior to holoenzyme assembly, leading to efficient inhibition of telomerase. The RNA template of telomerase was targeted by peptide conjugated derivatives of a PNA pentamer (Figure 10). It was shown that the presence of cationic peptides at the ‘N’ terminus of the PNA resulted in enhanced inhibition of telomerase activity. The inhibition was dependent on the specificity of PNA recognition. PNAs complementary to the 11-base template of hTR were shown to be potent inhibitors of human telomerase \textit{in vitro} and PNAs were found to be 10-50 times more efficient inhibitors in comparison with phosphorothioate oligomers.
Figure 10: Design of PNA-peptide conjugates for inhibition of human telomerase.

1.3 BIOLOGICAL APPLICATIONS OF PNA

1.3.1 In situ hybridization (PNA-FISH)

The efficiency of PNAs as hybridization probes has also been demonstrated in fluorescence in situ hybridization (FISH) applications. Because of their neutral backbone, PNA probes present in situ show a high specificity, require low concentrations and short hybridization times. The PNA-FISH technique was first developed for quantitative telomere analysis. Using a unique fluorescein-labelled PNA probe, Lansdorp et al. performed the in situ labelling of human telomeric repeat sequences and the data obtained allowed accurate estimates of telomere lengths. Subsequently, telomeric PNA probes were used in several in situ studies of cancer and ageing.

1.3.2 Solid-phase hybridization techniques

PNAs can be used in many of the same hybridization applications as natural or synthetic DNA probes but with the added advantages of tighter binding and higher specificity. This leads to faster and easier procedures in most standard hybridization techniques.
1.3.3 PCR and Q-PNA PCR

PNA probes have no direct interaction with DNA polymerase but PNAs can terminate the elongation of oligonucleotide primers by binding to the template or competing with the primers. Furthermore, PNA-DNA chimeras can be recognized by the DNA polymerase and can thus be used as primers for PCR reactions. The high affinity binding of PNAs has also been used for detecting single base pair mutations by PCR. This strategy, named PNA directed PCR clamping, uses PNAs to inhibit the amplification of a specific target by direct competition of the PNA targeted against one of the PCR primer sites and the conventional PCR primer. This PNA-DNA complex formed at one of the primer sites effectively blocks the formation of the PCR product. The procedure is so powerful that it can be used to detect single base-pair gene variants for mutation screening and gene isolation. More recently, novel automated real-time PCR has been developed using PNAs. In this method, named Q-PNA PCR, a generic quencher labelled PNA (Q-PNA) is hybridized to the 5'-TAG sequence of a fluorescent dye-labelled DNA primer in order to quench the fluorescence of the primer. During PCR, the Q-PNA is displaced by incorporation of the primer into amplicons and the fluorescence of the dye label is liberated.

1.3.4 Anti-cancer agent

PNA-peptide:DNA duplexes, which can penetrates into cells, have been used in anti-cancer applications. In this manner, telomerase activity in human melanoma cells and tumour specimens was inhibited by PNA conjugated with Antennapedia derived peptide (Antp) at nanomolar concentrations. Since telomerase is almost ubiquitously expressed in human tumours, the data point out the potential use of PNAs as anticancer drugs. Applications of PNAs as anticancer agents were also reported with PNA complementary to various sequences of bcl-2.
1.3.5 PNA as delivery agents

A major limitation of non-viral gene therapy is the low efficiency of gene transfer into target cells. PNAs can be used as adapter to link peptides, drugs or molecular tracer to plasmid vectors.67 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 barrier to gene transfer and fixing drugs to plasmid in order to enhance the gene delivery or tissue-specific targeting. Using a triplex forming PNA as linker, Brandén et. al.68 observed an eight times higher nuclear localization signal than did the free oligonucleotide.

1.4 BIOTECHNOLOGICAL APPLICATIONS OF PNA

1.4.1 PNA as a molecular-biological tool

Peptide nucleic acids also exhibit potential for use as a tool in biotechnology and molecular biology. Here we will mainly present indications of PNA becoming an important molecular biology tool.

1.4.1a Enhanced PCR amplification

The polymerase chain reaction (PCR) has been widely used for various molecular genetic applications including the amplification of variable number of tandem repeat (VNTR)69 loci for the purpose of genetic typing. PNA has been used to achieve an enhanced amplification of VNTR locus D1S80. Small PNA oligomers are used to block the template, and the latter becomes unavailable for intra- and interstrand interaction during reassociation. On the other hand, the primer extension is not blocked; during this extension, the polymerase displaces the PNA molecules from the template and the primer is extended toward completion of reaction. This approach shows the potential of PNA
application for PCR amplification where fragments of different sizes are more accurately and evenly amplified. Since the probability of differential amplification is less, the risk of misclassification is greatly reduced.

1.4.1b PNA-assisted rare cleavage

Peptide nucleic acids, in combination with methylases and other restriction endonucleases, can act as rare genome cutters. The method is called PNA-assisted rare cleavage (PARC) technique. It uses the strong sequence-selective binding of PNAs, preferably bis-PNAs, to short homopyrimidine sites on large DNA molecules, e.g., yeast or I DNA. The PNA target site is experimentally designed to overlap with the methylation/restriction enzyme site on the DNA, so a bound PNA molecule will efficiently shield the host site from enzymatic methylation whereas the other, unprotected methylation/restriction sites will be methylated. After the removal of bis-PNA, followed by restriction digestions, it is possible to cleave the whole DNA by enzymes into limited number of pieces.

1.4.1c Determination of telomere size

The conventional method for the determination of telomere length involves Southern blot analysis of genomic DNA and provides a range for the telomere length of all chromosomes present. The modern approach uses fluorescein-labeled oligonucleotides and monitor in situ hybridization to telomeric repeats. However, Lansdorp et al. shown a more delicate approach resulting in better quantitative results is possible by using fluorescein-labeled PNAs. This PNA-mediated approach permits accurate estimates of telomeric length. In situ hybridization of fluorescein-labeled PNA probes to telomeres is faster and requires a lower concentration of the probe compared to its DNA counterpart.
1.4.1d Nucleic acid purification

Based on its unique hybridization properties, PNAs can also be used to purify target nucleic acids. PNAs carrying six histidine residues have been used to purify target nucleic acids using nickel affinity chromatography. Thus, short PNAs can also be used as generic capture probes for purification of large nucleic acids. It has been shown that a biotin tagged PNA-thymine heptamer could be used to efficiently purify human genomic DNA from whole blood by a simple and rapid procedure.

1.4.1e Isolation of mRNA

PNAs composed of trans-4-hydroxy-L-proline monomers and phosphono PNA monomers were utilized to achieve improved recovery of mRNA molecules with secondary structure at their 3’ end as well as RNAs with short polyA tails. By this method, mRNA free of genomic DNA contamination could be isolated. PNA has also been utilized to capture ds DNA of a particular sequence of interest by affinity capture in the form of linear, non-supercoiled molecules. The classical biotin-streptavidin recognition is utilized for this process (Figure 11).
Figure 11: a The PD-loop consists of duplex DNA, an ODN and two PNA "openers". The ODN binds to the complementary DNA target via Watson-Crick pairing and carries biotin to provide capture on the affinity support. b The key steps of the procedure for dsDNA biomagnetic isolation.
1.4.1f **PNAs as artificial restriction enzymes**

PNAs in combination with a non-specific nuclease, such as S1 nuclease, have been used as artificial restriction enzymes to cut target DNA at desired positions.\(^7\) Double stranded DNA is cleaved at a site created by PNA strand displacement (Figure 12). This cleavage efficiency is enhanced more than 10 fold when a tandem PNA site is targeted, and additionally if this site is in *trans* rather than *cis* orientation. Thus, the single strand specific nuclease S1 behaves like a pseudo restriction endonuclease. The tethering of an artificial nuclease like Gly-Gly-His to PNA exhibits a similar effect. Binding to complementary DNA and in the presence of Ni, cleaves the DNA duplex in its *proximity*.

![Figure 12: Schematic model for PNA-targeted S1 nuclease ds cleavage of DNA. (a) Single target. (b) Double target in *cis* orientation. (c) Double target in *trans* orientation. Arrows indicate S1 attack.](image)

1.4.1g **PNA as primordial genetic material**

The currently widely-accepted theory of the origin of biomolecular life implies that RNA evolutionarily preceded all the other biomolecules, *viz.*, DNA, proteins and carbohydrates.\(^7\) The theory gained credibility because RNA provides the link between DNA and proteins. Moreover, RNAs have been discovered to possess catalytic activity (Ribozymes). However, RNA is highly unstable and it is suspectable how prebiotic life could have relied on such a fragile molecule as its genetic material. Miller\(^7\) and Oro demonstrated that under the conditions prevailing on the primitive earth, nucleobases and
amino acids can be easily obtained, whereas, ribose and nucleosides are extremely
difficult to obtain under the same conditions. Recently,\textsuperscript{77} it was shown that the PNA
precursors are possible prebiotic products. In addition, information transfer between PNA
and RNA is also possible, although with low efficiency.

\subsection{Plasmid labeling}

In this approach, a highly fluorescent plasmid DNA is made by hybridizing
fluorescently labeled PNA to it. Importantly, the plasmid is neither functionally nor
conformationally altered. For this, a PNA homopurine binding site was cloned into a
reporter gene plasmid in a region that is not involved in transcription regulation so that
PNA-based probes could bind to the plasmid without affecting reporter gene expression.\textsuperscript{78}
The PNA clamp conjugated to reporter molecules like biotin, fluorescein or rhodamine did
not affect the supercoiled conformation, nuclease sensitivity or transcription ability of the
plasmid.\textsuperscript{79} This method was employed to study the biodistribution of the plasmid upon
transfection into cells. By using this system in a plasmid expressing green fluorescent
protein (GFP), it was possible to simultaneously follow the delivery of the DNA and the
expression of its transgene in real time in living cells.

\subsection{PNA as a diagnostic tool}

The high-affinity binding of PNA oligomers has led to the development of new
applications of PNA, especially as a diagnostic probe for detecting genetic mutations;
applications are possible for the detection of genetic mutation and mismatch analysis that
can use its unique hybridization properties. The following sections will highlight some of
the recent developments related to the use of PNA as a probe to detect genetic mutations
and corresponding mismatch analysis confirming its potential as a diagnostic tool for
clinical applications.
1.4.2a Single base pair mutation analysis using PNA directed PCR clamping

Amplification of the target nucleic acid by the PCR technique is considered an important step for detection of genetic diseases. The higher specificity of PNA binding to DNA, higher stability of a PNA–DNA duplex compared to the corresponding DNA–DNA duplex, and its inability to act as a primer for DNA polymerases is the basis for this novel technique. The strategy includes a distinct annealing step involving the PNA targeted against one of the PCR primer sites. This step is carried out at a higher temperature than that for conventional PCR primer annealing where the PNA is selectively bound to the DNA molecule. The PNA/DNA complex formed at one of the primer sites effectively blocks the formation of a PCR product. PNA is also able to discriminate between fully complementary and single mismatch targets in a mixed target PCR. Sequence-selective blockage by PNA allows suppression of target sequences that differ by only one base pair. Also, this PNA clamping was able to discriminate three different point mutations at a single position, as demonstrated in a model system by O’rum et al. Thiede et al. have reported a novel approach for simple and sensitive detection of mutations in the ras proto-oncogenes. A schematic representation of the strategy for the PCR cycle involving PNA-directed for mutation analysis using PNA-directed PCR clamping is shown in Figure 13. In the case of the normal (wild-type) DNA, the bound PNA will sterically hinder annealing of a partially overlapping primer sequence, thus preventing the normal sequence from appropriate PCR amplification. In the case of mutant alleles, the melting temperature of the PNA/DNA is reduced and the primer can out-compete PNA annealing to carry on preferential amplification of mutant sequences.
1.4.2b Screening for genetic mutations by capillary electrophoresis

In capillary electrophoresis, the separation is generally carried out using a long, thin fused silica capillary (typically 50–80 cm long, inner diameter : 10–300 mm). A portion of the coating, close to one end of the capillary, is removed to allow optical detection of the analyte. The analyte passes the detection window during a separation process and can be visualized by online automated UV, or laser-induced fluorescence (LIF) detection systems. A novel diagnostic method for the detection of genetic mutation using PNA as a probe for capillary electrophoresis has been reported by Carlsson et al. The method is sensitive enough to detect a single mismatch in the sample DNA.
1.4.2c **PNA as a probe for nucleic acid biosensor**

The DNA biosensor technology holds promise for rapid and cost-effective detection of specific DNA sequences. A single-stranded nucleic acid probe is immobilized onto optical, electrochemical, or mass sensitive transducers to detect the complementary (or mismatch) strand in a sample solution. The response from the hybridization event is converted into a useful electrical signal by the transducer. The use of PNA as a novel probe for sequence-specific biosensors holds great promise for use as the recognition layer in DNA biosensors.86

1.4.2d **BIAcore technique**

The PNA hybridization and corresponding mismatch analysis can be studied using a BIAcore (biomolecular interaction analysis) instrument,87 which can evaluate a real-time biomolecular interaction analysis using optical detection technology. The probe molecule is attached directly to the surface and the analyte molecule is free in solution. A biotinylated PNA \([\text{biotin-(egl)}_3-\text{TGTACGTCAACA}-\text{NH}_2]\) probe was immobilized on the surface by using the strong coupling between biotin and streptavidin. Short spacer molecules, e.g., mercaptohexanol, can be used together with the ligand (probe) to form the PNA monolayer at the top of the sensor (gold) surface to prevent DNA from being nonspecifically adsorbed to the surface.

1.4.2e **Quartz crystal microbalance (QCM)**

The quartz crystal microbalance has been used for some time to monitor mass or thickness of thin films deposited on surfaces, study gas adsorption and deposition on surfaces in the monolayer and sub monolayer regimes.88 A recent report by Wang and co-workers89 on quartz crystal microbalance biosensor, based on peptide nucleic acid probes,
showed that the system can differentiate between a full complementary and single mismatch oligonucleotide.

**1.4.2f MALDI-TOF mass spectrometry**

MALDI-TOF mass spectrometry has been used successfully in PNA-based diagnostic research to study discrimination of single-nucleotide polymorphisms (SNPs) in human DNA. Human genomic and mitochondrial DNA contains many SNPs that may be linked to diseases. Rapid and accurate screening of important SNPs, based on high-affinity binding of PNA probes to DNA, is possible by using MALDI-TOF mass spectroscopy.

**1.4.2g Potentiometric measurements**

Wang et al. have also reported the use of PNA as a recognition probe for the electrochemical detection of the hybridization event using chronopotentiometric measurements. The method consists of four steps: probe (PNA) immobilization onto the transducer surface, hybridization, indicator binding, and chronopotentiometric transduction. A carbon paste electrode is in this process containing the immobilized DNA or PNA probe. The hybridization experiment was carried out by immersing the electrode into the stirred buffer solution containing a desired target, followed by measurement of signal.

**1.4.2h PNA microarray**

The basic requirement for the array system is the ability of all different probes to hybridize to their target sequences with high specificity at single temperature. As PNAs are neutral, they can hybridize with nucleic acids in absence of counter ions needed to stabilize pure nucleic acid duplexes. It has been demonstrated that PNA probes can effectively discriminate between single base mismatches in the target sequence at temperature near the optimum for hybridization. They can also be used to analyze and
roughly quantify the amount of target molecules over a considerable concentration range. PNA arrays can be reused much more often than conventional oligonucleotide array, since the PNA molecules are extremely stable under conditions, which natural DNA cannot withstand.

1.4.2i Antiviral PNAs

One of the key enzymes in the life cycle of retroviruses (such as HIV), reverse transriptase, is very sensitive to PNA “Antisense Inhibition’. Reverse transcription of the RNA template is effectively arrested by PNA oligomers bound to the template. This finding has raised hope that DNA antiviral drugs could be developed with the demonstration that HIV replication in cell culture can be inhibited by PNA targeting gag-pol gene. However, very high PNA concentrations were required emphasizing the need of an efficient cell delivery system for PNA.

1.4.2j Antibacterial PNAs

Inhibiting translation through directly interfering with the ribosome will shut down all protein synthesis, providing a very potent antibiotic strategy. Good and Nielsen demonstrated that PNAs targeted to two regions of ribosomal RNA, the alpha-sarcin loop and the peptidyl transferase center, were capable of causing cell death in bacterial colonies. Homopyrimidine bisPNAs were more effective than the monoPNAs, indicating that triplex formation was important for the observed antibiotic activity. It has been reported that 9-12 mer PNAs attached to the cell wall/membrane-active peptide (KFF)3K provide improvement in antisense potency in E. Coli amounting to two orders of magnitude while retaining target specificity. Peptide-PNA conjugates targeted to rRNA and to mRNA encoding the fatty acids biosynthesis protein Acp, prevented cell growth of E. coli K12 without any apparent toxicity to human cells. This indicates that the peptide
can be used to carry antisense PNA agents into bacteria. Such peptide-PNA conjugates open exciting possibilities for anti-infective drug development and provide new tools for microbial genetics. These results bear promise that PNA could be developed as ‘generic antibiotics’.

1.4.3 Cellular uptake of PNA

PNAs do not readily enter cultured cells unless present at high concentrations in the media and unlike DNA/RNA, they cannot be complexed directly with cationic lipids because they are uncharged. However, Corey et. al.\textsuperscript{101} have reported a novel method for \textit{in vitro} cellular delivery of PNAs using cationic lipid. The cationic lipid is capable of associating with the negatively charged phosphodiester backbone of DNA and RNA and fusion with the cell membrane allows the oligonucleotides to enter into the cell. Desired PNA oligomers are hybridized to overlapping oligonucleotides and the complex is mixed with cationic lipid. The cationic lipid-DNA-PNA complex thus formed can be internalized and the partially hybridized PNA is imported into the cell. Cellular uptake of PNAs can also be achieved by the attachment of peptide sequence that promotes translocation across cell membranes. Peptides such as Trojan peptide and penetratin have been used as carriers for cellular delivery of PNA.\textsuperscript{98} Another strategy that has been adapted to improvise the delivery of PNA \textit{in vitro} is to incorporate it into delivery vehicles (vesicles), e.g. liposomes. Nucleic acid- PNA chimeras are reported to be taken up even at lower extracellular concentration (1μM), so PNA-DNA chimera may be better antisense agent.\textsuperscript{102} At higher concentrations of PNA, cytotoxic effects could also be observed.
1.5 CHEMICAL MODIFICATION OF PNA

The structure of the classical PNA monomer has been subjected to a variety of rational modifications with the aim of understanding the structure-activity relations as well as obtaining PNA oligomers with specifically improved properties for various applications in medicine, diagnostics and molecular biology.\(^{103}\) The limitations of PNA for such applications include low aqueous solubility, ambiguity in DNA binding orientation and poor membrane permeability. Structurally, the analogues can be derived from modifications in the ethylenediamine or glycine part of the monomer, linker to the nucleobase, the nucleobase itself or a combination of the above. The strategic rationale behind the modifications are (i) introduction of chirality into the achiral PNA backbone to influence the orientational selectivity in complementary DNA binding, (ii) rigidification of PNA backbone \(\text{via}\) conformational constraint to pre-organize the PNA structure and 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 the PNA to improve water solubility, (iv) modulate nucleobase pairing either by modification of the linker or the nucleobase itself for effective binding at physiological conditions and (v) conjugation with ‘transfer’ molecules for effective penetration into cells. In addition to improving the PNA structure as above for therapeutics, several modifications are directed towards their applications in diagnostics. Some of the modifications are discussed below.

1.5.1 Construction of non-bridged PNA structures

To improve the binding affinity and to enhance aqueous solubility of PNA while retaining its basic acyclic structure, various chemical modifications have been carried out. Improvement of aqueous solubility of PNAs has been achieved by the introduction of charges within the molecule or by the introduction of ether linkages in the backbone (Figure 14).\(^{104}\)
Making PNA anionic also aided in increasing the water solubility as in the case of the phosphonate analogs, but was accompanied by a decrease in the binding affinity to complementary nucleic acid sequence (Figure 15).\textsuperscript{104b} The chiral versions of these analogs similar to original PNAs led to excellent aqueous solubility properties. PNAs composed of monomers derived from serine and homoserine coupled by ether linker with glycine or alanine, were able to bind sequence specifically to RNA, though with much weaker affinity.

Novel class of cationic PNA (DNG/PNA) which binds to DNA/RNA targets with high affinity has been also reported (Figure 16a).\textsuperscript{102} In another report guanidium functional group was introduced into the PNA backbone, which exhibited remarkable cellular uptake properties while maintaining Watson-Crick recognition with complementrary DNA strand (Figure 16b).\textsuperscript{103}
Another type of modification involved interchange of various CO and NH groups on the peptide linkages leading to retro-inverse, peptoid and heterodimeric analogs (Figure 17). Except for the heterodimer analogue (Figure 17c), these exhibited a lower potency for duplex formation with complementary DNA/RNA suggesting that in addition to geometric factors, other subtle requirements such as hydration and dipole-dipole interactions that influence the environment of backbone, may be involved in effecting efficient PNA:DNA hybridization.

In another case, PNA backbone was extended by inserting a methylene group either in aminoethyl part or in the linker to the nucleobase (Figure 18). The thermal stability of the hybrids between these PNA oligomers and complementary DNA oligonucleotide was significantly lower than that of the corresponding aegPNA. However, the sequence selectivity was retained. Thymidyl decamers with these modified units were
unable to hybridize to the complementary dA_{10} oligonucleotide, while PNA decamer containing only ethylenecarbonyl linkers between the nucleobases showed weak affinity for complementary DNA.\textsuperscript{107}

![Figure 18: Backbone and side chain extended PNA.](image)

1.5.2 Construction of bridged PNA structures

Any favorable structural reorganization of PNA may trigger a shift in equilibrium towards the desired 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 curtailed by bridging the aminoethyl/glycyl acetyl linker arms to give rise to cyclic analogs with preorganized structure. Additionally, the introduction of chemical bridges into aeg-PNA to provide cyclic structure may help in controlling the rotameric population by fixing the nucleobase orientation. Such modifications also introduce chiral centers into PNA monomeric units with the possibility of further fine-tuning the structural features of PNA to mimic DNA.\textsuperscript{108}

1.5.2a PNA with 5-membered nitrogen heterocycles

The naturally occurring amino acid \textit{trans}-4-hydroxy-L-proline, a five-membered nitrogen heterocycle with useful substituents and easily manipulated stereochemistry, is a versatile, commercially available starting material amenable for creating structural diversity to mimic the DNA/PNA structures. Many researchers have exploited \textit{trans}-4-
hydroxy-L-proline for the synthesis of a wide variety of chiral, constrained and structurally preorganized PNAs. Depending on the construction strategy and the presence or absence of the tertiary amine group in monomers; the modifications afford either positively charged or uncharged cyclic PNA analogues.

**Aminoprolyl 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 aegPNA resulted in 4-aminoprolyl PNA, with the introduction of two chiral centers (Figure 19). Upon partial substitution of these monomers into PNA oligomer, these exhibited tendency to hybridize with nucleic acids similar to that of unmodified PNA. Interestingly, inclusion of even one 4-aminoproline unit into a PNA sequence, either at the N-terminus or in the interior resulted in a very interesting CD profile and lead to stabilization of derived PNA-DNA hybrids simultaneously effecting 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 completely failed to form duplexes. In another report, alternating 4-aminoprolyl and glycine units stabilize the complex suggesting that in the homo-oligomer, inter-nucleoside distances are too low.

![Figure 19: Aminoprolyl PNA.](image)

**Gly-Pro-Peptide PNA:** The methylene bridge was inserted between the α-carbon atom of the glycine unit and the β'-carbon atom of the nucleobase linker of aeg-PNA (Figure...
Unlike other PNAs, this has a tertiary amide group with the amide nitrogen part of a cyclic ring system on the backbone. This leads to highly rigid structures that are not poised for effective duplex formation.

\[\text{Aminoethylprolyl (aep) PNA}\] The replacement of the tertiary amide carbonyl on the backbone by a methylene group relieves strain to generate aep-PNA (Figure 21). These show remarkable biophysical properties in terms of triplex stabilities. Hitherto unprecedented higher melting of the derived PNA:DNA hybrids reflected very significantly enhanced DNA affinity while retaining the base pair discriminating power. The mixed pyrimidine hairpin sequences with cytosine and N-7 guanine aepPNA units exhibited directional discrimination in binding to parallel/antiparallel DNA sequences.
**Pyrrolidinone (pyrr) 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 aegPNA (Figure 22). The synthesis of all the four diastereomers of adenin-9-yl-pyrPNA was accomplished and the oligomers incorporating the (3S,5R) isomer were shown to have highest affinity for RNA compared to DNA. The fully modified decamer bound to rU10 with a small decrease in the binding efficiency relative to aegPNA.

![Figure 22: Pyrrolidinone PNA.](image)

**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 23).

![Figure 23: (a) prolyl-2-amino cyclopentanecarboxylic acid, (b) prolyl-β-alanine, c) prolyl-D/L-aminopyrrolidine carboxylic acid.](image)
**Pyrrolidine PNA and pyrrolidine PNA-DNA chimera:** Insertion of a methylene bridge in aegPNA, linking the α-carbon atom of the aminoethyl segment and the β-carbon atom of the tertiary amide linker, afforded the pyrrolidine PNA (Figure 24a). 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 (Figure 24b). The chimeric PNA:DNA bound to the target DNA with decreased efficiency relative to the native DNA.

![Figure 24: (a) Pyrrolidine PNA. (b) Pyrrolidine PNA-DNA chimerae.](image)

**A cyclopentane conformational restraint for a peptide nucleic:** Based on molecular modelling studies (1S, 2S) cyclopentadiamine ring was used for conformational restraint of the C2-C3 dihedral angle of the PNA backbone. The trans cyclopentane modification improves the stability of PNA-DNA triplexes and PNA-RNA duplexes for a poly-T PNA. Recently cyclopentyl PNA s having cis isomers have been reported (Figure 25). The results suggest that these have a stereochemistry dependent stabilization effect on binding both DNA and RNA. The cpPNA s have a better selectivity for mismatch DNA sequence and a higher binding to complementary DNA sequence than the unmodified PNA.
Thiazane and thiazolidine PNA: Bregant, et. al.\textsuperscript{121} introduced rigidity by induction of ring containing thiazane and thiazolidine in the backbone of PNA (Figure 26). With the presence of sulfur in ring, both PNAs showed improved solubility, but, the derived PNA/DNA triplexes were destabilized.

Peptide ribonucleic Acids, PRNA: The synthesis of poly-L-glutamic acid in which ribonucleoside units are attached to the side chain as pendant groups through an amide linkage between the $\gamma$-carboxyl function of the side chain and the 5-amine of the 5-amino-5-deoxyribonucleoside afforded the $\alpha$-peptide ribonucleic acid $\alpha$-PRNA (Figure 27a).\textsuperscript{122a} The strategy was to actively control the function of these oligomers through an external factor. Unfortunately, the efficiency of the external control was not very high because of the mismatched distance of the nucleobases and as a result, the complexes were all
destabilized. The strategy was further improved by the synthesis of isopoly-L-glutamic acid in which the ribonucleoside units were attached as pendant groups through an amide linkage between the α-carboxy function of the glutamic acid and the 5'-amine of the ribonucleoside. This gave rise to the γ-peptide ribonucleic acid (Figure 27b),\textsuperscript{122b} in which the nucleobases were in the correct positions for RNA/DNA recognition. The 1:1 complex of homothymine γ-PRNA with complementary DNA was considerably more stable than the unmodified PNA-DNA complex. The mixed base sequence was also able to exhibit high directional selectivity, the antiparallel complex being more stable than the parallel one. The presence of the ribose sugar could favourably improve the water solubility of γ-PRNA. The concept of external control on DNA/RNA, recognition through duplex formation is quite interesting and may have potential in the next generation of antisense molecules.

![Figure 27: (a) α-PRNA with polyglutamate backbone, (b) γ-PRNA with polyisoglutamate backbone](image)

### 1.5.2b PNA with six membered ring structures

Six-membered ring structures exhibit unique conformational preferences, and the binding abilities of hexose sugar phosphate containing oligonucleotide have been extensively studied by Eschenmoser et al.\textsuperscript{123} 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 (Figure 28). Conformations in the six membered ring structures are rigid, in contrast to the relatively
flexible five membered rings, and hence their influence on the stability of the resulting PNA-DNA/RNA complexes may be expected to make important contributions to the stabilities of the DNA/RNA complexes.

![Locked 3'-endo conformation in LNA.](image)

**Figure 28:** (a) Locked 3'-endo conformation in LNA. (b) Frozen 3'-endo conformation in hexitol and altritol.

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**Glucosamine Nucleic Acids (GNAs):** The six membered glucosamine ring appeared to fulfill the requirement of optically pure and constrained conformational scaffolding for the attachment of nucleobases (Figure 29). The homopyrimidine and mixed base sequences using GNA monomer were constructed. The binding affinities and selectivities of these oligomers to DNA and RNA targets indicated selective recognition of RNA by Watson–Crick hydrogen bonding. The entropy changes were found to be smaller for GNA-DNA/RNA than for DNA-DNA/RNA, consistent with idea that the GNA oligomer was preorganized for binding to the target sequences.

![Dimer of GNA](image)

**Figure 29:** Dimer of GNA
**Aminopipecolyl PNA, pip-PNA:** In the quest to produce a PNA analogue with favourable geometry for optimum binding to the target DNA sequences, a PNA analogue with a six-membered ring was synthesized. This structure was arrived at by introducing a methylene bridge between the γ-carbon atom and the α-carbon atom of the aminopropylglycyl PNA (Figure 30). It was envisaged that the increased conformational freedom and the internucleobase distance in the aminopropylglycyl PNA could be effectively curtailed by a bridged system in the monomeric unit. This was synthesized and incorporated into the homo-pyrimidine aegPNA. The complexes with target DNA were found to be destabilized and the additional methylene groups caused reduced water solubility in the modified oligomers.

![Pipecolyl PNA](image)

*Figure 30: Pipecolyl PNA based on γ-α-methylene bridge in an aminopropylglycyl backbone.*

**Aminoethyl pipecolyl PNA:** The α carbon atom of the glycyl unit and β carbon atom of linker to a nucleobase are bridged by an ethylene unit to get six membered aminoethylpipecolyl PNA (Figure 31). When introduced into PNA oligomers, UV-Tm studies indicated that (2S,5R)-1-(N-Boc-aminoethyl)-5-(thymin-1-yl)pipecolic acid, aepip-PNA, stabilize the resulting complex with complementary DNA.
Piperidinone PNA: Introduction of an ethylene bridge between the α carbon atom and β' carbon atom in the ethylenediamine and acetyl linker resulted in a six-membered ring structure piperidinone PNA (Figure 32).\textsuperscript{127} \((3R,6R)\) and \((3S,6R)\) adenine monomers were synthesized and incorporated into \(\text{aegPNA}\) which resulted in a large decrease in the duplex stability.

Cyclohexyl PNA: Introduction of local conformational constraint in the \(\text{aegPNA}\) resulted in the chiral cyclohexyl-derived backbone (Figure 33).\textsuperscript{128} The aminoethyl segment of the \(\text{aegPNA}\) was replaced with a 1,2 diaminocyclohexyl moiety, either in the \((S,S)\) or \((R,R)\) configuration. The oligomers with \((S,S)\)-cyclohexyl residues were able to hybridize with DNA or RNA, with little effect on thermal stability. Molecular modeling studies revealed that \((S,S)\) isomer can be accommodated more easily in duplex than \((R,R)\) isomer. In contrast, incorporation of the \((R,R)\) isomer resulted in a drastic decrease in the stability of PNA-DNA/RNA complexes. The complexes formed by the two isomers were of the opposite handedness, as evident from CD spectroscopy. The synthesis of ethyl \textit{cis-} (\textit{1S,4R/1R,2S})-2-aminocyclohex-1-yl-N-(thymin-1-yl-acetyl) glycinate waws reported via
enzymatic resolution of the 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$S$,2$R$) isomer preferred to bind RNA and (1$R$,2$S$) isomer showed higher affinity towards DNA in homothymine sequences leading to stereodiscrimination in recognition of DNA and RNA.$^{129}$

![Figure 33: Cyclohexyl PNA](image)

**Aminoethyl-amino-cyclohexanoic acid:** Rigidity was introduced into the aeg-PNA by replacing the glycyl segment in the backbone by $\alpha$-amino cyclohexanoic acid (Figure 34).$^{130}$ Incorporation of these monomers into oligomers and their DNA/RNA binding properties has not yet been reported.

![Figure 34: Aminoethyl-amino cyclohexanoic acid](image)
**Morpholino PNA:** The set of morpholino analogues with phosphonate esters, amide or ester linkages between the morpholino nucleoside residues was synthesized. Preliminary results indicated that amide-linked morpholino PNAs were better accommodated in the complexes than the ester or the phosphonate linked oligomers (Figure 35).\textsuperscript{131}

![Figure 35: PNA with morpholino amide and ester backbones.](image)

**APNA and PNA-APNA chimera:** An aromatic PNA analogue in which the backbone O-aminophenylbutanoic acid derivative carries the nucleobase through an ether linkage has been synthesized (Figure 35).\textsuperscript{132} The direct incorporation of aromatic rings in the backbone renders at least three bonds in the backbone coplanar. Furthermore, the internucleobase distance was altered by additional methyl substitution in the glycyl segment, affording N-(2-aminobenzyl)-(R or S)-alanine or by replacing it with β-alanine, as in N-(2-aminobenzyl)-β-alanine backbones. The incorporation of these modified units in aegPNA produced PNA-APNA chimera. The modified oligomers exhibited decreased binding affinities relative to the pure PNA. An N-(2-aminobenzyl)-glycine unit in the
aegPNA resulted in the smallest decrease in the thermal stability of the triplexes with DNA and RNA while maintaining the selectivity of base pairing recognition.

Figure 35: Aromatic peptide nucleic acid, APNA-I
APNA-II-PNA chimera.

1.5.3 Modified nucleobases

Non-natural nucleobases could aid in understanding of the recognition process between the natural nucleobase pairs in terms of factors such as hydrogen bonding and internucleobase stacking. They could also generate new recognition motifs with potential applications in diagnostics. Only a few nucleobase modifications have been reported in the PNA context (Figure 37). 2,6-Diaminopurine\(^{133}\) offers increased affinity and selectivity for thymine and pseudocytosine mimics the C\(^+\) recognition pattern for triplex formation. 2-Aminopurine\(^{134}\) can hydrogen bond with uracil and thymine in the reverse Watson-Crick mode and being inherently fluorescent, can be used to study the kinetics of the hybridization process with complementary nucleic acids. Replacement of aeg PNA with thiazole orange afforded a PNA probe that fluoresced upon hybridization.\(^{135}\) The E-base, hypoxanthine, \(N^4\)-benzoylcytosine and 6-thio guanine\(^{136}\) represent some more examples of modified nucleobases. Thiouracil along with 2,6-diaminopurine has been utilized as a non-natural base pair in PNA-DNA recognition and was shown for the first time to lead to a phenomenon termed as ‘double duplex invasion’. 
1.6 PNA CONJUGATES

Covalent hybrids of PNA with other molecules have been constructed to overcome the limitations of PNAs such as aggregation, solubility and cell uptake and to impart abilities to enable therapeutic applications like RNase H activation, cell uptake, etc.

1.6.1 PNA-DNA chimerae

Several PNA-DNA chimerae (Figure 38) have been reported till date. Conjugation of PNA to the 5'-end of DNA led to PNA-(5')-DNA chimerae while conjugation to the 3'-end led to the DNA-(3')-PNA chimerae. An advantage of attaching the PNA to the 3'-end of DNA is imparting stability towards the most common 3'-exonucleases. Other advantages of such chimerae are their improved solubility in aqueous media, improved cellular uptake and a lower tendency to self-aggregate. The thermal stability of the complexes of these with complementary nucleic acids was however, lower than that of the complexes with PNA.
Since, PNA is incredibly stable against degradation by nuclease, it constitutes serious limitations with respect to certain applications, i.e. PNA is not accepted as substrate for polymerases, DNA kinases or DNA ligases. Further, PNA cannot induce RNase H cleavage of target RNA that in many cases support biological efficacy of antisense agents. The combination of PNA and DNA in one-molecule resulted in PNA-DNA chimeras with new properties (Figure 37). The PNA-DNA chimeras obey the Watson-Crick rules on binding to complementary DNA and RNA. Binding affinity of PNA-DNA chimeras strongly depend on the PNA:DNA ratio. PNA-DNA chimeras show improved solubility in aqueous solution as compared to pure PNA. Due to the negative charges in DNA part, it can be analyzed and purified by polyacrylamide gel electrophoresis (PAGE) and ion exchange chromatography similarly to oligonucleotide. Interestingly, PNA/DNA chimeras can also assume biological function, e.g. they can serve as primers for DNA polymerases, or upon binding to RNA they can mediate RNase H cleavage.\textsuperscript{138}

### 1.6.2 PNA-Peptide chimerae

There are varied reports in literature of the conjugation of PNA with peptides and proteins to gain an application advantage in biological systems. For example, the presence of cationic peptides at the N-terminus of the PNA resulted in an enhanced inhibition of
human telomerase activity. Another example of PNA-peptide chimerae can be found in the PNA-NLS peptide corresponding to the SV40 core nuclear localization signal. This conjugate increased the nuclear uptake of oligonucleotides and enhanced the transfection efficacy of plasmids.

Shuttle proteins upon conjugation with PNA were used to ‘smuggle’ the target PNA into cells and across the cellular membranes. This significantly increased the inhibition of target RNA expression compared to PNAs alone. Cationic peptides linked to PNAs were found to enhance the strand invasion capability of PNAs into target DNA duplexes. This complex formation was also found to prime DNA strand elongation by oligonucleotide-peptide conjugates at sequences where elongation was hitherto undetected.

1.6.3 PNA-Liposome chimerae

PNAs were conjugated to lipophilic groups and incorporated into liposomes. As predicted, these enhanced the cellular uptake and distribution. These favourable properties increased with the introduction of an amino side-chain into the PNA backbone.

1.6.4 PNA-Polyamine conjugates

PNAs were conjugated to polyamines like ethylene diamine and spermine in order to improve their solubility properties in aqueous media. Spermine accelerated the association of PNA with anionic DNA by electrostatic interaction. It was envisaged that the conjugation of biologically relevant polyamines such as spermine would enhance the cellular uptake of PNAs through polyamine receptor mediated mechanisms. However, such studies are yet to be reported.
1.7 PRESENT WORK

The preceding sections give an overview of the peptide nucleic acids (PNA) which are DNA analogues with a homomorphous but chemically different backbone consisting of \( N \)-(2-aminoethyl)-glycine units in contrast to the sugar-phosphate backbone of DNA. In spite of this, PNAs bind to complementary nucleic acid oligomers obeying the Watson-Crick hydrogen bonding rules for PNA:DNA duplexes and Hoogsteen hydrogen bonding mode for third strand binding in a triplex. The attractive binding properties of PNAs, both in terms of affinity and specificity, coupled with their strand invasion potential have promoted PNA as a useful tool in molecular biology, diagnostics, and as a possible candidate for antisense/antigene drug therapy. The major factors restricting the applications of PNA have been its poor water solubility, insufficient cell uptake, self-aggregation and ambiguity in the binding orientation. Moreover, the strand invasion phenomenon is restricted to low salt concentrations.

In order to overcome these limitations, several modifications of PNA have been carried out in literature. PNAs have also been linked to helper molecules in various chimeras in an endeavor to improve their properties. The work presented in this thesis involves the design, synthesis and biophysical evaluation of these backbone modified, chiral, ring constrained PNA analogues: \textit{aepone}-PNA, a new isomer of \textit{aep}-PNA, conformational study of prolyl ring of \textit{aep}-PNA monomers, tetraplexing properties in \textit{aep}-PNA and \( i \)-motif formation by \textit{aeg}-PNA and foldamers formed by aminoethylprolyl amino acids, a new class of amino acids.

**Chapter 2**

Chapter 2 describes the synthesis of a novel modified PNA monomer (\textit{aepone}-PNA), which was envisaged to confer constrained flexibility on the relatively more flexible PNA backbone (Figure 39). The modification introduces two chiral centers per
unit and retaining carbonyl group of aeg-PNA, which is not present in aep-PNA. The carbonyl group may improve orientational problems of unmodified PNA conceived by bridging the β-carbon atom of the glycine moiety in PNA and the β-carbon of the linker to the nucleobase by a methylene group. The chiral monomers were synthesized from the naturally occurring and easily available 4(R)-hydroxy-2(S)-proline. The synthesis of the chiral monomers bearing each of the four natural nucleobases is described. In addition, a new isomer of aep-PNA is also synthesized. These aminoethylpyrroolidinine and prolyl PNA monomers have been incorporated into PNA oligomeric sequences by solid phase peptide synthesis. Cleavage of the synthesized oligomers from the solid support, their subsequent purification procedures, followed by suitable characterization is also detailed.

\[ \text{Figure 39: (a) aeg-PNA: aminoethylglycyl PNA. (b) aep-PNA: (Aminoethylprolyl PNA). (c) aepone-PNA (aminethypyrroolidinone PNA).} \]

Chapter 3

This Chapter presents analysis of the conformation of prolyl ring in aep-PNA monomers by NMR. All four monomers of aep-PNA were synthesized by reported procedures and fully assigned by different 2D-NMR spectroscopy techniques. The derived
vicinal coupling constants of protons in the prolyl ring were used in PSEUROT software to obtain ring-puckering information. It was seen that the conformation of prolyl ring significantly depends on the nature of the nucleobase, unlike in natural nucleosides.

Chapter 4

In this chapter, the binding properties of the synthesized PNA oligomers containing the aminoethylpyrrolidinone PNA monomers are studied. The biophysical effects of the modification have been elucidated by sequentially increasing the number of modified units in the oligomer. Temperature-dependent UV and CD spectroscopic studies were used to evaluate the binding affinities of the PNA oligomers for complementary DNA sequences. The results of the above studies are discussed along with implications and potential for future work.

Chapter 5A

This section demonstrates G-tetrad formation by ae-PNA. Here, the G-rich sequences of ae-PNA with different lengths were synthesized by solid phase synthesis and their tetraplexing properties were traced by UV spectroscopy and mass spectroscopy techniques.

Chapter 5A

This section illustrates the hitherto unknown tetraplexing properties i.e. t-motif formation of C-rich sequences of aeg-PNA by UV spectroscopy and mass spectroscopy methods and determination of pKa value of N3 in protonated cytosine in aeg-monomer monomer by UV (Figure 40)
Chapter 6

This section is devoted to the synthesis of a new class of amino acid δ-aminoethylproly amino acid (δ-aep) and their peptides (Figure 41), to study the probable secondary structure like 8-helix, 10-helix and 14-helix adopted by these molecules using CD and IR spectroscopic techniques.

Figure 40: Hydrogen bonding Pattern in two Cytosine

Figure 41: Left. Chemical structure of δ-aep and (δ-aepone); Right. Their proposed secondary structure.
1.8 REFERENCES


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