Peptide Nucleic Acids: An Overview

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Peptide nucleic acid (PNA) is a synthetic mimics of DNA in which the deoxyribose phosphate backbone is replaced by a pseudo-peptide polymer to which the nucleobases are linked. It is capable of recognizing specific sequences of DNA and RNA obeying the Watson–Crick hydrogen bonding scheme, and the hybrid complexes exhibit extraordinary thermal stability and unique ionic strength effects. Since its discovery, PNA has attracted major attention at the interface of chemistry and biology because of its unique, interesting physico-chemical and biological properties and its potential to act as an active component for diagnostics as well as pharmaceutical applications. PNA exhibits superior hybridization characteristics and improved chemical and enzymatic stability relative to nucleic acids. The more recent applications of PNA involve their use as molecular hybridization probes. Since PNA is capable of inhibiting transcription as well as translation, so it can be used as a new tool for antigene and antisense therapy. Thus, several sensitive and robust PNA-dependent methods have been designed for developing antigene and anticancer drugs, modulating PCR reactions, detecting genomic mutation or labelling chromosomes in situ. Owing to its superior properties, PNA could replace DNA as a probe for many investigation purposes including broad spectrum of clinical assays and environmental tests that will utilize the PNA technology. PNA will also perform a great impact on areas of in situ hybridization, cytogenetics and industrial microbiology. In this paper the potential applications of peptide nucleic acids has been discussed.

Keyword: PNA; Diagnostics; Hybridization probes; Biosensors; Antisense therapy.

INTRODUCTION: Peptides are a family of short molecules formed from the linking of α-amino acids in a defined order as determined by the genetic code from two to 50 amino acids long. Amino acid residues are linked to each other with an amide bond, referred also as a peptide bond.

There are three large classes of peptides viz, Ribosomal Peptides (synthesized by translation of mRNA are linear in structure and generally function in higher organisms, as hormones and signaling molecules), Non-ribosomal peptides (synthesized using a modular enzyme complex and are confined primarily to unicellular organisms, plants and fungi) and Digested Peptides (result of non-specific proteolysis as part of the digestive cycle).
Peptide nucleic acids (PNAs) are oligonucleotide analogues in which the entire sugar-phosphate backbone has been replaced with a pseudopeptide [1]. These molecules have attracted interest due to their high affinity for DNA and their resistance to degradation by nuclease and protease enzymes. PNA was introduced in 1991 by four Danish scientists at Copenhagen University. PNAs have an achiral, pseudopeptide backbone consisting of N-(2-amino ethyl) glycine units, with purine or pyrimidine bases linked to each unit via a methylene carbonyl linker. Therefore, chemically they are more proteins (peptides) than nucleic acids [2-4]. Unlike DNA or other DNA analogues, PNAs do not contain any pentose sugar moieties or phosphate groups. They are depicted as peptides from the N terminus to the C terminus, corresponding to the 5’ to 3’ direction as in DNA. Because all intramolecular distances and the configuration of the nucleobases are similar to those in natural DNA molecules, specific hybridization occurs between PNA and DNA or RNA sequences by hydrogen bonding (Fig. 1).

The uncharged nature of the PNA oligomers enhances the stability of the hybrid PNA DNA/RNA duplexes in comparison to the natural homoduplexes. The non-natural character of the PNA makes these oligomers highly resistant to various protease and nuclease attacks. These favorable properties of PNA oligomers suggest that they could be efficient antisense or anti-gene agents. Indeed, peptide nucleic acids have already been applied to block protein expression on the transcriptional and translational level and PNA oligomers microinjected into the nuclei of cultured cells demonstrate a strong antisense effect. However, PNA oligomers are not efficiently delivered into the cell cytoplasm through the membrane like the “normal” nucleic acid, and until recently this has obstructed the application of PNA oligomers as antisense reagents [5].

APPLICATIONS OF PNA:
PNAs may be used in many of the same applications as synthetic DNA, but with the additional benefits gained from tighter binding and greater specificity. It has therefore become a versatile tool in genetic diagnostics and a variety of molecular biology techniques; particularly in situ hybridisation and PCR clamping, but also nucleic acid capture, plasmid vector tagging, duplex DNA targeting, and solution-phase hybridisation detection. 4 bis-PNAs, particularly, provide a tool for selectively targeting any short homopurine sequence in intact double stranded DNA with very high specificity and efficacy. The ability to bind to both DNA and RNA is a key feature of PNA, as compared to other analogues that favour RNA. In a typical in situ hybridisation probing experiment of mRNA, PNA probes offer faster hybridisation, higher signal, and better specificity.

1. PNA in Diagnostics & detection:
The excellent hybridization properties of PNA oligomers combined with its unique chemistry has been exploited in a variety of genetic
diagnostic techniques. For instance, PNA probes for in situ hybridization yield superior signal to noise ratios and often allow milder washing procedures resulting in morphologically better samples. Thus PNA-fluorescence in situ hybridization (FISH) techniques have been developed for quantitative telomere analyses, chromosome painting and viral and bacterial diagnostics both in medical as well as environmental samples [6-12].

In another very powerful application, PNA oligomers can be used to silent polymerase chain reaction (PCR) amplifications in single mutation analyses [13]. This technique is so powerful that it is possible to obtain a specific signal from a single mutation oncogene in the presence of a 1,000–10,000-fold excess of the non mutated wild-type normal gene [14-18]. Furthermore, various beacon [19-20] or light-up probe technologies have taken advantage of PNA chemistry [21-23]. PNA oligomers are perfectly suited for MALDI-TOF mass-spectrometry giving very high and distinct signals, and this property has elegantly been exploited in an array hybridization technique in which the hybridized DNA (or RNA) is detected by mass-spectrometry via secondary hybridization of a PNA tag [24]. Such tags are simply made with individual molecular weights, and the presence of a specific PNA tag in the MALDI thus identifies the presence of a specific hybridization and thus the gene variant. Most importantly many PNA tags can be analyzed in the same experiment. Finally, PNA oligomers can be used as capture probes for DNA or RNA purification and sample preparation [25-28].

**SNP detection using PNA-directed PCR clamping**

Single-base-pair mutation or single-nucleotide polymorphism (SNP) analysis is possible using the PCR technique if PNA is synthesised targeting the primer binding site [29-31]. Basically, in the PNA directed PCR clamping technique, at the annealing step the PNA is targeted against one of the PCR primer sites. The temperature set for this step is higher than that for normal PCR primer annealing where the PNA is selectively bound to the DNA molecule. The PNA, which binds to the primer binding site instead of the primer, effectively blocks the formation of a PCR product. PNA is able to discriminate between fully complementary and single-mismatch targets (mutations) in a mixed target PCR, because in the case of mismatches the PNA/DNA hybrid has a melting temperature much lower than the corresponding normal one. Hence the binding of primer will be favoured to out-compete PNA annealing. Consequently, mutated sequences will be preferentially amplified. This PNA clamping is able to discriminate three different point mutations at a single position.

**2. PNA as Hybridization Probes:**

Early interest in PNA came from the field of antisense research and there was also significant interest in the ability of homopyrimidine PNA oligomers to perform strand invasion. Although both these areas have experienced significant progress, the bulk of the interest in PNA in the last few years has come from the use of PNA as a molecular hybridization probe. PNAs may be used in many of the same applications as traditional synthetic DNA or DNA analogs, but with the added benefits of tighter binding (especially at low ionic strength) and greater specificity. For instance, PNAs labeled with biotin, fluorescein, or reporter enzymes are powerful probes in most hybridization based assay formats where oligonucleotide probes are commonly used. However, the greatest contribution of PNAs may come from the development of new applications that cannot be performed using oligonucleotides. The ability to bind to both DNA and RNA is a key feature of PNA, as compared to other analogs that tend to have a higher preference for binding to RNA. The majority of the analysis of nucleic acids is carried out at the DNA level and the high binding affinity of PNA to DNA is hence key to PNA’s application in molecular biology research. The neutral backbone also increases the rate of hybridization significantly in assays where either the target or the probe is immobilized. This permits significantly faster and simpler
Labeled PNAs can function as probes for membrane bound DNA and RNA, i.e. Southern and northern blotting. Probe binding is detected with chemiluminescence or fluorescence in exactly the same way as with labeled DNA probes. In Southern blotting PNA oligomers hybridize faster and withstand much higher washing temperatures, even under conditions where the Tm’s in solution are comparable. Furthermore, PNAs as short as 10-mer were found to give ample signal. The ability of short probes to hybridize efficiently and specifically could be useful when screening plaque lifts based on limited protein sequence information although this needs to be shown.

An alternative to Southern type analysis is the pre-gel hybridization technique. The basic concept is to hybridize a PNA probe to a denatured double stranded target DNA prior to loading on the gel. This is in contrast to conventional Southern (and northern) blotting were the probing occurs after gel electrophoresis, denaturation, and membrane transfer. Pre-gel hybridization exploits the unique ability of PNAs to hybridize to complementary DNA and RNA targets under low salt conditions.

Furthermore, as the PNA probe is uncharged excess PNA probe will not migrate in an electrical field and the electrophoresis therefore offers a facile separation of bound and unbound PNA probe. This allows for elimination of most of the steps involved in Southern hybridization. With this technique effective single base pair specificity has been demonstrated even though there is no stringency washes involved in the procedure. However, pre-gel hybridization cannot be used directly on genomic samples to detect a single copy gene because of the complexity of the genome and the relative low amount of the actual target present. One way to address the enormous complexity of a genomic sample is to use two PNA probes targeted to the same fragment. While coincidence detection addresses the issue of specificity effectively the detection limit requirements also changes dramatically.

There are few applications where the advantage of PNA probes is as apparent as for in situ hybridization. In the typical in situ probing experiment of chromosomes, PNA probes offer faster hybridization, higher signal, and better specificity. Typically a set of longer DNA probes with multiple labels can be substituted by a single PNA 15-mer carrying a single label. In general, using only one short probe improves the ability to discriminate between closely related sequences. With PNA probes the higher intrinsic specificity also adds to the overall specificity of the assay. Additional benefits of using PNA probes are lower background and indefinite stability of the probe solutions.

The benefits of using PNAs as hybridization probes are also seen in affinity capture and reverse dot blot applications. A most convincing set of data was generated using a His6-tag on PNA and nickel-NTA as the capture support. Using this system it was demonstrated that PNA capture proceeds with very high efficacy while maintaining efficient single base pair discrimination. This work has been expanded to include general DNA capture via the bis-PNAs. Likewise hybridization PNA-based biosensors have been constructed. These devices generate an electric signal in the presence of a complementary oligonucleotide. Although unmodified PNA oligomers cannot be used as primers in PCR and other amplification techniques, the improved hybridization characteristics of PNA can still be exploited in the so-called PCR clamping technique. PNA oligomers can block extension of a DNA primer by competing for binding at or around the primer site. It has been demonstrated that the superior specificity of the PNA competition probe results in an assay that allows for the detection of possible single base pair differences. This technique was the first indication of the role that
PNA might come to play as a molecular biology tool.

**Using PNA in southern hybridisation**

Southern hybridisation, which is a routine technique used daily in most molecular biology laboratories for predicting size and sequence information on DNA and information regarding the genetic context, possesses certain disadvantages such as a multistep washing procedure and sometimes poor sequence discrimination between closely related species. Pregel hybridisation of PNA provides a solution to reduce the number of steps in the process of southern hybridisation, as the cumbersome post separation, probing and washing steps are eliminated. Denatured DNA samples at low ionic strength are made to hybridise with fluorescent-labelled PNA oligomers. The mixture is thereafter subjected directly to electrophoresis for size separation, and single stranded DNA fragments are separated on the basis of length. The charge-neutral PNA allows hybridization at low ionic strength and renders higher mobility to the complex compared with the excess unbound PNA. The DNA/PNA hybrids are transferred onto a nylon membrane, dried, UV crosslinked and detected using standard chemiluminescent techniques. Under the same conditions a normal DNA/DNA duplex will tend to disrupt, whereas the PNA/DNA duplex will remain intact owing to the strong binding of PNA to DNA. This allows specific sequence detection with simultaneous size separation of the target DNA following a simple and straightforward protocol. Consequently, the analysis is much faster than with the conventional southern hybridisation technique [41].

3. **PNA as therapeutic drug**:

Because of their strand invasion property and being chemically and biologically stable, PNAs can be used to design gene therapeutic drugs [42-44]. The primary requisite is the identification of targets and a suitable cellular delivery system for them. There are basically two strategies involved in using PNAs as therapeutic drugs, namely antigene and antisense methods. In antigene strategy the PNAs’ nucleotide bases or their potential analogues are designed to recognize and hybridise to complementary sequences in a particular gene, whereby they should interfere with the transcription of that particular gene. PNAs are capable of arresting transcriptional processes by virtue of their ability to form a stable triplex structure or a strand invasion or strand displacement complex with DNA. Such complexes can create a structural hindrance to block the stable functioning of RNA polymerase and thus are capable of working as antigene agents. Evidence from *in vitro* studies supports the idea that such complexes are indeed capable of affecting the process of transcription involving both prokaryotic and eukaryotic RNA polymerases. PNA targeted against the promoter region can form a stable PNA/DNA complex that restricts the DNA access of the corresponding polymerase. PNA strand displacement complexes, located far downstream from the promoter, can also efficiently block polymerase progression and transcription elongation and thereby produce truncated RNA transcripts; the PNA (DNA polypurine) target must be present in the gene of interest.

Alternatively, in the case of antisense strategy the nucleic acid analogues can be designed to recognize and hybridise to complementary sequences in mRNA and thereby inhibit its translation. Normally, the PNA antisense effect is based on the steric blocking of either RNA processing, transport into cytoplasm, or translation. It has been concluded from the results of *in vitro* translation experiments involving rabbit reticulocyte lysates that both duplex-forming (mixed sequence) and triplex-forming (pyrimidinerich) PNAs are capable of inhibiting translation at targets overlapping the AUG start codon. Triplex-forming PNAs are able to hinder the translation machinery at targets in the coding region of mRNA. However, translation elongation arrest requires a 2PNA/1RNA 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. Although the use of PNA as a therapeutic lead compound looks promising, intracellular delivery of PNA is still a problem area, because, unlike most nucleic acid oligonucleotides that can easily be delivered into cells through endocytosis, PNAs penetrate poorly through the cell membrane [45]. This is partially due to the neutral charge property of PNA, whereas the nucleic acid oligonucleotide gains entry into the cell because of its negative charge property. To enhance the efficiency of PNA delivery, many strategies have been explored. Since a positive charge will enhance the attraction of molecules to the cell membrane, some studies have attempted to incorporate positively charged residues such as lysine and arginine into the PNA molecules to enhance the PNA delivery efficiency. Studies have also been done using ligands to enhance the attachment of PNAs to the cell membrane. For example, PNAs have been conjugated with short peptide sequences to enhance the PNA delivery efficiency. PNAs conjugated with other ligands such as antibodies or steroids have also been used to increase the PNA delivery efficiency.

4. Tool for molecular biology and functional genomics:

PNA for artificial restriction system
There are many sequences which are not recognized by the restriction enzymes; in such cases, PNAs are designed to have their oligonucleotides complementary to the recognition sequences. These PNAs hybridise to the complementary targets on dsDNA via a strand invasion mechanism and loop out the non-complementary DNA sequences [46]. The single-stranded looped-out fragments of DNA are then removed by S1 nuclease, 1 which cleaves single stranded nucleic acids, releasing 5′-phosphorylmonoor oligonucleotides. Therefore PNA can be used as a cutting tool in combination with S1 nuclease to make an ‘artificial restriction enzyme’ system. If two PNAs are used for this purpose and allowed to bind to two adjacent targets on either the same or opposite DNA strands, it will essentially open up the entire region, making the substrate accessible for the nuclease digestion and thereby increasing the cleavage efficiency [47].

PNA for rare enzyme cutting
For a long time there has been a problem in using restriction enzymes for cutting a DNA sequence at only one position if there are a number of restriction sites on the DNA sequence, since the result is cleavage of the DNA at more than one place. This problem can now be solved using PNAs. PNAs used in combination with methylases and other restriction endonucleases can act as rare genome cutters. This method is called the PNA-assisted rare cleavage (PARC) technique [48]. It uses the strong sequence-selective binding of PNAs, preferably bis-PNAs, to short homopyrimidine sites on large DNA molecules, e.g. yeast. 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 digestion, it is possible to cleave the whole DNA by enzymes into a limited number of pieces.

The DNA is efficiently protected from enzymatic digestion due to methylation at all sites except for those previously bound to PNA. Thus short PNA sequences, particularly positively charged bis-PNAs, in combination with various methylation/restriction enzyme pairs can constitute an extraordinary new class of rare genome cutters.

Enhanced PCR amplification
Molecular genetics applications such as amplification of VNTR (variable number tandem repeat) loci for genetic typing make large use of PCR (polymerase chain reaction), but it is seen in some cases that preferential amplification of small allelic products relative to large allelic products presents a problem. This may result in incorrect typing in a heterozygous sample. By using PNA, enhanced amplification of a specific
VNTR product is possible, and this has been done in the case of VNTR locus D1S80 [49]. For PCR amplification the template is blocked using a small PNA and becomes unavailable for intra- and inter-strand interactions during the reassociation step. Although reassociation is blocked by PNA, primer extension can occur. During extension the polymerase displaces the PNA molecules from the template, and the primer is extended towards completion of the reaction. This approach shows the potential of PNA application for PCR amplification where fragments of different sizes are required to be more accurately and evenly amplified. Since the probability of differential amplification is less, the risk of misclassification is greatly reduced. PCR has been widely used for various molecular genetic applications including the amplification of variable number of tandem repeat (VNTR) loci for the purpose of genetic typing [50-51]. However, in some cases preferential amplification of small allelic products relative to large allelic products presents a problem. This results in an incorrect typing in a heterozygous sample [52]. PNA has been used to achieve an enhanced amplification of VNTR locus D1S80 [53]. 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. Scientists [54] have demonstrated that PNA–DNA chimera [55] lacking the true phosphate backbone is capable of acting as a primer for the polymerase reaction catalyzed by DNA polymerases. The chimera (PNA) 19-TPG-OH, consisting of a 19 base PNA part linked to a single phosphate-containing dinucleotide (TPG-OH) with a free 39-OH terminus, when annealed with a complementary RNA or DNA template strand works as an efficient primer to catalyze the addition of nucleotide by polymerase enzymes. The primer is also recognized by reverse transcriptase and by the Klenow fragment of E. coli DNA polymerase I. The results suggested that the diameter of the duplex region rather than the presence of phosphate backbone of the template primer is the critical factor for a proper template-primer reaction and accommodating the enzyme within the binding domain. It also appears that the primer phosphate backbone may not be essential, at least not in this case, for the polymerase recognition and binding.

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, a more delicate approach resulting in better quantitative results is possible by using fluorescein-labeled PNAs [56]. 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. Low photo bleaching and an excellent signal-to-noise ratio make it possible to quantitate telomeric repeats on individual chromosomes in this way. Experiments suggest that variations of this approach can possibly be applied to other repetitive sequences.

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 [57]. Also, biotinylated PNAs in combination with streptavidin-coated magnetic beads may be used to purify Chlamydia trachomatis genomic DNA directly from urine samples. However, it appears that this simple, fast, and straightforward ‘purification by hybridization’ approach has
certain drawbacks. It requires the knowledge of a target sequence and depends on a capture oligomer to be synthesized for each different target nucleic acid. Such target sequences for the short pyrimidine PNA, i.e., the most efficient probe for strand invasion, are prevalent in large nucleic acids. Thus, short PNAs can also be used as generic capture probes for purification of large nucleic acids. It has been shown that a biotintagged PNA-thymine heptamer [58] could be used to efficiently purify human genomic DNA from whole blood by a simple and rapid procedure. However, this nucleic acid purification technique has certain problems: knowledge of the target sequence is required to synthesise the PNA, and different PNAs have to be synthesised in order to capture different nucleic acids.

5. Antigene and antisense applications of PNA
Peptide nucleic acids have promise as candidates for gene therapeutic drugs design. 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 potential analogs are designed to recognize and hybridize to complementary sequences in a particular gene whereby they should 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). PNAs are chemically and biologically stable molecules and have significant effects on replication, transcription, and translation processes, as revealed from in vitro experiments. Moreover, no sign of any general toxicity of PNA has so far been observed. As we shall see, PNA can interfere with the translation process, and PNA ds DNA strand displacement complexes can inhibit protein binding and block RNA polymerase elongation.

Inhibition of transcription
Peptide nucleic acids should be capable of arresting transcriptional processes by virtue of their ability to form a stable triplex structure or a strand-invaded or strand displacement complex with DNA. Such complexes can create a structural hindrance to block the stable function of RNA polymerase and thus are capable of working as antigene agents.

Evidence from in vitro studies supports the idea that such complexes are indeed capable of affecting the process of transcription involving both prokaryotic and eukaryotic RNA polymerases. PNA targeted against the promoter region can form a stable PNA–DNA complex that restricts the DNA access of the corresponding polymerase. PNA strand displacement complexes, located far downstream from the promoter, can also efficiently block polymerase progression and transcription elongation and thereby produce truncated RNA transcripts; the PNA (DNA poly-purine) target must be present in the gene of interest. It had been demonstrated that even an 8-mer PNA (T8) is capable of blocking phage T3 polymerase activity [59]. 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 [60-61]. The PNA2/DNA triplex arrests transcription in vitro and is capable of acting as an antigene agent. But one of the major obstacles to applying PNA as an antigene agent is that the strand invasion or the formation of strand displacement complex is rather slow at physiological salt concentrations [62-63].

Several modifications of PNA have shown improvement in terms of binding. Modifications of PNA by chemically linking the ends of the Watson-Crick and Hoogsteen PNA strands to each other [64], introducing pH-independent pseudoisocytosines into the Hoogsteen strand, incorporating intercalators [65], or positively charged lysine residues [64, 66] in PNA strand can drastically increase the association rates with dsDNA. PNA as well as the PNA–DNA chimera complementary to the primary site of the HIV-I
genome can completely block priming by tRNA3Lys [67]. Consequently, in vitro initiation of the reverse transcription by HIV-1 RT is blocked. Thus, oligomeric PNAs targeted to various critical regions of the viral genome are likely to have a strong therapeutic potential for interrupting multiple steps involved in the replication of HIV-1 [67].

It has been found that under physiological salt conditions, binding of PNA to supercoiled plasmid DNA is faster compared to linear DNA [63, 66]. This result is relevant to the fact that the transcriptionally active chromosomal DNA usually is negatively supercoiled, which can act as a better target for PNA binding in vivo. It has also been found that the binding of PNA to dsDNA is enhanced when the DNA is being transcribed. This transcription-mediated PNA binding occurs about threefold as efficiently when the PNA target is situated on the non template strand instead of the template strand. As transcription mediates template strand-associated (PNA)2/DNA complexes, which can arrest further elongation, the action of RNA polymerase results in repression of its own activity, i.e., suicide transcription [68]. These findings are highly relevant for the possible future use of PNA as an antigene agent.

Scientists have efficiently demonstrated that the looped-out single-stranded structure formed as a result of strand invasion is also capable of acting as efficient initiation sites for Escherichia coli and mammalian RNA polymerases in which the polymerase might start transcription using the single-stranded loop as a template [69]. This is consistent with the affinity of RNA polymerase for single-stranded DNA and its ability to transcribe single-stranded DNA.

**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 [70]. 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. Normally, the peptide nucleic acid antisense effect is based on the steric blocking of either RNA processing, transport into cytoplasm, or translation.

It has been concluded from the results of in vitro translation experiments involving rabbit reticulocyte lysates that both duplex- (mixed sequence) and triplex-forming (pyrimidine-rich) PNAs are capable of inhibiting translation at targets overlapping the AUG start codon [70]. 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) 2–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.

Mologni et al. [71] showed effects of three different types of antisense on the in vitro expression of PML/RARa gene. The first one was complementary to the first AUG (initiation) site. The second could bind to a sequence in the coding region that includes the second AUG, the starting site for the synthesis of an active protein. The third PNA was targeted against the 5′-untranslated region (UTR) of the mRNA, the point of assembly of the translation machinery. Together, these three PNAs could efficiently inhibit translation even at a concentration much below the critical concentration used for each individual. The result suggests that the PNA targeting of RNA molecules like PML/RARa requires effective blocking of different sequences on the 59 part of the messenger. A 59-UTR PNA target can also be used as efficiently as an initiation (AUG) target to achieve an antisense activity of PNA, and a more effective translation inhibition can be achieved by combining PNA directed toward 59-UTR and AUG regions.
Triple helix-forming PNAs can also hinder the translation process. Bis-PNA or clamp-PNA structures are capable of forming internal triple helical constructs. In principle, if targeted against the coding region of mRNA, PNA2/RNA triple helix-forming derivatives can also cause a stop in translation, which can be easily verified by the detection of a truncated protein [70]. However, this methodology requires a sequence optimization for each new target. Recent studies show that *E. coli* cells are somewhat permeable for PNA molecules. Good and Nielsen have shown that it is possible to achieve PNA antisense effects in the ‘leaky’ mutant strains of *E. coli*. PNAs targeted against the AUG region of the mRNA corresponding to β-galactosidase and β-lactamase genes were indeed capable of down-regulating the expression of these two genes [72]. Another study [73] demonstrated the effect of two bis-PNAs, targeted against the homopurine stretches in rRNA, either in the peptidyl transferase center or in the a-sarcin loop, in inhibiting the ribosome function in a cell-free system. The translation was arrested at submicromolar range of PNA concentration. The growth of a mutant strain of *E. coli*, namely, AS19, was also inhibited by using the same PNAs at low micromolar concentration.

**Inhibition of replication**

It is also possible by using PNA to inhibit the elongation of DNA primers by DNA polymerase. Further, the inhibition of DNA replication should be possible if the DNA duplex is subjected to strand invasion by PNA under physiological conditions or if the DNA is single stranded during the replication process. The PNA-mediated inhibition of the replication of mutant human mitochondrial DNA is a novel (and also potential) approach toward the treatment of patients suffering from ailments related to the heteroplasmy of mitochondrial DNA [74]. 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.

**Interaction of PNA with enzymes**

**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 an RNase H activity (e.g., phosphorothioate oligos) is considered a better antisense molecule (inhibitor) than one without the activity (methylphosphonates and hexitol nucleic acids) [75]. 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 are capable of stimulating RNase H activity. On formation of a chimeric RNA double strand, PNA/DNA can activate the RNA cleavage activity of RNase H.

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 corresponding oligonucleotides [75]. Thus, DNA/PNA chimeras appear by far the best potential candidates for antisense PNA constructs.

**Polymerase and reverse transcriptase**

In general, there is no direct interaction of PNA with either DNA polymerase or reverse transcriptase. However, different groups have shown indirect involvement of PNA in inhibiting these enzyme functions (activity) under *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 has been shown to be inhibited by introducing a PNA oligomer. In another experiment [76], demonstrated that the primer extension catalyzed by *Taq*-polymerase can be terminated by...
incorporating a PNA oligomer (PNA-H(t)10) into the system. The latter can bind to a (dA) 10 sequence in the template and thereby terminate the primer extension. Moreover, uncharged PNA primers with only a single 59-amino-29,59-dideoxynucleoside at the carboxyl terminus can be recognized by the Klenow fragment for DNA pol I and VentDNA polymerase (Thermococcus litoralis), and a linear amplification is possible with the use of an excess of PNA-DNA primer and suitable thermostable polymerases [77]. Moreover, the reverse transcription of gag gene of HIV I is also inhibited in vitro by PNAs [78]. The inhibition has been achieved by using a bis-PNA construct, which is more efficient than the corresponding mono PNA construct. Also, 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 [78].

**Telomerase**

Human telomerase, a ribonucleoprotein complex consisting of a protein with DNA polymerase activity and an RNA component, synthesizes (TTAGGG)n repeats at the 39 end of DNA strands. PNA oligomers that are complementary to the RNA primer binding site can inhibit the telomerase activity. Studies have shown that the telomerase inhibition activity of PNA is better than that of corresponding activity of phosphorothioate oligonucleotides. This is mainly due to a higher binding affinity of PNA compared to phosphorothiates [79]. Corey and co-workers [80] have demonstrated an efficient inhibition of telomerase after lipid-mediated delivery of template- and nontemplate-directed PNA into the cell.

**6. PNA as a probe for nucleic acid biosensor**

**Surface-attached PNA molecular beacons**

Molecular beacons are molecules with a fluorescent dye at one terminus and a quencher molecule at the other [81-82]. If a beacon is not hybridising, then its conformation is such that the fluorophore and quencher lie next to each other and the molecule does not emit a signal, since the emission spectrum of the fluorophore and the absorption spectrum of the quencher overlap and, because they lie close to each other, the energy emitted by the fluorophore is absorbed by the quencher. On hybridisation the molecule stretches out, separating the quencher and the fluorophore. Without the quenching effect the fluorescent dye emits a signal, thus reporting the occurrence of hybridisation. Molecular beacons represent a versatile tool in DNA diagnostics.

Although these molecules initially had a hairpin structure with a stem, it was later found, especially for PNA molecules, that a stem is not required for their functioning. In contrast to DNA molecular beacons, stemless PNA beacons are less sensitive to ionic strength, and the quenched fluorescence of PNA is not affected by DNA-binding proteins. This enables the usage of PNA beacons under conditions that are not feasible for DNA beacons. Immobilisation on both flat surfaces and optical fibres has been reported for both DNA and PNA beacons.

**Light-up probes**

PNA light-up probes are molecules that could be used instead of molecular beacons except on solid supports [83-84]. The PNA oligomer is labelled with a dye that is usually coupled to a flexible link at the N-terminal end. Upon binding to cDNA, the fluorescence quantum yield of the dye increases. With thiazole orange, for example, a fluorescence increase of ~50-fold was observed. In the case of DNA oligonucleotides there is an electrostatic attraction between the negatively charged sugar–phosphate backbone and the cationic dye; this effect is avoided with the neutral PNA backbone. The PNA-based technique is used successfully for quantification of real time PCR and for the discrimination of target sequences that differ by a single base only.

**PNA probe for microarray**

Microarrays usually make use of DNA probes, which can be synthetic oligonucleotides or longer enzymatically generated DNA, specifically PCR products and isolated clone DNA. The probes are immobilised sensor molecules of known sequence. Although DNA probes work well, they
have problems of selectivity, sensitivity and stability under various conditions. With DNA probes of longer length, such as PCR products, sensitivity is not a problem but selectivity is greatly reduced. Oligomers normally used for DNA sequencing by hybridisation have a relatively low duplex stability. Another common problem of using DNA probes is that DNA sequences form stable duplexes only in the presence of a salt, which is needed to counteract the inter-strand repulsion.

Such conditions, however, also stabilise secondary and tertiary structures within a target molecule. Sequences might therefore not be accessible and be prevented from hybridisation to the gridded DNA. The above problems can be overcome if PNA probes/chips replace DNA in manufacturing oligo-DNAchips [85-86]. Because of the superior characteristics of PNA compared with DNA, fast and reusable PNA chips with improvements such as longer storage and higher accuracy and reproducibility could be developed. 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. We describe here the use of PNA as a novel probe for sequence-specific biosensors and highlight some of the promise it holds to work as the recognition layer in DNA biosensors.

**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 real-time interactions are monitored on a sensor (surface) chip, which constitutes the core part of a BIAcore instrument. The probe molecule is attached directly to the surface and the analyte molecule is free in solution. The detection principle in BIA uses surface plasmon resonance [87]. The response signal of the BIAcore apparatus is proportional to the change in the refractive index at the surface and is assumed to be proportional to the mass of substance bound to the chip.

The first report regarding the study of PNA-DNA/RNA hybridization using the BIAcore technique came from the work of Jensen et al. [88] in 1997. The sensor chip used in this case was basically a thin gold surface covered with a layer of dextran and containing streptavidin chemically coupled to the dextran (Pharmacia sensor chip SA5). A biotinylated PNA (biotin-(eg13)-TGTACGTCAAACTA-NH2) probe was immobilized on the surface by using the strong coupling between biotin and streptavidin. The amount of bound substance (fully complementary as well as various singly mismatched RNA and DNA oligonucleotides) was measured as a function of time when a solution containing the complementary strands passed over the chip surface. In this way the association kinetics could be studied. The dissociation was subsequently studied by washing the surface with appropriate buffer and monitoring the time dependence of the mass decrease.

Assuming a two-state model, A 1 B N AB, analysis of the hybridization kinetics was carried out. The PNA surface can be regenerated in SA5 by removing the remaining hybridized products with HCl. Thus, consecutive studies could be carried out with the same immobilized PNA. Norden and co-workers have also explored the possibility of carrying out BIAcore measurements using a plain gold surface (e.g., BIAcore sensor chip J1) to which PNA molecules carrying cystein at the amino-terminal can be immobilized using the strong coupling between gold and sulfur. In this way, erroneous results due to nonspecific binding of ligands to the dextran layer, if any, can be eliminated. However, it should be kept in mind that DNA has a high affinity for gold and generally is nonspecifically adsorbed to the surface.
Direct addition of analyte DNA molecules onto the sensor surface to study its binding with PNA might facilitate its adsorption to the gold surface. 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.

**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 [89], research areas related to electrochemistry, or study protein adsorption. Only recently has this sensitive mass measuring device begun to be used in the area of biochemistry and biotechnology, such as for studying the hybridization of nucleic acids on surface [90-92]. The resonant frequency of the crystal changes due to a minute weight increase on the surface. It is expected that immobilized PNA strands (or probes) would show an improved distinction between the closely related target sequences compared to an immobilized DNA probe. A recent report by Wang and co-workers [94] 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. A rapid and sensitive detection of mismatch sequences is possible by monitoring the frequency/time response of the PNA-QCM biosensor. The PNA probes used in the above-mentioned study [93], which formed the monolayer onto the gold QCM surface, contained a cysteine attached to the PNA core with the help of an ethylene glycol unit. The remarkable specificity of the immobilized probe provides a rapid hybridization with corresponding oligonucleotides. Such a mismatch sensitivity of PNA-immobilized QCM biosensors could be of great importance for diagnostic applications, particularly for genetic screening and diagnosis of malignant diseases.

**MALDI-TOF mass spectrometry**

MALDI-TOF mass spectrometry [94-95] 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. The captured, single-stranded DNA molecules are PCR-amplified and thereafter hybridized with PNA probes in an allele-specific fashion. MALDI-TOF can rapidly and accurately detect (identify) these hybridized PNA probes. This provides a straightforward, rapid, accurate, and specific detection of SNPs in amplified DNA [96]. The detection of multiple point mutations using allele-specific, mass-labeled PNA hybridization probes is also possible by using a direct MALDI-TOF-MS analysis method [97]. The mass spectra will show peaks of distinct masses corresponding to each allele present, and in this way a mass spectral ‘fingerprint’ of each DNA sample can be obtained.

**Potentiometric measurements**

PNA [98] has also been used 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.

**CONCLUSION:**

Various types of applications have been reported, signifying the great interest of the PNAs for biotechnology and molecular genetics. Powerful applications of PNA have also emerged in microbiology, virology and parasitology. PNA-
based applications benefit from the unique physico-chemical properties of PNA molecules, enabling the development of simple and robust assays in molecular genetics and cytogenetics. PNAs are finding increasing uses as probes and other tools in genetics, because these molecules exhibit enhanced binding affinity, increased stability and resistance in biological fluids and reduced nonspecific effects.

New chemical modifications of the original PNA backbone may contribute to increasing the potentialities of PNAs and lead to the development of novel applications and PNA-dependent projects in many areas of biology and therapy & hence may prove to be a major bludgeon for the healthcare professionals in the coming century.

**REFERENCE:**


