APPLICATIONS OF PEPTIDE-NUCLEIC ACIDS AS ADVANCED TOOLS FOR
BIOMEDICAL APPLICATIONS

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ABSTRACT

PNAs have been discovered thirty years ago, reported as therapeutic synthetic analogs of natural nucleic acids. PNAs have repeating N-(2-aminoethyl) glycine units’ backbone which is linked by peptide bonds and methylene carbonyl linkages help in joining the purines & pyrimidines bases with the backbone. In RNA/DNA recognition, PNAs play an effective role in terms of selectivity and affinity. PNAs don’t have any pentose sugar just like DNA or their analogs rather these are neutral chemical entities. This uncharged PNA nature is responsible for its hybridization with DNA molecules as well as for thermal stability of PNA/DNA duplexes. Due to achiral nature of PNA backbone, PNA/DNA duplexes are very less effected in high ionic solutions. Due to inactivity as substrate in proteases, peptidases & nucleases, they exhibit strong biochemical stability. PNA has been reported as biosensors, antigene& antisense agents, biomolecular tools and molecular probes etc. PNAs show countless issues regarding cellular uptake mechanism, which can be easily resolved via chemical modification or via assemble conjugation with CPPs (cell penetrating peptides) with PNAs. They showed improvement in cellular drug delivery system by these chemical modifications and increases the drug delivery/design/development. Here, we have conveyed tremendous applications of PNAs in biomedical and genetic fields including: anticancer, antimicrobial, antigene, antisense, PCR, MALDI-TOF mass spectroscopy, FISH, diagnostics in biosensors & vivo imaging, antibiotic agents etc. There is all vision that in future, with the teamwork of biologists and chemists, PNA will realize in full of its massive applied potential.

Keywords: PNA, PCR, MALDI, FISH, antisense, antigene, biosensors.

1. Introduction

PNA (Peptide Nucleic Acid) commonly known as RNA/DNA analogs was discovered by collaboration of Peter Nielsen and an organic chemist Ole Buchardt in 1990s. PNAs have repeating N-(2-aminoethyl) glycine units’ backbone which is linked by many peptide bonds. The methylene carbonyl linkages help in joining the purines & pyrimidines bases with the backbone. In RNA/DNA recognition, PNAs play an effective role in terms of selectivity and affinity [1]. PNAs are supramolecules which have enormous role in cutting edge field, for example, they help in treatment of various diseases whose effective therapy is not available, helps in development of new diagnostic tools and by coding targeting micro-RNA it helps in modulation of gene expression etc. [2]. To contest the relative distance, the PNA design was considered where the backbone of sugar grips the significant mechanical/structural units, i.e., the nucleobase, 3’-OH and the 5’-OH. The linear chain of PNA backbone consisting six σ-bonds. Out of these six σ-bonds, nucleobase is linked with three σ-bonds linkers (methylene carbonyl). The modified backbone has elaborated the substitution at α, β and Y positions. The substitution on these positions of modified backbone increases chemical, enzymatic stability and the affinity of PNA for RNA and favors PNAs as anti-miRNAs [3]. PNAs don’t have any
pentose sugar just like DNA or their analogs rather these are neutral chemical entities. This uncharged PNA nature is responsible for its hybridization with DNA molecules as well as for thermal stability of PNA/DNA duplexes. Due to achiral nature of PNA backbone, PNA/DNA duplexes are very less effected in high ionic solutions. In acidic conditions they are highly stable and in basic medium acts as moderately stable whereas at high temperature show greater stability. Due to inactivity as substrate in proteases, peptidases & nucleases, they exhibit strong biochemical stability. PNA has been reported as biosensors, antitumor & antiviral agents, biomolecular tools and molecular probes etc. They are not so sensitive against pH, due to which PNAs can exhibit high constancy over an extensive range of pH, whereas, RNA or DNA are very subtle towards pH ranges. Thus, PNAs are highly sequence dependent too due to which PNAs are considered as far better than their native biological counterparts. Their structural comparison is shown in Figure 1 [5]. In drug discovery, PNAs showed conjugation with bacterial target proteins and acts as antiviral antimicrobials. Exact location of ribosome binding site and selection of protein target is necessary key for conjugation or inhibition of active side of protein target. For this criteria, optimum length should be around 10-12 nucleobases. It is found that in wild type *E. coli MG1655* & in *E. coli AS19*, the 10-mer of PNAs were found the most active antiviral antimicrobials, which signifies the bacterial uptake of PNA and peptide conjugation [4].

![Structural representation of PNA, DNA & RNA.](image)

**Figure 1**: Structural representation of PNA, DNA & RNA.

2. **Chemical Modifications of PNA Backbone**

As PNAs are neutral charged species, the stability and formation of duplex & triplex helices would be increased by introducing positive charge in PNA[6]. Apart from applications, PNAs have many stability issues too, which can be minimized by acetylating or blocking the N-terminus. PNAs show countless issues regarding cellular uptake mechanism, which can be easily resolved via chemical modification or via assembly conjugation with CPPs (cell penetrating peptides) with PNAs. Therefore, PNAs showed improvement in cellular drug delivery system by these chemical modifications and increases the drug delivery/design/development[7]. Chemical modifications in backbone of PNA enhances the antisense property along with numerous additional applications that is useful as applications in medicine, molecular biology and diagnostics. Strategies like introducing chirality into achiral backbone of PNA, by addition of cationic functional group so that aqueous solubility can be improved, by linker/nucleobase modification so that DNA/RNA binding can be controlled
at physiological conditions and so on are being appraised for PNA alterations. Apart from altering PNA backbone, it could also be modified through artificial nucleobases, i.e., 2,6-diaminopurine, pseudocytosine, 2-aminopurine, thiazole, hypoxanthine, thiouracil, and which provides PNA salient characteristics like enhanced affinity, thymine selectivity, via fluorescence as a probe which help in hybridization detection and so on, listed are merely some examples with characteristics of artificial nucleobases[14]. More over these alterations will outcome in greater aqueous solubility, increased cellular uptake and additional properties too; that appraise for greater technique in applications of biomedical[7]. The nucleobase and backbone of PNA alterations will outcome in upgraded applications of PNA. Hnedzko et al. synthesized (M) modified PNA and figured out that, PNA are appropriate ligands for dsRNA recognition in live cells and supplementary biotic systems [15]. Henceforth, modified PNA couples are likely positive study, show outstanding cellular uptake and minute cytotoxicity; that make it suited for varied range of PNAs biomedical applications. Here, some PNA backbone modifications are listed as per earlier reviews.

![Chemical structures: PNA positions for modification](image)

**Figure 2: Chemical structures: PNA positions for modification**

As it has been already found that modified backbone of PNA increases the thermal stability, binding affinity, enzymatic activity etc. and hence responsible for antisense antimicrobial activity of modified PNAs [1]. The basic modifications/substitutions have been carried on \( \alpha \), \( \beta \), \( \gamma \) positions and cyclic position (Figure 2). Nielsen et al. proposed the first modified PNA backbone as \( \alpha \)-PNA (\( \alpha \)-modified PNA). In this reported modification, L or D alanine is substituted in place of glycine during monomer synthesis. D-form was invented to increase the affinity for hybridization with complementary DNA rather than L-form for specific stereochemistry. Later, the main functional groups like lysine, arginine and serine etc. were incorporated on the \( \alpha \)-position to increase the cellular uptake of this modified PNA backbone. For versatile function of \( \alpha \)-PNA, phenylalanine, valine, tryptophan and leucine like bulky hydrophobic groups have been combined at \( \alpha \)-position. However, a new field \( \beta \)-PNA (\( \beta \)-modified PNA) is also developed to increase the significant changes in PNA backbone. PNA monomers having methyl groups on \( \beta \)-position have been explored for hybridization process. \( \gamma \)-PNA (\( \gamma \)-modified PNA) is also a new field which is more explored than \( \alpha \) & \( \beta \) position modifications. These \( \gamma \)-PNA modifications have been done to increase the physicochemical properties such as cell permeability, solubility and hybridization stability etc. First \( \gamma \)-PNA monomer was synthesized by reductive animation reaction in which alanine amino aldehyde is reacted with glycine. \( \gamma \)-position modified PNA synthesized from L-amino acids showed greater binding affinity as compared D-amino acids.
Figure 3: PNA modified nucleobases

Herein, in Figure 3, (a) shows G-clamp interactions with the protein target “G’ guanine, (b) shows uracil nucleobase modification with C5 alkyne chain, (c) shows modification of 5-(azidomethyl) and (d) shows PNA consisting pyrene units which is a monomer attained from 5-(azidomethyl) uracil. PNA modified (G-clamp), [figure 3 (a)] exhibit strong binding affinity & stacking capability by making additional Hoogsteen hydrogen bonds. This combination increases the selectivity as well as affinity for complementary DNA. These types of modifications showed fluorescence changes when interact with DNA, which directly allow hybridization detection. Whereas, 5-alkynyluracyl is a versatile nucleobase which is used in linking of functional groups with oligonucleotides [Figure 3(b)]. It can be produced very easily. For example, 5-iodouracil base is a PNA monomer, which is used in Sonogashira reactions due to which many 5-alkynyluracil derivatives obtained along with added groups. Similarly, another versatile precursor i.e. 5-azidomethyluracil, is used a PNA component [Figure 3 (c)]. The azido group is used in conversion to amine group. It is used in derivatization through coupling to carboxylic acids. It can be used in linking of alkyne-modified modules for click chemistry. It is also used PNA monomer component bearing two pyrenyl residues as shown in [figure 3(d)-(f)]. The stacking interactions of these pyrene units favor the excimer fluorescence emission [figure 3(e)-(f)]. The process is so selective[2].

A monomer whose glycine moiety has been replaced by protein alanine, was first reported by Nielsen et al. D and L enantiomers were introduced in different oligomers after synthesizing process. It is found that PNA-DNA duplexes containing glycine exhibit higher thermal stability as compared to L-form PNA-DNA duplexes. For PNA modification, lysine, serine and arginine proteins have been used on α-position rather than glycine. A PNA monomer containing lysine plays significant role in PNA-DNA duplex formation as lysine contains positive charge. A PNA containing D-lysine showed high Tm (thermal stability) as compared
with unmodified PNA monomer as lysine shows electrostatic interactions between the phosphate of DNA and ammonium group of lysine.

**Figure 4: α-position substituted PNA modified backbone.**

Fabani et al. first time reported that PNA-peptides and PNAs showed conjugation to target miRNA-122. They exhibited far better activity and binding affinity as compared to 2’-O-methyl oligonucleotides (Figure 4). Here, some modifications are listed in below Table 1 [3].

<table>
<thead>
<tr>
<th>Modification in PNA backbone</th>
<th>Target (Protein)</th>
<th>System (Cellular/Animal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRRQRRKKR-O-PNA</td>
<td>miR-16</td>
<td>HeLa cells</td>
</tr>
<tr>
<td>RRRQRRKKR-O-PNA</td>
<td>miR-21</td>
<td>HeLa cells</td>
</tr>
<tr>
<td>RRRQRRKKR-O-PNA</td>
<td>miR-24</td>
<td>HeLa cells</td>
</tr>
<tr>
<td>K-PNA-K₃</td>
<td>miR-122</td>
<td>Human hepatocellular carcinoma cells- primary rat hepatocytes</td>
</tr>
<tr>
<td>K-PNA-K₃</td>
<td>miR-155</td>
<td>LPS- activated primary B cell and mice</td>
</tr>
<tr>
<td>PLGA NP coated with R9-PNA</td>
<td>miR-155</td>
<td>KB cells</td>
</tr>
<tr>
<td>RQIKIWFQNRRMKWKK-PNA</td>
<td>miR-155</td>
<td>Mouse model of Lymphoma</td>
</tr>
<tr>
<td>Re-PNA</td>
<td>miR-210</td>
<td>K562 chronic myelogenous leukemia cells</td>
</tr>
<tr>
<td>Y-GPNA</td>
<td>miR-210</td>
<td>Alterations of erythroid differentiation</td>
</tr>
<tr>
<td>Rᵢ₀-PNA</td>
<td>miR-210</td>
<td>Breast cancer cells</td>
</tr>
<tr>
<td>RRRQRRKKKR-PNA</td>
<td>miR-375</td>
<td>Limb mesenchymal cell</td>
</tr>
</tbody>
</table>

Synthesis process has been carried out to produce a large number of non-natural nucleobases (PNA analogs). Bhoc (benzhydrloxycarbonyl group) or Fmoc (9-fluorenylmethoxycarbonyl group) protects the PNA monomer’s amino group (figure 5). Initially, methylbenzhydrylamine (Merrifield resin) is linked with PNA monomer in a fritted vessel. With the help of addition of piperidine solution (20% piperidine in dimethylformamide). Fmoc group is deprotected. This process is done by capping the unreacted groups present in resin. The above mentioned PNA monomers (figure 5) were connected to the resins with corresponding coupling reagents [22].
3. Biomedical Applications of Modified PNA

PNAs have been discovered 28 years ago, they have been reported as therapeutic chemical moieties. They are well known for their tremendous applications in biomedical and genetic fields. In DNA genotyping, they are using as HPAB (high performance affinity biosensors)[8]. They show an important role in DNA hybridization, antisense tool/technology, PCR (polymerase chain reaction) based systems which provide a tremendous concept to pharmacists and chemists for drug designing and development with more accurate properties[9]. DNA hybridization is a technique of molecular biology which is used in the measurement of genetic similarity between DNA sequences pools. It helps in genetic distance determination between two organisms. This is also used as in-silico studies partially or completely sequenced genomes. Antisense technology is a tool/technique which is used in gene expression inhibition process, whereas PCR (polymerase chain reaction) is a method of making copies of DNA sample in millions to billions of numbers. Scientists generally take small sample of DNA sample and make ample copies for further lab practices. Whereas, MALDI (matrix-assisted laser desorption) is an ionization technique which create ions from giant molecules by using laser energy-absorbing matrix with minimum fragmentation. Besides these properties, PNAs have been reported as a potential drug in novel antibiotics. They block the active sites of viral/bacterial active sites and terminate their protein synthesis process, thus helps as antimicrobial agents. PNAs act as good gene-activating mediators, fluorescent in-situ hybridization (FISH) for imaging, detection techniques, matrix-assisted laser desorption (MALDI) or Ionization-time of flight (TOF), antigene agents and anticancer agents etc. gene-activating agents (GTAs) are virus like particles, which contain DNA. These agents are produced by some archaea & bacteria. These mediate horizontal gene transfer. Ionization-time of flight (TOF) is a method of mass spectroscopy. It helps in determination of mass to charge ratio of an ion with the help of time-of-flight measurement. Antigene agents binds to complementary DNA sequence and helps in inhibition of transcriptional process whereas,
anticancer agents are the agents which prevent cancer cells to develop.[10]. PNA serves antisense activity as peptide conjugate in eukaryotic as well as prokaryotic, which has been exhibited a remarkable outcome in rat brain and in nerve cell-line[11]. In biomedical research’s field, the PNA’s properties offer wide range of applications, including: diagnostics in biosensors & in vivo imaging, antibiotic agents etc.[7]. The extraordinary properties are detailed in below segment.

3.1. PNA’s Stability
The complex structure of PNA’s make them immune to chemicals/enzymes like DNases and proteinases, due to which they won’t degrade within the cell and thus have great biostability which stands it as preferred choice for in-vivo examination. Even though its passage inside the cell is tough because of its limited lipid membrane diffusion [3]. An experiment done by Eriksson et al propose that PNA with thymine monomer at pH 11 with 37 days half-life go through N-acyl transfer rearrangement, and at pH9 or above they show sequential degradation and N-acyl transfer reactions. Because of its stability and complex structure, it has binding affinity in high proportion and specifically in targeting to DNA/RNA which makes it good choice for gene therapy-based applications. Further adaptation gives additional constancy, therefore biochemical variation in fusion with nanotechnology may aid PNA’s to enhance their intracellular delivery, that earlier was a notable challenge [12].

3.2. PNA’s as Antibacterial Agents
PNA’s acts as good antibacterial agents as they easily block the active site of bacterial protein via conjugation peptide. This conjugation directly affects the inner membrane of E.coli. It terminates the polymerase chain mechanism and helps in inhibition of gene expression of bacterial protein due to which PNA’s are of center attraction in research field for drug designing and development [15]. It has immense possibility to target the pathogen active site only rather than whole pathogen (non-targeted).

They exhibited the antibacterial agents against gram –ve bacteria, which is similar to various standard antibiotics. It shows antibacterial activity without disturbing the normal flora for oto-pathogens [16-17]. According to many research findings, PNA’s exhibited inhibition against extraand Intra-Cellular Salmonella enterica Serovar Typhimurium through the nonlytic Mode of Action, bacterial processivity aspect sliding etc. [18-19]. As PNA’s play key role in antisense properties, they help in termination of generic mutations as well as different infections. Monomer chain [series of (KFF)3- & (RXX)3] of PNA’s exhibited antisense antimicrobial activity in E. coli (Both wild type MG1655 and AS19) [20]. According to Gorska, et al., PNA oligomers successfully bound with 16S RNA and inhibit the growth of bacteria [21]. It is found that PNA effectively target the bacterial gene of E. coli species having MIC values of 2-4 μM. The highest strength is exhibited by anti-ΔpO & ΔpD PNA’s in case of Salmonella enterica serover Typhimurium. It is helpful tool for identifying and microbial genetic treatment [22-25].

3.3. PNA’s as Antiviral Agents
RNA replicase polyproteins synthesis is caused by PRF-1 signal in virus for genome replication. This is specially for coronavirus replication (severe acute respiratory syndrome). PNA’s play key role in targeting hepatitis B virus (HBV). APNA, a synthesized PNA chemical moieties directly attacks the target protein of HBV [7]. It has been reported that modified CPP-PNA’s (Cell penetration peptides-polypeptide nucleic acids) targets the chronic hepatitis B infections [26]. Antiviral activity of antisense PNA is reported against human hepatitis C.
virus. Delivery is done by gold nanoparticles [27]. According to a report, APNA found to be as targeting the hepatitis virus which is responsible for cirrhosis, chronic hepatitis and carcinoma etc. [28].

3.4. PNAs as Anticancer Agents
PNAs play major role in tumor treatment by inhibiting the overexpression of microRNA-155. According to a research report, radiolabeled PNAs are capable of inhibiting the overexpression in non-Hodgkin’s lymphoma and of B-cell leukemia. Blocking of DNA transcript, which is initiator of lung cancer SPC-A1 can be done by altering the physiological characteristics with the help of PNA antisense. It is one of the main inhibitory factor of human ovarian cancer cell lines SKOV3. So, we can say that PNAs are responsible for termination of cancer synthesizing process. For genome replication, PRF-1 signal is the reason for RNA replicas polyproteins synthesis. PNA antisense is responsible for the inhibition of this RNA replication. Recently, it has been reported that PNA antisense agents exhibited the inhibition of replication process of Hepatitis B virus [7].

As per Moccia et. al. tiny PNA analogues were find as effective RNA targeting agents. Their structural features are investigated. The interaction of PNA analogs of microRNA-34a(tumor-suppressor) with the binding sites on MYCNmRNA have been analyzed [29]. Due to uncharged nature of PNA backbone, PNA/DNA duplexes favors the stability, selectivity and higher efficiency. According Falanga et. al. PNA analogs easily counteract the B-cell lymphoma 2 (Bcl-2), which encodes for antiapoptotic protein. This gene is associated with human tumors [30]. According to an analyst article of royal society of chemistry, PNA-DNA probes are proving as diagnostic tool in identifying and differentiating three different types of lung cancer cell lines, i.e., PC-9, H1975 and A549 [31].

3.5. PNAs as FISH conjugation
FISH is Fluorescent in-situ hybridization used for imaging. It is a molecular cytogenetic technique which is used to find out the DNA sequences on chromosome with fluorescent probes. This technique is used in localization/detection of specific RNA targets. For identification of DNA sequences FISH uses PNA probes [32]. According to a report, PNA-FISH exhibited 60% sensitivity in detection of C. burnetii. It is found remedial in diagnosis for C. burnetii endocarditis and vascular infection [33]. It is found that PNA-FISH targeted gram positive bacteria species (S. epidermidis, L. innocua and B. cereus) and gram-negative bacteria species (E. coli and P. fluorescens) and showed superior results with gram positive species (p<0.05) [34]. According to a report, PNA-FISH is proved as diagnostic method in detection of H. pylori clarithromycin resistance. It is also found that this diagnostic method is one of the best methods for identification of H. pylori in clinical samples [35]. In another article, PNA-FISH reported as culture-independent and noninvasive diagnostic technique. P. aeruginosa and S. aureus general clinical pathogens are identified in burn wound infections with the help of PNA-FISH probes. Athird pathogen species Candida albicans is also identified by including calcofluor via PNA-FISH probes [36]. PNA fluorescent probes were used in identification/imaging of human telomeres in chromosomes [37]. PNA-FISH-AFC (PNA fluorescence in situ hybridization enhanced acoustic flow cytometry) is used for identification and detection of bacteraemia/bacteria E. coli, K. pneumoniae and P. aeruginosa species) in BC(blood culture) and incubation period for diagnosis is 7-10 hours for PCR based FilmArray assay & 12-24 hours for MALDI-TOF [38].
3.6. PNA as TOF

Ionization-time of flight (TOF) is a method of mass spectroscopy. It helps in determination of mass to charge ratio of an ion with the help of time-of-flight measurement. MALDI-TOF, a multiplexable, novel, fast and inexpensive method which is used for evaluation of colon cancer as methylation markers. The methylation of genes is evaluated in 12 colon cancer & 12 normal tissues. Methylated genes are Adenomatous Polyposis coli (APC), eyes absent 4 (EYA4) and glycogen synthase kinase-β-3 (GSK3β). After evaluation, it is found that GSK3β did not exhibited differential methylation whereas APC and EYA4 exhibited as differentially methylated found in colon cancer patients [39]. According to Duan, et. al. PNA based nanofabrication’s are more efficient as compared to DNA based nanofabrication in biosensing, bioengineering and drug delivery. PNA based nanostructures were confirmed by MALDI-TOF mass spectroscopy, FPLC and AFM [40]. According to Butler, et. al., MALDI-TOF mass spectroscopy for PNA probes is found superior to DNA matrices (3-hydroxy-picolinic acid, trihydroxy-acetophenone, picolinic acid etc.) [41].

3.7. Miscellaneous Activities

According to Kadam, et. al., PNAs act as fluorescent sensor of local dielectric constant and exhibited more hydrophobic character in major grooves as compared to DNA-DNA duplexes [42]. Due to uncharged nature, high binding affinity, stability, remarkable hybridization attributes, etc. PNAs acts as strong biosensors. They are used in detection of RNA, Viruses, bacteria, SNP (single-nucleotide polymorphism), DNA etc. [43]. PNA oligomers have well known reported for their tremendous applications as cell permeability (Conjugation of PNA to cell penetrating peptide), high stability in biological fluids and encapsulation into micro particles [44]. By including mega-nucleases, CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats), transcription activator-like effector nucleases and zinc-finger nucleases etc. genome editing is done via PNA probes (TFO) [45]. L. monocytogenes is a food borne pathogen, its recognition and evaluation have been done by peptide nucleic acid fluorescence in situ hybridization [46]. As per an opinion, PNAs are found to be as effective antisense agents [47]. The mismatch intolerance and sequence selective nature of PNA probes are responsible for making it diagnostic use in biosciences and biomedical remedies [48]. PNA-RNA duplex is found to be effective potential therapeutic applications [49]. 2-amino pyridine is found to be effective in detection of RNA editing [50].

4. CONCLUSION

PNAs are ‘magical’ chemical moieties which causes ample of applications in biology field. They have been discovered 28 years ago and reported as therapeutic chemical moieties. Here, we have conveyed tremendous applications of PNAs in biomedical and genetic fields including: anticancer, antimicrobial, antigen, antisense, PCR, TOF, MALDI, FISH, diagnostics in biosensors & vivo imaging, antibiotic agents etc.on unmodified PNA backbone due to their high binding affinity and high stability in biological fluids. There is all vision that in future, with the teamwork of biologists and chemists, PNA will realize in full of its massive applied potential. PNA derivatives can help the pharmacists and chemists in drug designing and development.

REFERENCES


