

Review

Peptide nucleic acid: Current perspectives and application for future therapeutics

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Peptide nucleic acid (PNA) is a deoxyribonucleic acid (DNA) mimic based on a polyamide backbone first synthesized and reported by Peter Nielsen in 1991. PNA shows remarkable hybridization properties and has many exciting applications. It is chemically and biologically stable and can readily conjugate with peptides and other useful molecules. The search for new chemical modification of the PNA is a very active field of research and new structures are continuously proposed. Conjugation of an oligonucleotide to some peptides that can move through the cell membrane would facilitate the passage of this conjugate across for delivery into the cell's cytosol. Oligonucleotides can also be conjugated to lipids to yield derivatives with lipophilic properties that are capable to pass across the cell membrane. Thus, PNA can have multiple roles to perform in molecular biology, antigene therapy, molecular diagnostics and nano-biotechnology.

Key words: Peptide nucleic acid, oligonucleotide, electroporation, ribonucleic acid, polyamide

INTRODUCTION

PNA contain nucleobases with a peptide-like backbone, making them resistant to both proteases and nucleases, and giving PNA/DNA complexes enhanced stability compared to DNA/DNA complexes due to the lack of negatively charged phosphodiester bonds (Figure 1). PNAs can form a triplex structure with DNA by strand invasion, inducing DNA repair, and thereby stimulating recombination of short donor DNA fragments near the PNA's binding site. It has been previously shown that bis-PNA-194 (IVS2-194), which targets a polypurine site in the second intronic sequence of the human β -globin gene, can stimulate site-specific gene modification when co-introduced with a short, single-stranded donor DNA encoding the desired modification (Chin *et al.*, 2008). PNAs do not readily cross the cell membrane, so special delivery methods are needed. Thus the PNAs are polyamidic oligonucleotide analogues which have been described for the first time almost twenty years ago and were immediately found to be excellent tools in binding DNA and RNA for diagnostics and gene regulation (Rogers *et al.*, 2002).

Their use as therapeutic agents have been proposed since early studies and recent advancements in cellular delivery systems and in the so called anti-gene strategy make them good candidates for drug development. The

search for new chemical modification of PNAs is a very active field of research and new structures are continuously proposed. This review focuses on the modification of the PNA backbone, and their possible use in medicinal chemistry with an update of this topics in view of emerging new trends and opening of new possibilities. In particular two classes of structurally biased PNAs have been described in recent studies i) PNAs with acyclic structures and their helical preference, which is regulated by stereochemistry and ii) cyclic PNAs with pre-organized structures, whose performances depend both on stereochemistry and on conformational constraints. The properties of these compounds in terms of affinity for nucleic acids, and several examples of their use in cellular or animal systems are presented, with exciting new fields of research such as microRNA targeting and gene repair, delivery mechanisms and use in nano-biotechnology (Eriksson and Nielsen, 1996).

PNA application in neuro-degenerative and musculo-skeletal disorders

Nanoparticles have the potential to deliver new drug

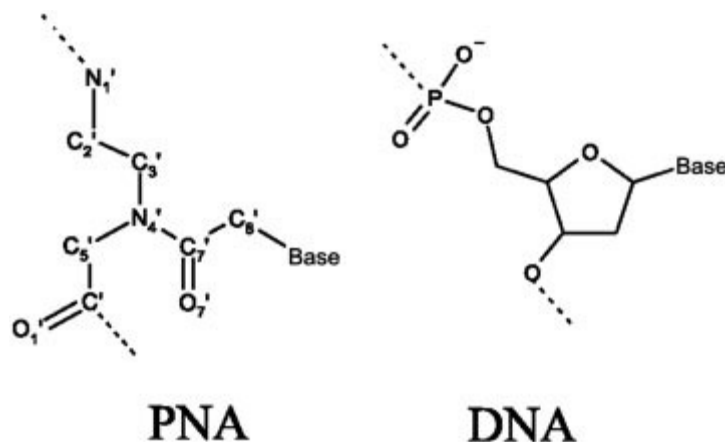


Figure 1: Structure of Peptide nucleic acid and Deoxyribonucleic acid

molecules across the blood brain barrier and are biodegradable in nature. The physicochemical properties of the NPs at different surfactant concentrations, stabilizers, and amyloid-affinity agents could influence the transport mechanism and have potential for usage in AD (Soppimath *et al.*, 2001, Roney *et al.*, 2005). Alzheimer's disease (AD) is a neurodegenerative disease associated with increased expression of amyloid precursor protein (APP) and the deposition of its proteolytic cleavage products, the amyloid- β peptides, $A\beta_{1-40}$ and $A\beta_{1-42}$. In this regard, the peptide nucleic acids have been shown to block the expression of proteins at transcriptional and translational levels. In this study, a sense and an antisense PNA specifically targeted to APP to inhibit the transcription and translation of APP by complementary binding to DNA or RNA, respectively were used. Using Western blotting techniques, APP showed a drastic decrease (50% and 90% reduction, in two separate experiments, as compared with saline control) with the injection of sense APP mRNA levels were higher at the same time point after injection of APP sense PNA, most probably because of a compensatory mechanism in response to the drop of APP that might have occurred at an earlier time point (0–1 h) and was reflected in a drop at the protein level at 1 hour. The injection of antisense PNA showed about 70% decrease in APP as estimated by Western blotting. Unmodified PNA can be used *in vivo* to reduce the levels of APP, which plays an important role in the development of AD (Boules *et al.*, 2004).

The ability of the sensor system to detect normal, wild-type human DNA sequences, and those sequences containing a single base mutation is of tremendous biological importance. Specifically, experiments were conducted with a PNA probe complementary to a region of the gene encoding the microtubule associated protein tau. The probe sequence covers a known point mutation implicated in a dominant neurodegenerative dementia known as fronto-

temporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), which has clinical and molecular similarities to Alzheimer's disease. By using an appropriate PNA probe, the conjugated polymer poly [(9,9-bis(6'-*N,N,N*-trimethylammoniumhexylbromide) fluorene)-co-phenylene] and S1 nuclease, unambiguous FRET signaling is achieved for the wild-type DNA and not the mutant sequence harboring the SNP. Distance relationships in the CP/PNA assay are also discussed to highlight constraints and demonstrate improvements within the system (Brent *et al.*, 2005). Alternatively, peptide nucleic acid (PNA) probes can be used for diagnosis of AD instead of DNA probes or as complementary probes to DNA. They exactly mimic DNA probes are therefore one of the most powerful tools for molecular biology and medical diagnostic analysis. PNA can bind to complementary strand of DNA and RNA sequences with high affinity and high specificity. PNA-based FISH analyses are used for quantitative telomere analysis using fluorescent-labeled PNA probes (Egholm *et al.*, 1993; Giesen *et al.*, 1998) Labeling analysis reveals human telomeric repeat sequences and also can accurately estimate telomere lengths. PNA can also form a triplex with the target double-stranded DNA (Pellestor and Paulasova, 2004).

Other studies demonstrate the usefulness of PNA in Amyotrophic lateral sclerosis. Novel antisense peptide nucleic acid constructs targeting p75NTR as a potential therapeutic strategy for amyotrophic lateral sclerosis (ALS) have been designed, synthesized and evaluated against phosphorothioate oligonucleotide sequences (PS-ODN). An 11-mer antisense PNA directed at the initiation codon dose-dependently inhibited p75NTR expression and death signalling by nerve growth factor in Schwann cell cultures. Inhibition of p75NTR production was not detected in cultures treated with the nonsense PNA or antisense PNA directed at the 3'-terminus sequence. The 19-mer PS-ODN sequences also failed to confer any activity against p75NTR but,

unlike the PNA sequences, were toxic *in vitro* at comparable doses (Irwin *et al.*, 2003).

PNA can also be useful for possible therapeutics of musculo-skeletal disorders like the Duchenne muscular dystrophy (DMD). DMD is the most common and severe form of muscular dystrophy, arising from mutations in the dystrophin gene that preclude the synthesis of functional protein. Antisense oligonucleotides (AOs) have been shown to induce specific exon skipping and thereby restore the reading frame and expression of functional dystrophin. In this report, investigation was done on the effects of peptide nucleic acid (PNA) oligonucleotides and PNAs conjugated with peptides including TAT, muscle-specific peptide (MSP), adenoassociated virus 6 (AAV6) functional domain (AAV6), and AAV8 functional domain (AAV8), on exon skipping *in vitro* and *in vivo*. Efficient skipping of targeted exon 23 was achieved in cultured *mdx* myoblasts with PNA and PNA-peptide conjugates. Furthermore, single intramuscular injections of PNA and all PNA-peptide conjugates resulted in significant numbers of dystrophin-positive fibers in the injected tibialis anterior (TA) muscles of *mdx* mice, with no apparent local toxicity. Similar effects of exon skipping and dystrophin expression were obtained in mice of all ages. PNA and PNA-AAV6, PNA-AAV8 conjugates induced dystrophin expression in a dose-dependent manner. The results demonstrate that PNAs have a higher efficiency of exon skipping than 2'-O methyl phosphorothioate AOs do, and have a potential use in AO chemistry for antisense therapy of DMD (Haifang *et al.*, 2008).

Soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) analogous PNA hybrid i.e the transmembrane domain was based on the native peptide sequence. Where as the SNARE identifying motif was replaced by PNA oligomers. PNA oligomers form stable and well-defined double-stranded nucleobase recognition complexes. The PNA recognition motif resembles the double-stranded DNA already used in a SNARE analogue. Nevertheless, PNA oligomers offer the advantage of the sequence-dependent formation of antiparallel as well as parallel duplexes with high thermal stability, sequence selectivity, and noncharged backbone, and protease resistance (Lygina *et al.*, 2011).

PNA as an Anti-sense Oligonucleotide

During the past decade, many biotechnology companies have showed an interest in antisense oligonucleotides for the treatment and prevention of disease. Anti-sense oligonucleotides are synthetic molecules that resemble pieces of single stranded DNA and incorporate any and/or all of the four bases. These oligonucleotides can then be used to either inhibit or promote gene expression by binding directly to target DNA or RNA.

Thus a particular sequence of antisense oligonucleotide could be designed specifically around a known mRNA transcript and then allowed to bind with that mRNA in order to control the production of a specific protein. Also, a plethora of information about any gene could be obtained by synthesizing a vast library of complementary oligonucleotide sequences. This is because of the hydrogen bonding that exists between the 6 complementary base pairs; it can provide necessary information for the design of an oligonucleotide which will target any gene with a known sequence. Greater control over binding specificity could be obtained as a result of targeting a defective gene with such oligonucleotides which in turn would give birth to a new strategy of drug design. Anti-sense RNA can be described as a naturally occurring phenomenon in which the cell transcribes a strand of RNA which is complementary to a specific segment of mRNA. These antisense RNA strands hybridized to the mRNA thereby inhibiting gene expression. In order to keep these antisense RNA strands in check, certain enzymes have important key roles in the regulatory process of these antisense RNA strands. It has been shown that studying a small portion of any gene can reveal its sequencing information thus providing scientists with the anti-sense sequence. The inhibition of gene expression through a natural process is already known for anti-sense RNA strands. However, a similar natural system involving anti-sense DNA is unknown at this time. In theory, the inhibition of a single gene target could be accomplished through the use of an oligomer 15 nucleotide bases in length that binds complementary to the mRNA strand in question (Gaglione *et al.*, 2011).

Cellular delivery of anti-sense peptide nucleic acid by electroporation

In 1978, Zamecnik and Stephenson discovered a new class of therapeutic agents called anti-sense oligonucleotides which specifically targeted mRNA. In their research, Zamecnik and Stephenson successfully showed that anti-sense could be used to inhibit viral replication using an anti-sense phosphodiester oligodeoxynucleotide (ODN), they observed that it bound to the mRNA strands of the Rous sarcoma virus which in turn inhibited replication. Earlier studies into the mode of this type of inhibition prove that the binding between the mRNA strands and the ODN's follow the antisense mechanism. These studies used two types of ODN's, control ODN's, which were sequences that were not complementary for the target mRNA and complementary ODN's (Joergensen *et al.*, 2011).

PNA may give the molecule several advantages over oligonucleotide-type compounds. Most important is the very high biological (and chemical) stability as well as easy access to a wide range of chemical modifications in a medicinal chemistry context. However, despite these apparent advantages, biological applications of

Table 1: Luciferase activity in PC 12 cells exposed to electrotransfer of PNA at different concentrations using the cuvette system.

No.	Treatment	Relative light units (RLU)	% Change
1	No PNA	393	
2	PNA (0.5 μ M)	2229	467.1
3	PNA (1.0 μ M)	4392	1017.5
4	PNA (1.5 μ M)	5487	1296.1
5	PNA (2.0 μ M)	6223	1483.4

PNA molecules are limited by their inherently poor cellular uptake, being a relatively hydrophilic molecule that does not readily cross cell membranes (Wittung *et al.*, 1995). Although several delivery methods have been described to enhance cellular uptake *in vitro*, including microinjection (Hanvey *et al.*, 1992), electroporation (Karras *et al.*, 2001), incorporation into liposomes (Wittung *et al.*, 1994; Shiraishi *et al.*, 2008), and conjugation to cell penetrating peptides (Koppelhus and Nielsen, 2003; Bendifallah *et al.*, 2006) or receptor targeted ligands (Basu and Wickstrom, 1997; Zhang *et al.*, 2001), and several studies have demonstrated *in vivo* effects in animal models of PNA conjugates (Sazani *et al.*, 2002; Ivanova *et al.*, 2008), a major challenge still exists in finding effective and easy *in vivo* delivery methods of PNA to be able to reach clinical trials and eventually clinical use.

Electroporation is a simple, transitory, and straightforward method for delivery of molecules, which may aid or substitute transfer, mediating modifications of the PNAs such as conjugation to delivery peptides or receptor-specific ligands. A study conducted by this author demonstrated that a 15-oligomer PNA which was introduced into PC 12 cells by electroporation showed increase in luciferase activity in a concentration gradient manner with maximal effect at 2 μ M (Table 1). Thus, electrotransfer may eliminate some of the problems (such as endosomal entrapment) encountered by exploiting other transport mechanisms across the membrane (Koppelhus and Nielsen, 2003; Lundin *et al.*, 2006).

By focused delivery, using electrotransfer, the target cells can be selectively exposed to the molecules, and cells in other tissues may remain unaffected. Further, electroporation is widely used for *in vivo* gene electrotransfer, and it has become an acknowledged method in clinical use for enhancing delivery of chemotherapeutics. Currently, electroporators for clinical use are approved and have been proven efficient with few side effects in several clinical studies (Gothelf *et al.*, 2003; Marty *et al.*, 2006; Mir *et al.*, 2006; Daud *et al.*, 2008). Thus, the necessary technical set up for future electrotransfer of PNA in a clinical setting is

ready once potential drug candidates have been identified.

Peptide nucleic acid and nano-biotechnology

The development of advanced biological sensors could impact significantly the areas of genomics, proteomics, biomedical diagnostics, and drug discovery. While both silicon nanowires (SiNWs) and nanotubes (NTs) have been used previously for detecting biological species, there has been a focus on SiNWs, since the electrical properties and sensitivity of SiNWs can be tuned reproducibly by controlling dopant concentration and NW diameter (Zhang, 2011). The modification of silicon oxide surfaces also has been well studied, and this information can be exploited for tailoring SiNW surfaces with biological or chemical receptors.

Recent reports suggest the use of SiNWs for real-time, label-free detection of DNA and DNA mismatches. It has been shown that SiNW sensors functionalized with peptide nucleic acid (PNA) receptors can distinguish wild-type from the CF508 mutation site in the cystic fibrosis transmembrane receptor (CFTR) gene. The p-type SiNWs used in these studies were synthesized using a gold nanocluster catalyzed chemical vapor deposition (CVD) approach described previously. SiNWs were assembled into sensor devices consisting of electrically addressable NWs with PNA surface receptors, where the receptors were linked to the NW surface using intervening avidin protein layer, and a microfluidic sample delivery structure (Hahm and Lieber, 2004). PNA was chosen as a recognition sequence in our studies, since it is known to bind to DNA with much greater affinity and stability than corresponding DNA recognition sequences, and has shown binding without either WT or MU DNA exhibited no substantial change in conductance (Cattani-scholz *et al.*, 2010).

The nanowires incorporated into the PNA have tremendous futuristic potential in genomics, proteomics and drug discovery. One strategic way of introducing these SiNW with PNA would be through electroporation

into blood cells and culturing these stable transfected cells under optimal conditions and injecting them into patients for therapeutic and diagnostic purpose. I presume this will happen in the decades to come.

Peptide-Based Strategy for *Ex Vivo* and *In Vivo* Oligonucleotide Delivery

Cellular uptake of therapeutic oligonucleotides and subsequent intracellular trafficking to their target sites represents the major technical hurdle for the biological effectiveness of these potential drugs. Accordingly, laboratories worldwide focus on the development of suitable delivery systems. Among the different available non-viral systems like cationic polymers, cationic liposomes and polymeric nanoparticles, cell-penetrating peptides (CPPs) represent an attractive concept to bypass the problem of poor membrane permeability of these charged macromolecules. Most cationic CPPs bind to cell-associated glycosaminoglycans and are internalized by endocytosis, although the detailed mechanisms involved remain controversial. Sequestration and degradation in endocytic vesicles severely limits the efficiency of cytoplasmic and/or nuclear delivery of CPP-conjugated material. Re-routing the splicing machinery by using steric-block ON (oligonucleotide) analogues, such as PNAs (peptide nucleic acids) or PMOs (phosphorodiamidate morpholino oligomers), has consequently been inefficient when ONs are conjugated with standard CPPs such as Tat (transactivator of transcription), R(9) (nona-arginine), K(8) (octalysine) or penetratin in the absence of endosomolytic agents (Laufer and Restle, 2008). The identification of new nucleic acid-based therapeutic molecules such as short interfering RNA (siRNA) and PNA analogues has provided new perspectives for therapeutic targeting of specific genes responsible for various pathological disorders. However, the inappropriate cellular uptake of nucleic acids together with the low permeability of the cell membrane to negatively charged molecules remain major obstacles to their clinical development. Numerous non-viral strategies have been proposed to improve the delivery of synthetic short oligonucleotides both in cultured cells and in vivo. Cell-penetrating peptides constitute very promising tools for non-invasive cellular uptake of oligonucleotides and analogs. Recent research studies demonstrate a non-covalent strategy based on short amphiphatic peptides (MPG8/PEP3) that have been successfully applied *ex vivo* and *in vivo* for the delivery of therapeutic siRNA and PNA molecules. PEP3 and MPG8 form stable nanoparticles with PNA analogues and siRNA, respectively, and promote their efficient cellular uptake, independently of the endosomal pathway, into a wide variety of cell lines, including primary and suspension lines, without any associated cytotoxicity (Scherf et al., 2000). These studies describe convenient protocols for

the use of MPG-8 or PEP-3-nanoparticle technologies for PNA and siRNA delivery into adherent and suspension cell lines as well as *in vivo* into cancer mouse models (Crombez et al., 2011).

CONCLUSION

The high affinity and sequence selectivity of PNA can be used in therapeutic and diagnostic applications based on hybridization nucleic acid strands such as DNA/RNA microarrays, polymerase chain reaction and fluorescent *in situ* hybridization techniques. Thus it can be suggested based on the current research status on PNA that it has multifaceted role to play in various fields of medical science and biotechnology. Novel applications of the PNA based on novel delivery systems will throw new vistas open in the future.

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