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Peptide nucleic acids: versatile tools for gene therapy strategies

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Abstract

Peptide nucleic acids, or PNAs, are oligonucleotide analogs in which the phosphodiester backbone is replaced with a polyamide structure. First synthesized less than 10 years ago, they have received great attention due to their several favorable properties, including resistance to nuclease and protease digestion, stability in serum and cell extracts, and their high affinity for RNA and single and double-stranded DNA targets. Although initially designed and demonstrated to function as antisense and antigene reagents that inhibit both transcription and translation by steric hindrance, more recent applications have included gene activation by synthetic promoter formation and mutagenesis of chromosomal targets. Most notably for gene delivery, they have been used to specifically label plasmids and act as adapters to link synthetic peptides or ligands to the DNA. Thus, their great potential lies in the ability to attach specific targeting peptides to plasmids to circumvent such barriers to gene transfer as cell-targeting or nuclear localization, thereby increasing the efficacy of gene therapy. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Peptide nucleic acid; PNA; Triplex; Antisense; Gene therapy; Transcription; Gene transfer; Nuclear import

Contents

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1. Introduction

Synthetic oligonucleotides have shown immense promise and have been extremely useful in gene activation and repression strategies over the past 20 years. However, several factors have limited their potential, most importantly the susceptibility to nuclease digestion. Less than 10 years ago, Neilsen and colleagues developed an oligonucleotide analog in which the phosphodiester backbone was replaced with a polyamide to create a 'peptide nucleic acid' or PNA [1]. This modified oligonucleotide displayed high affinity and specificity of binding to DNA and RNA and showed great resistance to both nucleases and proteases. Although initially designed to function as antisense and antigene reagents, these molecules have recently found more applications that make them ideal for the development of a variety of novel gene therapy approaches, including gene activation and plasmid delivery.

2. Structure and binding characteristics

PNAs are nucleic acid analogs in which the phosphodiester backbone has been replaced with a polyamide backbone made up of repeating *N*-(2-amnioethyl)glycine units [1]. The purine and pyrimidine bases are attached to the backbone and extend out in a conformation that is remarkably similar, both in spacing and geometry, to standard oligonucleotides (Fig. 1). One consequence of this polyamide backbone is that, unlike DNA and RNA, the backbone contains no phosphate groups. Because the backbone is not charged, there is very little repulsion when the PNA hybridizes to its target nucleic acid sequence, leading to a more stable complex. An additional semantic and structural consequence of the polyamide backbone is that PNAs do not have a 5′ to 3′ orientation as do phosphodiester backbones, but rather have an amino (NH_2) to carboxyl (CO_2H) orientation. Because the geometry and the spacing of the bases is nearly identical to that found in a native DNA or RNA strand, PNAs can hybridize to complementary sequences of DNA or RNA through classic Watson–Crick base pairing (Fig. 2A). PNAs can bind to either single-stranded DNA or RNA, in which the resulting hybrid resembles the B-form of DNA [2,3], or to double-stranded DNA. When bound to double-stranded DNA, the PNA invades the DNA double-stranded helix and hybridizes to the target sequence, thus displacing the second DNA strand into a 'D' loop (Fig. 2B) [1].

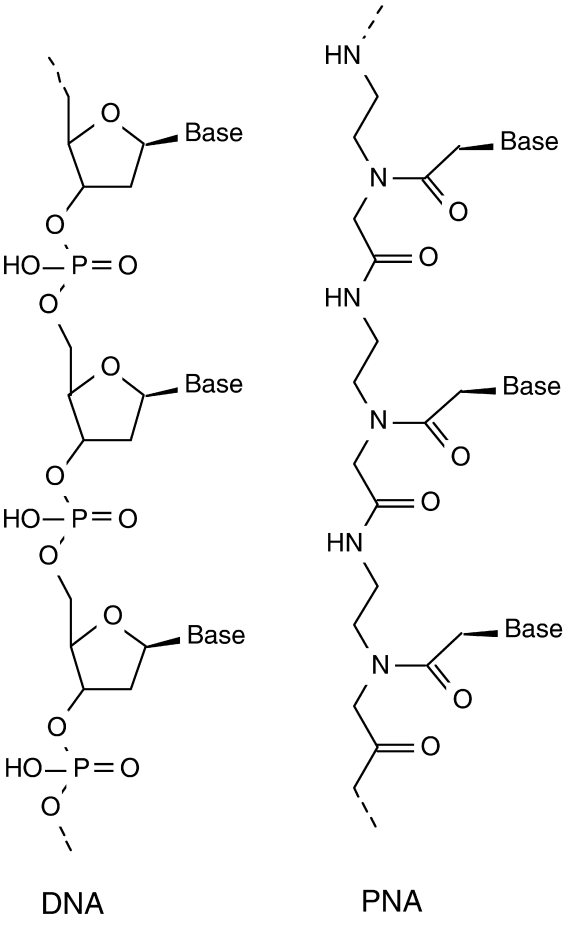


Fig. 1. Structures of single-stranded DNA and PNA.

Like DNA base-pairing, the affinity of the PNA–DNA or PNA–RNA complex is determined by the base pair composition. Thus, C–G base pairs are more stable than A–T base pairs, but these differences have little impact on the ability of the PNA to bind to a target sequence: PNAs can be designed to bind to essentially any target sequence. Sequence restrictions do apply, however, when designing PNAs that are desired to form triplex structures. In this case, the first strand of the PNA hybridizes to

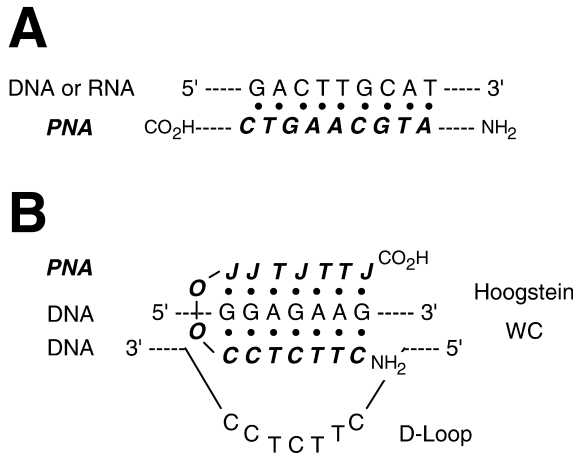


Fig. 2. Structures of duplex and triplex PNA complexes. (A) Duplex PNA–DNA or PNA–RNA complex. The target sequence present in either DNA or RNA is shown as the top strand, while the PNA (bold, italics) is shown as the bottom strand, in the antiparallel orientation. Base-pairing interactions are denoted by dots between the dases. (B) Triplex PNA_2–DNA complex. In this case, the DNA target sequence is depicted as the upper DNA strand and the displaced 'D-loop' is shown below the triplex structure, bulging away from the complementary DNA strand. The amino-terminal half of the PNA hybridizes to the target sequence using standard Watson–Crick base pairs (WC) and the carboxy-terminal half of the PNA uses Hoogstein base pairs to form the third strand. 8-amino-3,6-dioxaoctanoic acid linkers used to connect the two halves of the PNA are abbreviated as 'O'.

the target sequence using standard Watson–Crick base pairing rules, while the third strand of the triplex is formed by the PNA (either as a second independent PNA molecule or as a long PNA with two homologous sequences separated by a linker, see below) using Hoogstein base pairs (Fig. 2B) [4,5]. For such structures to be formed, the target sequence must be composed of a run of purine residues (As or Gs) simply because of the restrictions of triple base pairing. Each pyrimidine within the target sequence of a triplex–forming PNA has been shown to significantly reduce the binding affinity. Pseudo-isocytosine residues, denoted 'J', are often used in place of cytosines in the third strand because they can hybridize to the target sequence in a pH-independent manner and can increase the T_m of the structure by up to 20 degrees [6].

When added to double stranded DNA, the PNA strand invades the duplex and anneals to its target, thus displacing the second strand into a D-loop structure [1]. The first strand of pyrimidine PNAs can bind to their purine targets in triplex structures in two orientations. The parallel orientation is when the amino-terminus of the PNA binds to the 5′ end of the target sequence, whereas the antiparallel orientation occurs when the amino terminus of the PNA binds to the 3′ end of the target. In the latter orientation, the structure is drawn as in a normal DNA duplex, with the first PNA strand taking the place of the complementary DNA strand, as shown in Fig. 2B. Both orientations appear to have similar binding characteristics. For a typical PNA:DNA duplex hybrid, the binding constants for the hybridization reaction are in the range of 10^{-6} to 10^{-7} M for targets of 8–15 nucleotides. PNAs in triplex structures bind even tighter to their targets. Although the first strand (Watson–Crick) PNA binds to the target with an affinity similar to that of a duplex PNA:DNA hybrid, the formation of the third strand gives greater stability to the complex and increases the affinity in binding reactions to 10^{-6}–10^{-9} M, and the melting temperatures to between 50°C and 90°C, depending on the sequence and length [1,6].

Strand invasion of the duplex DNA target site during triplex formation by PNA is dependent on both salt concentration and pH. Strand annealing occurs optimally under low ionic conditions. It has been shown that whereas 100% of strand invasion occurred within 1 h at 37°C in 1 mM Tris (pH 8) and 0.1 mM EDTA, between 10 and 20% occurred within the same time when the buffer was changed to 50 mM Tris (pH 8), 50 mM NaCl, and 10 mM $MgCl_2$ [7]. Thus, the decreased annealing found at higher salt concentrations was thought initially to limit the use of PNA–DNA hybridization under physiological conditions. However, the fact that significant annealing can still be detected in vitro at 100 mM NaCl and higher when the concentrations of PNA and DNA are adjusted has made PNAs attractive and functional for applications involving in vivo target binding [8]. Binding of PNA to its target site has also been shown to be dependent on pH, with more stable binding occurring at neutral to slightly acidic pH [6]. In contrast, it has been demonstrated that triplex strands containing J instead of C form the third strand independent of pH [6]. Finally, as for any hybridization reaction, temperature and time are crucial to the formation of the complex. Conse-

quently, our laboratory routinely carries out PNA:DNA annealing (for both duplex and triplex formation) in TE buffer (10 mM Tris, pH 8, 1 mM EDTA) at 37°C–42°C for between 1 and 4 h [9].

Once annealed, PNA:DNA and PNA_2:DNA (triplex) complexes are extremely stable. Melting temperatures for triplex structures can range from 50 to 90°C in TE containing 100 mM NaCl, 10–30°C higher than their annealing temperatures [10]. Furthermore, under physiological conditions, PNAs are unlikely to dissociate from their target site to bind to a second site. Elegant experiments from Phil Felgner's group using fluorescence resonance energy transfer (FRET) demonstrated that a PNA bound to its DNA target in a triplex formation would not dissociate from its target site upon incubation with 100–1000-fold molar excesses of charged and neutral lipids, histones, bovine serum albumin, competitor DNAs (containing or lacking the target site), tRNA, dATP, polyglutamic acid, spermidine, spermine, polylysine, or dextran sulfate (Fig. 3) [11]. Similarly, our laboratory has not been able to measure dissociation and/or transfer of a triplex-forming PNA from its bound target site upon incubation for 8 h at 37°C with 1000-fold molar excess of its target site [9]. Thus, once bound to their target site, PNAs, especially those in triplex structures, are very stable and well suited to many biological applications.

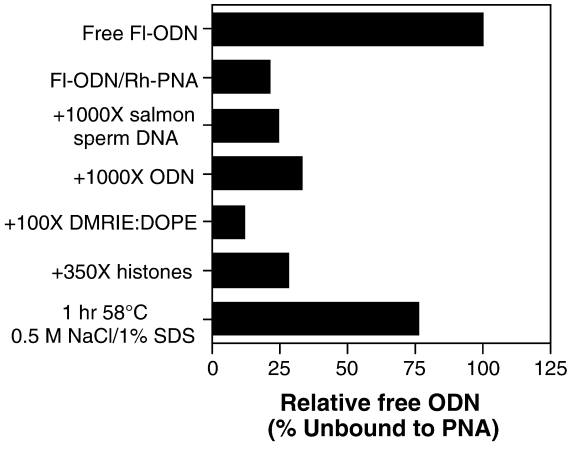


Fig. 3. Stability of PNA_2–DNA triplex. A fluorescein-labeled 20-mer oligonucleotide (Fl-ODN) was reacted with a triplex-forming rhodamine-labeled PNA (8 base target site; Rh-PNA) for 2 h at 37°C to form a stable 'PNA-clamp', and then incubated for at least 3 h with 100–1000-fold molar excess of competitors. Fluorescence resonance energy transfer (FRET) was used to measure the degree of dissociation of PNA from the fluorescein-labeled oligonucleotide. Peak fluorescence intensity of the free Fl-ODN at 520 nm is shown. Thus, 100% of the Fl-ODN is free when unincubated with PNA, whereas 25% of the Fl-ODN is free after the 2 h reaction with the PNA. Values above 25% therefore represent dissociation of the PNA from the Fl-ODN. Competitors included 1000-fold molar excess of salmon sperm DNA or unlabeled target site-containing oligonucleotide (ODN), 350-fold molar excess of histones, or 100-fold molar excess of the DMRIE:DOPE liposomes. The triplex complex was also incubated for 1 h at 58°C with 50 mM Tris–HCl (pH 8.9), 0.5 M NaCl, 10 mM EDTA, and 1% SDS. This Fig. was adapted from data in [11].

As stated above, one consequence of the polyamide backbone of PNA is that the structure is highly resistant to either nuclease or protease digestion. Incubation of PNA with S1 nuclease or DNase I has no effect on the PNA. Similarly, PNAs are not proteolyzed or degraded upon incubation with proteinase K or porcine mucosal peptidase [12], thus resulting in long biological half-lives for PNAs in vivo. Further, PNAs are stable in both serum and cellular extracts [12]. In contrast to the PNA itself, the hybridized DNA or RNA target is not protected from degradation, nor is the displaced D-loop [11,13]. Thus, while the PNA itself is resistant to degradation by a variety of enzymes, its targets remain sensitive.

3. PNAs as gene expression modifiers

3.1. Use of PNAs as anti-sense agents

Due to their ability to bind to both DNA and RNA targets with high specificity and affinity, the first application of PNA was as anti-sense or anti-gene agents. Researchers at Glaxo demonstrated that a 15-mer PNA whose target sequence was on the transcribed strand of a DNA template expressing the SV40 large T antigen, inhibited RNA polymerase II-mediated transcription in cell extracts by 90–100% [7]. By contrast, when the PNA was targeted to the corresponding complementary strand (non-transcribed), less than 50% inhibition was obtained. The inhibition was both sequence-specific, showing no alteration of expression of other reporter genes

lacking the target sequence, and dose-dependent, with half maximal inhibition of transcription seen around 500 nM. Moreover, inhibition of expression could also be demonstrated in microinjected cells, although this inhibition required higher concentrations of PNA (1–5 μM) and PNAs of longer length. Whereas 10- and 15-mer PNAs induced transcriptional inhibition in extracts, the 10-mer showed no effect on in vivo expression, even at up to 10-fold higher concentration, while the 15-mer was effective. The most likely explanation for this is due to the decreased binding of PNAs to their target sequences in the presence of high salt and counterions. Furthermore, because of the decreased strand invasion at physiological salt concentrations, it is likely that the inhibition of expression detected in the microinjected cells was due to translational arrest caused by PNA binding to the messenger RNA, rather than transcriptional inhibition. Indeed, this appears to be the predominant mode of antisense activity by PNAs.

Subsequent studies into the mechanism(s) of antisense activity displayed by PNAs, demonstrated that they inhibited expression differently than phosphate-backbone derived oligonucleotides. When antisense RNA was originally introduced to inhibit expression, it was believed that the major mechanism would be steric hindrance: the oligonucleotide would bind to its complementary RNA sequence, thus blocking initiation or elongation by ribosomes. However, it was quickly determined that this was a minor mechanism, used only by a few types of modified oligonucleotides [14,15]. The major route of RNA- and modified oligonucleotide-mediated antisense activity occurs through RNaseH-mediated degradation of the mRNA/oligonucleotide hybrid, resulting in decreased message levels [15–17]. By contrast, because PNAs are not substrates for RNaseH or other RNases, the major antisense mechanism of PNAs is through steric interference [15,18]. This interference can be at several levels. PNAs can bind to DNA to inhibit RNA polymerase initiation and elongation [7,19–22]. They have also been designed and shown to bind to targets corresponding to transcription factor binding sites within the promoter, thus inhibiting the binding and action of the corresponding transcription factor [23]. PNAs can bind to RNA in the 5′ untranslated region and within the coding sequence to inhibit either ribosome binding or translational elongation [7,15,18,24–26]. Finally, it is also possible that PNAs may bind to regions of the RNA that influence export from the nucleus to the cytoplasm, an essential step in the ultimate synthesis of proteins.

A rather interesting use of PNAs to inhibit global gene expression was devised by Good and Nielsen [27]. Duplex- and triplex-forming PNAs were targeted to several regions of the *Escherichia coli* 16S rRNA, including the peptidyl transferase and mRNA binding domains and the alpha sarcin loop. In a cell-free translation system, PNAs against all three sites inhibited translation at concentrations between 50 and 100 nM. Because global translation was inhibited, these PNAs were also tested for their ability to act as antibiotics. When added to cultures of *E. coli*, PNAs that inhibited translation in the cell-free system also inhibited cell growth, but at concentrations that were 10 to 100-fold higher. This need for higher concentrations in living cells most likely represents the permeability barrier to the PNAs presented by the bacterial cell wall.

When compared with traditional oligonucleotides and their analogues, including phosphorothioate and methyphosphonate, there appears to be no consensus in the field as to whether PNAs or other oligonucleotides are more efficient. For example, in one study, PNAs inhibited in vivo expression to the same extent as did propyne-phosphorothioate oligonucleotides, but required 10-fold higher concentrations to do so [15]. By contrast, others have reported that PNAs inhibit gene expression in vivo and in vitro much better than oligonucleotides [7,28]. Even if the concentrations of PNA needed to achieve reasonable antisense activity are significantly higher than those of traditional oligonucleotides, the resistance to degradation, stability, and specificity of PNAs make them a reasonable choice for antisense applications.

3.2. Additional targets of PNA anti-sense technology

Because triplex PNA_2/DNA and PNA_2/RNA structures are extremely stable, they have the ability to act as molecular road blocks for enzymes acting on DNA or RNA. This explains the inhibition of transcription and translation by PNA binding in antisense strategies. Using this same rationale, several groups have come up with novel cellular targets

for PNA inhibition. By designing PNAs recognizing the RNA component of telomerase, it was shown that they could inhibit enzyme activity in the nanomolar range, suggesting the potential for their use in cancer therapy [29]. PNAs have also been used to inhibit the reverse transcriptases from Moloney murine leukemia virus, avian myelosarcoma virus, and HIV-1 in a dose-dependent manner [30]. Using an in vitro assay, complete arrest of HIV-1 reverse transcriptase could be achieved at a 6-fold molar excess of PNA over the HIV RNA target, and at concentrations well below those needed to inhibit translation. Further, phosphorothioate oligonucleotides of identical sequence gave no effect on reverse transcriptase activity. Finally, PNAs have been used to inhibit genomic and mitochondrial DNA replication [31], and Herpes virus DNA helicases that are necessary for viral replication [32].

3.3. PNAs as mutagens

One consequence of the tight binding of triplex-forming oligonucleotides and PNAs to cellular homopurine DNA targets is that they can induce DNA repair pathways within the cell. Thus, the PNA_2/DNA complex can be interpreted by the cell to be a DNA lesion in need of repair. One possible outcome of this is the production of site-specific mutants at, or proximal to, the site of PNA binding. Faruqi and co-workers reported that PNAs with binding affinities of around 10^{-7} M were able to be taken up by streptolysin-O permeabilized murine fibroblasts, bind to their target sites within a SupF1 reporter gene integrated into the genome, and induce point mutations or single base deletions or insertions in the target sequence or within 5 bp of the site [33]. The frequency of mutagenesis observed with the PNAs was 8×10^{-4}, compared to a background mutagenesis frequency of 9×10^{-5}. Similar results have been obtained with tightly binding triplex-forming phosphorothioates, leading to the prediction that triplex complexes in general can induce transcription-coupled DNA repair pathways [34]. Presently, this mutagenic capacity is being developed as a technique to selectively mutagenize and repair single point mutations important in certain genetic diseases, including sickle cell disease [35].

3.4. Use of PNAs as inducers of endogenous gene expression

Although most of the focus on PNA applications has revolved around antisense strategies, several groups have instead studied the ability of PNAs to turn on gene expression [8,36]. When PNA triplex structures are formed on one strand of the DNA duplex, the opposing DNA strand is displaced to form a D-loop. When sufficiently large, this D-loop resembles a transcriptional bubble or initiation/elongation loop. Using bacterial and rat spleen cell nuclear extracts and triplex-forming PNAs that bound to adjacent sites on a DNA template, Møllegaard and colleagues found that transcription was more efficient if two adjacent PNAs were bound to the same strand of DNA, thus giving rise to a 26 base D-loop. In this case, transcript initiation sites mapped to a site just downstream of PNA binding [36]. When the PNAs were bound to opposite strands, but producing the same sized D-loop, two divergent transcripts were generated, each of which initiated at the site of the PNA bound to the non-template strand.

More recently, work from the Wang laboratory has extended these findings and shown that PNAs can be used in cultured cells to activate endogenous gene expression [8]. Using promoter-less GFP reporter constructs containing homopurine PNA target sites just upstream of the GFP initiation codon, Wang was able to demonstrate specific transcriptional initiation in vitro using HeLa cell extracts. In these experiments, designed to explore gene therapy approaches for sickle cell disease and thalassemias, the homopurine target sequence was from the -300 region of the human Gγ-globin promoter. As seen by Møllegaard [36], transcript start sites mapped to the PNA target site and used the D-loop strand as template. When the PNAs were hybridized to the reporter plasmid under low salt conditions and microinjected into the nuclei of cells, GFP expression was detected within 20 h of injection. By contrast, no GFP expression was detected in cells microinjected with plasmid alone. Wang also demonstrated that K562 erythroleukemia cells could be efficiently transfected with PNAs using electroporation and induce the endogenous γ-globin gene threefold over background. Moreover, this induction was

clearly a specific effect of the PNAs since the start sites for the transcripts mapped to both the endogenous promoter and to the PNA binding sites, 300 bp upstream. Thus, despite the presence of physiological salt concentrations and potential chromatin structures, the PNAs were able to bind to their target sites in the chromosome and promote transcription initiation, providing proof of principle for this approach of gene therapy.

3.5. In vitro vs. in vivo use of PNA

Perhaps the limiting factor for the in vivo use of PNAs is their accessibility to the intracellular environment. Using unilamellar phospholipid vesicles as model membranes, Wittung and co-workers demonstrated that PNAs and DNA oligonucleotides had very low efflux rates from liposomes, when trapped inside [37]. Indeed, the efflux half-times for two 10-mer PNAs were between 5 and 11 days. From these experiments it was concluded that entry of PNAs into cells by passive diffusion is very slow. Indeed, in subsequent experiments from other laboratories, it was demonstrated that PNA entry into certain cells and cell lines was exceedingly slow or undetectable [15,38,39]. Similarly, although fluorescently-labeled PNAs could be detected in vesicles within cells after prolonged incubation, no antisense effects of the PNAs were ever detected in cells including CV1, W138, and Rat1 cells, whereas the same PNAs microinjected into the cytoplasm or nucleus caused robust inhibition of gene expression [15]. This suggested that, at least in these cells, PNAs had limited access to the cytoplasm.

By contrast, several groups have found that certain cells are amenable to PNA entry, suggesting specific transport mechanisms for the molecules [19,20,24,27,31,38,40]. In one set of experiments comparing the uptake of different oligonucleotides, PNA was found to be imported into cells and achieve intracellular concentrations approximately 25–50% of those seen with phosphorothioate oligonucleotides [38]. The intracellular concentration of PNA after 24 h incubation with HL60 cells or H-*ras*-transformed murine fibroblasts was equal to those found with the charged DNA and RNA analogs, 3′-alkylamino oligonucleoside phosphodiester and 2′-methyl oligoribonucleoside methylphosphonate, and was 2 to 5-fold higher than that achieved by unmodified methylphosphonate oligonucleotides [38]. PNAs have been reported to enter rat neuronal cells, in both studies using cultured cells and in animals. In cultured rat neurons, not only were PNAs taken up by the cells over time, but they also showed a time- and dose-dependent inhibition of expression of their target gene, prepro-oxytocin [20]. Uptake by neurons has also been shown in vivo. When PNAs were injected into the rat brain, they were able to downregulate target gene expression by antisense activity [40]. Moreover, several groups also have demonstrated that PNAs, injected either intravenously or intraperitoneally, can cross the blood–brain barrier and enter neurons in vivo, eliciting an antisense response [19,20].

Myotubes also have been suggested to take up PNAs from the culture medium. Using biotinylated PNAs, Taylor et al. [31] observed a punctate intracellular staining of PNAs within 4 h that became diffuse over time, leading the authors to suggest that the PNA was gaining access to the cytoplasm. The observed punctate staining is similar to that seen in CV1 cells by Bonham [15], who concluded, based on the absence of antisense effects of the added PNAs, that PNA did not access the cytoplasm in these cells. Thus, to confirm whether myoblasts indeed transport PNAs into the cytosol, functional assays must be performed.

In order to deliver relevant amounts of PNAs to a variety of cell types, several approaches have been taken. First, we and others have demonstrated effective delivery of PNAs to cells using cationic liposomes [8,11,41]. Using this approach, not only do PNAs have access to the cytoplasm, based on fluorescence and EM [11,41], but the free PNAs also have further access to the nucleus, based on their ability to induce endogenous transcription from the chromosome [8].

A second approach to increase cell delivery of free PNAs has been to modify them by addition of targeting molecules or peptides to the polyamide backbone. Working in a rat model, Pardridge and colleagues found that when an unmodified PNA was injected intravenously, negligible uptake by the brain was detected [39]. It should be noted that the concentrations used by Pardridge were 10,000-fold lower than those used by Tyler [19], who found PNA

uptake into the brain, and could thus account for the apparent discrepancy in results. Based on the fact that the blood–brain barrier contains a high concentration of transferrin receptor, Pardridge coupled a streptavidin-labeled monoclonal antibody reactive against the rat transferrin receptor to a biotinylated PNA. When injected intravenously, uptake of the PNA conjugate by the brain increased by almost 30-fold, suggesting that the addition of the anti-transferrin receptor antibody to the PNA caused increased access to the blood–brain barrier and to cells of the brain.

Using a different type of conjugate, Rabié's group was able to show that conjugation of a 'retro-inverso' peptide to the PNA caused a 2-fold increase in PNA uptake by cultured cerebral cortical neurons. This is a 16-residue synthetic peptide composed of D-amino acids that corresponds to a peptide from the antennapedia homeodomain [42]. The antennapedia peptide has the relatively unique ability to transverse membranes with ease and efficiency, by a receptor-independent mechanism [43], even when fused to other proteins [44]. Several other peptides, including a 35 residue peptide from HIV Tat, appear to have the same property [45,46]. By using D-amino acids, the peptide is extremely resistant to cellular proteases, thus a perfect companion for the PNA. Similarly, the 16-residue antennapedia peptide and another peptide called transporfan, consisting of the first 12 residues of galanin followed by a lysine and the first 14 residues of mastoparan [47], have both been fused to PNAs and their penetration of cells was measured in cell culture and in rats [48]. In cultured cells, addition to the media of either the antennapedia or transporfan peptides coupled to a PNA inhibited target gene expression by 50% at 100 nM external concentration, whereas PNAs conjugated to scrambled peptides gave no effect even at 100 μM. Using expression and physiological responses as measures of PNA cellular delivery and activity, the antennapedia and transporfan peptide-PNA conjugates also exhibited dose-dependent inhibition of gene expression in intrathecally injected rats. These results suggest that the membrane internalization domain of these proteins can aid the translocation of both proteins and oligonucleotides across the cell membrane, leading to therapeutic effects.

Based on these results, additional ligands could be conjugated to PNAs to increase delivery to cells systemically or to specific cell types. For example, many cancer cells express high levels of folate receptor on their surface [49]. This fact has been used by several groups to develop cancer-targeting liposomes for the delivery of plasmids specifically to these cells [50,51]. By attaching folate to a PNA using standard chemical crosslinking reagents, the PNA could be delivered with some degree of cell-specificity to tumor cells. Any number of ligands could be attached to PNAs and used in this manner to selectively target the molecules to the desired cell types or tissues. Alternatively, ligands whose receptors are expressed at certain times in development or are upregulated due to certain environmental or therapeutic stimuli could be utilized to develop temporally active or regulated PNA reagents, respectively.

3.6. PNAs as delivery vehicles for gene therapy

Because of their high affinity for DNA, their specificity of binding, and the ability to covalently link other molecules to their polyamide backbone, PNAs offer the ability to be used as adapters, linking plasmid vectors to peptides, proteins, drugs, and molecular tracers. Moreover, it has been clearly demonstrated that the coupling of PNAs to plasmids at specific sites has no effect on the transcription of genes located elsewhere on the plasmid, or on the plasmid's physiological activities [9,11]. Thus, PNAs can be used to selectively label DNA for experiments designed to follow biodistribution of DNAs or the cell biology of gene transfer. Alternatively, they can be used to attach peptides or drugs to plasmids to aid in gene delivery and tissue-specific targeting.

3.6.1. PNAs as tools for cell biology

Nonviral gene delivery systems offer great promise for gene therapy of a variety of diseases. However, at present, they are much less efficient than their viral counterparts in terms of stability in vivo, specific targeting to cells, and levels and duration of gene expression. In order for these areas to be optimized, efficient tools for the real-time study of plasmids in cell systems are required. Indeed, our laboratory has studied the nuclear import of plasmids as one of the rate-limiting steps in gene transfer [52–54]. Although we have made progress elucidat-

ing the mechanisms of plasmid nuclear import using in situ hybridization to follow the movement of plasmids, this method is limited by its ability to detect DNA only in fixed samples. The ideal substrate would be fluorescently-labeled DNA itself. In order to produce such a substrate, we and others have tried a variety of labeling techniques including incorporation of fluorescent nucleotide analogues (e.g., fluorescein-dUTP) by PCR or nick-translation followed by ligation to form intact plasmid, covalent and non-covalent high affinity intercalating dyes (e.g., ethidium bromide monoazide and TOTO-1), and reaction of supercoiled plasmid with photoactivatable fluorophore conjugates. Unfortunately, all of these methods have produced either very low quantities of labeled plasmid (most incorporation techniques resulted in linear DNA), or plasmid that was inactive for transcription or nuclear import. The use of fluorescently-labeled PNA-plasmid complexes circumvented both of these problems [9,11].

Using fluorescently-labeled, triplex-forming PNAs to label plasmids, Felgner and colleagues were able to follow the distribution of plasmid in cationic liposome-transfected CV1 cells [11]. They were able to demonstrate that liposomes efficiently delivered fluorescently-labeled PNA-complexed plasmids to the cells, and that the plasmids localized to vesicles within the cytoplasm at early times after transfection (3 h). Interestingly, no nuclear localization of fluorescent plasmid was observed until after cell division, whereas the majority of fluorescently-labeled PNA/oligonucleotide complexes delivered into the cells by liposomes localized to the nucleus within 3 h. The most likely explanation for this discrepancy is that the much smaller PNA-oligonucleotide complexes, once in the cytoplasm, could enter the nucleus through the nuclear pore complex by diffusion. The plasmids used in this study, however, lacked DNA sequences that we have shown to be necessary for sequence-specific nuclear import [52,53]. Thus, when such a sequence was cloned into the plasmid used in the Zelphati study, nuclear import of the fluorescent DNA was observed within 6–8 h, well before cell division (D. Dean, unpublished observation). Due to the high affinity of the triplex-forming PNA for its target and the stability of the PNA in serum and cell extracts [12], the plasmid bound PNA was neither released from the plasmid within 48 h inside the cells, or degraded [11]. Finally, they were able to show that addition of the PNA to the plasmids had no effect on the plasmids ability to direct gene expression from a reporter gene elsewhere on the plasmid.

Our laboratory also has utilized fluorescently-labeled PNA clamps to label plasmids in order to follow their nuclear localization [9]. By microinjecting PNA/DNA complexes into the cytoplasm of cells, we were able to follow the movement of the plasmid into the nuclei of individual cells in real time. Thus, unlike all other previously tested labeling techniques, PNA-labeled plasmids behaved indistinguishably from native plasmids in their ability to enter the nucleus in a sequence-specific manner, in the absence of cell division. Because the DNA was directly labeled, we could easily take advantage of a permeabilized cell system in which nuclear import and export could be reconstituted from cell extracts and purified import factors. Using this approach we were able to demonstrate that plasmids enter the nucleus in a sequence-specific manner and use the same pathways as do nuclear localization signal (NLS)-containing proteins. However, unlike NLS-mediated protein nuclear import, additional proteins provided by nuclear extracts (presumably DNA-binding transcription factors) were required for plasmid nuclear localization [9]. In order to identify these proteins involved in DNA nuclear import, another novel use of PNAs has been developed by Munkonge and colleagues [55]. We have taken advantage of the polyamide backbone of a triplex-forming PNA to react it with cyanogen bromide-activated Sepharose and immobilize the intact, supercoiled plasmid to the column matrix. Plasmid–PNA interactions are maintained on the column under non-denaturing conditions, with elution of intact plasmid only possible with 0.75 M NaCl. Such columns can now be employed to capture plasmid binding proteins (transcription factors and other proteins) from mammalian cell cytosol and nuclear extracts under non-denaturing conditions, thereby elucidating the mechanisms of plasmid nuclear entry.

3.6.2. PNAs as delivery agents

One of the major problems limiting the success of non-viral gene therapy vectors is their low efficiency of gene transfer to target cells. Many barriers exist for the efficient transfer of genes to cells, including the extracellular matrix, the endosomal/lysosomal

environment, the endosomal membrane, and the nuclear envelope [56]. In contrast to naked DNA, viruses have developed ways to circumvent many of these barriers using proteins within the viral particles. As such, proteins within viral envelopes often act to direct the virus to the appropriate receptor-bearing cell. Similarly, some viral envelope proteins also possess membrane fusion activity so that the core viral particle can escape the endosome and be deposited into the cytoplasm. Many viruses also use their capsid proteins or other genome-associated proteins to target the viral DNA or RNA to the nucleus during an infection. Thus, perhaps the best way to optimize nonviral vectors is to incorporate viral features and proteins (or portions thereof) into nonviral vector systems. To this end, PNAs offer the ability to act as scaffolds for building these features into new vectors. The most developed example of this approach to date is in the area of plasmid nuclear delivery.

We and others have shown that the nuclear import of DNA plays a significant role in gene expression in the absence of cell division [52–54,56,57]. It also has been shown that the level of gene expression is directly proportional to the amount of plasmid in the nucleus [53,58]. Thus, to increase gene expression, the amounts of plasmid reaching the nucleus need to be increased. One approach used to do this has been to devise ways of attaching nuclear localization signals (NLS) to plasmids. The NLS is a short stretch of amino acids carried within proteins that localize to the nucleus [59]. To date, three main classes of NLS have been identified. The classical SV40 large T-antigen NLS is a run of 5 basic residues (KKKRK) that can be positioned anywhere within a protein. The bipartite NLS, exemplified in the Xenopus protein nucleoplasmin, is made up of two shorter groups of basic residues, separated by 10 to 20 intervening residues. The third major NLS type is called the M9 sequence and was identified in the hnRNP A1 protein that shuttles into and out of the nucleus. This sequence contains both import and export activity within a 38 residue domain. All three classes of NLS interact with receptors called importins in the cytoplasm that form complexes that are transported into the nucleus through the nuclear pore complex (for a review of nuclear import mechanism, see [60]). Because very large proteins and even ribosomal subunits can translocate across the nuclear pore in an NLS-dependent manner, it follows that by attaching one or more NLS to DNA, its nuclear localization should be enhanced.

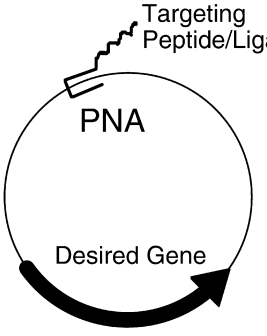
Several approaches have been taken to attach single or multiple NLS peptides to DNA. A series of experiments in zebrafish embryos and cell extracts by Philippe Collás and Peter Alestrom reported increased nuclear localization, gene expression, and integration into the host chromosome when synthetic NLS peptides were non-covalently complexed to plasmid DNA at an optimal ratio of 100 peptides per plasmid [61–64]. Similarly, others have complexed the M9 peptide to DNA using the highly basic SV40 T-antigen NLS to bind to the DNA and have shown that in liposome-transfected cells, over a 60-fold increase in gene expression was detected [65]. A second approach by Jon Wolff and colleagues used a novel crosslinking reagent to link peptides, through N-terminal cysteine residues, to DNA [66]. Using a digitonin-permeabilized cell system to study nuclear import [67], they were able to show that the DNA localized to the nucleus in an importin- and NLS peptide-mediated manner. At least 100 NLS peptides conjugated to the plasmid were required for nuclear import to be detected. Unfortunately, such high levels of conjugation to the DNA (1 chemical crosslink every 700 bp) abolish transcription and expression of genes on the plasmid. To circumvent this, they ligated an unmodified reporter plasmid to a piece of DNA that had been labeled with NLS peptides. However, there was no benefit to reporter gene expression, suggesting that this approach may not be suitable for gene delivery. Other NLS peptide-plasmid cross-linking studies also have shown direct interaction of the complex with importins, confirming that these peptide–DNA complexes use the normal nuclear import machinery for their nuclear localization [68]. More recently, a single NLS peptide fused to a linear piece of DNA that was capped at both ends by DNA hairpins was shown to increase gene expression by a factor of 10–1000 when delivered by polyethylenimine [69]. These experiments used an NLS peptide conjugated to a base within the hairpin at one end of the DNA. Although clearly demonstrating that the addition of an NLS to a piece of DNA will increase gene expression, presumably by increasing the amount of DNA

reaching the nucleus, a peptide-free, capped, linear DNA was over 100-fold less transcriptionally active than supercoiled plasmid. Thus, to achieve maximal expression, it appears that supercoiled plasmid should be the form of choice.

Because the number and location of PNA target sites on a plasmid can be controlled, so can the exact number and location on the plasmid of PNA-linked peptide(s). As previously mentioned, PNAs placed at a distance from a promoter and expression cassette have no effect on their activities. Thus, they provide an excellent tool to attach peptides to DNA (Fig. 4). Using this approach, Brandén and coworkers attached an NLS to a triplex-forming PNA and then complexed the PNA to oligonucleotides and either a LacZ- or GFP-expressing plasmid [70]. When PNA-NLS complexed oligonucleotides were transfected into the cells by polyethylenimine, they localized to the nucleus between 2- and 8-fold better than did free oligonucleotides. When a non-functional NLS was bound to the PNA, no increased nuclear localization was observed. Similarly, when bound to reporter plasmids, gene expression in transfected cells also increased 8-fold, providing proof of principle.

Although not yet optimized, the use of PNAs to attach peptides that increase nuclear localization of plasmids will clearly lead to increases in gene expression. At present, most are focusing on the attachment of the classical SV40 T-antigen NLS to PNAs on plasmids. However, because it is estimated that this class of NLS may actually represent a small fraction of NLS sequences [59], it may be prudent to test other NLS sequences for their enhancement of plasmid nuclear import. In this vein, the importin nuclear import receptor for the classical NLS is made up of two proteins: importinα, which binds to the NLS, and importin β, which binds both importin α and the NPC and mediates translocation. Alternative importin β homologs bind directly to certain NLSs to mediate nuclear import. Based on this, it may be better to attach a peptide to the PNA corresponding to the importin β-binding domain from importinα or one corresponding to the NPC-binding domain from importin β. That these peptides are less basic and thus less likely to interact and/or interfere with the DNA may make them more suitable candidates for increasing nuclear import.

Apart from nuclear targeting, one can imagine using PNAs to specifically attach peptides to plasmid for a variety of other functions or targeting abilities. For example, it has been demonstrated that PNAs can create transcription initiation bubbles and initiate transcription [8,36]. However, initiation is still rather weak compared to a normal strong promoter. By attaching peptides that can recruit transcription factors to either these artificial PNA-induced promoters or to standard promoters that contain a PNA-binding site, it may be possible to increase the levels of transcription from these promoters by building 'designer' transcription factor complexes. Examples of peptides that could be used are leucine zippers,



Targeting Peptide/Ligand	Site of Action
NLS	Nuclear Import
Importinß-Binding Domain	Nuclear Import
Leucine Zipper	Nuclear Import/Transcription
MADS box	Nuclear Import/Transcription
Antennapedia/Tat	Cell Membrane Translocation
Mitochondrial Presequence	Mitochondrial Import
HA Fusion Peptide	Endosomal escape
Folate	Cancer cell Targeting
Transferrin	Blood-Brain Barrier Access

Fig. 4. Use of peptide–PNA conjugates as targeting reagents for plasmids. A generic transgene-expressing plasmid is shown with a triplex-forming PNA–synthetic peptide conjugate bound to its target site at a unique location. Several potential targeting peptides or ligands are listed along with their activities or sites of action. Such PNAs can be used alone or in any combination on the plasmid by varying the number of PNA-binding sites on the plasmid, thus creating a wide variety of non-viral vectors optimized for different aspects of delivery and function.

responsible for the dimerization of many transcription factors including Fos and Jun (AP1), or the MADS box, a protein–protein interaction domain found within transcription factors such as Serum Response Factor (SRF).

Intracellular organelles can also be targeted using PNA technology. It has been shown that by attaching the mitochondrial presequence peptide from COX VIII to a PNA, the PNA was taken up by either isolated rat liver mitochondria or mitochondria in intact cells [71]. Moreover, it was previously demonstrated that fusion of double-stranded oligonucleotides to such a presequence peptide resulted in the mitochondrial import of the DNA in an intact form [72]. Thus, it may be possible to target plasmids to the mitochondria in an attempt to repair mutations within the mitochondrial genome or express genes that are deficient in certain diseases that have a mitochondrial component. Finally, as mentioned above, peptides from HIV Tat and the antennapedia homeodomain have the ability to cross membranes in a receptor- and energy-independent manner [43–46]. Given that proteins at least up to 120 kilodaltons can traverse the membrane when associated with these peptides [44], it is intriguing to speculate that peptide–PNA-coupled plasmids may also have increased access to the cell. Recently, PNAs and vectors containing the appropriate target sequences have become commercially available (Gene Therapy Systems, San Diego CA). PNAs can be labeled with fluorescent molecules, biotin, gold, and a cross-linking agent for the attachment of peptides [11,41]. This system shows great promise and it should be straight forward to screen peptides and other molecules for novel delivery activities.

Perhaps the most powerful aspect of PNA-mediated targeting, is the ability to engineer multiple distinct complexes on the same plasmid. Such complexes can be constructed in one of two ways. First, plasmids can be designed to have multiple, tandem PNA target sequences within a defined region of the DNA. The pGeneGrip plasmids from Gene Therapy Systems contain 10 identical, tandem PNA binding sites, allowing binding of 8 PNAs [11,41]. By combining identical PNAs that contain different bound peptides in varying ratios, complexes can be produced that have two or more different peptides attached to the DNA. A second approach would be to use distinct PNAs that bind to unique target sequences, with each PNA having a different peptide bound to it. The target sequences could be placed at desired sites within the plasmid to take advantage of individual peptide activities. Thus, for example, an NLS-PNA could be placed away from the gene to be expressed on the plasmid to enable nuclear import of the DNA, while a PNA conjugated to a transcription factor multimerization domain could be positioned within the promoter to enhance gene expression. Using these approaches, one could create a multifunctional plasmid complex designed to overcome the multiple barriers to gene delivery and expression.

In summary, the progress made over the past 8 years in devising applications for PNAs has been good. Although initially thought of as stable oligonucleotide analogs for antisense applications, it is clear that they have activities far beyond this limited scope. Their activity as synthetic promoters that act on exogenous as well as endogenous genes, their ability to induce or repair mutations in the genome, and their capacity to act as adapter molecules and targeting agents for gene delivery make them quite promising additions to the field of gene delivery and therapy.

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