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Targeting Peptide Nucleic Acid–Protein Conjugates to Structural Features Within Duplex DNA

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Abstract—A convenient small scale synthesis has been developed for obtaining peptide nucleic acid oligomers (PNAs). PNAs have been conjugated to a protein, staphylococcal nuclease, through disulfide exchange between a cysteine at the 3'-(carboxy) end of the PNA and an introduced cysteine on the surface of the nuclease. Site specific DNA cleavage by the attached nuclease has been used to examine the Watson–Crick hybridization of the PNAs to duplex DNA. Substantial affinity cleavage occurred when target sites contained inverted repeats which have the potential to form non B-DNA structures such as cruciforms. No affinity cleavage was observed at a site lacking apparent potential for non B-DNA structures. These results indicate that the Watson–Crick hybridization of PNAs to duplex DNA by strand displacement is favored by the presence of potential alternative secondary structures within the target sequence.

Introduction

The ability of oligonucleotides to recognize specific DNA or RNA sequences affords a versatile approach to control of gene expression. However, native DNA or RNA oligomers are susceptible to endogenous nucleases and lack the membrane permeability necessary for many *in vivo* applications. As a result, various chemical strategies have been used to synthesize modified oligomers with increased resistance to nuclease digestion and optimized membrane permeability.[1,2] One such strategy is the development of peptide nucleic acids (PNAs).[3] PNAs are DNA analogs in which the phosphate backbone has been replaced by (2-aminoethyl) glycine units that are linked to the nucleotide bases via the glycine amino nitrogen and methylenecarbonyl linkers. PNAs bind to both single-stranded DNA and RNA by Watson–Crick base-pairing. The melting temperatures of such PNA:DNA and PNA:RNA hybrids are higher than analogous DNA:DNA and DNA:RNA hybrids.[4] By nuclear microinjection PNAs have been shown to inhibit translation *in vivo*[5–7] and may be well suited for this and other *in vivo* applications since they are not substrates for endogenous nucleases and/or proteases.[8] PNAs may also be potential anti-gene agents and have been shown to recognize polypurine–polypyrimidine sequences within duplex DNA by either triple helix formation, or by triple helix formation in combination with strand invasion in which one PNA strand hybridizes by Watson–Crick base-pairing to one strand of a DNA duplex while a second PNA strand hybridizes to the PNA–DNA hybrid by Hoogstein base-pairing.[9–13] The extension of targeting PNAs to sequences within duplex DNA which are not practical targets for triple helix formation would reveal new targets for inhibition of gene expression.

One strategy to increase the range of potential target sequences is the non-triple helix dependent hybridization of PNAs within complementary duplexes by Watson–Crick base-pairing and D-loop formation. PNA recognition of sequences within duplex DNA, however, is limited by native base-pairing of the duplex. At some sequences the strength of native base-pairing can be overcome because superhelical tension of negatively supercoiled DNA drives the formation of structural elements which have a tendency to be partially single-stranded.[14–16] Such elements are often in promoter regions or at origins of replication where their partially single-stranded nature makes the initiation of strand invasion by short oligomers more likely.[17,18] In the present study we have developed a convenient and economical small scale method for the synthesis of PNAs. We have efficiently coupled PNAs to staphylococcal nuclease to afford conjugates whose hybridization can be monitored via affinity cleavage. We observe that hybridization and affinity cleavage by the PNA-nuclease conjugates is most efficient at sequences which contain inverted repeats.

Results

PNA synthesis

The relatively small scale (10–20 μmol) synthesis of PNAs (Fig. 1A) was accomplished manually using the apparatus shown in Figure 1B using methods adapted from those previously described.[19] Standard N-blocked amino acid synthesis resin (t-boc-Cys (Cl-Z) OCH_3 PAM Resin) (Applied Biosystems) was used with a 10-fold excess of capped resin as a carrier to facilitate experimental manipulations. The coupling, deprotection, and washing were performed in a 15 mL

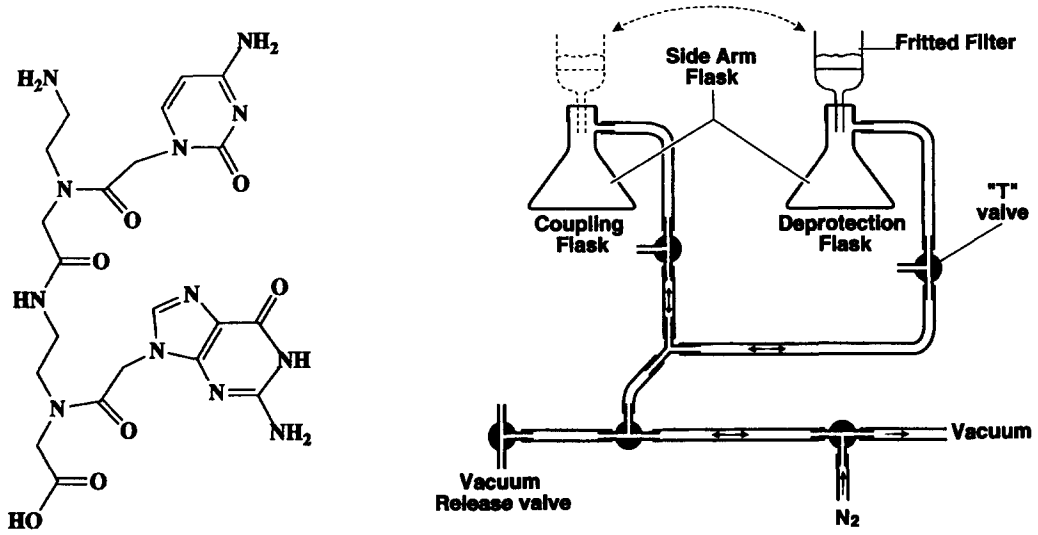

Figure 1. (A) Structure of a PNA dimer CG showing the *N*(2-aminoethyl)-glycine backbone with the bases linked to the backbone by a methylene carbonyl linkage. (B) Apparatus for PNA monome and amino acid coupling.

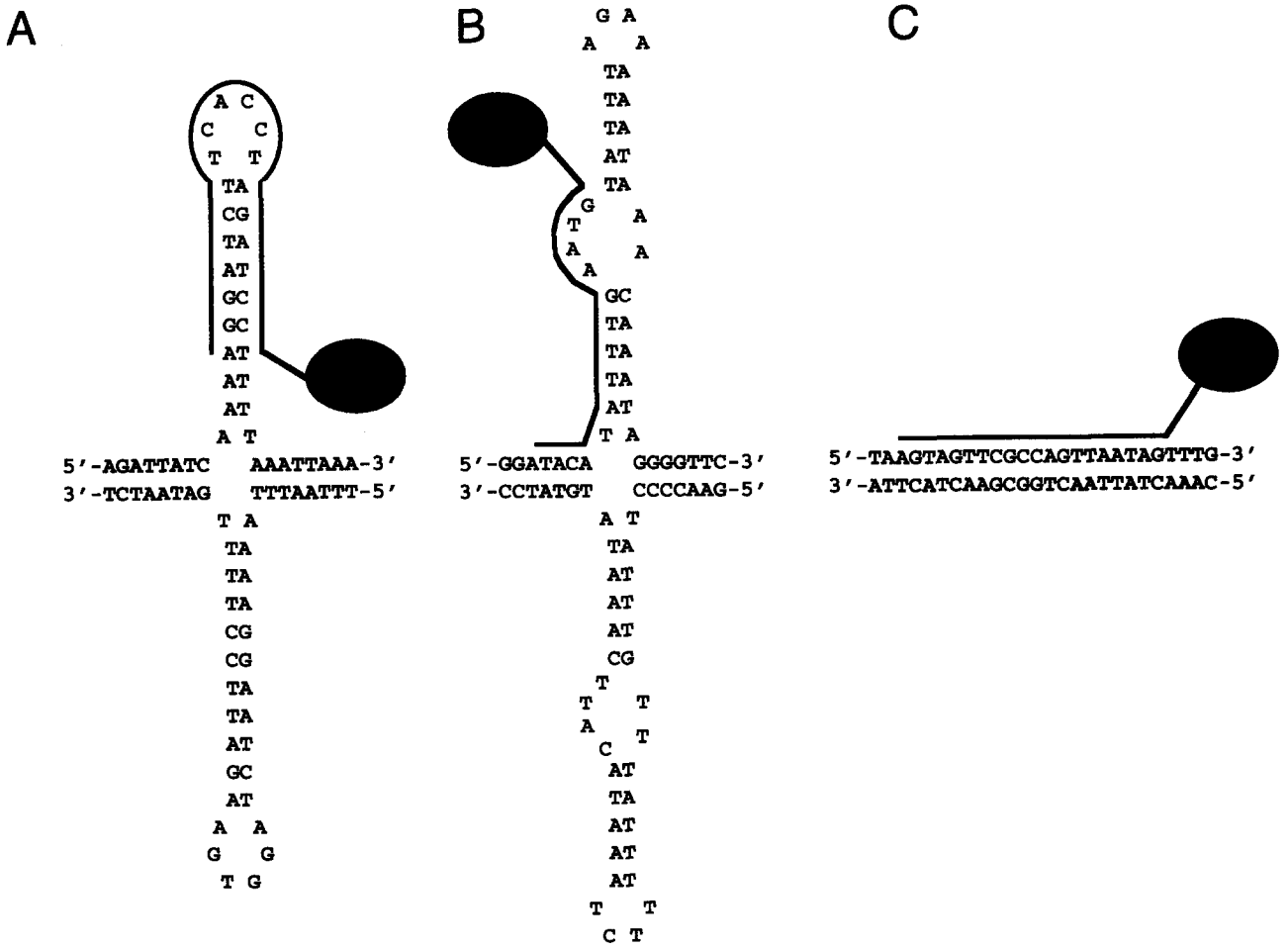

Figure 2. Sequences and potential cruciform structures of target regions for the hybridization of PNA–nuclease conjugates. The PNA sequence is shown in blue. The conjugate is shown as a blue line with the attached nuclease shown in orange. (A) Possible cruciform structure of the inverted repeat located at bases 1531 to 1573 of pUC19 the sequence of PNA I is indicated along with the orientation of the PNA–nuclease conjugate **Ia**. (B) Possible cruciform structure of the inverted repeat located at bases 2542–2587 of pUC19 the sequence of PNA II is indicated along with the orientation of the PNA–nuclease conjugate **IIa**. (C) Shows the sequence of pUC19 located at bases 1895–1920 the sequence of PNA III is indicated along with the orientation of the PNA–nuclease conjugate **IIIa**.

medium fritted glass filter. Efficient mixing was performed by forcing nitrogen backwards through the filter. Solvents were removed by applying a vacuum to the filter. Two flasks were employed, one for manipulations with trifluoroacetic acid (TFA) and one for the coupling reactions. The second flask was needed to prevent contamination of the coupling chemistry by TFA. The PNA monomers were activated by addition of diisopropylethylamine (DIPEA), 1-hydroxybenzotriazole (HoBT), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate for (HBTU) prior to addition to resin. After coupling, the primary amine group was deprotected with TFA so as to be available for addition of the subsequent PNA monomer. Generally, 10 to 12 couplings could be performed per day, and no loss of coupling efficiency was observed if incomplete products were stored overnight. Upon completion of synthesis a TFMSA:TFA:*m*-cresol:thioanisole solution was utilized to cleave PNAs from the resin and to remove the base protecting groups.

Purification of PNAs

PNAs were purified by C_{18} RP-HPLC using a gradient of acetonitrile and either aqueous 0.1 M triethylammonium acetate (TEAA) pH 7.0 or aqueous 0.1% TFA. Normally, the TFA-based system was preferred because the complete removal of TEAA from purified PNA could not be achieved. PNAs **II** (Gly-ATACATATTTGAATGT-Cys) and **III** (Gly-AGTAGTTCGCCAGTTAATAG-Cys) (Fig. 2B, C) lacked the potential for substantial base-paired secondary structure and eluted as single peaks under either condition. Additional products were occasionally observed that corresponded to incompletely deprotected oligomers. In these cases, a second deprotection successfully increased the yield and purity of the desired product.

PNA **I** (Gly-AGGATCTTCACCTAGATCCT-Cys) possessed self-complementarity allowing for the formation of a hairpin or a bulged duplex because it was designed to anneal to both strands of an inverted repeat sequence of pUC19 (Fig. 2A). C_{18} RP-HPLC analysis of PNA **I** using the TFA-based solvent system revealed multiple broad peaks (Fig. 3A). Upon mass-spectral analysis, all collected fractions derived from these peaks were shown to contain material of the appropriate molecular weight for correctly synthesized PNA **I**, suggesting that aggregation and differing secondary structures were resulting in chromatographic heterogeneity. To purify PNA **I** as a single peak C_{18} RP-HPLC was performed using the TEAA-based solvent system (Fig. 3B). Presumably, under these conditions of neutral pH, the PNA is purified as a hairpin or a bulged duplex. Subsequent chromatography using the TFA-based solvent system was sufficient to remove residual TEAA as monitored by one dimensional NMR. Purified PNA **I** exhibited a melting temperature of greater than 88 °C.

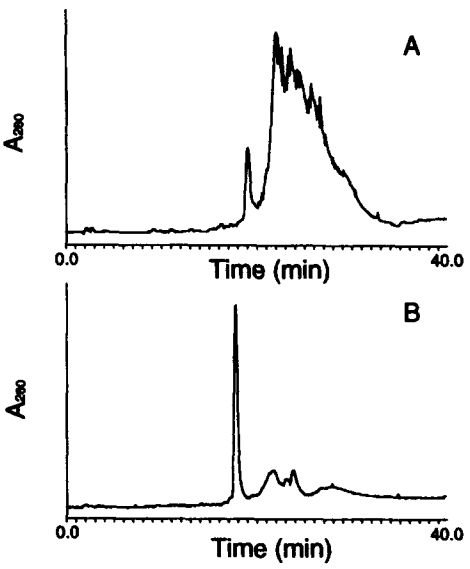

Figure 3. Aggregation of PNA **I** monitored by HPLC. HPLC was performed at 50 °C with a flow rate of 1 mL min^{-1} and detection at 260 nm. PNA **I** was dissolved in 140 mM NaCl, 10 mM $MgCl_2$, and 10 mM Na_2HPO_4 pH 7.4, heated to 75 °C for 15 min. (A) Resolution of PNA **I** using a buffer system of water:0.1% TFA (buffer A) in acetonitrile:0.1% TFA (buffer B). The method employed 1 min with 100% buffer A followed by a gradient of 0–35% buffer B in 35 min. Buffer B was then increase to 100% over 1 min and maintained at 100% for 2 min followed by return to 0% buffer B within 1 min. (B) Resolution of PNA **I** using a buffer system of 100 mM TEAA in water (buffer A) and acetonitrile (buffer B) employing the method described above.

Synthesis of PNA-staphylococcal nuclease conjugates

Cysteine-containing K116C staphylococcal nuclease was isolated as a disulfide linked dimer and reduced to monomeric form by incubation with 25 mM dithiothreitol (DTT). The nuclease was purified by Mono S cation exchange chromatography and the cysteine activated with 2,2'-dithiodipyridine.[20] We chose to activate the nuclease rather than the PNAs to avoid multiple manipulations of the PNAs and resultant decreased recoveries. The 3'-*S*-thiopyridyl-nuclease was purified by further Mono S cation exchange chromatography and could be stored for days in the activated form.

PNAs **I–III** were synthesized to contain a cysteine at the carboxy terminus. The PNAs were reduced with DTT to ensure the presence of free thiols. Unreacted DTT was removed by C_{18} RP-HPLC and the reduced PNAs were collected and immediately mixed with 3'-*S*-thiopyridyl staphylococcal nuclease. Crosslinking reactions were monitored at 342 nm following release of the 3'-*S*-thiopyridyl group (Fig. 4). A slight excess of the relevant PNA was added to minimize residual unreacted nuclease. Previously DNA–staphylococcal nuclease conjugates had been purified by Mono S cation exchange chromatography.[20] We found that this purification was not possible as the PNA–nuclease conjugates bound irreversibly to the Mono S column matrix. Complete loss of material was also observed during attempts to isolate PNA conjugates using a G-25

sizing resin. Therefore, following crosslinking, PNA-nuclease conjugates **Ia–IIIa** were purified away from reactants by diphenyl RP-HPLC (Fig. 5). The purified material exhibited an absorbance maxima at 260 nm, derived from the PNA, and phosphodiesterase activity derived from the nuclease. Mass spectral analysis confirmed that the product of crosslinking was a 1:1 conjugate of PNA **I** to nuclease (Fig. 6). Similar results were obtained for conjugates **IIa** and **IIIa**.

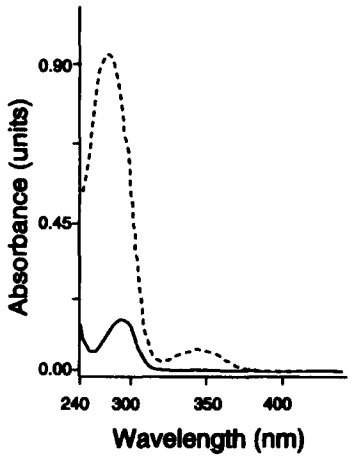

Figure 4. UV spectral changes during crosslinking between PNA **I** and staphylococcal nuclease. Solid line: spectrum of 3'-*S*-thiopyridyl staphylococcal nuclease. Dashed line: spectrum after mixture of reduced PNA **I** with 3'-*S*-thiopyridyl staphylococcal nuclease.

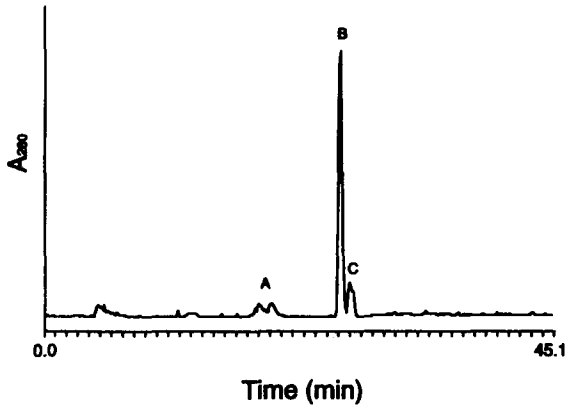

Figure 5. HPLC trace showing purification of PNA–nuclease conjugate **Ia**. HPLC was performed diphenyl column with a flow rate of 1 mL min^{-1} and detection at 260 nm. Resolution of the adducts was obtained using a buffer system of 0.1% TFA in water (buffer A) 0.1% TFA in 99.9% acetonitrile (buffer B). The method was as described in Figure 3. The identity of each peak was confirmed by mass spectral analysis (A) Uncrosslinked PNA. (B) PNA–nuclease conjugate. (C) Unmodified staphylococcal nuclease

Selective cleavage of supercoiled plasmid by the PNA–nuclease conjugates

Supercoiled pUC19 plasmid was utilized to determine the ability of the PNA–nuclease conjugates to hybridize with and cleave duplex DNA.[21] DNA was not denatured prior to incubation with the PNA–nuclease conjugates. The PNA domains of conjugates **Ia** and **IIa** were complementary to sequences within pUC19 which contain inverted repeats (Fig. 2A, B). Conjugate **IIIa** was complementary to a sequence lacking any elements likely to contribute secondary structure (Fig. 2C). The conjugates were incubated at 37 °C for 15 min with equimolar concentrations of plasmid. Following incubation, the solutions were cooled on ice and the nuclease domain of the conjugate was activated for DNA hydrolysis by addition of $CaCl_2$. After 1–2 s inactivation of the nuclease was achieved by addition of excess EGTA to chelate the calcium. The products of the cleavage caused by PNA–nuclease conjugates were subsequently digested with BamHI for conjugates **Ia** and **IIIa** or Eam1105I for conjugate **IIa**. These restriction enzymes were chosen based on their being only one site present in pUC19 and that fragments of discrete size could be detected by gel electrophoresis. Analysis of the ethidium bromide stained agarose gel revealed that PNA–nuclease conjugates **Ia** and **IIa** yielded duplex cleavage products that were consistent with hybridization by the PNAs via Watson–Crick base-pairing at the target site and subsequent affinity cleavage (Fig. 7, lanes 4 and 5). PNA–nuclease conjugate **IIIa** did not yield any selective PNA-directed duplex cleavage when present at an equimolar ratio (Fig. 7, lane 6) or when present at a 10-fold excess, although background cleavage at inverted repeats and other sites was observed at higher concentrations of conjugate.

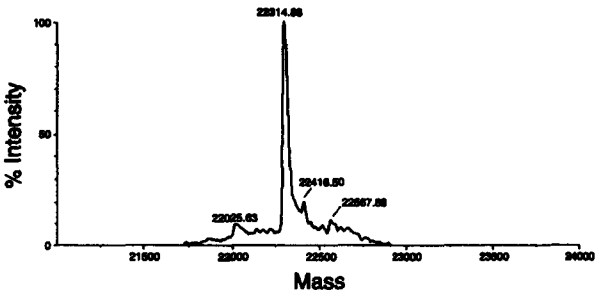

Figure 6. Electrospray mass spectral analysis of PNA–nuclease conjugate **Ia**.

Synthesis of PNA–peptide conjugates

To examine the generality of our crosslinking protocol, PNA **I** was crosslinked via disulfide exchange to a cysteine-containing peptide sequence, CGGSPRKSPRK, known to bind DNA independently at A/T rich regions.[22–24] Peptide was reduced by DTT, purified by analytical C_{18} RP-HPLC, and activated with 2,2'-dithiodipyridine. The 3'-*S*-thiopyridyl peptide was purified by C_{18} RP-HPLC and incubated with reduced PNA **I**. Crosslinking was monitored at 342 nm following release of the 3'-*S*-thiopyridyl group. The PNA–peptide conjugate was purified from the reactants by C_{18} RP-HPLC. Mass spectral data showed that the product was a 1:1 conjugate of PNA to peptide.

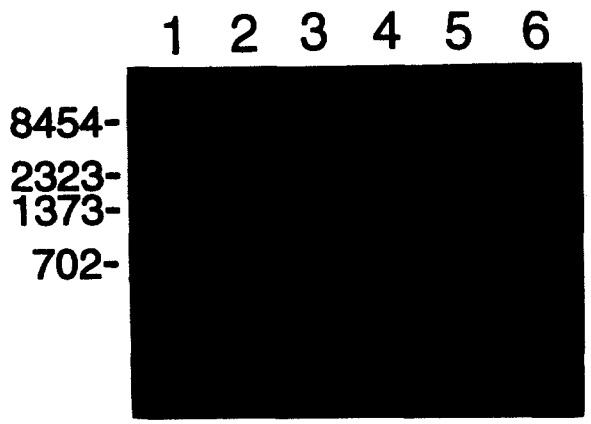

Figure 7. Cleavage of supercoiled plasmid DNA by PNA–nuclease conjugates. pUC19 (0.026 μM) was mixed with oligonucleotide-nuclease adducts (0.026 μM), incubated at 37 °C for 15 min and cooled to 0 °C prior to initiation of cleavage. Following nuclease cleavage the products were digested with the restriction enzyme indicated. Lane 1: BstII λ DNA ladder (New England Biolabs), Lane 2: supercoiled pUC19, Lane 3: linearized pUC19, Lane 4: pUC19 cleaved with PNA conjugate **Ia** and digested with BamHI, Lane 5: pUC19 cleaved with PNA conjugate **IIa** and digested with Eam1105I, Lane 6: pUC19 cleaved with PNA conjugate **IIIa** and digested with BamHI. Substrate DNA had a superhelical density (σ) of −0.056 ± 0.014 as measured by the band counting method of Keller.[34]

Discussion

To evaluate the potential for PNAs to be used as antigene therapeutics it is important to understand their hybridization to duplex DNA. PNAs hybridize by Watson–Crick base-pairing to DNA or RNA in spite of their dramatically different internucleoside linkages, and are resistant to nuclease or protease degradation. These properties make them interesting *in vitro* experimental probes and potential *in vivo* therapeutic agents. In this report we have developed a scheme for conjugating PNA oligomers to proteins and peptides. This allows stable and selective hybridization of PNAs to be combined with useful properties of either peptides or proteins to create hybrid molecules possessing novel functions.

To facilitate crosslinking of PNAs to a series of peptides and proteins, we developed a small scale manual synthesis of PNA oligomers, which routinely allows us to obtain 10–15 μmol of 15–20 base PNAs over a 2 day period. Upon deprotection and purification, the PNAs were obtained in high yield. Interestingly, PNAs which contain self-complementary sequences appeared to form heterogeneous aggregates by HPLC analysis, but can be purified as single peaks given appropriate buffer conditions (Fig. 3A, B). Surprisingly, while the hairpin structure complicates HPLC analysis and results in a measured melting temperature of greater than 90 °C, the preexisting intramolecular base-pairing of the hairpin does not prevent intermolecular base-pairing to plasmid templates. This result suggests that hairpin PNAs, in spite of their double-stranded character, may be useful probes for hybridization to complementary targets. This may prove a significant observation since hairpin PNAs may have different membrane permeabilities and other properties relative to linear PNAs and may be more effective probes for some applications.

We chose to develop a crosslinking protocol to attach PNA oligomers to staphylococcal nuclease, an enzyme capable of cleaving single stranded or double-stranded DNA, both because staphylococcal nuclease is an efficient probe for affinity cleavage, and to afford a general strategy for the synthesis of PNA–Protein conjugates. Because of the tendency for PNA oligomers to aggregate, their non-ionic character, and their incompatibility with Mono S cation exchange and G-25 resins, the synthesis of PNA–nuclease conjugate formation was not a straightforward extension of known protocols for crosslinking oligonucleotides and proteins.[18] In spite of these complications, our optimized protocol allowed for the rapid synthesis of PNA conjugates. Affinity cleavage by the conjugate revealed that our crosslinking protocol yielded active hybrid macromolecules and provided a useful probe for the ability of PNAs to hybridize to DNA target sequences. With limited modifications, the crosslinking protocol was readily adaptable to the coupling of PNAs and peptides. Of course, PNA synthesis can directly be combined with peptide synthesis, but disulfide crosslinking allows the rapid combination of pre-made peptides with a single existing PNA.

In previous studies, the recognition of duplex DNA by PNAs have been limited to polypurine–polypyrimidine target sequences. We found that PNAs could also be targeted to inverted repeats with varied sequences within supercoiled plasmid DNA, where hybridization presumably occurs by Watson–Crick base-pairing and D-loop formation. We did not observe affinity cleavage at a sequence lacking an inverted repeat. The efficient hybridization at inverted repeats suggests that PNA uptake may be most efficient at duplex DNA sequences that tend to form alternate structures (e.g. cruciforms) possessing potentially single-stranded character. PNA–nuclease conjugates can be used as probes for these sequences, since the hybridization of the PNA would be readily revealed through sequence-specific substrate cleavage by the nuclease. The efficient synthetic methods described here facilitate the rapid synthesis of diverse PNAs for use as probes for hybridization to various target sequences.

PNA uptake at inverted repeats requires that DNA substrates be negatively supercoiled. Presumably negative supercoiling increases the likelihood of transient strand melting of duplex DNA and increases the opportunity for nucleation of base-pairing by an invading strand. The need for supercoiling is a general limitation relative to hybridization via triple helix formation. However, it is not an obstacle for many applications of PNAs as probes of DNA structure, or for control of enzyme activity. DNA can be negatively supercoiled *in vivo*,[25–28] particularly in regions which are transcriptionally active. Also, inverted repeats and

other unstable structures are common within regulatory regions of DNA. Negative supercoiling favors the unwinding of B-form DNA and has been shown to cause the conversion of inverted repeats to cruciforms. Negative supercoiling can also stabilize other structures, including left handed Z-DNA, and H-form triplexes.[29,30] These and other structural motifs, such as AT-rich unwinding elements, encourage DNA strand separation which is required for a number of important genetic processes, including the initiation of transcription and DNA replication, and may be appropriate targets for anti-gene PNAs.

Conclusion

PNAs offer a dramatically different chemical and structural motif for the sequence specific delivery of agents to DNA and RNA. This study shows both probe and target structure are important for the delivery of PNA oligomers to double stranded DNA by Watson–Crick base-pairing. Generally, conjugation of proteins and peptides to PNAs is possible and the attachment of staphylococcal nuclease to PNAs yields functional hybrid macromolecules. Specifically, hybridization and selective cleavage of DNA by PNA–nuclease conjugates allows for rapid evaluation of the efficiency and feasibility of PNA uptake at the sequence being investigated. The chemistry involved in these studies can be readily adapted to attach other proteins to PNAs, and such adducts may afford wide experimental and therapeutic utility.

Experimental

Materials and methods

PNA monomers, dichloromethane (DCM), and diisopropylethylamine (DIPEA) were supplied by Millipore. 1-Hydroxybenzotriazole (HoBT), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HBTU), triethyl ammonium acetate pH 7.0, unmodified PAM resin, and t-boc-Cys (Cl-Z) OCH_3 PAM resin were purchased from Applied Biosystems. Unmodified PAM resin was capped with acetic anhydride for use as a carrier. *N,N*-Dimethylformamide (DMF), acetonitrile, and trifluoroacetic acid (TFA) were supplied by Fisher. Pyridine, *m*-cresol, thioanisol, and trifluoromethanesulfonic acid (TFMSA) were supplied by Aldrich. Tris(hydroxymethyl)aminomethane hydrochloride (Tris), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ethylenebis(oxyethylene-nitrilo)tetraacetic acid (EGTA), 2,2'-dithiodipyridine and dithiothreitol (DTT) were obtained from Sigma. Bio-Rex 70 cation exchange resin was purchased from Bio-Rad. Plasmid pUC 19[31] was prepared either by cesium chloride gradient centrifugation or by chromatography (Qiagen). Mass spectral analysis of PNAs and conjugates was acquired using a VG 30-250 Quadrapole mass spectrometer (Altrincham, U.K.) employing a standard VG electrospray source.

RP-HPLC conditions

Analytical and small scale preparative RP-HPLC was performed on a Rainin HPXL dual pump system using a Vydac C_{18} 4.6 × 150 mm 300 Å column enclosed in a water jacket at 50 °C with a flow rate of 1 mL min^{-1} and detection at 260 nm. Water was purified using a Milli Q Plus system (Millipore). PNAs were resolved using buffer system (1) which consisted of H_2O:0.1% TFA (buffer A) and acetonitrile:0.1% TFA (buffer B). The method employed consisted of 1 min buffer A followed by a gradient of 0–35% buffer B in 35 min. Buffer B was then increase to 100% over 1 min and maintained at 100% for 2 min followed by return to 0% buffer B within 1 min. Alternatively, buffer system (2) was used which consisted of 100 mM aqueous TEAA, pH 7.0 (buffer A) and acetonitrile (buffer B), employing the method described above. For large scale purification a Waters Delta Pak C_{18} 7.8 × 300 mm 300 Å semi-preparative column was used employing the described conditions with the flow rate increased to 3 mL min^{-1}.

Synthesis of PNA oligomers

PNAs Gly-AGGATCTTCACCTAGATCCT-Cys **(I)**, Gly-ATACATATTTGAATGT-Cys **(II)** and Gly-AGTAGT-TCGCCAGTTAATAG-Cys **(III)** were synthesized following basic t-boc peptide chemistry with the following modifications. t-boc-Cys (Cl-Z) OCH_3 PAM resin was weighed out with a 10-fold excess of capped resin added as carrier, and swollen for 1 h in DMF. All reactions are carried out in a 15 mL medium grade fritted funnel inserted into a 125 mL side arm Erlenmeyer flask through a single hole rubber septum (Fig. 1). Two flasks were used to separate the deprotection and coupling reactions with the fritted funnel being exchanged between them. The side arms were connected to vacuum and nitrogen sources through a series of T-valves; in this way, solvents could be mixed by forcing nitrogen through the frit, and removed by applying vacuum.

The swollen resin was added to the fritted funnel which was then placed in the dedicated deprotection flask and vacuum applied to remove the solvent. The primary amine group was deprotected by addition of 1 mL 95% TFA:*m*-cresol (95:5) for 3 min during which time nitrogen was bubbled through the glass frit. The resin was then washed with 1:1 DCM:DMF (2 × 1 mL) followed by a second 3 min incubaton with TFA:*m*-cresol (95:5). During the deprotection steps, the monomer was resuspended in DMF (final monomer concentration 0.05 mM) and then activated by addition of five equivalents (eq.) HoBT, 10 eq. HBTU, and 10 eq. DIPEA. Monomer was dissolved in the DMF/activation mixture by vigorous vortexing. After the second deprotection step the resin was washed with 1:1 DCM:DMF (6 × 1 mL) and then washed once with 1 mL of pyridine. The fritted funnel was transferred to

the coupling flask and monomer solution added to resin. Nitrogen was forced through the glass frit and the solution was incubated for 20 min. Following coupling the resin was washed with 1:1 DCM:DMF (6 × 1 mL) and the fritted funnel transferred back to the deprotection flask. The procedure was repeated for each coupling step until all monomer reactions had been completed. Following complete synthesis, or for overnight storage if synthesis was extended over more than one day, the resin was washed with 1:1 DCM:DMF (6 × 1 mL), DCM (6 × 1 mL) and finally with MeOH (6 × 1 mL). The resin was then stored in the fritted funnel under house vacuum in a desiccator. To continue the synthesis the following day, the resin was again swollen for 1 h in DMF and the coupling protocol was restarted with primary amine deprotection. Once the synthesis was complete the fritted funnel was placed into a clean side arm Erlenmeyer flask and the cleavage and deprotection was carried out by the addition of 1 mL TFMSA:TFA:*m*-cresol:thioanisole (2:6:1:1) (CAUTION: TFMSA is highly corrosive, one of the strongest acids known, note that upon mixing these four solutions they turn a dark green to black color). Optimal results were obtained with newly opened TFMSA. The reaction was incubated with very slow nitrogen bubbling through the glass frit for 1 h. Following cleavage and deprotection the product was drawn into the side arm flask, and the resin was washed once with a 1 mL volume of TFA. The product was precipitated from the solution by addition of 4 volumes of ice cold Et_2O. The slurry was collected into a 15 mL conical tube and centrifuged for 3 min, 3500 rpm in a Beckman G310 table top centrifuge at 4 °C. The product was then washed three times with 10 mL of ice cold ether. PNA purity was evaluated by analytical C_{18} RP-HPLC using the buffer system (1) and by mass spectral analysis. To reduce aggregation, PNA **I** was dissolved into 140 mM NaCl, 10 mM $MgCl_2$, and 10 mM Na_2HPO_4 pH 7.4, heated to 80°C for 15 min and evaluated by C_{18} RP-HPLC using buffer system (2). The calculated and observed molecular weights were 5232.96 and 5233.75 ± 0.98 for PNA **I**, and 4526.28 and 4526.62 ± 0.21 for PNA **II**, and 5635.18 and 5636.81 ± 0.46 for PNA **III**. The PNA products were purified on a large scale using the semi-preparative C_{18} RP-HPLC column described above. Only PNA **I** was purified to completion and exhibited a purified yield of 60%.

Melting temperature determination

The melting temperature for the hairpin (PNA **I**) was determined using a Peltier temperature controller attached to a Hewlett Packard 8452 diode array spectrophotometer. The sample was dissolved into a 5 mM Tris pH 6.5 solution with a final volume of 2 mL and an initial absorbance at 260 nm of 1.73. The sample was then placed into the temperature controller cell holder and stirred continuously at 250 rpm. The temperature was ramped from 30 °C to 100 °C in 2 °C increments with a 2 min hold time at each temperature. The change in absorbance is monitored at 260 nm and plotted against the temperature. The melting temperature (T_m) was calculated from this curve by software supplied with the system using the midpoint and the first derivative of the temperature trace.

Synthesis of cysteine-S-thiopyridyl-activated peptide

The peptide CGGSPRKSPRK was synthesized on a Symphony Multiplex synthesizer (Rainin). The presence of predominantly one species was confirmed by C_{18} RP-HPLC using buffer system (1). The peptide was characterized by fast atom bombardment mass spectrometry. The calculated and observed masses were 1172.4 and 1172.4 daltons respectively. The peptide was fully reduced by treatment with 20 mM DTT incubated overnight at 37 °C. The reduced peptide was purified by C_{18} RP-HPLC as described above and collected directly into an equal volume of 10 mM 2,2'-dithiodipyridine in acetonitrile and incubated at room temperature for 30 min to form the peptide-*S*-thiopyridyl adduct. The solution was extracted with diethyl ether (6 × 3 mL) to remove unreacted 2,2'-dithiodipyridine. The 3'-*S*-thiopyridyl peptide was purified using the C_{18} RP-HPLC employing buffer system (1). The presence of the thiopyridyl group was confirmed and the concentration of activated peptide quantitated by monitoring release of thiopyridyl anion at 342 nm (ε = 7060) upon treatment with DTT.

Synthesis of PNA-peptide conjugates

Two to four milligrams of dry PNA **I** were weighed out, dissolved in H_2O and incubated overnight at 37 °C with 10 mM DTT to ensure that all material was present in monomeric form. The reduced PNA was purified by C_{18} RP-HPLC using buffer system (1), neutralized with 1/5 volume 100 mM Tris–HCl pH 10.2, and added to a 1 mL quartz cuvette containing the 3'-*S*-thiopyridyl peptide. The reaction was monitored at 342 nm utilizing a Hewlett Packard 8452 diode array spectrophotometer. Sufficient PNA **I** and peptide were present in the cuvette to ensure a distinct peak at 342 nm (>0.05 OD_{342}) upon completion of the reaction. The PNA **I**–peptide conjugate was then purified by C_{18} RP-HPLC using buffer system (1).

S-*Thiopyridyl activation of staphylococcal nuclease*

Plasmid DC1, a pONF1 derivative encoding staphylococcal nuclease containing the K116C mutation was provided by Dr Peter G. Schultz (UC Berkeley). K116C staphylococcal nuclease was expressed behind a *lac* promoter and an *omp*A signal sequence within plasmid pDC1.[32] Following expression and isolation of the periplasmic fraction, the nuclease was purified by cation exchange chromatography using Bio-Rex 70 resin. As isolated, the enzyme was primarily a disulfide-linked dimer and was completely reduced to monomer by treatment with 50 mM DTT for 8 h at 37 °C in 10 mM Tris–HCl, pH 8.0. Monomeric enzyme was separated from DTT by Mono S cation exchange chromatography (Pharmacia) in 50 mM Na-HEPES, pH 7.5, 1 mM EGTA and a gradient of 0 to 1.0

M NaCl, with a flow rate of 1 mL min^{-1} with monitoring at 280 nm. The reduced nuclease was added to 10 mM 2,2'-dithiodipyridine in acetonitrile (0.5 volumes) and incubated at room temperature for 30 min. The solution was extracted with Et_2O (6 × 3 mL) to remove unreacted 2,2'-dithiodipyridine. The solution was frozen and residual diethyl ether was removed under reduced pressure for 30 min. Freezing was necessary to prevent loss of material through vigorous evaporation of ET_2O. The 3'-*S*-thiopyridyl nuclease was purified by Mono S cation exchange chromatography as described above. The presence of the thiopyridyl group was confirmed by treatment with DTT and monitoring the release of thiopyridyl anion as described above.

Synthesis of PNA-staphylococcal nuclease conjugates

Two to four milligrams of dry PNA **I**, **II**, or **III** were dissolved in H_2O and incubated overnight at 37 °C with 10 mM DTT to reduce all material to monomeric form. The reduced PNA was purified by C_{18} RP-HPLC using buffer system (1), neutralized with 1/5 volume 100 mM Tris–HCl pH 10.2, and added to a 1 mL quartz cuvette containing the 3'-*S*-thiopyridyl nuclease. The reaction was monitored at 342 nm utilizing a Hewlett Packard 8452 diode array spectrophotometer. The PNA was added to the cuvette until no further change in absorbence at 342 nm was observed. The PNA-nuclease conjugates **Ia**, **IIa** and **IIIa** were then purified by RP-HPLC on a diphenyl column (Vydac) using the buffer system (1), with detection at 280 nm, and the collected fractions containing the conjugate were neutralized by addition of 1/5 volume 100 mM Tris, pH 10.5.

Affinity cleavage of DNA

DNA cleavage by PNA-nuclease conjugates was essentially performed as described.[20,21,33] PNA–nuclease conjugates (0.026 μM) were annealed to substrate DNA (0.026 μM) for 15 min at 37 °C in 5 mM Tris–HCl, pH 6.5, in a total volume 45 μL. The mixtures were chilled on ice and cleavage reactions initiated by addition of 4.5 μL 50 mM $CaCl_2$ and were terminated after 1–2 s by addition of 4.5 μL 100 mM EGTA. The solution was neutralized by the addition of 9 μL 100 mM Tris, pH 8.0. The DNA was then digested to completion with a restriction enzyme to generate discrete products of identifiable size which were analyzed by gel electrophoresis through a 1.4% agarose horizontal slab gel.

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