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Bifacial PNA complexation inhibits enzymatic access to DNA and RNA

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bifacial; nucleobase mimic; bPNA; hairpin; triplex

We have previously reported that synthetic melamine-displaying α -peptides, termed bifacial peptide nucleic acid (bPNA), can selectively bind thymine-rich DNA into synthetic heterotriplex structures composed of two T-rich DNA strands assembled onto a bPNA strand.^[1] When T-rich domains are separated by an intervening oligonucleotide sequence of 4–10 nt, a hairpin triplex stem-loop structure is formed with high affinity ($K_d \sim 2$ nM). We hypothesized that these high melting complexes ($T_m = 57^\circ\text{C}$) would be competitive with protein enzymes whose native substrates are DNA or RNA. Herein we report the effective *in vitro* inhibition of transcription, reverse-transcription and exonuclease function via the formation of synthetic bPNA-nucleic acid triplex structures formed by folding of DNA or RNA around a single bPNA template strand. These studies demonstrate versatile and selective bPNA targeting of both DNA and RNA substrates with native-like oligonucleotide composition, suggesting possible application of bPNAs as synthetic regulators of nucleic acid function.

Many native nucleic acid processes are controlled via induction of structure in DNA or RNA substrates, by protein complexation,^[2] small molecule metabolite binding^[3] or both. This mechanism for repression of enzymatic “read-through” has been observed in transcriptional, reverse-transcriptional and translational regulation; further, the increased stability of folded nucleic acid structures towards chemical and enzymatic degradation is well-known. Moderate substrate affinity appears to be sufficient to modulate nucleic acid function, as observed in native riboswitches with nanomolar to micromolar range affinity to metabolite targets.^[4] Metabolite binding toggles native riboswitch sequences between folded conformations with distinct function, thus regulating transcription, translation initiation, mRNA degradation and splicing. A similar strategy of sequence-encoded conformational control is found natively in prokaryotic transcriptional regulation wherein palindromic transcripts signal transcriptional termination by folding into RNA hairpin structures.^[5] We hypothesized that bifacial peptide nucleic acid (bPNA), which binds T-rich DNA and induces the formation of synthetic bPNA-DNA triplex-stem loop (hairpin) structures, could be used in a similar fashion as an artificial repressor of nucleic acid function.

Peptide nucleic acids from α -amino acids presenting nucleobases on derivatized sidechains are synthetically more convenient relative to the well-studied PNA backbone reported by Nielsen and Buchardt,^[6] which features a non-native peptide backbone *N*-amide acylated

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with a nucleobase moiety. A neutral PNA backbone^[7] results in enhanced stability of PNA hybrids with DNA and RNA relative to native hybrids, due to reduced interstrand electrostatic repulsion.^[6] Increased hybrid stability permits strand invasion of native duplexes to form PNA hybrid duplex and triplex structures, which suggested potential utility as antigene and antisense therapeutic agents. Notably, Nielsen demonstrated inhibition of enzymatic restriction upon complexation of an A₁₀ tract proximal to restriction sites by oligo-T PNA;^[8] PNA-DNA triplex formation via Watson-Crick and Hoogsteen base-pairing thus blocked enzymatic access to restriction sites. Corey^[9] and Nielsen^[10] independently reported the design of bis-PNA “tail-clamps” which target pyrimidine-rich duplex sites for triplex invasion; this strategy has been shown to be effective in transcriptional repression.^[9, 11]

Bifacial peptide nucleic acid (bPNA) presents an alternative approach to block enzymatic access via non-native structuring of nucleic acid substrates. Conventional PNA triplex hybrids mimic DNA triplexes^[12] by replacing two DNA strands with PNA: two PNA strands bind to a native oligopyrimidine via both Watson-Crick and Hoogsteen base-pairing, which can also lead to intramolecular folding, as in G-quartets.^[13] In contrast, melamine-based bPNA strands do not fold intramolecularly and serve as the *template* strand, binding two native pyrimidine nucleic acid (NA) strands on their Watson-Crick faces to form a bPNA-NA triplex structure (Figure 1). This approach is similar to “Janus-wedge” nucleobase-pairing introduced by Lehn,^[14] but addresses mismatch sites in which the two nucleobases are identical. It has been previously demonstrated that 2-fold symmetric triazine bases can base-pair with DNA when displayed on a PNA backbone^[15] and recognize T-T and U-U mismatch sites when coupled to an intercalator.^[16] Eschenmoser and Krishnamurthy have demonstrated that synthetic triazine bases displayed at alternate residues on α -peptide backbone and peptoid backbones can effectively form duplex structures with DNA and RNA partners.^[17] A single-stranded oligonucleotide with two separated oligothymidine^[1a] or oligouracil tracts is folded upon bPNA binding into a hairpin secondary structure with a bPNA-nucleic acid triplex stem. The recognition nucleobase mimic on bPNA is 1,3,5-triazine-2,4,6-triamine (melamine) which is attached to the bPNA backbone via lysine sidechain. Small molecules, peptides, lipids and polymers^[18] displaying melamine derivatives have been previously shown to avidly bind to synthetic hydrogen bonding complements in organic^[19] and aqueous solution.^[1a, 20] A bPNA sequence was designed with alternating residues of melamine-modified lysine (M*) and glutamic acid (E) to yield peptides of the general form (EM*)_n, where repeat units (n) of 6, 8 and 10 were studied. This polyanionic design was expected to impart greater water solubility and ease of purification relative to conventional PNA, and minimize non-specific interactions with nucleic acids while binding and folding thymine-rich DNA sequences into hairpin structures. The biophysical underpinnings of this interaction are similar to DNA triplex formation,^[21] with assembly driven by exothermic base-stacking proceeding in highly predictable fashion driven by interface length-matching.^[1] Prior bPNA binding studies revealed low nanomolar affinity to dT₁₀ tracts with non-interacting C₁₀ linkers, and related studies on triazine polymers suggested uracil nucleobase binding should be similarly robust. Control studies indicated undetectable binding to oligo-A and oligo-C sequences,^[1a] with complex T_m directly dependent on thymine content.^[1b] We tested bPNA **10** affinity to longer DNA sequences (~127 nt) encoding a T7 RNA polymerase promoter region, tRNA-Lys and a dT₁₀C₁₀T₁₀ domain. Fluorescence anisotropy binding isotherms generated with fluorescein-labeled **10** indicated low nanomolar (~21 nM) affinity to the large, heterogeneous DNA strand while no binding was observed to a similar DNA construct with a random unstructured sequence in place of the bPNA binding site (Figure 2). Similar 127 nt DNA templates were designed with 5'-T₁₀(CA)₂T₁₀-3' domains placed at 4 different positions throughout the template: directly 3' and 5' of the T7 promoter site, in the middle of the template, at the 5' terminus. These templates were all designed to host bPNA strands

with 10 triazine rings (**10**) targeting the 10 T-T pairs presented in a DNA hairpin conformation. An additional DNA template was designed in which two T₁₀ tracts flanked the 21 nt T7 promoter sequence, with the notion that a triplex stem-loop would be formed that constrained the promoter into the loop region. All templates exhibited a clean gel-shift upon bPNA binding and cooperative thermal transitions (~55°C) by UV spectroscopy, supporting discrete and specific 1:1 recognition, in line with prior studies on smaller DNA strands (Figure 2). Quantitation of native electrophoretic mobility shift assays (EMSA) of DNA treated with unlabeled bPNA **10** yielded binding curves that fit well to 1:1 binding models.^[22] As with the dC₁₀ loop, bPNA **10** exhibited ~20 nM binding affinity to the DNA template with 4 nucleotide dCACA spacer in between T-tracts, in the context of a t-RNA-Lys encoding DNA sequence. Interestingly, despite the potential for higher entropic penalty on association, the DNA template with the 21 nt T7 promoter sequence flanked by T₁₀ tracts exhibited a tighter binding to **10** ($K_d=4$ nM), suggesting strain in the shorter loops or the existence of stabilizing interactions within the stem-loop folded promoter sequence. Affinity for **10** in all template designs, including the promoter stem-loop design, was unaffected by whether or not the promoter region was single or double-stranded, though it is unclear whether or not bPNA binding resulted in duplex dissociation.

Having established that longer native-like DNA templates exhibited similar bPNA binding affinity and specificity as previously found with shorter substrates, we considered bPNA **10** as an artificial transcriptional repressor. The effect of bPNA targeting on transcription was studied using the same DNA substrate templates and a T7 RNA polymerase run-off transcription assay. In the absence of **10**, run-off transcription yielded full-length RNA transcripts as expected. Prior incubation with bPNA **10** resulted in significant, though wide-ranging, transcriptional repression, as judged by total transcript production (Figure 3). Weakest repression (50%) was observed with the 5' hairpin triplex, which resulted in production of truncated transcript. Electrophoretic mobility of the truncated product corresponded to aborted transcription at the 5' hairpin site, indicating that T7 disengages with the template upon encountering the artificial folded structure. Up to 90% repression was observed when the bPNA hairpin site was placed proximal to the promoter sequence and when the promoter sequence was folded into the loop domain of the hairpin, suggesting that transcriptional initiation is especially sensitive to competing bPNA-DNA folded structures. Effective repression from placement of the 21 nt T7 promoter into the loop of the triplex hairpin structure directly demonstrates that the synthetic hairpin structure can prevent T7 binding and transcriptional initiation, and underscores the stability of DNA hairpin triplexes with 4 to 21 nt loop domains.

The ability of bPNA folded DNA and RNA structures to limit enzymatic access suggested resistance to nuclease degradation. We tested the stability of both RNA-bPNA and DNA-bPNA hairpin complexes towards exonuclease degradation. It was not possible to eliminate background degradation of long RNA substrates, so our RNA studies were limited to bPNA complexes of rU₁₀C₁₀U₁₀. As expected, discrete gel-shifts on binding bPNAs **8** and **10** were observed with rU₁₀C₁₀U₁₀ (Figure 4). While the free RNA substrate was rapidly consumed by exonuclease T (exo T) in less than one minute, the hairpin complexes remained detectable for over 4 hours, as judged by the quantity of free bPNA observed under saturating conditions. Gel quantification revealed that while ~40% of bPNA **8** was released over 4 hours, negligible quantities (<3%) of bPNA **10** from their respective RNA complexes upon exoT treatment. This difference is likely reflective of the greater stability afforded by a longer bPNA-RNA binding interface. Longer DNA substrates were studied with bPNA **10** and dT₁₀C₄T₁₀ targeting sites placed in middle, 3' and 5' termini. Following formation of triplex stem-loop structures, these complexes were exposed to exonuclease T. Exonuclease T operates in the 3' to 5' direction, and thus hairpin structures placed at the 3' end of a single-stranded RNA or DNA were largely resistant to exo T digestion while 5' hairpin

modified oligonucleotides were degraded down to a structure with the electrophoretic mobility of the hairpin structure alone. DNA or RNA substrates with hairpin triplexes in the middle of the sequence showed digestion was halted at the position of the synthetic secondary structure as well (Figure 4).

Inhibition of RNA-substrate based processes such as reverse-transcription was similarly effective. Initial attempts to study ribosome displacement using a toe-print assay on a well studied RNA sequence^[23] were unsuccessful as 70S bacterial ribosome binding was sensitive to U-rich bPNA binding sites^[24] even in the absence of bPNA. We instead utilized the 142 nt mRNA containing a mid-sequence bPNA binding site (rU₁₀CAAU₁₀) as a substrate for reverse-transcription inhibition. This system could accept bPNA with lengths varying from 10, 8 and 6 melamine-displaying repeat units (Figure 1), which yield bPNA-RNA complexes of decreasing stability as the size of the recognition interface is decreased, similar to what was observed with exo T resistance. The RNA substrate was produced by run-off transcription, purified, annealed with 5' ³²P end-labeled DNA primer, and subjected to reverse-transcription conditions with AMV reverse transcriptase. Each system exhibited a bPNA concentration-dependent suppression of full-length cDNA product. As expected, the most effective suppression of reverse-transcription was shown by the most tightly binding bPNA **10** and weaker, though significant, suppression was found with bPNAs **8** and **6** (Figure 5). As the yield of full length cDNA product decreased, increased yield of a truncated cDNA was observed; this truncation product corresponded to halted reverse-transcription at the rU₁₀CAAU₁₀ site. As the same bPNA binding site was being targeted, the identical truncated DNA reverse-transcript product was observed with bPNAs **6**, **8**, and **10**, suggestive of aborted enzymatic processing upon encountering the bPNA triplex. Interestingly, full-length cDNA production was sharply decreased to ~10% on treatment with **10**, and exhibited no further change at an approximate 1:1 mole ratio to the RNA, as expected based on the molecular recognition interface (Figures 1 & 5). Similar, but less pronounced, saturation effects were observed with bPNAs **6** and **8**, likely due to weaker binding.

Overall, we find that bPNAs presenting 6, 8 and 10 bifacial nucleobases can selectively target T and U-rich sites on long DNA and RNA substrates of heterogeneous composition. Control experiments indicate that bPNA has no effect on enzymatic function when substrates without a bPNA binding site (Supporting Information). Nanomolar range bPNA binding affinity provides sufficient hairpin stability to compete with enzymes for their native DNA and RNA substrates, greatly limiting substrate access in the context of transcription, exonuclease digestion and reverse-transcription. These results are remarkable considering the non-native nucleobase employed in bPNA. Though bPNA forms triplex hybrid structures with polypyrimidine nucleic acid much like conventional PNA, the bifacial triamino triazine bPNA recognition element generates a unique hybrid fold that is the inverse of the PNA triplex; two native nucleic acid strands are templated along a synthetic bPNA strand to form a triplex has no native or artificial cognate. This mode of recognition drives a significant structural reorganization in the nucleic acid, transforming unstructured single-stranded nucleic acids into well-folded triplex hairpin loops. The studies herein demonstrate that structure-nucleation^[25] upon formation of bPNA-nucleic acid hybrids may be used to limit substrate access and thereby modulate the functional output of enzymes involved in nucleic acid transcription, reverse transcription and degradation, suggesting the possible utility of bPNA as a tool for biotechnology.

Experimental Section

General

All DNA oligonucleotides were purchased from Integrated DNA Technology (IDT). DNA concentrations were measured at 260 nm using extinction coefficient provided by IDT. For DNA oligonucleotides with significant secondary structure at room temperature, the concentrations were measured at 50 °C in order to make sure there was no interference from the secondary structure. T7 RNA polymerase, Exonuclease T, AMV reverse transcriptase and SYBR gold stain were purchased from Invitrogen. Binding experiments and transcriptional repression experiments were performed in 1× RNA polymerase reaction buffer (NEB), supplemented with 10 mM DTT and 15mM Mg²⁺. Peptides were synthesized as previously reported using standard Fmoc solid phase peptide synthesis.^[1a] Fluorescently-labeled bPNAs were prepared by *N*-termination with carboxyfluorescein on a β-alanine residue. All bPNAs were purified to homogeneity on reversed phase HPLC and their identity confirmed by mass spectroscopy.

Sequences

The DNA sequence encoding tRNA-Lys was used in transcriptional repression and exonuclease resistance assays: 5'-CAC TGT AAA GAG GTG TTG GTT CTC TTA ATC TTT AAC TTA AAA G*GT TAA TGC TAA GTT AGC TTT ACA GTG GGC CCC TAT AGT GAG TCG TAT TAA TTT C-3' with the T7 RNA polymerase promoter sequence underlined. Templates (Figure 1) for studying 5' and 3' bPNA hairpin formation were constructed by placing T₁₀(CA)₂T₁₀ 5' and 3' respectively, to the sequence shown. The asterisk indicates the site for insertion of T₁₀(CA)₂T₁₀ to generate the mid-sequence DNA template. Anisotropy binding studies were carried out with a 5' bPNA binding site featuring a 10 base loop instead (T₁₀(CA)₅T₁₀), while a control sequence replaced this T-rich sequence with AGC TCT GGT ACT CGC ACT CTC GTA AAC GAT. The mRNA sequence^[23] used in reverse transcription (RT) repression studies with the bPNA binding site shown in bold and RT primer site underlined is as follows: 5'-GGU AAA GUG UCA UAG CAC CAA CUG UUA AUU AAA UUA AAU UAA AAA GGA AAU AAA AAU GUU UUU UUU UUC **AAA UUU UUU UUU** UAA CUC GCU GCA CAA AUG GCU AAA CUG AAU GGG AAU AAA GGU UUU UCU UUC UGA AGA UAA A-3'.

bPNA binding analysis

Native electrophoretic mobility shift assays (EMSA) and fluorescence anisotropy assays were performed to determine bPNA-NA binding affinity. DNA concentration was held constant at 100 nM in the EMSA experiments while bPNA concentration was varied from 0 nM to 1600 nM. All native PAGE samples were equilibrated at room temperature for 1hr before analysis. Gels were stained by using SYBR® gold for 20 minutes and scanned with a Typhoon gel scanner (excitation/emission=488/526 nm) followed by quantification using Image Quant software. For anisotropy experiments, carboxyfluorescein-labeled bPNA concentration was maintained at 60nM while DNA concentration was varied from 0 nM to 1440 nM. Samples were equilibrated at room temperature shielded from light for 1hr before analysis with a SpectraMax® M5 plate reader (excitation/emission 492/525 nm). EMSA and anisotropy data were fit to a 1:1 binding model^[22] with the general form:

$$[bPNA \bullet NA] = \frac{1}{2}(K_d + bPNA_0 + NA_0) - \frac{1}{2} \sqrt{(K_d + bPNA_0 + NA_0)^2 - 4bPNA_0NA_0}$$

Where K_d , $bPNA_0$ and NA_0 represent the dissociation constant, initial bPNA concentration and initial concentration of nucleic acid (NA), respectively. For anisotropy data sets, A_s replaces NA_0 as anisotropy of saturated binding.

Transcriptional repression

Transcription run-off assays were performed using 2 μ M promoter-duplexed DNA templates with and without 8 equivalents of bPNA, annealed in a 1:1.1 ratio of DNA/T7 promoter (5 minutes at 95 °C, cooled slowly to room temperature in 1mM EDTA, 10mM Tris-Cl and 50mM NaCl). After 1hr equilibration, 3.75 unit/ μ L T7 RNA polymerase was added, followed by addition of 4mM of each NTP and incubation at 37°C. Reaction aliquots were quenched in 200mM EDTA at 3, 8, 15, 30, 60 and 90 minute time points, analyzed on 3% EtBr/agarose gel and visualized by UV.

Exonuclease T resistance

All experiments were conducted in 1 $\times$ NEBuffer 4 (50mM Potassium Acetate, 20mM Tris-Acetate, 10mM Magnesium Acetate, 1mM DTT, pH7.9), supplemented with 10% glycerol. Saturated binding conditions of bPNA to DNA and RNA substrates were determined by an EMSA, titrating 100 nM carboxyfluorescein (Cbf) labeled bPNA against DNA or RNA. Exonuclease T (20 units, NEB) was added into a 150uL reaction solution under conditions in which complete bPNA complexation was observed (100nM bPNA to 200 nM DNA/ RNA) and incubated at 37°C for 4 hours. Reaction aliquots were quenched with 250mM EDTA at various time points and on native PAGE gel (RNA) or native gradient PAGE (DNA), and quantified using SYBR gold stain.

Inhibition of Reverse Transcription

Reverse transcription primers (100 pmol) were end-labeled by mixing with T4 polynucleotide kinase (50 units) and ATP γ -³²P in 1 $\times$ T4 PNK buffer (70mM Tris-Cl, 10mM MgCl₂, 5mM DTT, pH 7.6) at 25°C, followed by incubation at 37°C for 30 minutes and enzyme inactivation at 65°C for 20 minutes. Labeled DNA primer was purified using a Sephadex G15 column and radioactivity was diluted to 200,000 CPM. RNA substrate was prepared using standard T7 run-off transcription and purification methods (MEGAscript T7 and MEGAclear kits, Invitrogen) from the appropriate DNA template annealed to the 17 nt sense promoter sequence. Purified RNA (50nM) was incubated with excess radiolabeled RT primer and varying concentrations of bPNAs **6**, **8** or **10**. Following heat denaturation (65 °C, 10 minutes) and ice-cooling, reaction was initiated by addition of AMV reverse transcriptase (RT) to a final concentration of 0.4unit/ μ L. Reactions were incubated at 37°C for 30 minutes before being quenched with EDTA-formamide dye. Reactions were analyzed using denaturing PAGE (6%, 7M urea) after drying, overnight exposure to a phosphor image plate and Typhoon gel scanner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Design of DNA constructs

bPNA hairpin site: $T_{10}(CA)_2T_{10}$ or $T_{10}(CA)_5T_{10}$
Promoter loop site: T_{10} -promoter- T_{10}

Figure 1.

(Top) Structure of bPNAs **6**, **8** and **10** used in this study, corresponding to repeat units n of the same number, and the melamine-thymine/uracil interaction driving bPNA triplex formation. Folding of a T/U-rich sequence in DNA or RNA in a bPNA triplex hairpin blocks enzyme processing. (Bottom) Design of DNA constructs used in transcriptional repression and exonuclease resistance. A control construct features a sequence encoding tRNA-Lys flanked by an unstructured 5' sequence and 3' T7 RNA polymerase promoter (boxed). Binding sites for bPNAs are inserted as shown (dashed box) at locations 5' of tRNA-Lys, mid-sequence, post-promoter (pp) and 3' of the promoter. Decathymidine tracts are separated by the 21 nt promoter sequence in the hairpin promoter loop (HPPL) design.

Figure 2.

(Top) Anisotropy change describing association of Cbf-labeled bPNA **10** with the 5'-hairpin DNA transcription template (Figure 1, T₁₀C₁₀T₁₀) (—) or control sequence (---). Binding to DNA with double stranded (○) and single stranded (●) promoter regions is shown. (Center) Binding curves obtained by EMSA and calculated K_d are shown, of **10** to DNA transcription templates HPPL (■, hairpin promoter loop binding site) and 3'HP (◆, 3' hairpin binding site). All binding experiments were performed in triplicate. (Bottom) SYBR gold stained EMSA indicating binding saturation and full gel shift of the 3'HP DNA to complex upon treatment with bPNA **10**.

Figure 3.

(Top) Illustration of aborted T7 RNA polymerase transcription and initiation upon encountering a bPNA hairpin structure in the middle of the template or sequestering the promoter sequence (black) as a loop. Transcriptional efficiency of T7 as labelled, with 100% efficiency defined as product produced in the bPNA-free control run-off at 4 hours.

Corresponding SYBR gold stained gels of run-off transcription product (*) produced from template (<) over time are shown at right with gels A-E representing no bPNA (all templates similar), 5' hairpin, 3' hairpin, promoter-loop hairpin and post-promoter hairpin structured templates, respectively, as defined in Figure 1.

Figure 4.

Resistance of bPNA-DNA and RNA complexes to exoT degradation. (Left, Top) Complete gel shift of carboxyfluorescein labelled bPNA **10** (>) upon treatment with rU₁₀C₁₀U₁₀ to form bPNA-RNA complex (*) that remains detectable over 4 hours of exoT exposure. (Left, Bottom) Gel quantitation of bPNAs **8** and **10** released from their respective complexes with rU₁₀C₁₀U₁₀ over time. (Top, Right) Complete gel shift of carboxyfluorescein labelled bPNA **10** (>) upon treatment with DNA featuring a T₁₀CACAT₁₀ bPNA binding site in the middle of the sequence, to form bPNA-DNA complex (*) indicated above that remains detectable over 4 hours of exoT exposure, following digestion to the hairpin structure indicated. (Bottom, Right) Gel quantitation of exoT degradation of bPNA **10** complexes with DNA featuring 3' and 5' bPNA binding sites, as described in Figure 1.

Figure 5.

Production of cDNA from reverse transcription of RNA template bearing a bPNA $U_{10}CAAAU_{10}$ binding site, showing repression as function of bPNA length and mole ratio to RNA. Data obtained by EMSA quantitation, with a representative gel shown at right, where full length cDNA product (*) and truncated cDNA product (<) are indicated. Assays are run at constant RNA substrate concentration (50 nM).