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Bifacial Peptide Nucleic Acid as an Allosteric Switch for Aptamer and Ribozyme Function

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Supporting Information

ABSTRACT: We demonstrate herein that bifacial peptide nucleic acid (bPNA) hybrid triplexes functionally substitute for duplex DNA or RNA. Structure–function loss in three non-coding nucleic acids was inflicted by replacement of a duplex stem with unstructured oligo-T/U strands, which are bPNA binding sites. Functional rescue was observed on refolding of the oligo-T/U strands into bPNA triplex hybrid stems. Bifacial PNA binding was thus used to allosterically switch-on protein and small-molecule binding in DNA and RNA aptamers, as well as catalytic bond scission in a ribozyme. Duplex stems that support the catalytic site of a minimal type I hammerhead ribozyme were replaced with oligo-U loops, severely crippling or ablating the native RNA splicing function. Refolding of the U-loops into bPNA triplex stems completely restored splicing function in the hybrid system. These studies indicate that bPNA may have general utility as an allosteric trigger for a wide range of functions in non-coding nucleic acids.

We recently reported a new class of bifacial[1] α-peptide nucleic acids (bPNAs),[2] derived from studies on artificial recognition.[3] Bifacial PNA utilizes a synthetic triazine[4] base with two identical hydrogen-bonding faces available. This enables bPNA to simultaneously dock[5] two oligothymidines (Figure 1) with low nanomolar affinity.[2a,b] Two T/U-rich tracts separated by 4–25 nucleotides of random sequence may thus be folded into triplex stem–loop (hairpin) structures upon binding to a single bPNA strand. These unique bPNA hybrid structures are stable enough to block enzymatic access to DNA and RNA substrates.[2c] Bifacial PNA is distinct from conventional PNAs[6] in terms of its α-peptide backbone[4b,7] as well as its use of a non-native triazine recognition interface.[4a,b,5a,8] While PNA triplex[9] hybrids are formed from sequestration of native oligonucleotides with two strands of PNA, bPNA serves as a template to assemble two native oligonucleotides. Generally, PNA targeting has been applied as a *dissociative* operation, wherein native duplex[10] or quadruplex[11] folds are disrupted in favor of PNA hybrid structures. In contrast, bPNA targeting is *associative*, uniting two non-interacting strands on a bPNA bridge. We hypothesized that bPNA-driven association could be used as a synthetic allosteric switch[12] for non-coding nucleic acid function.[13] To test this notion, known aptamer and ribozyme sequences were functionally crippled by replacement of a critical duplex element with T/U tracts. We predicted that triplex hybridization of the T/U tracts with bPNA would mimic the native duplex and tighten the overall fold to restore function in an allosterically coupled domain. Three systems were chosen to represent a breadth of function: molecular recognition of protein, molecular recognition of small molecule, and chemical catalysis.

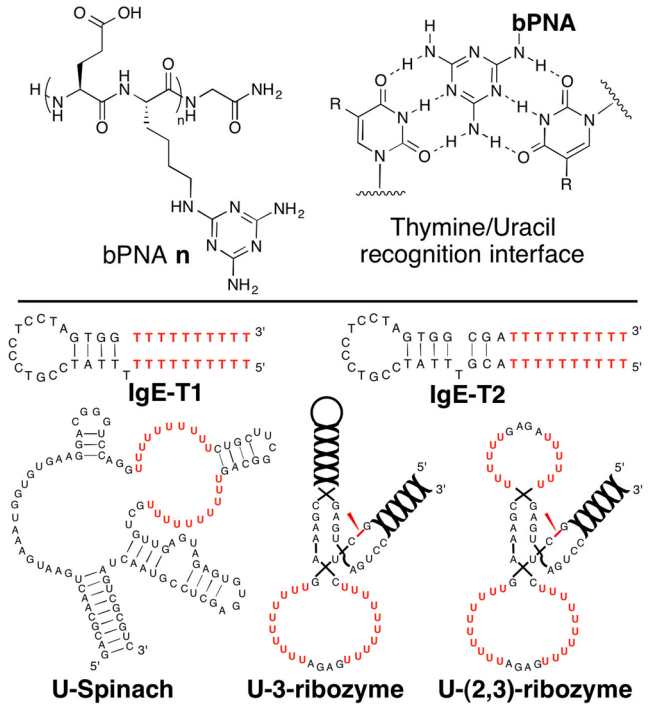

Figure 1. (Top) Structure of bPNAs studied, with n = 6, 8, and 10 corresponding to **6**, **8**, and **10**, showing the recognition interface between bPNA nucleobase melamine (M) and thymine/uracil. (Bottom) Aptamer and ribozyme sequences engineered with bPNA binding sites (red) are indicated with predicted fold based on the original sequence. The conserved catalytic site of the hammerhead ribozyme is shown, and the scissile bond is indicated with a red arrow; t-RNA Lys attached to the 3′ end is not shown.

A protein-binding DNA aptamer (D17.4) has been derived via SELEX that binds immunoglobulin E (IgE) with 7 nM affinity.[14] This compact 60-nt stem–loop aptamer is an ideal framework for bPNA structure–function manipulation: the 25-nt loop forms the primary molecular recognition module, which

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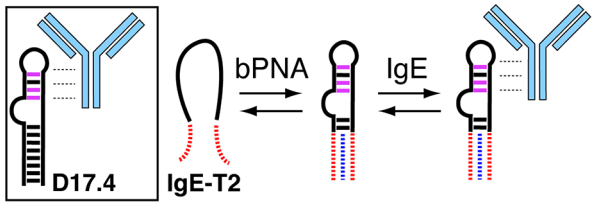
is held in place by a sequence-mutable duplex stem. The stem structure was ablated by replacement with two T_{10} tracts to yield **IgE-T1** (Figure 1). As expected, this mutant did not exhibit a thermal melting transition and was retained on nitrocellulose filters, indicative of an unfolded DNA structure. Binding to IgE was evaluated using a nitrocellulose filter-binding assay after DNA radiolabeling.[14] As reported, analysis of D17.4 binding to IgE yielded a K_d value of 7 nM, while a null binding result was observed for the unstructured **IgE-T1**. Stem-ablated DNA **IgE-T1** was treated with bPNA **10**, which was anticipated to serve as a folding "splint" to structure the terminal T-tracts into a triplex hybrid stem[2a,b] and restore the IgE recognition interface (Scheme 1). Indeed, addition of

Scheme 1. Rescue of Aptamer–Protein Binding[a]



[a] (Left) Binding of known IgE (blue) DNA aptamer D17.4 is shown with predicted fold and wobble base-pairs shown in purple. (Right) Illustration of bPNA refolding of **IgE-T2** into a functionally identical bPNA-DNA complex that binds IgE.

bPNA **10** to **IgE-T1** resulted in clean complexation to a single product, as judged by electrophoretic mobility shift assay (EMSA) and nominal retention on nitrocellulose filters (Supporting Information). However, IgE association by the complex **10·IgE-T1** was extremely weak. Inspection of the original D17.4 sequence suggested that predicted wobble base-pair interactions within the recognition loop may have been disrupted due to register-shifted bPNA complexation resulting from asymmetric T-tract lengths at the 3′ (T_{10}) and 5′ (T_{13}) termini (Figure 1). Stem replacement was redesigned as sequence **IgE-T2**, which punctuated the 5′ T_{13} tract with two CG/GC base-pairs found in the original D17.4 sequence (Figure 1). This new DNA sequence, **IgE-T2**, was itself also unfolded and ineffective in binding IgE. Complexation with bPNA **10** indicated sub-nanomolar (K_d = 0.2 nM) affinity (Figure 2), an order of magnitude tighter than previously found

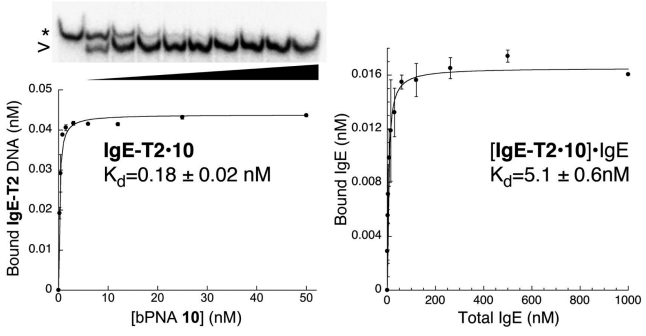
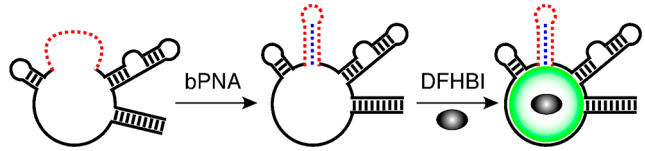


Figure 2. Binding isotherms of (left) **IgE-T2** binding to **10** and [**IgE-T2·10**] complex binding to IgE (right) obtained by gel shift (top, left) and filter-binding assays. Free **IgE-T2** and the [**IgE-T2·10**] complex are indicated on the gel by * and >, respectively. Binding data were obtained in triplicate and fitted to a 1:1 binding model to yield the dissociation constants indicated.

with T-tract hairpin loop complexes.[2a,b] Importantly, the redesigned **10·IgE-T2** triplex stem–loop structure exhibited low nanomolar affinity (5 nM) to IgE, similar to that reported for D17.4 (7 nM), indicating full recovery of molecular recognition via bPNA triplex stem replacement. Thus, bPNA rescue of DNA aptamer function revealed good tolerance of duplex–triplex replacement, as well as intolerance of a two base-pair shift in triplex site. These findings demonstrate the general utility of duplex replacement as well the precision of bPNA T-tract targeting.

Successful rescue of protein recognition in the relatively simple D17.4/**IgE-T2** "stem–loop" DNA fold prompted further investigation of aptamers with multiple secondary structural elements. Of particular interest was the "Spinach" aptamer, an RNA mimic of green fluorescent protein (GFP) recently disclosed in an elegant series of studies by Jaffrey and co-workers.[15] This RNA aptamer binds to a family of small-molecule fluorophores that closely mimic the luminescent nucleus of GFP (Scheme 2). The compound 3,5-difluoro-4-

Scheme 2. Rescue of RNA Aptamer–Small Molecule Binding[a]



[a]Stem II of the Spinach RNA aptamer is subject to replacement with $rU_{10}GAGAU_{10}$ (red dash). Fluorescence (green) and apparent DFHBI binding are abolished on stem replacement and restored by bPNA triplex stem refolding.

hydroxybenzylidene imidazolinone (DFHBI) is weakly emissive in solution but becomes strongly luminescent when bound by Spinach. Fluorophore binding (and fluorescence) may be disrupted by insertion of unstructured domains into the Spinach framework, with stem II particularly sensitive to modification. Jaffrey[16] and Hammond[17] independently demonstrated how a Spinach fluorescence read-out could be coupled to recognition of other substrates by insertion of a second aptamer domain into stem II of Spinach. Conformational tightening of the inserted aptamer fold upon target binding; restores Spinach structure and turns on DFHBI binding and emission. This design was used to couple bPNA binding to Spinach fluorescence by replacement of stem II with U_{10} tracts to yield U-Spinach. As expected, loss of stem II completely abolished Spinach fluorescence. Gratifyingly, treatment of U-Spinach with bPNAs **6**, **8** or **10** restored DFHBI binding to approximately 50% of fluorescence intensity. Notably, the spectral features of DFHBI on binding the bPNA-U-Spinach complex were identical to those with unmodified Spinach complex (Figure 3). Fluorescence activation of DFHBI by U-Spinach as a function of bPNA **6** concentration was measured, yielding an apparent K_d of bPNA-RNA binding of 0.3 μM, though the actual affinity of bPNA to U-Spinach is likely much tighter, given the thermal stability of analogous bPNA-RNA hairpin complexes (Supporting Information). Fluorescence activation of DFHBI by the RNA·bPNA complexes was comparable to that reported for the original Spinach aptamer (0.7 μM, Supporting Information), underscoring the functional similarity of a bPNA-triplex to a duplex stem structure.

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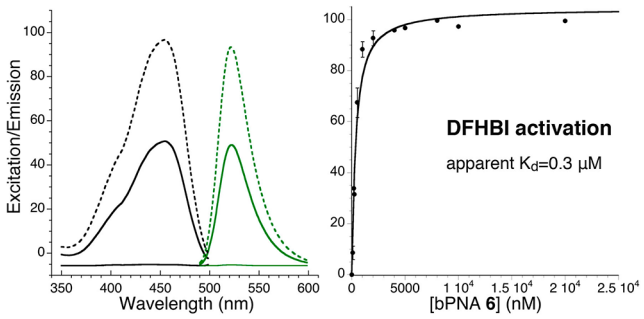
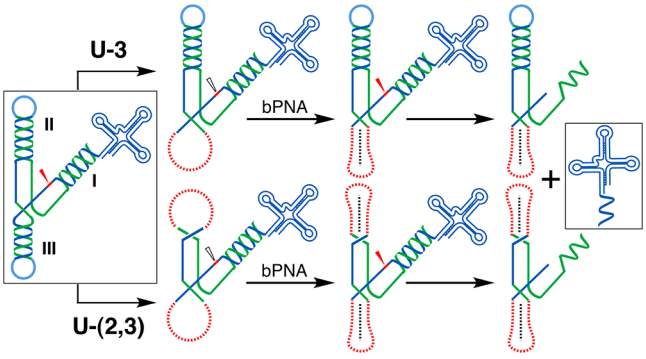


Figure 3. (Left) Excitation (black) and emission (green) of the original Spinach sequence (---) and the U-Spinach·**6** complex (—) bound to DFHBI. (Right) Fluorescence activation curve of DFHBI by the U-Spinach·**6** complex, with apparent DFHBI dissociation constant indicated.

Aptamer stem-replacement studies indicated the ability of bPNA triplex hybrid stems to support recognition of both protein and small-molecule targets by DNA and RNA aptamer folds. We hypothesized that chemical catalysis, another non-coding nucleic acid function, could be similarly placed under bPNA control, much like an artificial riboswitch.[18] To this end, a minimal type I hammerhead ribozyme fold[19] was sequence-engineered to install bPNA-sensitive catalytic function. The self-splicing hammerhead RNA features a conserved catalytic nucleus supported by three duplex stems (Scheme 3).

Scheme 3. U-Ribozyme Rescue by bPNA[a]



[a]Mutants of the hammerhead ribozyme fold, with replacement of stem III with $rU_{10}GAGAU_{10}$ to yield **U-3** and replacement of stems II and III with rU_6GAGAU_6 and $rU_{10}GAGAU_{10}$, respectively, to yield **U-(2,3)**. Splicing function is greatly diminished or ablated on stem replacement (red dash) and may be rescued by triplex refolding with bPNA (blue dash).

Structure–function studies indicate that the duplex stems may be varied in sequence without loss of splicing activity. Stems II and III of the minimal type I hammerhead fold were subject to replacement with rU_6CACAU_6 and $rU_{10}CACAU_{10}$ sequences, with single- and double-stem replacements yielding **U-3** and **U-(2,3)**, respectively. Stem knock-out "U-mutants" **U-3** and **U-(2,3)** of this ribozyme system (Figure 1, Scheme 3) were produced as tRNA–Lys fusions by run-off transcription from the appropriate DNA template. While the structurally intact ribozyme was quantitatively spliced during run-off transcription conditions, it was possible to isolate each of the U-ribozyme–tRNA fusions as full-length transcripts in high yield. On splicing, well-folded ribozyme and t-RNA products were readily detectable with minimal degradation by gel electrophoresis analysis. The **U-3** ribozyme exhibited weak background splicing activity only under high Mg^{2+} concentration (Figure 4), while the double-stem knock-out **U-(2,3)**

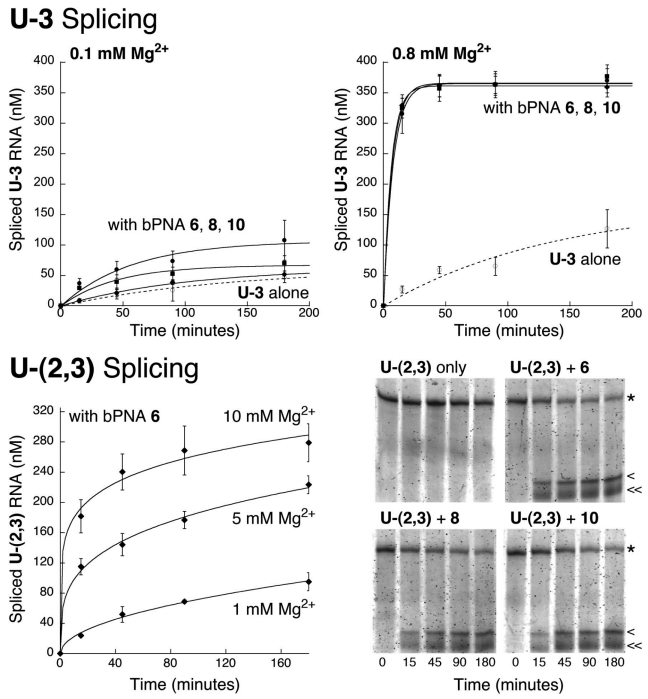


Figure 4. Rescue of self-splicing function by bPNAs **6** (◆), **8** (■), and **10** (●) in **U-3** and **U-(2,3)** hammerhead ribozymes. (Top) **U-3** splicing activity observed with bPNA as indicated, and (left) 0.1 mM and (right) 0.8 mM Mg^{2+} alone (---). (Bottom) **U-(2,3)** splicing can be triggered by bPNAs **6**, **8**, and **10** and also exhibits Mg^{2+} dependence, with representative data from bPNA **6** shown (left). **U-(2,3)** splicing was analyzed by urea-denaturing PAGE for bPNAs **6**, **8**, and **10** at 10 mM $MgCl_2$ (right). Background cleavage of **U-(2,3)** alone (*) was undetectable, while the tRNA (<) and ribozyme (≪) cleavage products could be clearly observed.

was not detectably active in self-splicing at any Mg^{2+} concentration. Triplex refolding of the U-loop by addition of bPNA to **U-3** resulted in allosteric turn-on of splicing activity, significantly above background (Figure 4). Interestingly, bPNAs **6**, **8**, and **10** all gave similar rate enhancements for **U-3** splicing, restoring cleavage rates to the reported 1 min^{-1} rate, despite the previously reported length-dependent bPNA affinity.[2b] This finding supports the notion that bPNA targeting can benefit from cooperative refolding, increasing affinity beyond that observed with unstructured nucleic acids. Targeting and ribozyme activation by bPNA could be accomplished even under the heterogeneous conditions of the run-off transcription reaction, with quantitative *in situ* splicing of **U-3** observed upon addition of bPNA **10** (Supporting Information). Though a triple-stem replacement mutant was prepared, it did not exhibit splicing on bPNA complexation. In contrast, splicing of the double-stem knockout **U-(2,3)** could be completely restored by bPNA to native activity (Figure 4). Surprisingly, all bPNAs again elicited identical splicing rates and identical Mg^{2+} dependence, despite the two different sizes of the U-loop bPNA binding sites in **U-(2,3)**. The smaller stem II site (U_6CACAU_6) was designed to be addressed exclusively by **6**, while the larger stem III site ($U_{10}CACAU_{10}$) was expected to preferentially bind **10**. The identical **U-(2,3)** splicing rates

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when complexed to bPNAs **6**, **8**, and **10** suggest high tolerance for bPNA length mismatching[2b] at these sites, leading to catalysis with both over-saturation and sub-saturation of the U−U sites by bPNA.

In summary, efficient allosteric control[20] of non-coding nucleic acid function by bPNA has been demonstrated in three distinct systems. Protein and small-molecule recognition by DNA and RNA aptamers may be turned on by bPNA triplex stem refolding. Catalysis of self-splicing by a hammerhead ribozyme may also be triggered by bPNA triplex refolding. The breadth of function and fold topology represented by these three nucleic acid systems suggest the possibility that bPNA may provide a *general* strategy to synthetically switch on nucleic acid function, provided there is a structurally important and mutable duplex stem that could be subject to duplex−triplex hybrid replacement. Nucleic acids designed with a bPNA binding site structurally coupled to a functional nucleus may thus be turned on by a bPNA allosteric switch. It is anticipated that further development of the bPNA-based methodology presented herein will yield broadly useful tools for study and control of nucleic acid function.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed experimental procedures, complete sequence information, additional binding data, T_m data, fluorescence spectra, and analysis of ribozyme splicing by gel electrophoresis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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