

# Peptide Ligation and RNA Cleavage via an Abiotic Template Interface

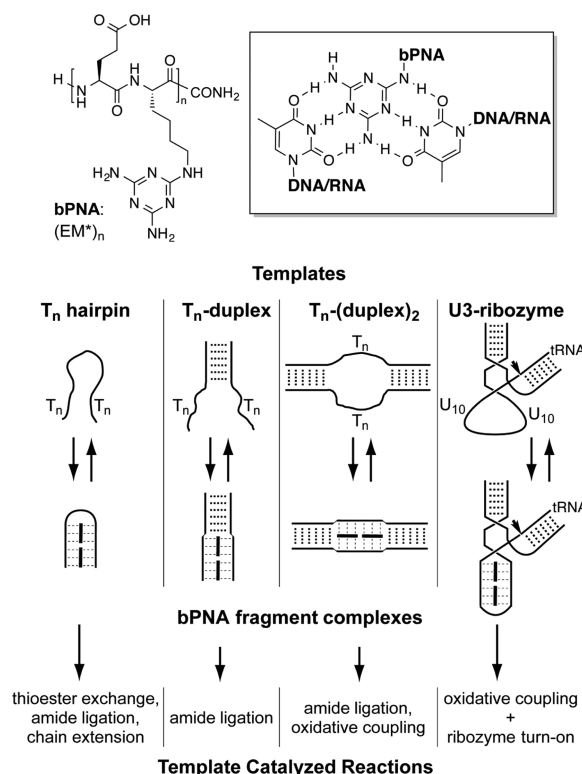
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**S** Supporting Information

**ABSTRACT:** We report herein DNA- and RNA-templated chemical transformation of bifacial peptide nucleic acid (bPNA) fragments directed by an abiotic triplex hybrid interface. Assembly of one bPNA strand with two unstructured oligo T/U strands enables facile insertion of DNA and RNA template sites within partially folded nucleic acids; this template topology is not easily accessed through native base-pairing. Triplex hybridization of reactive bPNA fragments on DNA and RNA templates is shown to catalyze amide bond ligation and controlled bPNA chain extension. RNA-templated oxidative coupling of bPNA fragments is found to result in the emergence of ribozyme cleavage function, thus establishing a connection between engineered and native reaction sites. These data demonstrate the use of new topologies in nucleic acid-templated chemistry that could serve as chemically sensitive DNA and RNA switches.

DNA recognition has been exploited to direct the chemistry of native and artificial<sup>1</sup> macromolecules. Watson–Crick base-pairing can be taken out of context to enable group transfer,<sup>2</sup> nucleic acid detection,<sup>3</sup> chemical library selection,<sup>4</sup> and self-replication.<sup>5</sup> In addition, base-pairing templates can code for synthesis of non-native scaffolds<sup>6</sup> and multisite macromolecular modification.<sup>7</sup> The abiotic base triple interface between bifacial peptide nucleic acid (bPNA)<sup>8</sup> and nucleic acids provides an opportunity to explore alternative template topologies. Triazine<sup>9</sup> bases in bPNA simultaneously engage two oligo T/U strands to form an obligate triplex hybrid (Figure 1). Unlike conventional PNA, which dissociatively invades native structures,<sup>1e</sup> bPNA recognition is an associative process that unites non-interacting native domains. Though symmetric<sup>10</sup> two-strand<sup>11</sup> recognition of this type has no cognate in extant biology, amino and oxo 2,4,6-substituted triazines<sup>12</sup> recapitulate the Watson–Crick hydrogen-bonding patterns, fueling the speculative notion of triazine-derived precursors<sup>13</sup> to DNA and RNA. We have previously demonstrated that bPNA hybridization can trigger DNA and RNA chemistry.<sup>8d</sup> We report herein that hybridization can likewise trigger bPNA chemistry. Single-stranded and partially structured DNA/RNA topologies were found to serve as templates to catalyze bPNA coupling and controlled chain extension (oligomerization) of bi-reactive bPNAs. Furthermore, integration of a template site into a ribozyme fold renders RNA splicing dependent on oxidative ligation of bPNA; this could serve as a blueprint for chemically sensitive nucleic acid switches<sup>14</sup> and gates<sup>15</sup> with applications in DNA/RNA



**Figure 1.** (Top) T/U-rich tracts can bind bPNA via melamine-derivatized lysine (M\*). (Bottom) The four template topologies shown catalyze thioester exchange, fragment ligation, chain extension, and oxidative coupling with activation of ribozyme splicing function with bPNA substrates (dark lines).

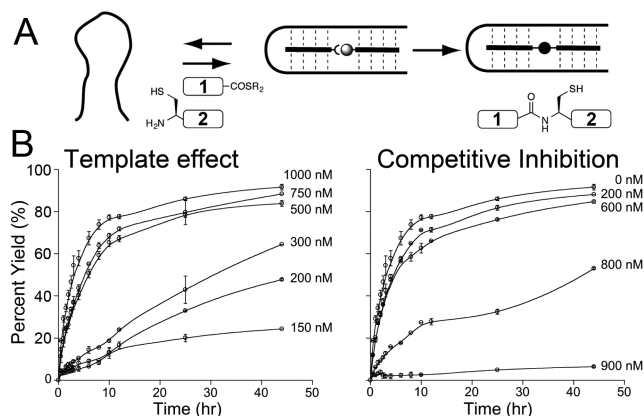
nanotechnology.<sup>16</sup> Overall, these data demonstrate readout and transformation of non-native macromolecules through an abiotic template interface in DNA/RNA template topologies that are not accessible via native base-pairing.

An *n*-mer of bPNA has the general form (EM\*)<sub>*n*</sub>, wherein M\* = melamine-modified lysine and E = glutamic acid (Figure 1). Hybridization of bPNA with dT<sub>*n*</sub>C<sub>4</sub>T<sub>*n*</sub> DNA results in triplex stem loop (hairpin) structures with *n* thymine–melamine–thymine (TMT) base triples. Binary, ternary, and quaternary bPNA–DNA complexes can be formed with one, two, or three bPNAs bound to a single dT<sub>*n*</sub>C<sub>4</sub>T<sub>*n*</sub> DNA strand.<sup>8b</sup> Successful preorganization of DNA into hairpin configurations with weakly binding 4mer bPNAs prompted investigation of

Received: January 13, 2015

Published: March 6, 2015

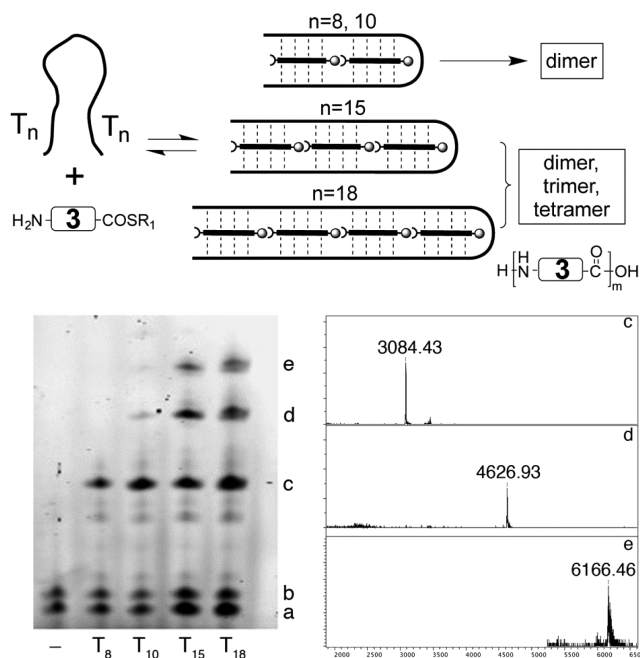
DNA hairpin templates as catalysts for native chemical ligation<sup>17</sup> of two bPNA 4mer fragments. Nominal background ligation of 4mer bPNAs **1** (C-terminal thioester) and **2** (N-terminal cysteine) was observed over several days at 200 nM fragment concentration (Figure 2). Remarkably, a strong



**Figure 2.** (A) Native chemical ligation of **1** (Cbf- $\beta$ -(EM\*)<sub>4</sub>G-COSR<sub>2</sub>) and **2** (CM\*(EM\*)<sub>3</sub>G) (200 nM each) with dT<sub>10</sub>C<sub>4</sub>T<sub>10</sub> (Cbf = carboxyfluorescein; R<sub>2</sub> = (CH<sub>2</sub>)<sub>2</sub>SO<sub>3</sub>Na);  $\beta$  =  $\beta$ -alanine. (B) Ligation yield with template (left) and (right) inhibitor concentration indicated. 10mer (EM\*)<sub>10</sub> bPNA was used as inhibitor at 1000 nM fixed DNA template. Lines are drawn to guide the eye.

template effect was observed with the T<sub>10</sub> hairpin DNA template, despite the modest binding affinity of 4mer bPNA. Consistent with the key role of the TMT interface, ligation yield dropped sharply with T→C substitutions in the DNA template (SI, Figure S7). The reaction profile is consistent with product inhibition,<sup>18</sup> further underscored by competitive inhibition of ligation with 10mer bPNA (Figure 2). As the reaction progresses, a well-defined UV transition emerges at ~50 °C, similar to the thermal stability observed with an 8-mer bPNA–DNA complex. Notably, optimum reaction temperature is at the T<sub>m</sub> of the 4mer–DNA complex (25 °C), with decreased rate at higher and lower temperatures, suggestive of the importance of dynamic complexation (SI, Figure S2). Despite the modest DNA affinity of the fragments, the T<sub>10</sub> hairpin template increased ligation rates by 2500 fold over background; increased effective molarity<sup>19</sup> in the bPNA–DNA ternary complex can readily account for this rate acceleration.

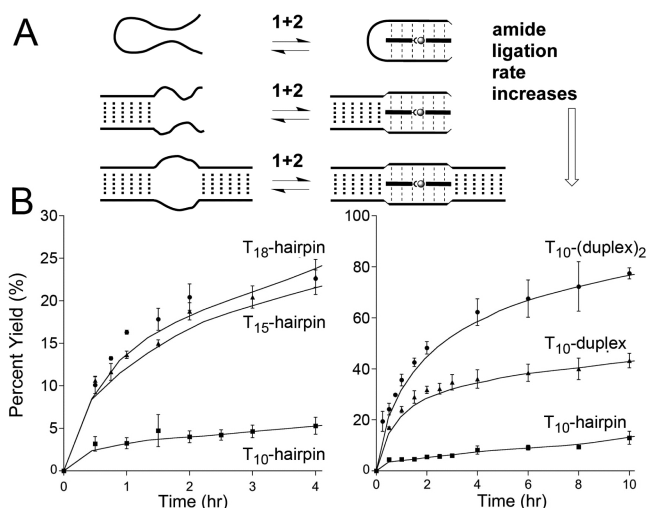
DNA-templated native chemical ligation suggested the possibility of chain extension (oligomerization) through multiple on-template couplings of bi-reactive bPNAs. This appeared reasonable as the thermal stability of bPNA–DNA complexes increases from binary to ternary to quaternary.<sup>8b</sup> Rapid cyclization<sup>20</sup> of bPNAs bearing N-terminal cysteine and C-terminal thioester functionality prompted investigation of cysteine-free amide coupling of bPNAs. Though direct peptide aminoacylation with thioesters is low yielding reaction in aqueous milieu, amino acid side chains can greatly influence reaction rate and yield.<sup>21</sup> Accordingly, a 4mer bPNA (**3**) fitted with N-terminal glycine and C-terminal histidine thioester was prepared for on-template chain extension. The reaction mixture was spiked with 5 mol% thioester **1** (Figure 3) to fluorescently label the products for PAGE analysis. While background coupling was insignificant, ligation was observed on incubation with dT<sub>n</sub>C<sub>4</sub>T<sub>n</sub> hairpin templates ( $n = 8, 10, 15, 18$ ), with 25–50% overall conversion wherein higher yields corresponded to



**Figure 3.** (Top) Peptide chain extension of **3** (GM\*(EM\*)<sub>3</sub>H-COSR<sub>2</sub>; R<sub>2</sub> = (CH<sub>2</sub>)<sub>2</sub>SO<sub>3</sub>Na) with dT<sub>n</sub>C<sub>4</sub>T<sub>n</sub> DNA. (Lower) Fluorescence-stained denaturing PAGE of extension reactions with hairpin template T-tract indicated. Product bands were identified as (a) hydrolyzed **3**, (b) **3**, (c) dimer, (d) trimer, and (e) tetramer by MALDI-MS (c–e are C-terminal acids).

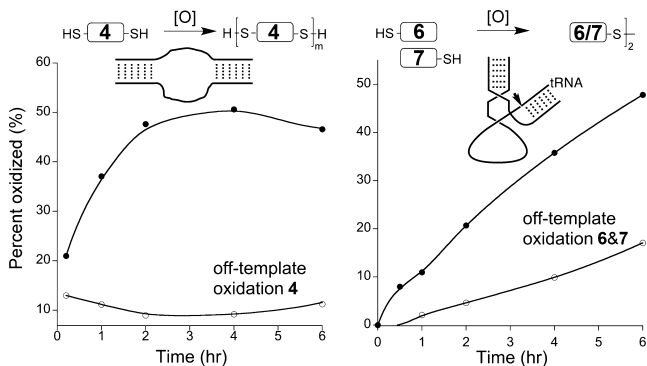
the longer templates (SI, Figures S5 and S6). Furthermore, longer ligation products were observed with longer templates (Figure 3). For  $n = 8$  and 10, dimer was the dominant outcome, consistent with the notion that two 4mer bPNAs could fit on the template at once. Longer trimer and tetramer bPNA products from two and three on-template couplings were clearly detected as major products with the dT<sub>15</sub>C<sub>4</sub>T<sub>15</sub> and dT<sub>18</sub>C<sub>4</sub>T<sub>18</sub> templates, commensurate with higher order complex formation. The identity of the oligomers was confirmed by band isolation and MALDI-MS. Though fluorescence labeling was used to image the gel, isolated bands yielded masses corresponding to the unlabeled, thioester hydrolyzed population. Thus, on-template, direct aminolysis of thioester fragments from ternary, quaternary, and apparent quaternary bPNA–DNA complexes leads to bPNA chain extension by virtue of the length-matching abiotic TMT interface.

Just as chain extension was higher yielding with longer templates, native chemical ligation of bPNA cysteine and thioester fragments was also significantly faster with T<sub>15</sub> and T<sub>18</sub> hairpin templates relative to T<sub>10</sub> (Figure 4). Two factors likely contribute to this effect: (1) length-enhanced binding of 4mers and (2) template preorganization by first coupling product, leading to enhanced binding and catalysis of subsequent fragments. To probe the effect of pre-structuring, duplex-organized DNA templates were tested in bPNA native chemical ligation. Indeed, ligation rates of bPNAs **1** and **2** increased as the T<sub>10</sub> tracts were buttressed by one (T<sub>10</sub>-duplex) and two duplexes (T<sub>10</sub>-duplex)<sub>2</sub>. Duplex presentation of the T-tracts likely increases coupling efficiency by decreasing the entropic cost of bPNA triplex hybridization (Figure 4). Constraining both ends of the unstructured T-tracts in T<sub>10</sub>-duplex)<sub>2</sub> results in further enhancement of ligation.



**Figure 4.** Native chemical ligation of bPNAs **1** and **2** using  $T_{10}$ -hairpin,  $T_{10}$ -duplex and  $T_{10}$ -(duplex)<sub>2</sub> templates. Rate increases with longer hairpin templates (left) and with duplex-organized templates (right). Lines are drawn to guide the eye.

In addition to acceleration of amide bond coupling, oxidation of bPNA dithiol **4** with  $T_{10}$ -(duplex)<sub>2</sub> was also significantly accelerated over background (Figure 5). While thiol oxidation

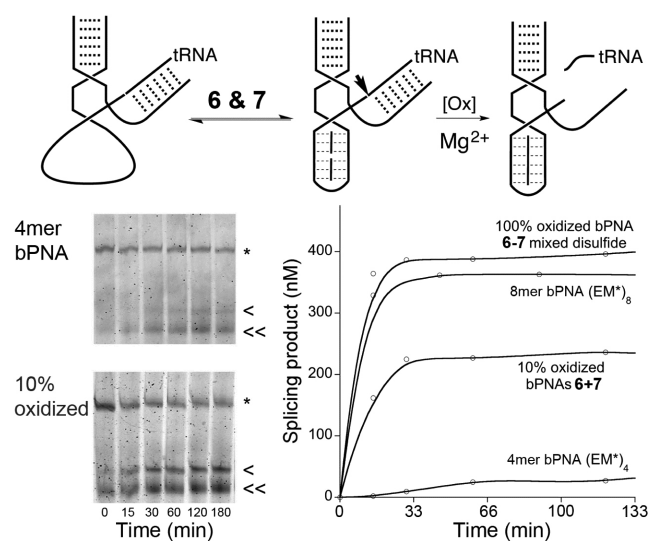


**Figure 5.** (Left) Oxidation of bPNA **4** (Ac-CM\*(EM\*)<sub>3</sub>C) alone (○) and with 50 mol%  $T_{10}$ -(duplex)<sub>2</sub> DNA (●), followed by PAGE. (Right) Oxidation of bPNAs **6** (Mpa-(EM\*)<sub>4</sub>G) and **7** (EM\*)<sub>4</sub>C alone (○) and with 12 mol% U3-ribozyme (●), followed by Ellman's test. Mpa = mercaptopropionamide. Lines are drawn to guide the eye.

is more facile than amide bond formation under these conditions, the duplex-constrained template limited products formed to dimeric and trimeric extension; in contrast, a wide range of oxidation products were formed off template (SI, Figures S10 and S11). Unlike amide bond chain extension (Figure 3), three bPNA fragments may be oxidatively coupled on a  $T_{10}$  template; this is perhaps due to the increased flexibility of the disulfide linkage. Successful catalysis with partially folded DNA templates prompted investigation of  $U_n$ -template loops imbedded within RNA folds. This notion was tested using a minimal type I hammerhead ribozyme<sup>22</sup> in which stem III was replaced with an  $rU_{10}$ CACAU<sub>10</sub> loop (U3-ribozyme).<sup>8d</sup> It was initially thought that the constrained U-loop would exhibit orientational bias with respect the RNA template; thus, two 4mer bPNAs bearing *N*-terminal (**6**) and *C*-terminal (**7**) thiols were prepared and studied. However, both thiols and their mixture, gave identical template-enhanced oxidation profiles with U3-ribozyme (Figure 5). Interestingly, the U3-ribozyme

was able to achieve a similar yield of oxidation at lower catalyst loading (12%) compared to the DNA (50%), suggesting a higher exchange rate off the RNA loop template, though more quantitative measurements are needed to pinpoint the origin of this difference.

The U3-ribozyme sequence has ablated self-cleavage activity due to the loss of stem III structure. We have previously demonstrated that duplex stems in aptamers and ribozyme folds can be functionally replaced with bPNA triplex hybrid stems when base-pairing sequences are replaced with T/U tracts. This allows bPNA to be used as an allosteric switch for both aptamer affinity and ribozyme catalysis. We hypothesized that native nucleic acid function could report on the coupling of short bPNA fragments. This notion was tested using oxidative thiol coupling since amide bond ligation occurs on a time scale similar to RNA degradation. While 4mer bPNA (EM\*)<sub>4</sub> and bPNAs **4**, **6**, and **7** only weakly activate cleavage of the U3-ribozyme (Figure 6), oxidative coupling produces an ~8mer



**Figure 6.** (Top) U3-ribozyme cleavage upon oxidation of bPNA thiols **6** and **7** and addition of  $Mg^{2+}$ . (Bottom) Denaturing PAGE of RNA cleavage triggered by 4mer bPNA (EM\*)<sub>4</sub> and 10% oxidized **6** and **7**, with full-length RNA (\*), tRNA (<), and ribozyme (<<) cleavage products indicated, along with gel quantification, with the bPNA additives indicated. Lines are drawn to guide the eye.

bPNA disulfide product that binds more tightly to the template and strongly activates function.<sup>8d</sup> Oxidation conditions are more concentrated than those for ribozyme cleavage; thus, reactions were studied by dilution of partially oxidized samples into ribozyme cleavage conditions with 1 mM  $Mg^{2+}$ . PAGE analysis of the reaction indicated the formation of two RNA products upon 10% bPNA oxidation, which were identified as the tRNA fusion and the hammerhead ribozyme components (Figure 6). Positive control experiments using 8mer bPNA (EM\*)<sub>8</sub> and fully oxidized and purified mixed disulfides of **6** and **7** gave higher yields of splicing. Ribozyme catalytic activity therefore may be used to report on fragment oxidative coupling, indicating two-way communication between an engineered abiotic template site and a native RNA splicing site. This connection makes possible functional selection<sup>2,3</sup> and optimization of template and redox-switchable ribozymes.

Overall, these data collectively demonstrate that effective molarity increases on DNA and RNA templates can catalyze

acyl transfer and oxidative coupling as well as chain extension of bPNA fragments. Insertion of template sites into folded nucleic acids is uniquely achieved through the thymine–melamine–thymine triplex interface with bPNA. Native nucleic acid function can thus be linked with engineered reactivity through allostery and template effects using the abiotic TMT interface. It is anticipated that facile inclusion of partially folded template topologies in nucleic acid directed chemistry will have use in DNA/RNA nanotechnology.<sup>16</sup>

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

Detailed experimental procedures, detailed sequence information, additional reaction analysis, UV melt data, and compound characterization. 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.

## ■ ACKNOWLEDGMENTS

Research was supported in part by NSF-DMR. Facilities were provided by The Ohio State University.

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