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Synthetic bPNAs as allosteric triggers of hammerhead ribozyme catalysis

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Abstract

The biochemistry and structural biology of the hammerhead ribozyme (HHR) have been well elucidated. The secondary and tertiary structural elements that enable sugar–phosphate bond scission to be catalyzed by this RNA are clearly understood. We have taken advantage of this knowledge base to test the extent to which synthetic molecules, may be used to trigger structure in secondary structure and tertiary interactions and thereby control HHR catalysis. These molecules belong to a family of molecules we generally call “bPNAs” based on our work on bifacial peptide nucleic acid (bPNA). This family of molecules displays the “bifacial” heterocycle melamine, which acts as a base-triple upon capturing two equivalents of thymine or uracil. Loosely structured internal oligouridylylate bulges of 4–20 nucleotides can be restructured as triplex hybrid stems upon binding bPNAs. As such, a duplex stem element can be replaced with a bPNA triplex hybrid stem; similarly, a tertiary loop–stem interaction can be replaced with a loop–bPNA–stem complex. The ability to control RNA structure–function facilitates elucidation of these critical aspects of RNA recognition. In this chapter, we discuss how bPNAs are prepared and applied to study structure–function turn on in the hammerhead ribozyme system.

1. Introduction

This chapter describes the application of bPNAs for triggering RNA structure–function in the hammerhead ribozyme, by virtue of the melamine base triple. Melamine hydrogen bonding has been studied extensively in organic solvents (ten Cate, Huskens, Crego-Calama, & Reinhoudt, 2004; Whitesides et al., 1995) and the solid state (Ranganathan, Pediredi, & Rao, 1999). The energetic signatures of assembly (Ma & Bong, 2011a) are identical to those of nucleic acid duplexes (Kool, 2001; SantaLucia & Hicks, 2004; Yakovchuk, Protozanova, & Frank-Kamenetskii, 2006). Accordingly, melamine recognition of native bases (Arambula, Ramisetty, Baranger, & Zimmerman, 2009; Ma & Bong, 2011b, 2011c; Zhou & Bong, 2013) in a range of contexts has been observed (Ariga & Kunitake, 1998; Kawasaki, Tokuhito, Kimizuka, & Kunitake, 2001; Ma & Bong, 2013), presaging the possibility of triplex formation with melamine and oligo T/U domains. Unlike triplex formation with native nucleic acids (Duca, Vekhoff, Oussedik, Halby, & Arimondo, 2008; Felsenfeld, Davies, & Rich, 1957; Pilch, Levenson, & Shafer, 1990; Soto, Loo, & Marky, 2002) involving both Watson–Crick (WC) and Hoogsteen base pairing at high salt (Rougee et al.,

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1992; Xodo, Manzini, & Quadrifoglio, 1990) and peptide nucleic acids (Nielsen, 1999) which invade native duplex structures, melamine triplex hybridization unites two noninteracting oligo-T/U domains. Our group found that α -PNAs (Mittapalli et al., 2007) displaying melamine at alternate residue sidechains form triplex hybrids with oligo T/U DNA/RNA; we called these peptides bifacial peptide nucleic acid (bPNA) (Piao, Xia, & Bong, 2013; Zeng, Pratumyot, Piao, & Bong, 2012). These bPNAs bind T-rich DNA to form thermally stable ($T_m \sim 58^\circ\text{C}$) triplex domains that can block enzymatic processing of DNA and RNA (Piao et al., 2013; Xia, Piao, Fredrick, & Bong, 2013). The triplex hybrids can functionally replace stems in RNAs and DNAs, serving as triggers of molecular recognition and catalysis (Piao, Xia, Mao, & Bong, 2015; Xia, Piao, & Bong, 2014). The bPNA family of melamine-bearing molecules has expanded beyond peptides to include peptoids (Mao & Bong, 2015), polyacrylates (Zhou, Xia, & Bong, 2015) and synthetic small molecules (Mao, DeSantis, & Bong, 2017). Herein, we focus on the use of bPNA peptides and small molecules (Fig. 1) to trigger folding and function in the hammerhead ribozyme RNA (HHR) scaffold to facilitate a deeper understanding of RNA recognition and catalysis.

The mini hammerhead ribozyme system (Robertson & Scott, 2007; Uhlenbeck, 1987) features a 19 nt catalytic core supported by three structural stems. The function of the HHR is to cleave itself, and this catalytic function is dependent on well-folded secondary structure in the stems and a tertiary contact between the loop of stem 2 and stem 1. It is possible to use bPNAs to trigger both secondary and tertiary structure in the HHR scaffold, replacing these native structures with bPNA triplex hybrids. Below we provide detailed protocols for the synthesis of bPNAs and design of HHRs under the control of bPNA triggers.

2. Synthetic protocols

Preparation of bPNAs is relatively straightforward but requires close attention. The following synthetic procedures have been developed to avoid chromatography where possible, but final purification of peptides requires HPLC. Oligoethyleneimine scaffolds (tren-derived) may be purified without column chromatography.

2.1 Materials and instrumentation for chemical synthesis

All chemicals were used without further purification from commercial sources, unless otherwise noted. Solid-phase peptide synthesis was carried out under nitrogen on an AAPTEC 96 automated peptide synthesizer equipped with a 40-well reaction block. MALDI-Mass spectra were acquired on Bruker Microflex MALDI-TOF instrument using RP mode. Peptide samples were dissolved in ACN/H₂O 1:99 (v/v) with 0.1% TFA while α -cyano-4-hydroxycinnamic acid was saturated in ACN/H₂O 1:1 (v/v) with 0.1% TFA used as the matrix. Peptide and matrix were mixed in 1:1 ratio and then spotted on MSP 96 target ground steel (Bruker Daltonics) for mass determination. Electrospray mass spectroscopy was acquired on a Bruker MicroTOF equipped with an electrospray ionization source under positive mode. Mass spectrometry instruments were provided by a grant from the Ohio BioProducts Innovation Center. NMR spectra were acquired on a Bruker Avance DPX 400 instrument.

2.2 Synthesis of bPNA amino acid derivatives

2.2.1 Boc-K^M-OH—This lysine derivative is prepared from Boc-Lys-OH, and must be converted to the Fmoc derivative prior to automated solid-phase peptide synthesis. It is not possible to start from the Fmoc derivative due to basic conditions required for installation of the melamine ring. However, the derivatization from Boc-Lys-OH is high yielding (95%), straightforward, and does not require chromatography. Commercially available Boc-Lys-OH (10 g, 40.6mmol) was suspended in 120mL H₂O with NaOH (2 eq, 3.25 g, 81.2mmol) and 2,4-diamino-6-chlorotriazine (1.25 eq, 7.4g, 50.7mmol) and heated to 85°C for 5h. Thin layer chromatography (5,1 dichloromethane-methanol) confirmed full consumption of starting material. The reaction was cooled to room temperature and filtered to remove unreacted chlorotriazine. The aqueous solution was cooled in an ice bath and the pH was adjusted to 5 using 1NHCl, resulting in a white precipitate, which was collected via filtration and washed with cold water then dried under vacuum. This material is the derivative Boc-Lys(M)-OH, or Boc-K^M-OH, and it should be analytically pure at this point.

2.2.2 Fmoc-K^M-OH—The Boc-K^M-OH (10 g, 28mmol) deprotected by dissolution in neat TFA (80mL) and 1mL of water was added as scavenger; alkylated product was never observed, and addition of water may be unnecessary. The mixture was stirred at room temperature until complete as judged by electrospray mass spectroscopy and TLC (~1h). The reaction was concentrated to an oil under reduced pressure, but still contains significant quantities of TFA, which is difficult to fully remove. This residue was resuspended in 200mL water and the solution was neutralized to pH 7 using NaHCO₃. The solution was rendered basic with another 1.5 eq of NaHCO₃ and cooled in an ice bath. A solution of Fmoc-OSU (14 g, 42mmol) in 200mL dioxane was also ice-bath cooled and added portionwise to the lysine derivative. The resulting mixture was stirred at 0°C for 1h and allowed to warm to room temperature overnight. The reaction was worked up via removal of dioxane under reduced pressure, addition of water (150mL), and extraction of the aqueous layer with ethyl acetate (2×100mL). The aqueous layer was acidified to pH 1 with 1NHCl and extracted again with EtOAc (3×120mL). The combined organic layers were concentrated under vacuum. The resulting residue was purified CH₂Cl₂ to yield Fmoc-K^M-OH as a white solid (8g, 60%). Alternatively, a lower yielding purification (44%) may be performed by trituration of the crude residue with ethanol (~200mL).

2.3 Solid-phase synthesis of bPNAs

Peptide synthesis was performed on an AAPPTEC Apex 396 Peptide Synthesizer using Rink Resin LS (100–200 mesh) equipped with a 40-well synthesis block. The melamine base does not require protection from SPPS or cleavage conditions and generally can be handled like any other Fmoc-amino acid derivative. To minimize aggregation on resin due to the melamine rings a low-loading resin (0.2mmol/g) is recommended. Standard solid phase conditions may be used. The following solutions should be prepared:

- A. 0.25M solution of Fmoc amino acid with 0.25 M HOBt in NMP. (HOBt is commercially available as the hydrate and this is acceptable)
- B. 20% Piperidine in NMP (by volume)

C. Diisopropylcarbodiimide (DIC), neat

Approximately 100–150mg of resin is added to a reaction well, which accommodates a recommended maximum ~2mL liquid volume when resin is swollen. Prior to synthesis, resin is swollen in DMF for 45min then drained under N₂ pressure and deprotected with piperidine solution B. The swelling, deprotection, washing, coupling, shrinking, and cleavage protocols are as follows.

2.3.1 Swelling—1× 45min. Two mL of DMF is delivered to the resin in the reaction chamber and agitated for 45min at 475rpm, then drained under N₂ pressure for 2min.

2.3.2 Deprotection cycle—2× 5min. Two mL of piperidine solution is delivered via liquid handler to reaction chamber and reaction block is agitated at 475rpm for 5min, then drained under N₂ pressure (2min). This is repeated. A wash cycle follows.

2.3.3 Wash cycle—3× 2min. Two mL DMF is delivered to the reaction chamber and reaction block is agitated at 475rpm for 2min, then drained under N₂ pressure (2min).

2.3.4 Coupling cycle—1×45–60 min. Two mL (0.5mmol) of amino acid/HOBt solution (A) is added to washed resin, followed by 75μL (0.48 mmol) DIC solution (C). Reaction block is agitated at 475rpm for 45–60min. Standard amino acid derivatives (e.g., Fmoc-Glu(OtBu)-OH) are coupled for 45min while Fmoc-K^M-OH is coupled for 60min. Coupling may be extended to 90min for longer peptides. After coupling is complete, reaction is drained under N₂ pressure (2min).

2.3.5 Methanol wash—3×5 min. Two mL methanol is added to washed resin and agitated for 5min at 475rpm, then drained for 2min. This is repeated two additional times, then drained under N₂ pressure for 15min to dry the shrunken resin.

Synthesis procedures are typically the following sequence:

1. Swell
2. Deprotection
3. Wash
4. Coupling
Repeat
5. Final wash
6. Methanol wash

Note that there is no wash after coupling as this is not necessary. After the methanol wash, the peptide resin should then be dry, but clumped together and easy to transfer by spatula into a preweighed 20mL glass scintillation vial. This resin is then washed three times with diethyl ether, removing ether each time with a glass pipette. The resin should be further shrunken at this time. The vial is covered with a clean kimwipe which is secured over the mouth of the vial with an elastic band, and then evacuated in a lyophilization bell to dry the

resin completely. The vial is then weighed to obtain the weight of the peptide-resin. It is important to obtain an accurate weight in order to perform the peptide cleavage from resin. The following cleavage protocol is followed:

2.3.6 Peptide cleavage from resin—For every 100mg of peptide resin, 1 mL of cleavage cocktail is used. For bPNAs containing only K^M and glutamic acid or lysine, there is no need for a complex scavenger mix. Peptides were cleaved for 1h at RT using a 95:5 volume ratio of TFA to water. The cleavage reaction including resin was filtered through cotton into a 50-mL conical tube and the peptides were precipitated from TFA by addition of 30–50mL chilled ether then centrifuged to yield a white pellet. This pellet was resuspended in chilled ether and repelleted three times (3×20mL) to remove as much TFA as possible.

2.3.7 HPLC purification—Crude peptides were dissolved in solvent A and purified by HPLC on a semiprep (8mL/min) C₁₈ reversed phase column using a two solvent gradient. Solvent A=0.1% TFA, 1% acetonitrile, 99% water; solvent B=90% acetonitrile, 10% water, 0.07% TFA. bPNA peptides typically elute around 40% B, depending on sequence. Typical UV detector settings for peptides are at 230nm while melamine itself can be observed as a distinct peak at 238nm under acidic conditions. Most purifications are set to observe 238nm, with a final check for purity at 230nm. Purified peptide fractions were concentrated under vacuum to remove acetonitrile then lyophilized. Though peptides are purified on a standard gradient optimized for concentrated elution and efficiency, peptide purity is evaluated on an analytical column with an optimized isocratic gradient and confirmed by MALDI-TOF using α -cyano-4-hydroxycinnamic acid matrix.

2.4 Oligoethyleneimine bPNAs by nucleophilic aromatic substitution

There are generally two methods employed in the preparation of oligoethyleneimine scaffolds displaying melamine: (1) nucleophilic aromatic substitution (NAS) of chloride for amine, or (2) reductive alkylation of amine using melamine acetaldehyde (Fig. 1). The first method is more straightforward and can proceed using only commercially available materials, but affords less control over substitution. The second involves more steps but can be highly controlled. We discuss both approaches below.

2.4.1 t3M from NAS—This molecule can bind to a tetrauridylate bulge but only uses 2 of 3 rings in the binding. This binding event can serve as a folding trigger for RNA. If the presence of a third nonparticipating melamine ring is acceptable, then it is recommended to prepare this molecule, as it is the easiest to synthesize. Commercially available tris(2-aminoethyl)amine (0.75mL, 5mmol) was dissolved in 30mL H₂O, followed by addition of 2,4-diamino-6-chlorotriazine (4.36 g, 30mmol) and NaHCO₃ (1.5 g, 18mmol). Diaminochlorotriazine may be purchased but the synthesis from cyanuric chloride and ammonium hydroxide in water is straightforward. The reaction was heated to 85°C and left to stir overnight. After cooling to room temperature, the solution was filtered, the solid was washed with water twice, and dried under vacuum (1.63g, 73%). The crude solid was further purified by prep HPLC using gradient of 0% to 15% acetonitrile over 40min.

2.4.2 Boc-tren—In order to prepare a tren scaffold with only two melamines (t2M), a protected derivative, mono-Boc tren, is required. Though this starting material is only available in modest yield, the preparation is convenient. Tris(2-aminoethyl)amine (6mL, 40mmol) was dissolved in 50mL DCM and cooled in an ice bath. A solution of Boc anhydride (1.09 g, 5mmol) in 20mL DCM was added dropwise, followed by dropwise addition of triethylamine (0.7mL, 5mmol) in 20mL DCM. The reaction was warmed up to room temperature slowly and stirred overnight. After removal of the solvent under reduced pressure, the crude oil was resuspended in 50mL H₂O and extracted with DCM (50mL×5). The organic phase was dried over Na₂SO₄ and solvent removed under reduced pressure. Chromatographic purification of the crude product (DCM:MeOH=5:1, 2% concentrated ammonium hydroxide) yielded 700mg (57%) of a light yellow oil.

2.4.3 t2M from NAS—6-chloro-2,4-diamino-1,3,5-triazine(550mg, 3.78mmol) and NaHCO₃ (500mg, 5.95mmol) were added into a 40mL H₂O solution of mono-Boc protected tren (220mg, 0.89mmol). The slurry was heated to 85 °C and reacted overnight. The reaction was then cooled to room temperature and filtered. The solid was washed twice with water and dried under vacuum. The crude product was then purified by silica gel chromatography using DCM:MeOH:conc NH₄OH =80:20:2 to yield Boc-t2M as a white solid (90mg, 22%).

Boc t2M was dissolved in trifluoroacetic acid (TFA) and reacted at room temperature for 10min. The TFA was evaporated under N₂ flow, the residue was dissolved in H₂O, and solution lyophilized to obtain the TFA salt of t2M.

2.4.4 t2M^{Me} from NAS—An appropriate control molecule to evaluate selective binding of melamine to T/U domains is the *N*-methylated melamine derivative. These molecules have the same overall electrostatics and similar shape, but with hydrogen bond donors replaced with methyl groups. Though this control changes steric interactions somewhat, this is unavoidable. The tetramethylated chlorotriazine can be prepared in a manner similar to the diamino chlorotriazine by reaction of cyanuric chloride with dimethylamine instead of ammonium hydroxide. Thus, 6-chloro-*N*²,*N*²,*N*⁴,*N*⁴-tetramethyl-2,4-diamino-1,3,5-triazine (80mg, 0.4mmol) and NaHCO₃ (84mg, 1mmol) were added into 10mL 1,4-dioxane:H₂O =1:1 solution of mono Boc-protected tren (40mg, 0.162mmol). The slurry was heated to 85°C and reacted overnight. The reaction was then cooled to RT and extracted with DCM twice. The organic layer was then dried and condensed to obtain a crude mixture which was purified by silica gel chromatography using 2% MeOH in DCM with 2% concentrated ammonia in MeOH. The product was obtained as a white solid (45mg, 48.4%). Boc t2M^{Me} was dissolved in TFA and reacted at room temperature for 10min. The TFA was evaporated under N₂ flow, the residue was dissolved in H₂O and lyophilized to obtain the TFA salt of t2M^{Me}.

2.5 Oligoethyleneimine bPNAs from reductive alkylation

Notes. The reductive alkylation procedure to prepare oligoethyleneimine bPNAs is efficient but requires careful attention. Like all reductive alkylation/aminations, the reaction requires slightly acidic conditions, but acidity <3 will result in significant quantities of aldehyde reduction. The aldehyde used is melamine acetaldehyde, and the corresponding alcohol is

difficult to remove completely from the oligoethyleneimine bPNA product, and thus its production should be minimized. We have found that sequential addition of substoichiometric aliquots of melamine acetaldehyde to the amine substrate and reaction at RT or 50°C for 30min generates the imine, which can then be completely reduced with NaBH₃CN in 30min. Thus, it is recommended to aliquot reagents in this alternating sequence, allowing imine formation at elevated temperature, then cooling to RT for the addition of reductant. Though laborious, this procedure allows for complete conversion to the per-alkylated scaffold within several hours, with minimum alcohol production, and the product may be purified by trituration. Additionally, given the sensitivity of the reaction to acid, care must be taken to completely remove excess acid from starting materials. The hydrochloride salt of the melamine acetaldehyde is preferred, but this starting material should be completely free of excess HCl used in the production of the free aldehyde from protected acetal. These reactions may be followed by analytical RP-HPLC (Fig. 2).

2.5.1 Boc-t2M—Dissolve Boc-ethylenediamine (0.15 g, 0.94mmol) in 20mL of methanol in a 50-mL round bottom flask. Prepare four aliquots of melamine acetaldehyde hydrochloride (0.11 g, 0.54mmol per aliquot) and NaBH₃CN (33.9mg, 0.54mmol per aliquot). Add two aliquots of melamine acetaldehyde hydrochloride to the methanol solution and stir for 30min at room temperature to allow imine formation to proceed. The hydrochloride salt is preferred here to generate the slightly acidic conditions favorable for reductive alkylation. Add aliquots of aldehyde and borohydride sequentially every 30min over 3–4h. A major side product of the reaction is the alcohol from reduction of melamine acetaldehyde, which can be difficult to separate from the desired product. If the pH drops to <3, then significant aldehyde reduction is observed. It was empirically determined that addition of aldehyde and reductant in aliquots over time minimizes formation of this side product as compared to addition of larger portions at the start of the reaction. A typical treatment is shown below:

Reaction start:

1. 2 Aliquots (1.15 eq) melamine acetaldehyde with Boc-ethylenediamine, stir 30min
2. 1 Aliquot NaBH₃CN added, stirred 30min
3. 1 Aliquot melamine acetaldehyde added, react 30min
4. 1 Aliquot NaBH₃CN added, react 30min
5. 1 Aliquot melamine acetaldehyde and 1 aliquot NaCNBH₃ added, react 30min
6. Final aliquot of NaCNBH₃ added

Monitor the reaction by analytical HPLC to follow the progression of the alkylation (Fig. 2). The imine intermediate may be detected by HPLC/MS (ESI). To work up the reaction, reduce the amount of methanol to ~4 mL and transfer to two 2-mL-centrifuge tubes. Centrifuge to obtain the solid and discard the methanol solution. The residue will be sticky, but can be triturated and sonicated in acetone three times, using 3mL acetone for each wash. Each time after trituration, centrifuge to obtain the solid and discard the acetone. Combine

the solid into one centrifuge tube and repeat the trituration procedure with ~0.5mL of neutral water to wash away inorganic salts. A final trituration with 0.5mL of ethanol yields a solid that can be air-dried to yield 210mg white powder (0.45mmol, yield =48%).

2.5.2 Boc-t4M—The procedure to produce Boc-t4M is similar to Boc-t2M, using portionwise addition of melamine acetaldehyde and NaBH₃CN to an amine scaffold to minimize production of the reduced acetaldehyde side product. To prepare Boc-t4M, dissolve Boc-tren (0.2g, 0.81mmol) in 40mL methanol. Prepare five aliquots of melamine acetaldehyde (0.166g, 1 eq, 0.81mmol per aliquot) and NaBH₃CN (51mg, 1eq, 0.81mmol). Reactants are aliquoted into the reaction every 30min until addition is complete. The reaction rate slows considerably as it progresses, resulting in small quantities of trialkylated material in addition to the desired t4M. This may be separated, or further reacted by addition of more aldehyde and borohydride with extended reaction times. Work up the reaction as described for t2M, by concentration, sedimentation and washing with acetone and water. Isolate the final product and wash with 5mL of ethanol, then air-dry to obtain 480mg of white powder (0.56mmol, yield =69%). The reaction can be followed by RP-HPLC (Fig. 2), though HPLC is not needed for purification.

3. Design and preparation of U-site HHR constructs

3.1 Materials, instrumentation, and general notes for transcription

DNA templates were purchased from integrated DNA technologies (IDT) purified by gel prior to use in transcription. SYBR® gold was purchased from Thermo Fisher Scientific. DNA and RNA stock solutions were prepared in deionized water and concentrations were determined by measuring solution absorbance at 260nm by Thermo Fisher Nanodrop 2000. The promoter sequence in each DNA template is underlined and does not appear in the final RNA transcripts. Buffers were prepared in house with the following composition: 1× PBS (137mM NaCl, 2.7mM KCl and 10mM PO₄³⁻, pH 7.4), Tris-Cl (50mM tris(hydroxymethyl)aminomethane, pH 7.6, adjusted with HCl). RNA transcription buffer (10×) was 1 M HEPES-KOH pH 7.5, 100mM MgCl₂, 20mM Spermidine-HCl, 400mM DTT.

3.1.1 General protocol for transcription—RNAs were prepared via runoff transcription using wild-type T7 RNA polymerase (Milligan & Uhlenbeck, 1989). A general starting point for transcriptions used the following conditions: 1× transcription buffer with an additional 10mM DTT, 10mM MgCl₂, 20mM rNTP, 7.5% glycerol, 350nM DNA template, and 2μL of T7 polymerase stock per 100μL of transcription reaction solution. These conditions require optimization for new template sequences. The transcriptions were run for 2h at 37°C, then quenched with 1.5 eq of EDTA and an equal volume of 2×TBE/urea loading buffer. Samples were heated at 95°C for 5min, cooled to room temperature, and loaded onto acrylamide (5% bisacrylamide) denaturing (8 M urea) gel (8.3cm×7.3cm×1.5mm). The RNA product bands were visualized on the gel by UV shadowing and cut from the gel. Desired RNA can be isolated from the cut band by standard “crush and soak” (gel is forced through a syringe and RNA extracted into water by overnight agitation at RT) or electroelution, which generally gives higher recovery. Extracted or

electroeluted RNA was precipitated by addition of 10% volume of 5 M NH₄OAc pH 5.2 and 2.5 volume equivalents of 200 proof ethanol, and incubation at −20°C for at least 2h. RNA was centrifuged down at high speed for 10min at 4°C and the pellet was washed with 500μL of ice-cold 70% ethanol in water and centrifuged again at high speed for 10min at 4°C. RNA pellets were then dried in a speed-vac at room temperature for 20min and suspended in water and stored at −20°C. After confirming RNA purity by denaturing gel, this pelleted material can be used to prepare stock solutions.

3.1.2 General design considerations and challenges in transcription—

Transcription targets containing extended oligo-U domains can be challenging as these domains are considered “slippery” for the polymerase and can also be read as termination signals (Martin & Tinoco, 1980; von Hippel, 1998; Yarnell & Roberts, 1999), thus leading to less pure transcription reactions with truncations and closely eluting products. This problem is exacerbated when the starting point for transcription is the U-domain. Yield and purity of transcription are greatly improved by the addition of several non-U nucleotides (preferably a GC rich sequence) prior to the U-site. While it is difficult to predict which transcripts will be the most problematic, it is generally true that shorter, internal oligo-U sites give better results than longer sites near start of transcription. If it is unavoidable to have the U-site near the start of the transcript, then addition of a few nts prior can be very helpful. In addition, a broad search of conditions for optimization of transcription can dramatically improve the yield. In some cases, the use of T7 mutants (Lyon & Gopalan, 2018) can improve purity and allow useful quantities of U-domain transcripts to be obtained when it is not possible using wild-type T7, though reaction rates, and sometimes overall yield, are decreased. The transcripts described below were amenable to our standard transcription protocol using wild-type T7, despite extended oligo-U domains.

3.2 Design of modified HHR transcripts with U-sites for allosteric binding

Installation of an allosteric site that is sensitive to triggering by bPNA requires replacement of a structural element with a oligo-U site that is typically less structured. Upon hybridization with a bPNA molecule, the duplex stem is replaced with a triplex stem. This concept appears to be largely backbone independent. In the minimal HHR scaffold, there are three structural stems that support a catalytic site of ~19nts. The sequence identity of the stems is not critical, though there needs to be structure. Full activity of the HHR requires an additional tertiary interaction between loop 2 and stem 1 that creates a more closed HHR structure, and further twists the substrate strand sugar–phosphate backbone into a reactive conformation for cleavage at the active site. This tertiary interaction is a Hoogsteen base pair between U and A (Robertson & Scott, 2007). HHR activity is greatly reduced by stem replacement with oligo-U loops and fully restored by bPNA peptide triplex hybridization. Further, the tertiary interaction between loop and stem may also be ablated by replacement of the loop and stem sequence with oligo-U sequences and restored by binding of oligoethyleneimine bPNAs (t2M, t4M) that bridge the loop and stem domains to restore the tertiary contact. Below, we describe our design methodology for these two systems.

3.3 Design and use of stem/loop replacement HHR sequences

Replacement of stem 2 alone with an oligo-U loop does indeed decrease HHR self-cleavage in the minimal HHR, but significant activity remains at 10mM Mg^{2+} . Replacement of two stems with oligo-U yields U-(2,3) HHR, which is not detectably functional in self-cleavage at high magnesium ion concentration (Fig. 3). This U-mutant was produced by transcription. The DNA template is shown below, with the promoter region underlined and the regions corresponding to stem 2 and 3 regions shown in bold.

3.3.1 Double stem replacement HHR: The U-(2,3) ribozyme sequence—This type I HHR mutant was designed to retain the sequence of the catalytic site while replacing duplex stems 2 and 3 with oligo-U loops (Fig. 3). We replaced all three stems (1,2,3) that support the catalytic domain, but this system did not refold into a catalytically active form by triplex hybridization with bPNA. We speculate that the replacement of all three stems with oligo-U domains may result in misfolding upon triplex hybridization. It may yet be possible to design a triple stem-replaced construct that could fold into a catalytically active form. However, a DNA template for the U-(2,3) HHR can be designed as indicated below. Two different binding sites were prepared, of different sizes. Stem-loop 2 was replaced with 16nt loop, comprised of a U6-GNRA-U6 domain. It was anticipated that the U6-domains would form a triplex stem with bPNA, capped with a stable GNRA (GAGA) loop. Similarly, stem-loop 3 was replaced with a larger 24nt loop, U10-GAGA-U10. Stem 1, formed from the 5' terminus and the domain following the substrate sequence, was left intact. An autonomously folding tRNA-Lys module was fused to the 3' end of stem 1, so that the HHR cleavage reaction would produce a stable, structured RNA leaving group that could be easily detected and stained on gel. The DNA template for T7 polymerase transcription, and the transcription product is shown below, with the stem replacement domains shown in bold and T7 promoter underlined.

DNA template: 5'-TCA CTG TAA AGA GGT GTT GGT TCT CTT AAT CTT
TAA CTT AAA AGG TTA ATG CTA AGT TAG CTT TAC AGT GCG ACA AAA
AAA AAA TCT CAA AAA AAA AAG TTT CGA AAA AAT CTC AAA AAA
CTC ATC AGG CAC TGC CTA TAG TGA GTC GTA TTA ATT TC-3'.

RNA transcript: 5'-GG CAG UGC CUG AUG AGU UUU UUG AGA UUU UUU
CGA AAC UUU UUU UUU UGA GAU UUU UUU UUU GUC GCA CUG UAA
AGC UAA CUU AGC AUU AAC CUU UUA AGU UAA AGA UUA AGA GAA
CCA ACA CCU CUU UAC AGU GA-3'.

3.3.2 Tertiary contacts and stem replacement: S-U4n and S-U4n-2bp HHR—

Stem restructuring has effects at both ends of the stem. In an HHR stem 2 replacement experiment, loss of structure destabilizes the catalytic site at the base of the stem, but also results in loss of a critical tertiary interaction between the loop of stem 2 and stem 1. This tertiary contact is a Hoogsteen AU base pair formed between an A in the loop atop stem 2 and a U in stem 1. This contact is restored upon triplex hybridization with oligoethyleneimine bPNA (t4M), a notion that was tested in the two constructs, S-U4n HHR and S-U4n-2bp HHR. As mentioned previously, a single stem 2 replacement results in significant retention of cleavage activity at 10mM Mg^{2+} concentration; however, minimal

cleavage is seen at 0.1mM Mg^{2+} . The construct S-U4n HHR is a stem replacement mutant of HHR in which the four base pair stem is replaced with two U4 domains, while retaining the native GUGA loop (Fig. 4). This stem replacement is a much smaller perturbation than in.

U-(2,3) HHR, and is designed to accommodate t4M binding. To stabilize the loop, a GC base pair is retained at the base of the loop as a clamp structure. Notably, addition of t4M can restore a significant degree of bond scission at 0.1mM Mg^{2+} , while t4M^{Me}, which has base-triple formation blocked by methylation, has no effect on catalysis. This indicates that t4M base recognition of the U-site rescues HHR function. The S-U4n-2bp HHR was designed to test the extent to which this restructuring affects the known tertiary contact between the GUGA loop of stem 2 and stem 1 by using the identical stem 2 design, but simply increasing the length of stem 1 by 2 base pairs. This 2 bp insert extended the distance between the loop and the uracil in stem 1 that was known to a Hoogsteen base pair with the adenine in the loop of stem 2, and proved to be sufficient to completely shut down HHR cleavage. The ability to tune HHR reactivity through this subtle adjustment remote from the active site attests to the amenability of the HHR scaffold to engineering. The DNA templates and transcript sequences for the S-U4n and S-U4n-2bp HHRs are shown below, with the genetically encoded bPNA binding site shown in bold, tertiary loop-stem Hoogsteen contact shown in double underline, tRNA-Lys sequence italicized and T7 promoter region underlined.

S-U4n HHR. DNA template: 5'-GCC GGG GGT GGG ATT TGA ACC CAC GTA AGG CGG ATC TGC AGT CCG CTG CCT AGC CCC TAG ACT ACC CCG GCT GGT AGA CTG TGA CCC AGT CTC CTG GGT TTC **GAA AAC TCA CGA AAA** CTC ATC AGA CAG TCT ATA GTG AGT CGT ATT A-3'

S-U4n HHR. RNA transcript: 5'-G ACU GUC UGA UGA GUU **UUC GUG** A^{L4}GU *UUU* CGA AAC CCA GGA GAC UGG GUC ACA G]UC U¹⁻⁷AC-CAG CCG GGG UAG UCU AGG GGC UAG GCA GCG GAC UGC AGA UCC GCC UUA CGU GGG UUC AAA UCC CAC CCC CGG C-3'

S-U4n-2bp. DNA template: 5'-GCC GGG GGT GGG ATT TGA ACC CAC GTA AGG CGG ATC TGC AGT CCG CTG CCT AGC CCC TAG ACT ACC CCG GCT GGT AGA GCC TGT GAC CCA GTC TCC TGG GTT TCG **AAA ACT CAC GAA AAC** TCA TCA GAC AGG CTC TAT AGT GAG TCG TAT TA-3'

S-U4n-2bp. RNA transcript: 5'-GAG CCU GUC UGA UGA GUU **UUC GUG** A^{L4}GU *UUU* CGA AAC CCA GGA GAC UGG GUC ACA G]GC UC U¹⁻⁷ AC-CAG CCG GGG UAG UCU AGG GGC UAG GCA GCG GAC UGC AGA UCC GCC UUA CGU GGG UUC AAA UCC CAC CCC CGG C-3'.

3.4 Design and use of U-loop replacement binary HHRs

Transcription of fully functional HHRs is challenging because the transcript will immediately cleave in transcription buffer, which contains 10mM Mg^{2+} that favors bond

scission. Stem replacement HHRs discussed above may be transcribed as a single RNA strand because the loss of secondary structure serves to suppress self-cleavage during the reaction and permit isolation. For loop replacement studies, the duplex stems supporting the catalytic site are intact, and thus a full-length HHR will completely self-cleave prior to isolation. For this reason, loop replacement studies must be carried out on binary HHRs, wherein one strand is an enzyme strand, and the other is a substrate strand. These binary HHRs exhibit significant rate dependence on the sequence identity of the stem 2 loop. However, though a GUGA loop is found in the wild-type HHR, with the adenine base of the loop interacting with uracil base in stem 1, a GUUU loop also functions well in cleavage. This suggests that the HHR loop-stem interaction may be quite plastic, and may accept alternative noncanonical interactions such as a GU wobble pair in addition to the AU Hoogsteen pair. To study the turn on of tertiary interactions with t4M, we transcribed the enzyme strand for a binary HHR in which the GUGA loop of stem 2 was replaced with UUUU. To provide a baseline of reactivity, the HHR enzyme strand with the native GUGA loop was also transcribed. It is convenient to follow this reaction by fluorescently tagged RNA substrates, which were purchased from IDT that are annealed with the enzyme strand (Fig. 5), and unreactive until triggered with Mg^{2+} ions and t4M bPNA. This avoids potential problems of refolding the enzyme and substrate strands together with a large tRNA fusion on the substrate strand. The DNA templates and transcript sequences are shown below, with the promoter sequence underlined and the critical loop shown in bold. The relevant nucleotide modifications in enzyme and substrate strands are shown in double underline.

- *WT HHR enzyme strand* DNA template: 5'-TGG GTT TCG TCC TCA CGG ACT CAT CAG ACA GTC TAT AGT GAG TCG TAT TA-3'
- *WT HHR enzyme strand*: 5'-G ACU GUC UGA UGA GUC **CGU GAG** GAC GAA ACC CA-3'.
- *L-U4 HHR enzyme strand* DNA template: 5'-TGG GTT TCG TCC AAA AGG ACT CAT CAG ACA GTC TAT AGT GAG TCG TAT TA-3'
- *L-U4 HHR enzyme strand*: 5'-G ACU GUC UGA UGA GUC **CUU UUG** GAC GAA ACC CA-3'
- *Substrate strands* (Scissile bond: C-A.)
WT-HHR: 5' Cy3-UGGGUCAC-AGUCUCCAAUCC-3'
2U: 5' Cy3-UGGGUCAC-AGUC**UU**CAAUCC-3'
5U: 5' Cy3-UGGGUCAC-AGUC**UUUUU**-3'

4. Triggering HHR catalysis with bPNAs

4.1 General reaction protocols

4.1.1 Single-stranded HHRs—A stock solution of ribozyme (625nM) and tren derivatives (12.5 μ M) in 1.25 $\times$ Tris–Cl buffer (pH 7.6) was annealed at 75°C. An aqueous $MgCl_2$ solution was added to the annealed solution to initiate the reaction at 37°C. The final concentrations were: 500nM ribozyme, 10 μ M tren derivatives and 10mM Mg^{2+} in 1 $\times$ Tris–Cl buffer (pH 7.6). Aliquots were taken at 0, 10, 45, 90, 150min, quenched with urea-EDTA, and frozen immediately on dry ice. Samples were analyzed on 12% denaturing acrylamide

gel and stained with SYBR® gold. This strategy has been demonstrated with both peptide bPNAs (Xia et al., 2014) and oligoethyleneimine bPNAs (Mao et al., 2017). We focus our protocol description on the oligoethyleneimine scaffold, but the procedures for use with peptide are identical.

4.1.2 Binary HHRs—Samples for single turnover experiments were prepared by making a stock solution with concentrations of the following species: 5 μ M of enzyme strand, 400 nM of the Cy3 labeled substrate strand, and 10 μ M tren derivatives in 1.25 $\times$ Tris–Cl buffer (pH 7.6). The mixture was heated at 75 °C for 2 min and equilibrated at 27 °C for 10 min. MgCl₂ was then added to initiate the reaction at 27 °C. The final concentrations were: 4 μ M enzyme strand, 320 nM Cy3 labeled substrate strand, 10 μ M tren derivatives and 0.1 mM Mg²⁺ in 1 $\times$ Tris–Cl buffer (pH 7.6). Aliquots were taken at 0, 15, 30, 45 s and 1, 2, 3, 5, 15, 30, 60 min, quenched with urea-EDTA, and frozen immediately on dry ice. Samples were analyzed on 20% denaturing acrylamide gel.

4.2 Experimental design and optimizing conditions

4.2.1 Cleavage reactions of single-stranded HHRs—The single-stranded HHR U-mutants such as U-(2,3), S-U4n and S-U4n-2bp HHRs can be transcribed under normal conditions and do not self-cleave appreciably, despite 10 mM MgCl₂ in the reaction. However, their reactivity remains sensitive to Mg²⁺ concentration, and the combination of both t4M and Mg²⁺ is required to trigger RNA cleavage. The presence of the tRNA fusions in these constructs yields two RNAs of comparable size upon cleavage, the HHR itself and the tRNA. These two RNAs may be easily observed by gel (Fig. 4). Addition of the methylated t4M molecule generally does not cause HHR cleavage. This control is essential to rule out the effects of the oligoamine backbone itself; molecules of this type can affect RNA function, but typically at much higher concentrations (Pingoud et al., 1984; Quigley, Teeter, & Rich, 1978). Reactions should be run in triplicate and gels quantified using standard software (Imagequant) to obtain HHR cleavage rates (Fig. 4). Reactivity should be very reproducible for each construct, resulting in relatively tight error bars. If the reaction rate does not allow monitoring by reasonable data time point sampling, the rate may be tuned by decreasing or increasing Mg²⁺ concentration to decrease or increase reaction rate, respectively. Decreasing Mg²⁺ to 0.1 mM enabled convenient monitoring of HHR cleavage in the sU4n HHR as a function of t4M concentration. Typically, cleavage reactions were monitored at a single t4M concentration over time. In addition, rates were measured as a function of increasing t4M concentrations. In this way, it can be clearly established that the t4M molecules can directly influence HHR cleavage.

4.2.2 Cleavage reactions of binary HHRs—Binary HHRs were annealed together prior to use in cleavage reactions as described above. Interestingly, it is possible to mix different substrate strands with each HHR enzyme strand, yielding cleavage rates reflective of their dependence on t4M as well as substrate–enzyme matching. While both loop modified L-GU3 and LU4 HHRs do not effectively cleave the native substrate, L-GU3 can cleave a substrate strand that features U's at both position 1.7 (native) and 1.8 in stem 1, which suggests that the L1 guanine may be forming a tertiary contact with the U 1.8. The

labeled substrate strand provides a convenient way to quantitatively follow the cleavage reaction, signaling unreacted and cleaved substrate with the same dye (Fig. 5).

5. Summary

The synthetic bPNAs described herein can bridge native and artificial architectures, uniting nucleic acid biotechnology (Burnett & Rossi, 2012) with new materials (Roh, Ruiz, Peng, Lee, & Luo, 2011). This protocol provides a general guide on the synthesis of bPNA peptides and oligoethyleneimine derivatives that have been used to trigger cleavage in engineered hammerhead ribozymes. This strategy enables additional control over RNA structure; this facilitates elucidation of critical aspects of RNA recognition that contribute to RNA functions, including catalysis.

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Fig. 1.

(A) Base-triple formation of T (X = CH₃, R = DNA) or U (X = H, R = RNA) and melamine attached to bPNA. (B) Methylated Boc-t2M and t3M structures. Synthetic approaches to the oligoethyleneimine (tren) bPNA scaffold from mono-Boc tren using (C) nucleophilic aromatic substitution to form Boc-t2M and (D) reductive alkylation to form Boc-t4M.

Fig. 2.

Analytical C₁₈RP-HPLC traces of reaction progress for reductive alkylation with melamine acetaldehyde of (A–D) Boc-t2M from Boc-ethylenediamine and (E–H) Boc-t4M from Boc-tren. t2M gradient: 0%B (5min); 0–20%B (5min); 20%B (10min); 20–100%B (5min); 5min B-wash. t4M gradient: 10%B (5min); 10–30%B (10min) 30%B (5min); 30–100%B (2min); 5min B-wash. Early products observed and identified by ESI-MS in (A) are: monoalkylated (15.9min), dialkylated (16.5min), and monoalkylated + imine (18.2min). Early products in Boc-t4M synthesis identified in (B) are: monoalkylated (6.2min), dialkylated (8.8, 10.3min), trialkylated (13.1, 14.1min), tetraalkylated (14.7min), and trialkylated + imine (15.5min).

Fig. 3.

(A) Illustration of U-(2,3) HHR cleavage triggered by stem refolding. The U-domains (red) bind to t4M, inducing cleavage of the scissile sugar phosphate bond (dashed blue line). (B) Results of gel quantification, in triplicate. (C) Representative gel analysis of U-(2,3) HHR cleavage reactions at 10 mM MgCl₂. Top band indicates intact U-(2,3) ribozyme, bottom two bands indicate the cleavage products. (D) Negligible cleavage is observed under the same conditions without bPNAs or with methylated t4M.

Fig. 4. S-U4n (A) and S-U4-2bp (B) HHR cleavage reactions triggered by tren bPNAs at 0.1mM Mg^{2+} , analyzed by gel. Top band indicates intact S-U4n ribozyme, middle band indicates the tRNA cleavage product, bottom band indicates the ribozyme cleavage product. (C and D) S-U4n ribozyme rescued by t4M, under 0.1mM Mg^{2+} conditions at increasing t4M concentration. Much weaker cleavage is seen with (E) methylated t4M control molecule and (F) the S-U4n-2bp ribozyme.

Fig. 5.

(Top) Secondary structure representation of the binary HHRs. Scissile bond shown in red between C17 and A1.1. Modified bases in the loop and stem I shown in red. (Below) Representative gel image for L-U4/5U binary ribozyme cleavage. Top band is the intact Cy3 labeled substrate strand RNA, bottom band indicates the cleavage product, with 10 μ M t4M, under 0.1 mM Mg²⁺ conditions. Aliquots (left to right) were taken at 0, 15, 30, and 45s, and 1, 2, 3, 5, 15, 30, and 60min.