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A Generalizable DNA-Catalyzed Approach to Peptide–Nucleic Acid Conjugation

Chih-Chi Chu, On Yi Wong, and Scott K. Silverman*[a]

We report DNA catalysts (deoxyribozymes) that join tyrosine-containing peptides to RNA and DNA in one step and without requiring protecting groups on either the peptide or the nucleic acid. Our previous efforts towards this goal required tethering the peptide to a DNA anchor oligonucleotide. Here, we established direct in vitro selection for deoxyribozymes that use untethered, free peptide substrates. This approach enables imposition of selection pressure via reduced peptide concentration and leads to preparatively useful lower apparent K_m values of ~100 μM peptide. Use of phosphorimidazolide (Imp) rather than triphosphate as the electrophile enables reactivity of either terminus (5′ or 3′) of both RNA and DNA. Our findings establish a generalizable means of joining unprotected peptide to nucleic acid in one step by using DNA catalysts identified by in vitro selection.

Peptide–nucleic acid conjugates play key roles in many biological processes, including recombination,[1] viral propagation,[2] topoisomerization,[3] and activity of tumor suppressor proteins.[4] Artificial nucleopeptides have also been used in various applications.[5] To achieve site-specific side chain modification of biologically relevant peptide sequences, for which a fragment coupling approach is not feasible, chemical synthesis of these conjugates generally requires detailed and sophisticated approaches that involve extensive use of protecting groups.[6] Therefore, alternative synthetic approaches are valuable when they enable direct one-step conjugation of unprotected nucleic acids to particular side chains of unprotected peptide substrates.

We have initiated efforts to identify deoxyribozymes (DNA enzymes)[7] that catalyze various reactions of peptide substrates,[8] including DNA-catalyzed formation of peptide–nucleic acid conjugates.[9,10] To date, our in vitro selection experiments have used peptides that are covalently tethered to a DNA oligonucleotide anchor, which in turn is Watson–Crick base-paired to the invariant-sequence binding arm of the DNA pool (Figure 1 A). We have identified numerous deoxyribozymes that catalyze formation of peptide–RNA conjugates by reaction of a tyrosine (Tyr) or serine (Ser) hydroxy group with the α-phosphate of a 5′-triphosphorylated RNA oligonucleotide (5′-pppRNA).[9] Inclusion of the DNA anchor attached to the peptide enables a straightforward selection strategy based on

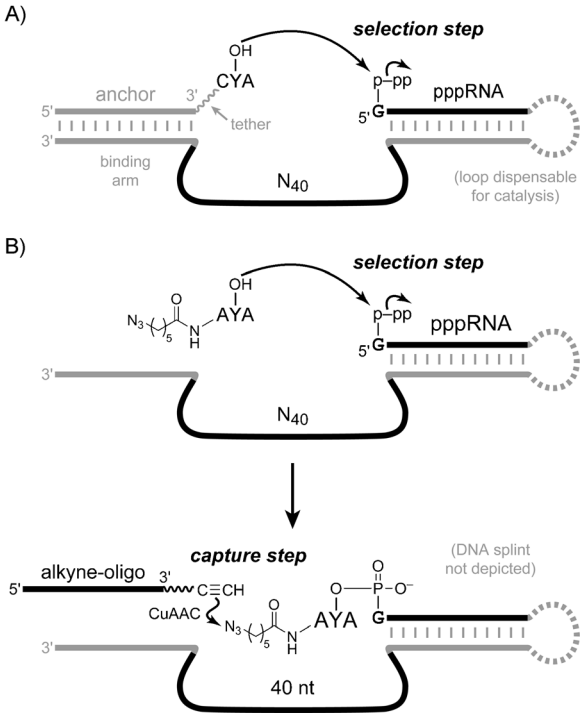

Figure 1. Selection strategies for DNA-catalyzed peptide–RNA conjugation. A) Previously used selection approach in which a DNA-anchored peptide is presented to the 5′-triphosphorylated RNA substrate, with the peptide joined at its remote 3′-terminus to the DNA pool to enable PAGE shift selection. This particular illustration shows a reaction of the disulfide-linked CYA tripeptide.[9c] B) Alternative selection approach established in this study, in which a free (non-anchored) peptide reacts with pppRNA. The peptide includes an N-terminal C_6-tethered azido group. After the DNA-catalyzed selection step, active DNA sequences are captured by DNA-splinted reaction of the azide with a 3′-alkyne oligonucleotide (CuAAC), leading to a substantial PAGE shift that enables their separation. See the Supporting Information for detailed procedures.

polyacrylamide gel electrophoresis (PAGE) shift. Catalytically active DNA sequences grow substantially larger due to the DNA-catalyzed attachment of the RNA strand to the DNA-anchored peptide, noting that the RNA strand is already preattached through its remote 3′-end to the DNA pool via a catalytically dispensable loop. This size increase upon peptide–RNA conjugation enables PAGE-shift separation of the catalytically active DNA sequences.

Despite such success, the resulting deoxyribozymes have several practical disadvantages. The inclusion of the DNA anchor means that the peptide is not presented freely to candidate DNA sequences, and catalytic activity of the resulting deoxyribozymes is generally dependent on the Watson–Crick binding interaction of the anchor oligonucleotide with the pool binding arm. Also, selection pressure for the DNA cata-

[a] C. Chu, O. Wong, Prof. S. K. Silverman
Department of Chemistry, University of Illinois at Urbana-Champaign
600 South Mathews Avenue, Urbana, IL 61801 (USA)
E-mail: scott@scs.illinois.edu

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lysts to function at low peptide concentration cannot be imposed by reducing the peptide concentration during a series of iterated selection rounds, because doing so would forgo the anchoring effect: the deoxyribozymes would be unlikely to bind their unanchored peptide substrates. As a consequence of these considerations, our previously identified deoxyribozymes often do not work at all when provided with the free peptide substrate that is unattached to its DNA anchor (even when the free oligonucleotide is additionally provided), and when they do function in this way, they require very high (greater than millimolar) free peptide concentration. Such failure of catalysis with the free peptide is likely observed because the deoxyribozymes are not required by the selection approach to interact sufficiently well with the peptide moiety itself, given the Watson–Crick binding energy separately provided by the DNA anchor.[11]

In the present work, we sought to establish a different selection approach in which entirely free (not DNA-anchored) peptides are presented to the DNA pool during the key selection step of each round. The immediate difficulty in achieving this goal is that using a free peptide substrate during the selection step results in an insufficient PAGE shift to enable physical separation of the DNA sequences that catalyzed the peptide–nucleic acid conjugation reaction. We therefore developed a new two-stage selection approach (Figure 1B). In each round, for the key selection step, the DNA pool is presented with a free Tyr-containing peptide substrate that incorporates an azido (N_3) group connected to the peptide N-terminus. Catalytically active DNA sequences can attach the Tyr of this azido-peptide to the nucleic acid (e.g., 5′-pppRNA), albeit without yet adding sufficient mass to induce a useful PAGE shift. Nevertheless, the catalytically active DNA sequences become covalently linked to the azido-peptide, due to the presence of the remote RNA–DNA junction (i.e., the azido-peptide is now joined to the RNA, and the RNA was already connected to the DNA). Therefore, in a subsequent "capture" step, the DNA pool is provided with a 3′-alkyne-modified oligonucleotide and Cu^+, such that copper-catalyzed azide–alkyne cycloaddition (CuAAC)[12] increases the mass of only the active DNA sequences by the size of the alkyne–oligonucleotide and thereby enables PAGE shift separation of these active sequences. Importantly, because the reaction step that involves DNA catalysis uses the entirely free peptide, the DNA sequences are directly challenged to interact well with this free peptide, without depending in any way upon Watson–Crick binding energy contributed by an (absent) anchor oligonucleotide. Furthermore, selection pressure for activity at low peptide concentration can be straightforwardly imposed simply by decreasing the free peptide concentration in a series of selection rounds.[13]

To implement the alternative selection approach of Figure 1B, we first performed an initial set of selection experiments to establish viability. These experiments used an Ala-Tyr-Ala (AYA) tripeptide, modified on its N-terminus with an azido group on a short C_6 tether, along with a random N_{40} DNA pool and 5′-pppRNA. Our previous experiments with DNA-catalyzed peptide–RNA conjugation used both Mg^{2+} and Mn^{2+} as catalytic cofactors,[9] whereas several of our unrelated selections have revealed the utility of Zn^{2+} for DNA catalysis.[8,14] Therefore, here we performed two selection experiments, using either all three of Mg^{2+}/Mn^{2+}/Zn^{2+} or only Zn^{2+} (for brevity, hereafter written as Mg/Mn/Zn or Zn, respectively). During each key selection step, the incubation conditions were 70 mM HEPES, pH 7.5, 40 mM $MgCl_2$ and 20 mM $MnCl_2$ (if included), 1 mM $ZnCl_2$, and 150 mM NaCl at 37 °C for 14 h. The free azido-AYA peptide substrate was provided at a concentration of 1 mM in each round. The Mg/Mn/Zn and Zn selections had 11% yield at round 8 and 4% yield at round 10, respectively (see Figure S1 in the Supporting Information for all selection progressions in this report).

Both selections were cloned, leading to individual deoxyribozymes whose catalytic activities were characterized (see Figure S2 for all deoxyribozyme sequences in this report). Altogether, three deoxyribozymes were identified. The Mg/Mn/Zn selection provided a single deoxyribozyme, 8XJ105,[15] that requires both Mn^{2+} and Zn^{2+} for optimal activity (even higher in the additional presence of Mg^{2+}), whereas either Mg^{2+}/Zn^{2+} or Zn^{2+} alone allows trace activity (Figure 2). 8XJ105 had 45% yield using 1 mM peptide, with k_{obs} 0.41 h^{-1} and apparent K_m of >1 mM peptide. The Zn selection led to two sequence-related deoxyribozymes that each require only Zn^{2+}, with lower but reproducible yields of 10–15% with 1 mM peptide (data not shown). Two primary conclusions from this initial set of selection experiments are as follows. First, the alternative selection approach of Figure 1B that uses the free peptide substrate is viable. Second, using different combinations of metal ions for this DNA-catalyzed reaction leads to functionally distinct deoxyribozymes. In particular, we have now found that Zn^{2+} can be a required catalytic cofactor for DNA-catalyzed synthesis of peptide–nucleic acid conjugates, noting that our previous experiments for this purpose used Mg^{2+} and Mn^{2+} but not Zn^{2+}.[9]

We also investigated the Mg/Mn/Zn ion combination with a different azido-peptide substrate, GPYSGN, which corresponds to the N-terminal sequence of the human rhinovirus 14 genome-linked (VPg) protein that is naturally connected at the Tyr to the 5′-end of viral ssRNA.[16] After 11 rounds using 1 mM peptide, the pool yield was 16%. Cloning revealed a single deoxyribozyme sequence, 11EM103, with 60% yield, k_{obs} of 0.34 h^{-1} at a peptide concentration of 1 mM, and apparent K_m value of 168 μM peptide (Figure 2). 11EM103 requires Mg^{2+}, Mn^{2+}, and Zn^{2+} for optimal activity; lesser but still strong activity is observed with Mn^{2+}/Zn^{2+} and Mg^{2+}/Zn^{2+}. Although identified by selection at 1 mM Zn^{2+}, 11EM103 was found to have optimum yield at 0.4 mM Zn^{2+} in the presence of Mg^{2+} and Mn^{2+} (Figure S4). Substantial yield was retained by 11EM103 even at only 10 μM peptide, which is a practical concentration for preparative experiments (see below). Because the round 11 population had already converged on a single sequence, further selection rounds with as little as 10 μM peptide led to the same sequence after cloning (data not shown). Assaying 11EM103 with GPFSGN and GPYAGN instead of GPYSGN showed equivalent reactivity with GPYAGN but no reaction with GPFSGN (Figure S6), consistent with nucleophilic reaction of the tyrosine (but not the adjacent serine) with the pppRNA.

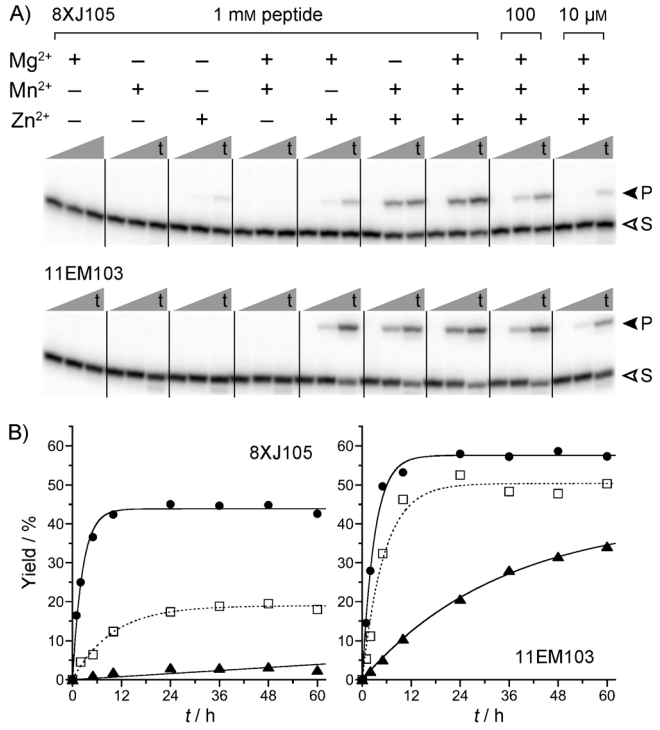

Figure 2. Deoxyribozymes from the initial experiments with the AYA and GPYSGN peptide substrates, to establish the alternative selection approach of Figure 1B. Assay conditions: 20 nM 3′-[^{32}P]-radiolabeled 5′-pppRNA, 0.5 μM deoxyribozyme, azido-AYA peptide as indicated (1 mM, 10 μM, or 100 μM), 50 mM ($-Zn^{2+}$) or 70 mM ($+Zn^{2+}$) HEPES, pH 7.5, divalent metal ions as indicated (chloride salts; concentrations as stated for panel B), and 150 mM NaCl at 37 °C. A) PAGE images for kinetic assays. Representative time points at $t = 30$ s, 2 h, 24 h. S = substrate; P = product. See Figure S3 for quantification of these data. B) Kinetic plots at 1 mM (●), 100 μM (□), and 10 μM (▲) peptide. k_{obs} values for 8XJ105 at 1 mM and 100 μM peptide, respectively: 0.41 and 0.10 h^{-1} (40 mM Mg^{2+}, 20 mM Mn^{2+}, 1 mM Zn^{2+}). The yield for 8XJ105 was optimal at 0.4–1.0 mM Zn^{2+} (Figure S4). k_{obs} values for 11EM103 at 1 mM, 100 μM, and 10 μM peptide, respectively, were 0.34, 0.19, and 0.029 h^{-1} (40 mM Mg^{2+}, 20 mM Mn^{2+}, 0.4 mM Zn^{2+}). The yield for 11EM103 was optimal at 0.4 mM Zn^{2+} (Figure S4). 11EM103 was clearly more active than 8XJ105 at lower peptide concentrations. See Figure S5 for apparent K_m plots.

Until this point, all of the experiments—including those from our past efforts for this type of conjugation reaction[9]—used only 5′-pppRNA as the electrophile for reaction with the Tyr hydroxy nucleophile. More generally, peptide–nucleic acid conjugates of both RNA and DNA are biologically relevant. Each of the peptide–5′-RNA,[2a,b] peptide–5′-DNA,[1,2c,d,3a] and DNA-3′–peptide[1,3b] junctions are formed naturally in various contexts. However, whereas 5′-pppRNA is readily prepared by in vitro transcription by using a double-stranded DNA template and a phage RNA polymerase, there are strict limitations on the 5′-end sequence (purine-rich, and must begin with G or A).[17] Several methods for solid-phase synthesis of 5′-pppRNA and 5′-pppDNA have been described, although their implementation requires access to an oligonucleotide synthesizer.[18] Preparation of 3′-triphosphorylated RNA or DNA is, to our knowledge, unreported. Therefore, deoxyribozymes that specifically require 5′-pppRNA as a substrate for peptide–nucleic acid conjugation have numerous limitations that would be relieved by using a different activated form of RNA or DNA.

Phosphorimidazolide (Imp; Figure 3A) reacts analogously to triphosphate (ppp), but the leaving group, upon nucleophilic attack at the α-phosphorus, is imidazole for Imp rather than pyrophosphate for ppp. Imp is generally more reactive than ppp by approximately 100-fold. We evaluated the use of Imp as an alternative to ppp as the electrophile, noting that either terminus of either RNA or DNA can be activated in solution as Imp from a simple and readily introduced 5′- or 3′-phosphate.[19] 5′-ImpDNA was prepared by reaction of 5′-phosphorylated DNA (5′-pDNA) with EDC and imidazole, followed by a desalting column. Two selection experiments were then performed with 5′-ImpDNA, azido-AYA (1 mM), and either Mg/Mn or Mg/Mn/Zn. The Mg/Mn selection led to 4% yield at round 13. The Mg/Mn/Zn selection led to 20% yield at round 11 and 8% yield at round 14 when the additional rounds were performed with 100 μM peptide. We cloned the three rounds for which yields were stated, and individual deoxyribozymes were characterized.

Selection with Mg/Mn led to five unique deoxyribozymes, each with $\leq$10% yield at 60 h (data not shown); these DNA catalysts were not pursued further. Selection with Mg/Mn/Zn led to six and five unique deoxyribozymes at rounds 11 and 14, respectively; of the latter five, four were the same as a sequence found in round 11, and one was a new sequence. Each of the deoxyribozymes had an optimum yield near 0.4 mM Zn^{2+} (Figure S4). Yields at 10 h ranged from 12 to 45% (Figure 3B); further incubation provided little further increase in yield. Apparent K_m values ranged from 93 μM to $>$1 mM peptide (Figure 3C). Notably, the sole deoxyribozyme that was uniquely identified in the round 14 population, 14EP125, has the lowest apparent K_m value of all of these deoxyribozymes (93 μM). These findings demonstrate that Imp is a viable and more general alternative to ppp as the oligonucleotide electrophile for DNA-catalyzed peptide–nucleic acid conjugation. Moreover, the imposition of selection pressure via decreased peptide concentration successfully led to a deoxyribozyme, 14EP125, that has a low apparent K_m value of ~100 μM for its peptide substrate.

The relatively high reactivity of Imp correlates with modest instability during both preparation and subsequent incubation. This instability is evident in the two closely spaced bands visible for the 5′-ImpDNA reactant in the PAGE images in Figure 3B. Additional assays (Figure S7) revealed that Imp is unstable to several treatments, including both annealing (heating/cooling) as commonly performed before deoxyribozyme assays, and incubation under the assay conditions. When the annealing step was omitted, the final yield increased by ~15%, limited by the instability of Imp under the assay conditions. These considerations should be noted for use of Imp substrates in such experiments.

The nine new deoxyribozymes of Figures 2 and 3 were each evaluated with four other tyrosine-containing peptide substrates (Figure S8). This was done to assess the catalytic activity of each deoxyribozyme with varying peptide sequences that were not used in the selection process itself. The results showed that 8XJ105 is highly tolerant of different amino acids near the reactive tyrosine, including those with potentially nu-

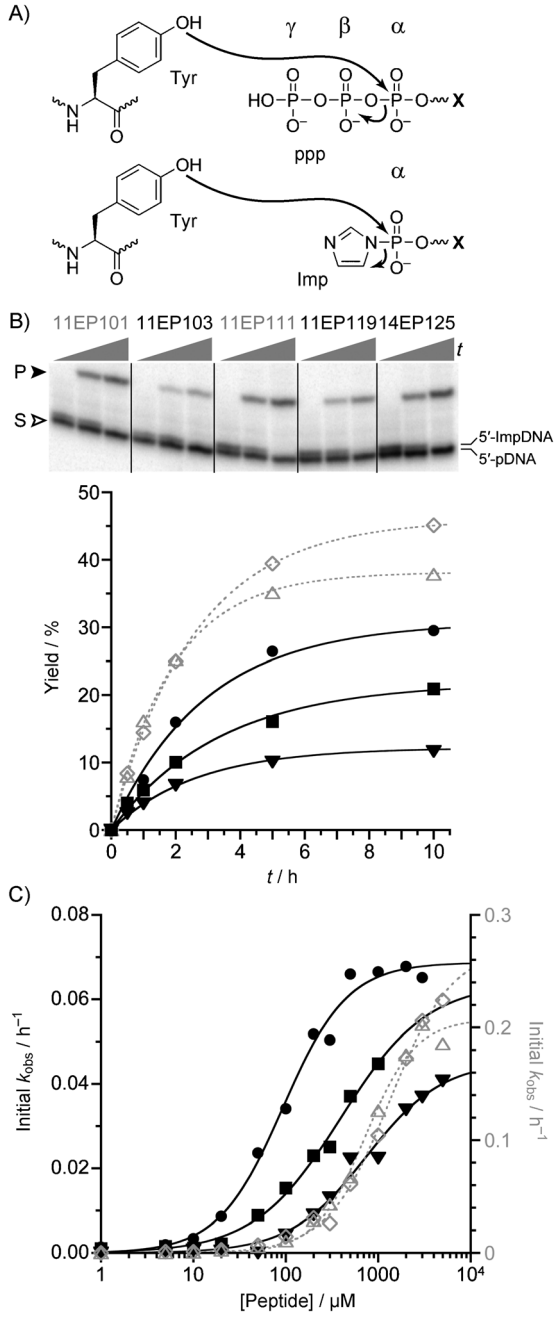

Figure 3. Alternative Imp electrophile, and deoxyribozymes for reaction of Tyr with 5′-ImpDNA. A) Structures of 5′-pppRNA and 5′-ImpDNA electrophiles, and their reactions with a Tyr nucleophile (**X** = RNA or DNA). B) Kinetic assays of deoxyribozymes for reaction of Tyr with 5′-ImpDNA. Assay conditions: 20 nM 3′-[^{32}P]-radiolabeled 5′-ImpDNA, 0.5 μM deoxyribozyme, 1 mM azido-AYA peptide, 70 mM HEPES, pH 7.5, 40 mM $MgCl_2$, 20 mM $MnCl_2$, 0.4 mM $ZnCl_2$, and 150 mM NaCl at 37 °C. t = 30 s, 2 h, 10 h for PAGE images (S = substrate, P = product). The 5′-ImpDNA substrate partially degrades to 5′-pDNA throughout the reaction, partially limiting the yield (see Figure S7 for details). Not shown are data for 11EP104 (very similar to 11EP101) and 11EP126 (very similar to 11EP103). k_{obs} values, top to bottom, in figure legend: 0.53, 0.38, 0.39, 0.30, and 0.34 h^{-1}. C) Determination of peptide apparent K_m values by using initial-rate kinetics. For (B) and (C): △: 11EP101, ▼: 11EP103, ◇: 11EP111, ■: 11EP119, ●: 14EP125. See Figure S5 for quantified data with error bars.

cleophilic side chains (Ser, Thr, Lys, His, Arg, Asp, Glu). 11EP104 is moderately tolerant to changing the nearby amino acids, and the remaining seven deoxyribozymes are much less tolerant or are intolerant. Therefore, the approach described here offers promise for sequence-general DNA catalysts for peptide–nucleic acid conjugation, although some of the deoxyribozymes are specific for particular tyrosine sequence contexts. Such specificity is important for covalently modifying peptide and protein substrates that have more than one tyrosine residue, where discrimination on the basis of sequence context is necessary. We have not yet tested these deoxyribozymes with nucleic acid substrates of varying type (i.e., RNA versus DNA) and sequence.

Finally, the 11EM103 deoxyribozyme was evaluated for larger-scale synthesis of a peptide–nucleic acid conjugate by using more realistic preparative concentrations and amounts of material. The GPYSGN peptide,[20] the 11EM103 deoxyribozyme, and 5′-pppRNA were incubated in 1.0:1.5:2.0 mole ratio in two parallel reactions, with 1 nmol of peptide at either 10 or 20 μM. Both reactions led to substantial yield of the peptide–RNA conjugate after PAGE and extraction (Figure 4). Based on

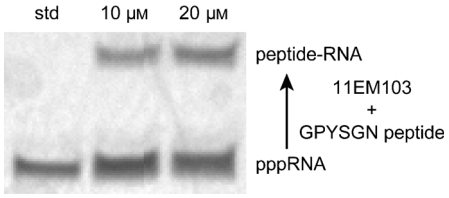

Figure 4. Preparative-scale reaction of the GPYSGN peptide with 5′-pppRNA, catalyzed by the 11EM103 deoxyribozyme. Reaction conditions: 10 or 20 μM azido-GPYSGN peptide, 15 or 30 μM 11EM103 deoxyribozyme, 20 or 40 μM pppRNA, 70 mM HEPES, pH 7.5, 40 mM $MgCl_2$, 20 mM $MnCl_2$, 0.7 mM or 1.0 mM $ZnCl_2$, and 150 mM NaCl at 37 °C for 60 h (100 or 50 μL total volume; 1.0 nmol peptide, 1.5 nmol deoxyribozyme, 2.0 nmol pppRNA). Zn^{2+} concentrations were optimized for each peptide concentration by using smaller-scale assays with 3′-[^{32}P]-radiolabeled 5′-pppRNA, where extraction was unnecessary and yields were 60–70% (Figure S9). The PAGE image was acquired by UV shadowing; the deoxyribozyme itself is evident higher in both reaction lanes (not shown). The respective amounts of peptide–RNA conjugate product and recovered pppRNA were 219 and 734 pmol (10 μM peptide) and 326 and 584 pmol (20 μM peptide). From these values, the efficiency of gel extraction was only 46–48%, indicating that the 22 and 33% yields of peptide–RNA conjugate are about twofold lower than the DNA-catalyzed chemical yields.

the peptide as the limiting reagent, the yields of peptide–RNA conjugate were 22 and 33%, respectively, for 10 and 20 μM peptide, limited in part by ~50% gel extraction efficiency. 11EM103 has apparent K_m value of 168 μM as described above; 10 or 20 μM concentrations of peptide were used because higher concentrations are generally impractical for preparative experiments, especially with longer peptides.

In summary, through a series of in vitro selection experiments, we have shown that free peptide substrates can be used directly to identify deoxyribozymes that create peptide–nucleic acid conjugates at Tyr residues. These deoxyribozymes can use the conventional 5′-ppp electrophile as the conjugation partner, or they can use the more general Imp electro-

phile. An Imp group can be placed readily at either the 5′-end or 3′-end of either RNA or DNA, and any of these activated oligonucleotide substrates can be incorporated readily into our selection approach.[21] Therefore, on the basis of the CuAAC selection design and the use of the Imp electrophile, both of which were established experimentally in this study, we anticipate that deoxyribozymes can be identified to form any desired Tyr–RNA or Tyr–DNA conjugate from the two corresponding unprotected peptide and nucleic acid substrates. It is likely that this approach can be expanded to include Ser (and Thr) as well,[9b] and our success with deoxyribozymes that have phosphatase activity[8] suggests that DNA-catalyzed nucleopeptide formation should be achievable with large proteins. In addition, the general ability to impose selection pressure by decreasing the peptide substrate concentration provides a rational means to seek DNA catalysts that function at low peptide concentrations. Our ongoing work aims to apply this class of deoxyribozymes to prepare and study biologically relevant peptide–nucleic acid conjugates.

Experimental Section

Peptides were prepared by solid-phase synthesis on Fmoc Rink amide MBHA resin as described.[8] To introduce the azido group on the peptide N terminus, the final coupling reaction was performed for 12 h with 6-azidohexanoic acid (1 mmol, 5 equiv; Chem-Impex), diisopropyl carbodiimide (DIC; 126 mg, 1 mmol, 5 equiv; Acros), and HOBt (135 mg, 1 mmol, 5 equiv; Acros) in anhydrous DMF (5 mL; Acros). At the end of the synthesis procedure, ethanedithiol was omitted in the step in which the peptide was cleaved from the resin, because no peptide used here included cysteine.

DNA oligonucleotides prepared by solid-phase synthesis were obtained from Integrated DNA Technologies (Coralville, IA) or synthesized on an ABI 394 instrument by using reagents from Glen Research. 5′-pppRNA oligonucleotides were prepared by in vitro transcription by using synthetic DNA templates and T7 RNA polymerase.[17a] All oligonucleotides were purified by denaturing PAGE with 1×TBE running buffer (89 mM each Tris and boric acid and 2 mM EDTA, pH 8.3), as described previously.[23] 5′-ImpDNA oligonucleotides were prepared by treatment of 5′-pDNA with EDC and imidazole followed by desalting, as described in the Supporting Information.

The in vitro selection and cloning procedures were performed essentially as described previously in related efforts,[9a,c] with full procedures given in the Supporting Information. Individual deoxyribozymes were prepared by solid-phase synthesis and assayed under single-turnover conditions by using 3′-[^{32}P]-radiolabeled substrates. For 5′-pppRNA, radiolabeling was achieved starting from the unlabeled RNA by using 5′-[^{32}P]pCp and T4 RNA ligase.[24] For 5′-ImpDNA, the unlabeled DNA was 3′-[^{32}P]-radiolabeled by using α-[^{32}P]dCTP and terminal deoxytransferase followed by heat inactivation, 5′-phosphorylation by using T4 polynucleotide kinase and ATP, PAGE purification, and finally formation of Imp by treatment with EDC and imidazole followed by desalting (see full procedure in the Supporting Information).

The general single-turnover assay procedure for each deoxyribozyme (Figures 2 and 3) was as follows. A sample (10 μL) containing 3′-[^{32}P]-radiolabeled 5′-pppRNA or 5′-ImpDNA substrate (0.4 pmol) and deoxyribozyme (10 pmol) was annealed in 5 mM HEPES (pH 7.5), 15 mM NaCl, and 0.1 mM EDTA by being heated at 95 °C for 1 min and cooled on ice for 5 min (for 8XJ105, 20 pmol of a short oligonucleotide complementary to the 3′-binding arm was additionally included). The peptide–nucleic acid conjugation reaction was initiated by bringing the sample to a total volume of 20 μL containing 50 mM ($-Zn^{2+}$) or 70 mM ($+Zn^{2+}$) HEPES, pH 7.5, 1–5000 μM azido-peptide, Mg^{2+}/Mn^{2+}/Zn^{2+} (chloride salts) as appropriate, and 150 mM NaCl. The sample was incubated at 37 °C, and aliquots (2 μL) were quenched at appropriate times with 5 μL of stop solution (80% formamide, 1×TBE, 50 mM EDTA, and 0.025% each bromophenol blue and xylene cyanol). For 8XJ105, a decoy oligonucleotide (complementary to the 40 nt deoxyribozyme catalytic region, along with 10 nt of binding arms on either side; 10 pmol) was added to each aliquot before PAGE to displace the deoxyribozyme from the substrate and product. Samples were separated by 20% PAGE and quantified with a PhosphorImager. k_{obs} values were obtained by fitting the complete yield versus time data directly to first-order kinetics. Alternatively, for initial-rate kinetics as used for all apparent K_m plots, data for the initial linear portion (up to 1 or 2 h) was fitted to a straight line.

The preparative-scale experiment on the 11EM103 deoxyribozyme (Figure 4) was performed as follows. For 10 or 20 μM peptide, a sample (50 or 25 μL) containing 11EM103 (1.5 nmol) and 5′-pppRNA (2 nmol) was annealed in 5 mM HEPES (pH 7.5), 15 mM NaCl, and 0.1 mM EDTA by being heated at 95 °C for 1 min and cooled on ice for 5 min. The reaction was initiated by bringing the sample to a total volume of 50 or 100 μL containing 70 mM HEPES (pH 7.5), 1 nmol azido-GPYSGN, 40 mM $MgCl_2$, 20 mM $MnCl_2$, 0.7 or 1 mM $ZnCl_2$, and 150 mM NaCl. The sample was incubated at 37 °C for 60 h, precipitated with ethanol, and purified by 20% PAGE. The product and recovered substrate were quantified after gel extraction by UV absorbance (A_{260}). The product was analyzed by mass spectrometry on a Bruker UltrafleXtreme MALDI-TOF mass spectrometer with matrix 3-hydroxypicolinic acid in positive ion mode at the UIUC School of Chemical Sciences Mass Spectrometry Laboratory (instrument purchased with support from NIH grant S10RR027109A). $[M+H]^+$ calcd 6268.7, found: 6272.3 (10 μM peptide) and 6268.2 (20 μM peptide), $\Delta = +0.06\%$ and -0.008%.

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Keywords: deoxyribozymes · DNA · in vitro selection · peptide–nucleic acid conjugate

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[11] Using a DNA "anchor" oligonucleotide that lacks base pairing to the pool binding arm but still enables direct PAGE-shift selection is not a viable strategy for two reasons. First, as a practical concern, the DNA–peptide conjugates are prepared on a nanomolar scale, and prohibitive amounts of these conjugates would be required to perform selections that use millimolar concentrations; in contrast, with a base-paired anchor sequence, only tractable micromolar concentrations are required. Second, as a conceptual consideration, we ultimately do not wish to require artificial attachment of a DNA oligonucleotide to the peptide substrate in order to achieve modification of the peptide side chain.

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[21] For 3′-Imp substrates, the loop in Figure 1 that enables the selection process can be switched from the right side to the left side, as we have already achieved in unrelated efforts.[8, 22]

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