

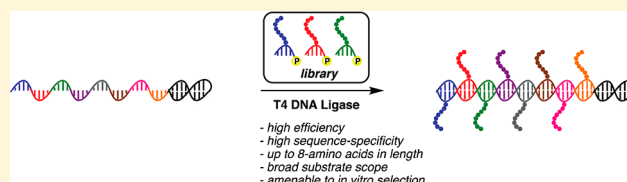
Sequence-Defined Scaffolding of Peptides on Nucleic Acid Polymers

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S Supporting Information

ABSTRACT: We have developed a method for the T4 DNA ligase-catalyzed DNA-templated polymerization of 5'-phosphorylated pentanucleotides containing peptide fragments. The polymerization proceeds sequence-specifically to generate DNA-scaffolded peptides in excellent yields. The method has been shown to tolerate peptides ranging from two to eight amino acids in length with a wide variety of functionality. We validated the capabilities of this system in a mock selection for the enrichment of a His-tagged DNA-scaffolded peptide phenotype from a library, which exhibited a 190-fold enrichment after one round of selection. This strategy demonstrates a promising new approach to allowing the generation and *in vitro* selection of high-affinity reagents based upon single-stranded DNA scaffolding of peptide fragments.



■ INTRODUCTION

Nature uses multivalency, the sum of low-affinity molecular interactions, to achieve specific and high-affinity molecular recognition.¹ Chemists have strived to apply this concept to the development of novel high-affinity reagents to address critical needs in biomedical research.^{2–4} Not surprisingly, the molecular scaffold is crucial for achieving precise multivalent display of ligands. Thus, the ability of nucleic acids to predictably and reproducibly form tertiary structures makes them an excellent scaffold for displaying ligands in a rigid and predefined spatial configuration.^{5–8} Indeed, DNA has been used to scaffold known ligands to generate multivalent high-affinity reagents with properties that rival those of traditional antibodies.^{9–14} Multivalent display of bioactive oligomers, such as peptides and glycans, has proven to be particularly effective; however, current approaches require existing knowledge of ligand binding and typically depend on homomultivalency. To fully realize the potential of DNA scaffolding for the development of novel high-affinity reagents, technologies that allow the sequence-defined display of a library of oligomeric ligands along a library of single-stranded DNA (ssDNA) at high densities are needed. This would enable the directed evolution of both the ssDNA scaffold architecture and the identity of the displayed oligomers to occur concomitantly to optimize recognition of molecular targets.

Inspired by the antigen-binding loops within the complementarity-determining regions of immunoglobulins,¹⁵ we hypothesized that single-stranded nucleic acid polymers decorated in a sequence-defined manner with short peptide fragments could mimic the surface of proteins and serve as a new class of high-affinity reagents (Figure 1a). The single-stranded nucleic acid component would function as a core scaffold to display multiple unique peptides, generating the heteromultivalency akin to hot spots presented at protein–protein interfaces.¹⁶ The T4 DNA ligase-catalyzed DNA-templated polymerization of 5'-phosphorylated trinucleotides provides a platform for the incorporation of multiple small ligands throughout a ssDNA polymer.¹⁷

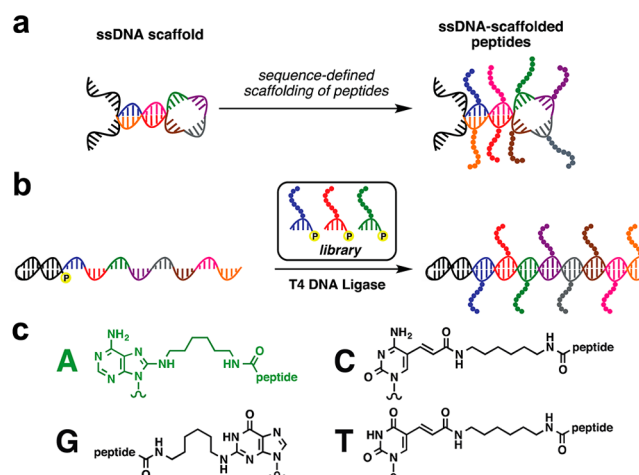


Figure 1. Generation of DNA-scaffolded peptides. (a) ssDNA-scaffolded peptides. (b) Sequence-defined T4 DNA ligase-catalyzed DNA-templated polymerization of peptide-modified oligonucleotides toward the synthesis of ssDNA-scaffolded peptides. (c) Peptide modification via commercial amino-modified nucleobases.

We reasoned that this approach could be optimized for the polymerization of 5'-phosphorylated oligonucleotides containing peptide fragments to readily assemble the desired DNA-scaffolded peptides (Figure 1b).

Herein, we report the development of a T4 DNA ligase-catalyzed DNA-templated polymerization of peptide-modified pentanucleotides that allows the sequence-defined scaffolding of peptide fragments on nucleic acid polymers. We examined and optimized this approach to achieve high-fidelity polymerizations with excellent efficiencies and peptide substrate scope. In addition, we validate the capabilities of the developed polymer-

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ization system by integrating it within a mock *in vitro* selection of functional DNA-scaffolded peptides. The findings from this work advance the field of DNA-templated polymerization and lay the foundation for the evolution of synthetic polymers for molecular recognition and catalysis that are based upon the heteromultivalent scaffolding of multiple unique peptide fragments on ssDNA.

RESULTS AND DISCUSSION

Polymerization Optimization for Dipeptide-Modified Pentanucleotides. We explored the use of peptide-modified pentanucleotides as minimal building blocks for T4 DNA ligase-mediated templated polymerization. We speculated that this oligonucleotide length would provide advantages over shorter codon lengths, including access to longer peptide modifications and a larger codon set, while still providing the desired density of scaffolding and hybridization specificity observed with shorter oligonucleotides.¹⁸ First, we characterized and optimized the ability of T4 DNA ligase to polymerize the 5'-phosphorylated ACTCT pentanucleotide modified with the Ac-Phe-Gly dipeptide at the first nucleobase position via an amino-modified dA (Figure 1c). The pentanucleotide building blocks were readily synthesized using automated oligonucleotide synthesis with commercially available amine-modified nucleoside phosphoramidites and 5'-phosphorylation reagents. The amine groups were used to install the dipeptide using well-established amide bond-forming chemistry.¹⁹ The desired dipeptide-modified 5'-phosphorylated pentanucleotides were furnished in high yield following purification by reverse-phase high-pressure liquid chromatography (HPLC).

An initial optimization screen for polymerization of the dipeptide building block was performed along a DNA template comprising a 5'-phosphorylated hairpin as the extension site followed by eight consecutive repeats of the corresponding pentanucleotide codon (Figure 2). Templates contained an

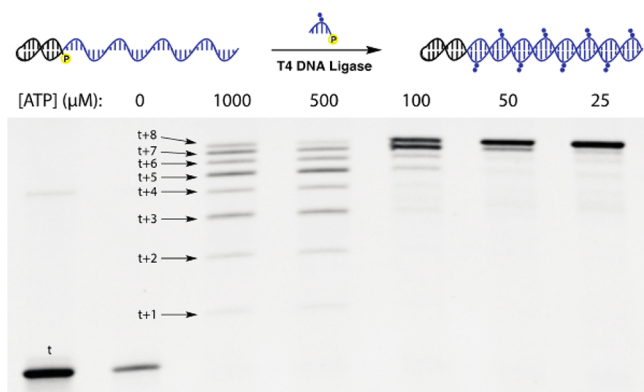


Figure 2. Optimization of ATP concentration for the T4 DNA ligase-mediated polymerization of Ac-Phe-Gly-modified pentanucleotide 5'-P-ACTCT. Conditions: 1 μ M template, 4 μ M/codon modified 5'-phosphorylated pentanucleotide, 20 units/ μ L T4 DNA ligase, 10% PEG 6000, and variable ATP for 24 h at 25 $^{\circ}$ C. t denotes template.

additional 3'-end nucleotide to preclude undesired blunt-end ligation. The initial polymerization conditions used were adapted from the trinucleotide polymerization system,¹⁷ which contained 10% PEG 6000 as a molecular crowding reagent²⁰ and 1 mM ATP for 24 h at 25 $^{\circ}$ C. These conditions resulted in incomplete polymerization, which was evidenced by a ladder of eight bands up to full-length product seen by denaturing polyacrylamide gel electrophoresis (PAGE) analysis (Figure 2, 1000 μ M ATP).

We hypothesized that T4 DNA ligase was inefficient at catalyzing the formation of the phosphodiester bond between the 5'-adenylated template and the 3'-hydroxyl group of transiently hybridized peptide-modified pentanucleotides. Under such conditions, the ligase would eventually dissociate from the template and readenylate itself in solution. Because adenylated ligase catalyzes adenylation of the 5'-phosphorylated template, while the unadenylated ligase catalyzes phosphodiester bond formation,²¹ we reasoned that the standard high concentration of ATP was inhibiting the polymerization by shifting the equilibrium of the reaction to a mixture of 5'-adenylated template and adenylated ligase, effectively shutting down the polymerization. Indeed, when the ATP concentration was decreased to 25 μ M, polymerization of the dipeptide-modified pentanucleotide proceeded smoothly to full-length product (Figure 2).

We next performed a positional scan for the modification site on the pentanucleotide building block. A series of pentanucleotides were synthesized with the Ac-Phe-Gly dipeptide at one of the five possible positions via an amino-modified dA nucleotide (Figure 3). PAGE analysis of the polymerization revealed that all

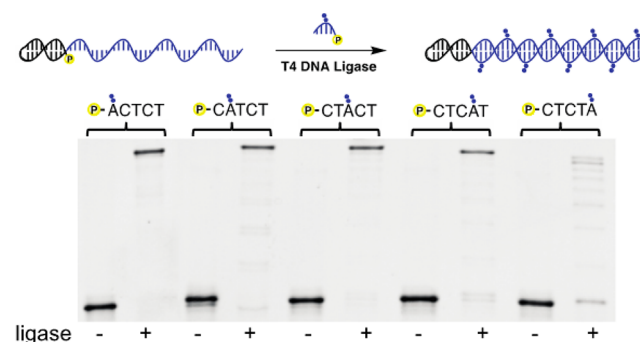


Figure 3. Positional scan of the modification site along the pentanucleotide. The dipeptide used was Ac-Phe-Gly, which was conjugated to the pentanucleotide via a C6 amino dA modifier.

modified positions except position 5 were tolerated by T4 DNA ligase; modification at position 5 resulted in extensive laddering up to full-length product. We also studied the nucleobase dependence at position 1 when it was modified with Ac-Phe-Gly via amino-modified dA, dT, dC, or dG (see Figure S1 of the Supporting Information). Surprisingly, only peptide modification of dA at position 1 was tolerated; however, a more thorough nucleobase screen across all positions of the pentanucleotide is required to fully understand the substrate tolerance of T4 DNA ligase.

Polymerization of Pentanucleotides with Longer Peptide Modifications. Building from the optimization studies described above, we sought to expand the scope of the polymerization process to include longer peptides. 5'-Phosphorylated pentanucleotide building block ACTCT was modified via an amino-modified dA with peptides ranging from two to eight amino acids in length and polymerized along a homo-octameric template containing a 5'-phosphorylated hairpin (Figure 4). We observed a steady decline in polymerization efficiency as peptide length increased, suggesting that T4 DNA ligase was sensitive to the size of the modification and that further optimization was required. We hypothesized that different template architectures, especially those that allow simultaneous extension from both a 3'-primer and a 5'-primer, might enable more efficient ligase-catalyzed polymerization. We chose to explore different template

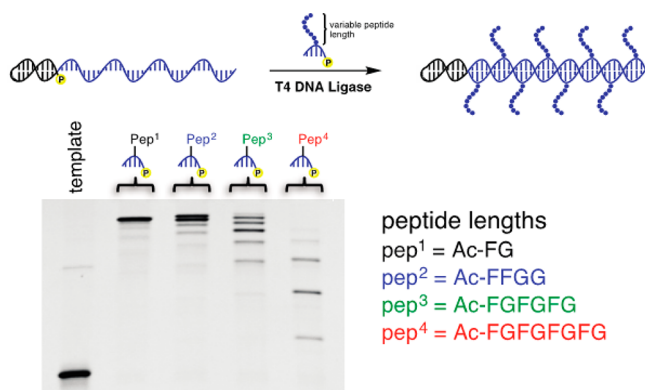


Figure 4. Denaturing PAGE analysis of polymerizations of pentanucleotides containing peptide modifications of increasing length along a 5'-hairpin DNA template.

architectures using the most challenging substrate, the octapeptide-modified pentanucleotide (Figure 5).

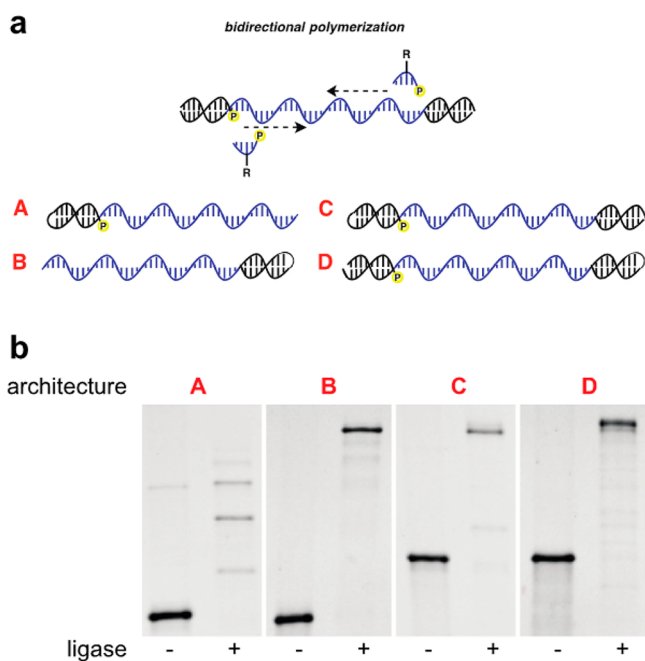


Figure 5. Influence of template architecture on the polymerization of pentanucleotides modified with Ac-FGFGFGFG. (a) Examined template architectures. (b) Denaturing PAGE analysis for a 24 h polymerization of octapeptide-modified pentanucleotide on different template architectures.

When comparing between polymerizations of the octapeptide-modified pentanucleotide along templates containing either the 3'-hairpin or 5'-hairpin architectures, we saw a dramatic change in efficiency (Figure 5b). Polymerizations that extended from the 3'-hairpin proceeded with excellent conversion into full-length product, while extensions from the 5'-hairpin resulted in a laddering of up to four pentanucleotide incorporations. Polymerizations on template architectures that contained both a hairpin and a primer-binding site for bidirectional extension proceeded efficiently regardless of the location of the hairpin. Importantly, 3'-hairpin templates allow displacement and display of modified single-stranded nucleic acids used during *in vitro* selections for molecular recognition.^{9,11,22}

We next challenged the polymerization system with octapeptides containing a diverse set of amino acids, including hydrophobic, hydrophilic, and charged residues (Figure 6). The

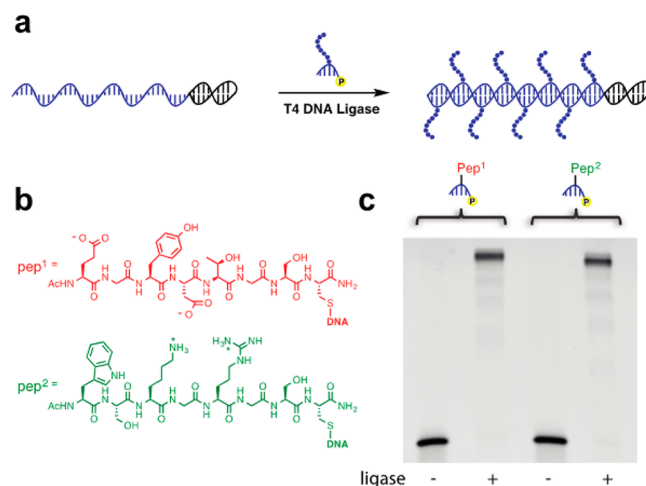


Figure 6. Scaffolding of densely functionalized peptides on DNA. (a) Optimized 3'-hairpin polymerization strategy. (b) Peptides used for polymerization. (c) Denaturing PAGE analysis of a 24 h polymerization.

cationic peptide Ac-WSKGRGSC and the anionic peptide Ac-EGYDTGSC were separately conjugated to the 5'-phosphorylated pentanucleotide ACTCT (Figure 6b). The conjugation was achieved by coupling the amino group of the C6 amino dA to the C-terminal cysteine residue of the peptide via a succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) linker. Polymerizations were performed on the corresponding 3'-hairpin templates using the optimized reaction conditions for 24 h (Figure 5a). Highly efficient polymerization into full-length product was observed for both peptide-containing substrates (Figure 5c). These data suggest that the developed polymerization system can accommodate a broad scope of peptide modifications.

Sequence Specificity of Polymerization. To evaluate the sequence specificity of peptide-modified pentanucleotide incorporation along a 3'-hairpin template, we used a polymerization inhibitor strategy. Because T4 DNA ligase requires 5'-phosphorylated pentanucleotides for the polymerization, the addition of pentanucleotides that lack a 5'-phosphate should inhibit polymerization and result in truncation products. If T4 DNA ligase is highly specific, then polymerization should terminate at the codon that specifies the nonphosphorylated pentanucleotide. If sequence specificity is poor, then polymerization should generate polymers of undesired lengths either by the misincorporation of a nonterminator substrate opposite the terminator codon or by nonspecific inhibition of polymerization by terminators at nonterminator codons.

We used a heterotetrameric template containing four codons with a GC content ranging from 0 to 40% and a 3'-hairpin as the extension site (Figure 7). Three 5'-phosphorylated pentanucleotides linked to the Ac-Phe-Gly dipeptide and one peptide-modified pentanucleotide lacking a 5'-phosphate were polymerized along the template, and the reaction products were analyzed by denaturing PAGE. One product at the anticipated molecular weight was observed for each terminator reaction, indicating that incorporation of all four peptide-modified pentanucleotides proceeds with a high degree of specificity. It is important to highlight the fact that nonphosphorylated

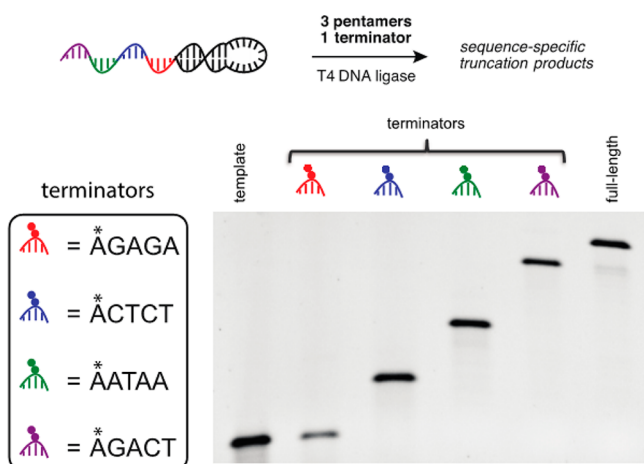


Figure 7. Sequence specificity of T4 DNA ligase-mediated templated polymerization of dipeptide-modified pentanucleotides. Asterisks designate sites of peptide modification.

pentanucleotides can only inhibit polymerization, as they cannot be incorporated into the polymer; despite a 24 h incubation, and a 4-fold excess of phosphorylated pentanucleotides, read-through of the termination codon was not observed.

In Vitro Selection of a Hexahistidine Phenotype from a Library of DNA-Scaffolded Peptides. Encouraged by the

efficiency and fidelity of the T4 DNA ligase-catalyzed DNA-templated polymerization of peptide-modified pentanucleotides, we sought to test its performance within the context of an *in vitro* selection cycle to enrich a known binder from a library of DNA-scaffolded peptides. Because the presence of the peptide fragments could potentially interfere with the polymerase chain reaction (PCR) amplification, we adapted a DNA display selection approach,^{9,11,22} which obviates the need for amplifying modified DNA by covalently linking genotype to phenotype (Figure 8a). As a model selection, we chose a positive control genotype that encoded a DNA-scaffolded peptide phenotype containing one instance of a hexahistidine peptide, which would allow it to survive a selection pressure based upon binding to Co^{2+} magnetic particles. For the selection system, we used the four pentanucleotide sequences that we previously demonstrated to be highly specific in the polymerization process and modified them with four different peptide fragments, including the hexahistidine tag (Figure 8b). We designed a DNA template (POS) comprising a 3'-hairpin as the extension site followed by seven codons that encoded pentanucleotides 2–4, and an eighth codon that encoded hexahistidine pentanucleotide 1; the reading frame was followed by a primer-binding site. A library of DNA templated (LIB) was also prepared, whereby the reading frame comprised only codons that encode pentanucleotides 2–4. The POS template had a unique *Xba*I digest site, so that its enrichment versus LIB could be monitored by restriction digest and PAGE

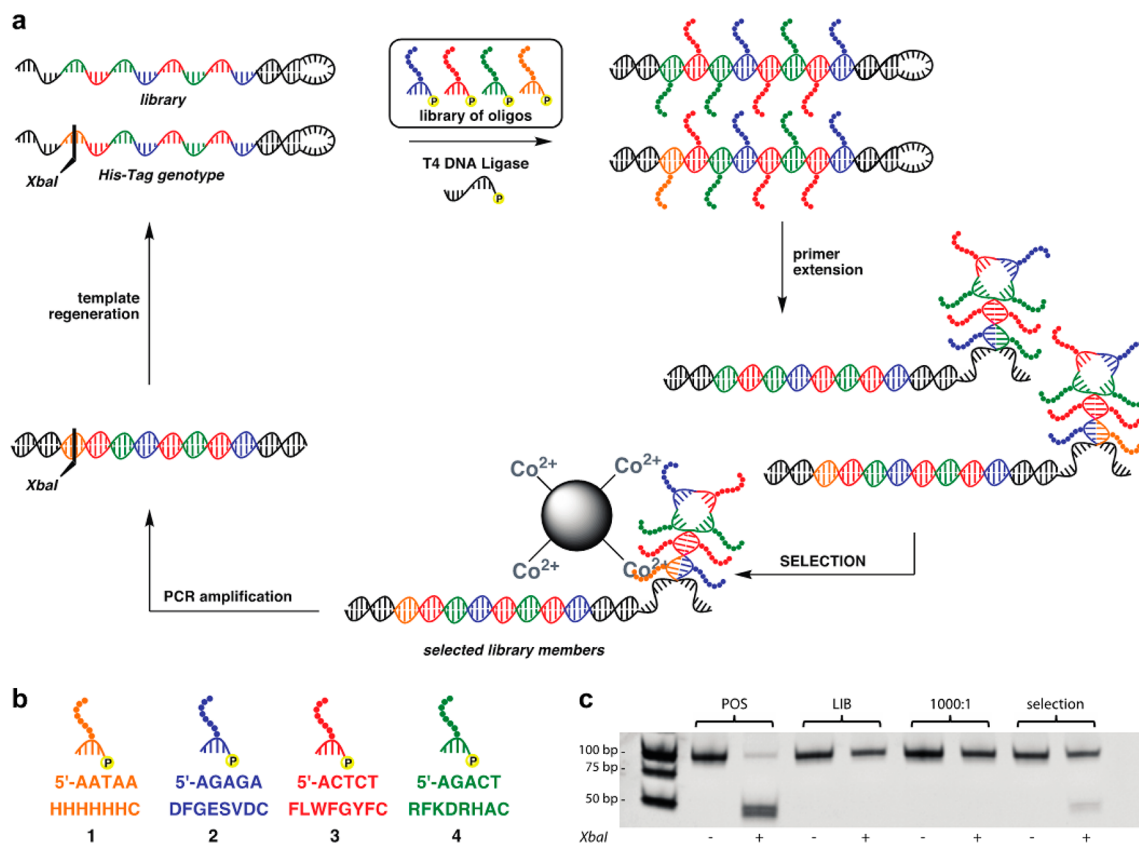


Figure 8. *In vitro* selection of DNA-scaffolded peptides for binding to Co^{2+} beads. (a) Complete cycle of *in vitro* selection of DNA-scaffolded peptides. The His-tagged genotype (POS) is diluted 1000-fold into library (LIB). The library is then subjected to polymerization, primer extension-mediated strand displacement by *Bst* polymerase, selection against Co^{2+} magnetic particles, polymerase chain reaction amplification, and digestion by the *Xba*I restriction enzyme. (b) Pentanucleotides used in the selection. The modification site is at the 5'-dA position. Conjugation of peptide occurs via a SMCC linker between the C6 amino dA nucleotide and the C-terminal cysteine residue of the peptide. (c) Nondenaturing PAGE analysis of *Xba*I digestion products after one round of selection, resulting in 190-fold enrichment of the genotype encoding the hexahistidine-tagged phenotype.

analysis. The POS template was diluted 1000-fold into LIB and subjected to one round of polymerization, primer extension, and selection. The surviving genotypes were amplified by PCR and subjected to digestion by *Xba*I, and the digestion results were analyzed by nondenaturing PAGE. Over the one round of selection, the POS genotype was enriched 190-fold, demonstrating the capability of the system to support the *in vitro* selection of DNA-scaffolded peptides against molecular targets.

CONCLUSIONS

In summary, we have developed a new approach for the generation of sequence-defined DNA-scaffolded peptides that uses T4 DNA ligase to catalyze the DNA-templated polymerization of peptide-modified 5'-phosphorylated pentanucleotides. Optimization of ATP concentration, template architecture, and peptide attachment to the pentanucleotide allowed efficient polymerization of these challenging substrates. Peptides ranging from two to eight amino acids in length with a wide variety of functionality were found to be within the scope of this method. A four-codon library with GC content ranging from 0 to 40% was used to demonstrate the high sequence specificity of the polymerization process. We anticipate expanding the codon set for this process to accommodate DNA scaffolding of a larger set of peptides. The ability to sequence-specifically incorporate multiple peptide fragments throughout an evolvable ssDNA polymer should allow the *in vitro* selection of ssDNA-scaffolded peptides that harness the power of heteromultivalency for molecular recognition of protein targets.

EXPERIMENTAL SECTION

Materials and General Procedures. Unless otherwise noted, all materials and compounds were prepared using commercially available reagents and used without further purification. Water was purified with a Milli-Q purification system. DNA pentanucleotides were synthesized on a Bioautomation Mermade 12 synthesizer. Peptides were purchased from Genscript. DNA oligonucleotides more than five nucleotides in length were purchased from Integrated DNA Technologies. All materials and reagents used for oligonucleotide synthesis were purchased from Glen Research. All oligonucleotides were synthesized and deprotected according to the manufacturer's protocols. Oligonucleotides were purified by reverse-phase high-pressure liquid chromatography (HPLC, Agilent 1260) using a C18 stationary phase (Eclipse-XDB C18, 5 μ m, 9.4 mm $\times$ 200 mm) and an acetonitrile/100 mM triethylammonium acetate gradient. Oligonucleotide concentrations were determined by UV spectroscopy using a Nanodrop ND2000 spectrophotometer. Noncommercial oligonucleotides were characterized by LC/ESI-MS using a C18 column at ambient temperature with a mobile phase of 100% 6 mM triethylammonium carbonate (TEAB) to 80% MeOH and 20% TEAB over 10 min, and a flow rate of 0.3 mL/min. Oligonucleotides >70 nucleotides in length were analyzed by denaturing PAGE.

Synthesis of Amino-Modified Pentanucleotides. Pentanucleotides were synthesized on a Mermaid 12 DNA synthesizer using a DMT-ON protocol on a 1 μ mol scale (1000 Å CPG column). Amine-modifier C6 dA (Glen Research catalog no. 10-1089), Amino-modifier C6 dC (Glen Research catalog no. 10-1019), N2-Amino-modifier C6 dG (Glen Research catalog no. 10-1529), Amino-modifier C6 dT (Glen Research catalog no. 10-1039), and Chemical Phosphorylation Reagent II (Glen Research catalog no. 10-1901) were incorporated as specified by the manufacturer.

Following synthesis, the oligonucleotide was cleaved from the resin by incubation at 65 °C in 500 μ L of a 1:1 mixture of ammonium hydroxide (30%) and methylamine (40%) for 15 min. The cleaved resin was removed by filtration, and the oligonucleotide was concentrated under reduced pressure using a speedvac. The residue was then taken up into 100 μ L of H₂O and purified using reverse-phase HPLC purification using

a 10% acetonitrile in 0.1 M TEAA (pH 7) to 80% acetonitrile in 0.1 M TEAA (pH 7) solvent gradient with a column temperature of 45 °C. The purified oligonucleotide was then incubated at room temperature in 500 μ L of 80% aqueous acetic acid for 1 h to cleave the DMT group, frozen, and lyophilized. The oligonucleotide was incubated in 500 μ L of 30% ammonium hydroxide at room temperature for 15 min to cleave the CPRII linker. Following deprotection, the oligonucleotide was concentrated under reduced pressure using a speedvac. The dried product was dissolved in 100 μ L of H₂O and subjected to reverse-phase HPLC purification using a 10% acetonitrile in 0.1 M TEAA (pH 7) to 80% acetonitrile in 0.1 M TEAA (pH 7) solvent gradient with a column temperature of 45 °C. The purified oligonucleotide was dissolved in water.

Synthesis of Pentanucleotides Modified with Carboxy-Terminal Peptides. To 215 μ L of DMSO were added 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC, free-base form) (1200 nmol in 12 μ L of water), sNHS (3333 nmol in 10 μ L of a 2/1 DMSO/water mixture), and peptide (1250 nmol in 12.5 μ L of DMSO), and the reaction mixture was incubated at room temperature for 45 min. After the initial incubation were added 5'-phosphorylated amino-modified pentanucleotide (25 nmol in 14.5 μ L of water) and 50 μ L of 500 mM NEt₃-HCl (pH 10), and the reaction mixture was incubated at room temperature overnight. The reaction was then quenched with 50 μ L of 500 mM Tris-HCl (pH 8) and the mixture incubated at room temperature for 1 h. The mixture was frozen and lyophilized to dryness. The crude product was dissolved in 100 μ L of water and then subjected to reverse-phase HPLC purification using a 10% acetonitrile in 0.1 M TEAA (pH 7) to 80% acetonitrile in 0.1 M TEAA (pH 7) solvent gradient with a column temperature of 45 °C. The average isolated yield of the product is 30%.

Functionalization of Amino-Modified Pentanucleotides with C-Terminal Cysteine Peptides. To 237 μ L of DMSO were added amino-modified pentanucleotides (25 nmol in 14.5 μ L of water), a SMCC solution (1250 nmol in 12.5 μ L of DMSO), and 50 μ L of 500 mM NEt₃-HCl (pH 10). The reaction mixture was incubated at room temperature for 30 min. The reaction was then quenched with 50 μ L of 500 mM Tris-HCl (pH 8) and the mixture incubated at room temperature for 1 h. The mixture was frozen and lyophilized to dryness. The crude product was dissolved in 100 μ L of water and then subjected to reverse-phase HPLC purification using a 10% acetonitrile in 0.1 M TEAA (pH 7) to 80% acetonitrile in 0.1 M TEAA (pH 7) solvent gradient with a column temperature of 45 °C. The purified product was dissolved in 200 μ L of 0.1 M KH₂PO₄ (pH 7.2); the peptide (100 nmol in 200 μ L of DMSO) was added to the solution, and the reaction mixture was incubated at room temperature for 3 h. The reaction mixture was frozen and lyophilized to dryness. The crude product was dissolved in 100 μ L of water and then subjected to reverse-phase HPLC purification using a 10% acetonitrile in 0.1 M TEAA (pH 7) to 80% acetonitrile in 0.1 M TEAA (pH 7) solvent gradient with a column temperature of 45 °C. The average isolated yield of the product over two steps is 20%.

General Procedure for DNA-Templated T4 DNA Ligase-Catalyzed Oligonucleotide Polymerization. To a PCR tube were added DNA template (15 pmol in 1.5 μ L of water), primer (22.5 pmol in 2.25 μ L of water), 10 μ L of ligation buffer [132 mM Tris-HCl, 20 mM MgCl₂, 2 mM dithiothreitol, and 20% polyethylene glycol (PEG 6000) (pH 7.6)], 2.75 μ L of water, and ATP (0.5 nmol in 0.5 μ L of water). The reaction mixture was heated to 90 °C for 2 min and then cooled to 25 °C at a rate of 0.1 °C/s. To this PCR tube were then added functionalized pentanucleotides (480 pmol in 1 μ L of water; 4 equiv/codon), BSA (2 μ g in 1 μ L of water), and 400 units of T4 DNA ligase (New England Biolabs, M0202L). The polymerization was performed at 25 °C for 24 h and then the mixture desalted by gel filtration using CENTRI-SEP Spin Columns (Princeton Separations) equilibrated with water. The crude polymerized material was separated for analysis using denaturing PAGE (15% TBE, 150 V, 55 °C), stained with ethidium bromide, and imaged by UV illumination.

Sequence Specificity Assay by Chain Termination. To a PCR tube were added 15 pmol of template (AGT CTT TAT TAG AGT TCT CTA CGC TGC CGT CCC CTT GGA CGG CAG CGT), 10 μ L of ligation buffer [132 mM Tris-HCl, 20 mM MgCl₂, 2 mM dithiothreitol,

and 20% polyethylene glycol (PEG 6000) (pH 7.6)], 2 μ L of water, and ATP (0.5 nmol in 0.5 μ L of water). The reaction mixture was heated to 90 $^{\circ}$ C for 2 min and then cooled to 25 $^{\circ}$ C at a rate of 0.1 $^{\circ}$ C/s. To this PCR tube were added three of the four 5'-phosphorylated modified pentanucleotides (60 pmol each) with the fourth modified pentanucleotide as an unphosphorylated terminator (60 pmol), BSA (2 μ g in 1 μ L of water), and 400 units of T4 DNA ligase (New England Biolabs, M0202L). The polymerization was performed at 25 $^{\circ}$ C for 24 h and then the mixture desalted by gel filtration using CENTRI-SEP Spin Columns (Princeton Separations) equilibrated with water. The crude polymerized material was separated for analysis using denaturing PAGE (15% TBE, 150 V, 55 $^{\circ}$ C), stained with ethidium bromide, and imaged by UV illumination. In four separate experiments, the terminator was incorporated from the first codon to the last codon.

In Vitro Selection Cycle. To a PCR tube were added DNA template (15 pmol in 1.5 μ L of water), primer (22.5 pmol in 2.25 μ L of water), 10 μ L of ligation buffer [132 mM Tris-HCl, 20 mM MgCl₂, 2 mM dithiothreitol, and 20% polyethylene glycol (PEG 6000) (pH 7.6)], 2.75 μ L of water, and ATP (0.5 nmol in 0.5 μ L of water, reaction concentration of 25 μ M). The reaction mixture was heated to 94 $^{\circ}$ C for 2 min and then cooled to 25 $^{\circ}$ C at a rate of 0.1 $^{\circ}$ C/s. The pentanucleotide library (120 pmol each), BSA (2 μ g in 1 μ L of water), and 400 units of T4 DNA ligase were added to make a 20.25 μ L reaction volume. After incubation for 24 h at 25 $^{\circ}$ C, the reaction mixture was purified with the MinElute reaction cleanup kit.

To the purified library in 10 μ L of water were added displacement primer (18.8 pmol), isothermal amplification buffer (1 $\times$), dNTPs (reaction concentration of 200 μ M), 4 units of *Bst* DNA polymerase (NEB, M0538L), and a volume of water of $\leq$ 18.75 μ L. The mixture was added to a preheated block at 65 $^{\circ}$ C for 5 min. The reaction mixture was purified with the MinElute reaction cleanup kit.

The DNA-displayed DNA-scaffolded library was taken up into 200 μ L of 1 $\times$ binding buffer [50 mM sodium phosphate (pH 8.0), 300 mM NaCl, and 0.01% Tween 20]. Two microliters of Co²⁺ magnetic beads (Dynabeads His-Tag Isolation and Pulldown, Invitrogen, 10103D) was added to the library sample and incubated on a rotary shaker for 30 min at 25 $^{\circ}$ C. The magnetic beads were isolated by use of a magnet, and the supernatant was discarded. The beads were then washed six times with 1 $\times$ binding buffer. After the binding buffer had been removed, 100 μ L of His elution buffer [300 mM imidazole, 50 mM sodium phosphate, 300 mM NaCl, and 0.01% Tween 20 (pH 8.0)] was added and the mixture then incubated on a rotary shaker for 5 min to elute the hexahistidine-tagged library members. The solution was purified with a CENTRI-SEP Spin Column (Princeton Separations) to remove the excess salt and imidazole prior to PCR amplification.

The eluted library members were subjected to PCR amplification. Thus, to the surviving genotypes in 10 μ L of water were added the forward and reverse primers (60 pmol each), thermopol buffer (1 $\times$), dNTPs (reaction concentration of 200 μ M), 4 units of Vent (exo-) polymerase (NEB, M0257L), and a volume of water of $\leq$ 200 μ L. The PCR cycles were as follows: 95 $^{\circ}$ C for 90 s to denature and then 95 $^{\circ}$ C for 15 s, 64 $^{\circ}$ C for 20 s, and 72 $^{\circ}$ C for 10 s for 20 cycles. Following the completion of 20 cycles, 15 units of Exo I and Exonuclease I (NEB, M0293L), reaction buffer (1 $\times$) was added to the amplification mixture and the mixture allowed to incubate at 37 $^{\circ}$ C for 30 min. The digest was subsequently purified using the MinElute reaction cleanup kit.

To assess the enrichment over the round, a digestion was performed using a restriction enzyme that specifically targeted the POS genotype. Thus, the amplified DNA library (1 pmol) was taken up into Cut Smart Buffer (1 $\times$), and 2 units of *Xba*I (NEB, R0145L) and water were added up to 10 μ L. The mixture was incubated at 37 $^{\circ}$ C for 20 h. The digested product was then purified with the MinElute reaction cleanup kit, and the digestion products were analyzed by 15% TBE nondenaturing PAGE.

Figures, supporting data, and detailed experimental methods (PDF)

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Notes

The authors declare no competing financial interest.

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ASSOCIATED CONTENT

Supporting Information

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