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REVIEW

Ligase-Catalyzed Oligonucleotide Polymerization (LOOPER): Evolution of chemically diverse aptamer libraries

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ABSTRACT

Expanding the chemical diversity of oligonucleotide libraries has allowed the evolution of synthetic nucleic acid polymers with enhanced molecular recognition and catalysis. Thus, methods that enable the sequence-defined incorporation of diverse chemical modifications are of particular importance in developing novel or improved nucleic acid polymers for diagnostics and therapeutics. In this review, we discuss the development of ligase-catalyzed oligonucleotide polymerization (LOOPER) as a method to increase the chemical diversity of oligonucleotide libraries, and its application towards the evolution of modified aptamers. An evaluation on the use of different ligases, scope and number of modifications, sequence space, and evolutionary outcomes from *in vitro* selections is provided, along with a critical lens on challenges to be addressed for the method to mature into a more widely adapted technology.

KEYWORDS: aptamers, ligase, ligase-catalyzed oligonucleotide polymerization, chemical diversity, molecular evolution, *in vitro* selection

INTRODUCTION

Within nature's hierarchy of molecular function, proteins represent its apex. Equipped with a wide array of functionality, proteins can achieve exceptionally tight binding (Stayton et al, 1999), diffusion-controlled catalysis (Koenig et al, 1972; Chou et al, 1982), and toughness rivaled only by man-made materials (Xu et al, 1990). On the opposite side of the spectrum lie nucleic acid polymers, which have evolved to display few functionalities as their overarching role remains largely within the domain of information storage and transfer. This notwithstanding, vestiges of a potential functional past (Gesteland et al, 2005) remain extant in the form of RNAs with binding and catalytic properties (Serganov et al, 2007). These properties prompted groundbreaking work on the laboratory evolution of nucleic acid polymers with binding (Ellington et al, 1990; Tuerk et al, 1990) and catalytic (Robertson et al, 1990) functions. Despite decades of research, natural nucleic acids have failed to reach the level of functionality of proteins. Accordingly, bridging the functional gap between proteins and nucleic acids has been a long-standing challenge within the nucleic acids community. The axiom of "diversity begets fitness" is universally accepted in the field of molecular evolution, and certainly greater sequence space has facilitated the evolution of functional nucleic acid polymers. However, chemical diversity is the crux of the functional dichotomy between proteins and nucleic acids. Indeed, expanding the chemical diversity of nucleic acids has been essential to improving molecular recognition and catalysis of nucleic acids (McKenzie et al, 2021). While generating modified nucleic acid polymers using polymerases has enabled the incorporation of up to four different modifications (Jäger et al, 2005; Ondruš et al, 2020), to attain the level of diver-

sity seen in proteins requires alternative approaches. This review will discuss the development, implementations, pitfalls, and outlook for using ligases to achieve greater diversity in aptamer libraries.

LIGASE-CATALYZED OLIGONUCLEOTIDE POLYMERIZATION (LOOPER)

The number of unique modifications that can be incorporated into aptamer libraries using polymerase-based approaches is theoretically limited to the number of nucleobases that define the genetic code, which canonically is four (A, C, G, and T/U). While up to four modifications have been used in primer extensions (Jäger et al, 2005; Ondruš et al, 2020), a maximum of three have been successfully implemented during in vitro selections (Hollenstein et al, 2009; Wang et al, 2018). To expand beyond this limitation requires an alternative approach. By using DNA ligases to ligate short, modified oligonucleotides, the number of modifications increases according to the size of the oligonucleotide fragment, such that the number of unique modifications equals 4n, where n is the length of the oligonucleotide. Thus, a trinucleotide can theoretically encode 64 modifications, whereas a pentanucleotide can accommodate up to 1024; this of course occurs at the expense of modification density. To achieve the sequence-defined ligation of short, modified oligonucleotide fragments, we developed Ligase-Catalyzed Oligonucleotide Polymerization (LOOPER). LOOPER begins with a library of templates which contain i) a reading frame comprising a random combinatorial sequence of codons, whereby each codon is the same length, and ii) flanking initiation and termination primers, which define the reading frame and are required for amplification during in vitro evolution Figure 1). During LOOPER, a 5'-phosphorylated primer anneals to the initiation primer site, which is then adenylated by a DNA ligase and functionalized anticodons are ligated in a DNA-templated manner along the reading frame. Subsequently, the duplex DNA can be strand separated to isolate the diversely modified ssDNA, which can be ported directly into traditional *in vitro* selection platforms. Thus, LOOPER enables access to highly functionalized aptamer libraries through sequence-defined heteromultivalent display of diverse modifications. Due to the critical role played by the DNA ligase in LOOPER, different ligases have been used, including *E. coli* DNA ligase (James et al, 1998) and T3, T4, and T7 DNA ligases (Hili et al, 2013; Lei et al, 2015; Chen et al, 2018) each demonstrating their own unique strengths and weaknesses for the process.

T4 DNA LIGASE IN LOOPER

T4 DNA ligase has been the standard ligase used for the LOOPER method due to its ready availability, high fidelity and specificity, compatibility with short oligonucleotides, and tolerance for oligonucleotide modifications. At the outset of the development of LOOPER, a crystal structure of T4 DNA ligase had yet to be reported (Shi et al, 2018), so initial efforts were devoted to systematically understanding the anticodon preferences of T4 DNA ligase during LOOPER. Several key early findings were critical to subsequent developments and implementation of LOOPER to *in vitro* selection. First, while T4 DNA ligase exhibited some activity with very short anticodons, such as trinucleotides (Hili et al, 2013; Lei et al, 2019), pentanucleotides were by-and-large the optimal length, with longer anticodons being prone to annealing and ligating out of frame, resulting in low yield of desired full-length products. Second, in a pentanucleotide anticodon, modifications on the Hoogsteen face of adenine, typically via position 8 (Figure 2), were tolerated at any position except the 3'-end. However, modifications of other nucleobases were poorly tolerated, including modifications via the 5-position of cytidine or thymine, or the N2-position of guanine (Guo et al, 2015). Third, high ATP concentrations

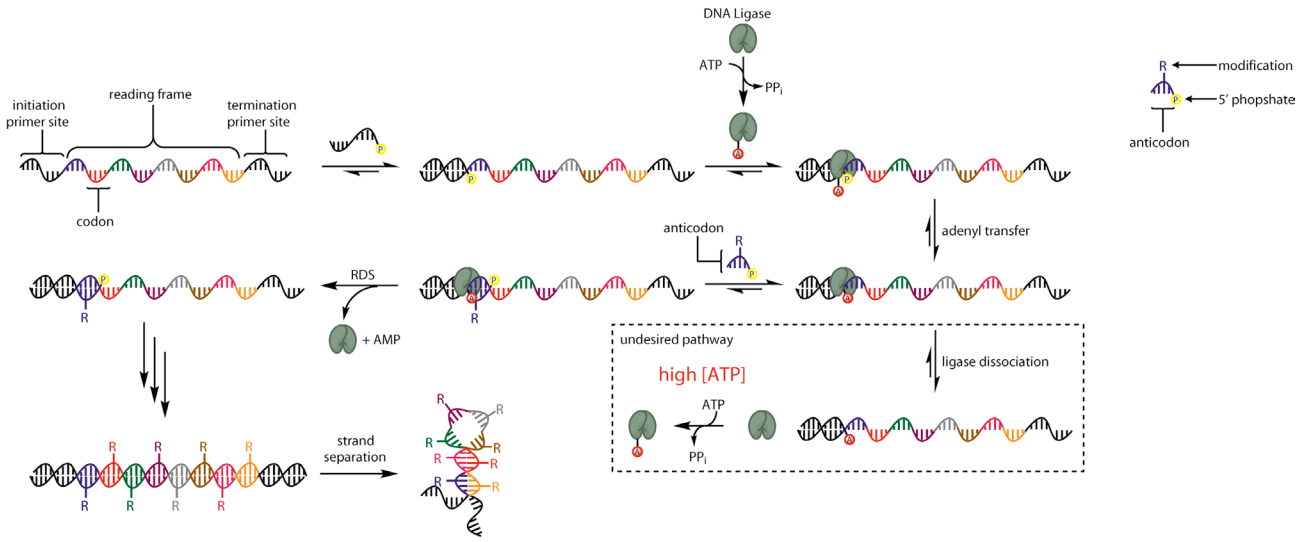

Figure 1. General process for ligase Catalyzed Oligonucleotide Polymerization (LOOPER). A DNA template library contains two primer binding sites, and a reading frame comprising multiple instances of codons are a specific length. Upon annealing of a primer, a DNA ligase catalyzes the phosphodiester bond formation of cognate anticodons with encoded modifications in a DNA-templated manner. Following LOOPER, modified dsDNA libraries can be strand separated to provide modified ssDNA libraries for *in vitro* selection. An undesired pathway (dotted line box) occurs with the ATP concentration is too high, resulting in truncated products.

resulted in a precipitous drop in polymerization efficiency, particularly with larger modifications relative to linker alone. This is hypothesized to result from dissociation of T4 DNA ligase from the adenylated duplex and subsequent readenylation of T4 DNA ligase by excess ATP. Since T4 DNA ligase must be unadenylated to catalyze rate-limiting phosphodiester bond formation, high ATP concentrations push the equilibrium to an over-adenylated state (Figure 1). To offset this issue, the ATP concentration had to be carefully controlled, with the optimal concentration found to be 25μM (Guo et al, 2015). Fourth, while LOOPER can proceed bidirectionally, as opposed to the unidirectional nature of polymerases, T4 DNA ligase exhibited considerably faster kinetics extending from the 3'-OH (Lei et al, 2015). Fifth, in pentanucleotide anticodons, T4 DNA ligase tolerates a large scope of functional groups except for aldehydes, and can accommodate large modifications such as octapeptides with a broad range of charge and hydrophobicity (Guo et al, 2017). Sixth, PEG 6000 was essential as a molecular crowding reagent to increase the effective molarity and thus the rate of LOOPER polymerization reactions (Hili et al, 2013). These early findings were essential toward the rational design of codon sets to be used in LOOPER and for porting the method into *in vitro* selections.

Designing a high-fidelity codon set

In designing a codon set for LOOPER, there are four essential requirements: *i*) tolerance of modifications on polymerized oligonucleotides; *ii*) high yield of full-length product; *iii*) broad coverage of sequence space; and *iv*) high-fidelity and low codon bias of DNA-templated polymerization. For the first two requirements, gel analysis consistently demonstrated that modified pentanucleotides resulted in the highest yields of full-length products. With the development of a duplex DNA sequencing method that allowed for template and polymer strands to be directly compared (Lei et al, 2015), the fidelity of LOOPER could be accurately calculated for various codon sets and anticodons, allowing for the optimization of the method ahead of its use for *in vitro* selections. Duplex DNA sequencing is compatible with standard high-throughput DNA sequencing instrumentation with full codon fidelity analysis achieved using *Analooper* (GitHub repository: https://github.com/HiliLab/analooper)

Using a codon set defined as NNNNN, and the corresponding 1024-membered unmodified anticodon library, LOOPER was able to incorporate the correct anticodon across from its cognate codon 81% of the time – a staggering feat considering the combinatorial complexity of the system. However, this fidelity remains too low for molecular evolution, as less than 20% of a 40-nt genotype would be correctly translated into the corresponding phenotype, resulting in an error catastrophe (Eigen, 1971). As anticipated, using smaller codon sets such as NNNNT, which requires the 256-membered anticodon library ANNNN, resulted in a higher LOOPER fidelity of 87%. Fortuitously, when modifications were incorporated into the anticodon at position 8 of the adenine base, fidelities increased sharply, ranging from 95-98% depending on the location of the modification along the anticodon. This striking observation has been suggested to result from an *anti* to *syn* conformational switch about the glycosidic bond to alleviate steric clash between the modification at position 8 of adenine and the adenylated phosphate backbone (Lei et al, 2015); such conformational switches have been observed in other C8-substitued adenosine derivatives (Sarma et al, 1974, Luyten et al, 1998). Since the *syn* conformation precludes Watson-Crick-Franklin interaction of the modified adenine of the anticodon with the thymine base in the codon, the anticodon hybridization is thus more sensitive to errors in the remaining four hybridizing nucleobases towards the 3'-end.

The location of a modification on an anticodon influences more than fidelity, it also had a large impact on codon bias during LOOPER. Codon bias is concerned with the frequency of a codon in the template as compared to its frequency in the product strand. Low codon bias is desirable and requires near equal frequencies of codon in the template and corresponding product. High codon bias can negatively impact selections as it convolutes the enrichment of sequence space. Generally, codon sets that result in high yield of full-length product have low codon bias. As seen in (Figure 3, the codon set NNNNT with the corresponding anticodon ANNNN with modification at the 5'-end has acceptable codon bias, yield, and fidelity, making it an ideal codon/anticodon system.

With high fidelity and low codon bias observed with the NNNNT codon set, the choice of how to encode the modification becomes important. Sequencing analysis of LOOPER with the NNNNT codon set revealed that most errors occurred within the two nucleotides at the 3'-end of the anticodon (Kong et al, 2016). This can be rationalized by the preference of T4 DNA ligase to extend from the 3'-end, thus errors occur distal to the site of ligation. To take advantage of these errors during in vitro evolutions, encoding of anticodon modifications were designated by the two 3'-end nucleotides of the anticodon, such that when an error occurred, not only was sequence space altered, but also the identity of the modification – akin to a missense mutation (Figure 4a). With a dinucleotide encoding system, up to 16 modifications can be incorporated throughout a

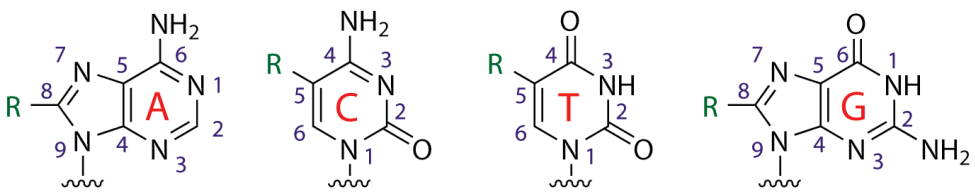

Figure 2. Common modification sites on nucleobases tested in LOOPER. "R" denotes modification site.

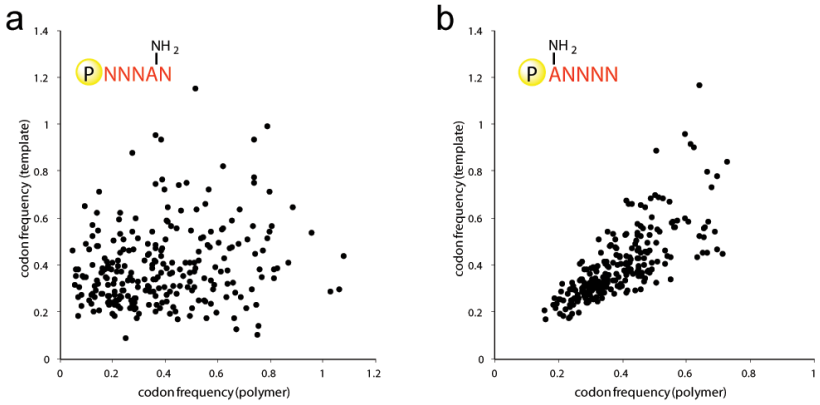

Figure 3. Codon bias observed with T4 DNA ligase mediated LOOPER using different modification sites on anticodons. Low codon bias is represented with datapoints lying along the diagonal. a) anticodon/codon set with high codon bias; b) anticodon/codon set with low codon bias. The amino modification was displayed as hexamethylenediamine at position 8 of adenine.

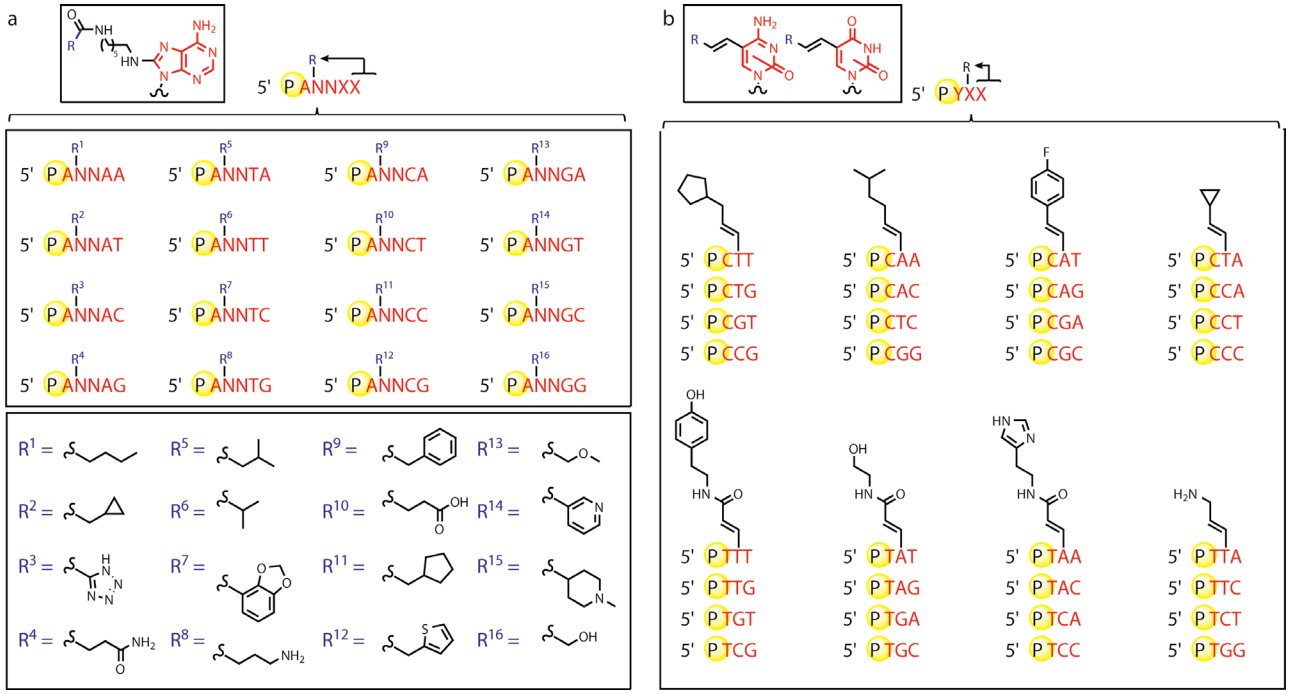

Figure 4. Modified anticodon sets used during LOOPER. a) 256-membered pentanucleotide anticodon set encoding 16 unique chemical modifications. b) 32-membered trinucleotide anticodon set encoding eight unique chemical modifications.

nucleic acid polymer. Furthermore, each modification has 16 degenerate codons due to the two non-encoding nucleotides in each anticodon. This affords the opportunity for each modification to be displayed within greater sequence space. This codon/anticodon set was used to generate oligonucleotide libraries decorated with 16 different functional groups, the most to date, and has been successfully employed during *in vitro* selection of aptamers.

Anticodon modifications play a significant role in target engagement during molecular recognition and catalysis, and influence the tertiary structures of aptamers. Thus, the choice of modifications is paramount and may be governed by the molecular target or type of catalysis. A variety of Brønsted acids and bases, polar and hydrophobic groups, and metal chelators have been successfully implemented in LOOPER during *in vitro* selections.

Fidelities ranging from 87-98% (average 94%) have been used during successful selections (Kong et al, 2016; Kong et al, 2017). Anticodons modified with peptide fragments have also been shown to be compatible with LOOPER. Peptides of up to eight amino acids in length, comprising charged, polar, or hydrophobic residues, are accommodated by T4 DNA ligase (Guo et al, 2015). Since the peptide-modified aptamer libraries could not be directly amplified with polymerase, a variant of duplex DNA sequencing was developed to analyze the fidelity of incorporation of these peptide-modified anticodons (Guo et al, 2017). Using oxidative cleavage to remove the peptide fragments ahead

of PCR, fidelities could readily be determined. Due to the size of the modification, some codon sets suffered from low full-length yields and concomitant codon bias; however, 16-membered codon sets resulted in good yields, low bias, and fidelities of up to 99% (Guo et al, 2017). Generation of DNA-scaffolded peptide libraries using LOOPER has been validated in mock selections (Guo et al, 2015), adapting previously developed (Brudno et al, 2010; McPherson et al, 2011) display approaches.

Recent advances in LOOPER with T4 DNA ligase

An increase in fidelity can benefit in vitro applications by increasing the enrichment factors and allowing access to larger reading frames. Considering the essential role that ATP plays in LOOPER, cofactor modifications can directly influence ligation kinetics, codon bias, and fidelity. 17 ATP derivatives were investigated as co-factors for T4 DNA ligase mediated LOOPER, including modifications on the nucleobase, ribose ring, and triphosphate (Lei et al, 2017) (Figure 5). Modifications on the ribose ring were not tolerated, resulting in no observable LOOPER. Nucleobase modifications were tolerated, including 2-amino ATP and 2-chloro ATP which provided 87% and 64% yield of full-length product at 25µM, respectively (versus 90% for ATP). N6-methyl ATP required high concentrations to effect LOOPER, yielding 70% full-length product at 1 mM (versus 60% for ATP). Importantly, both 2-amino ATP and N6-methyl ATP showed fidelities in excess of 96%, without compromising codon bias and yield, suggesting that they provide advantages over ATP in LOOPER and could serve as promising ATP derivatives in future iterations of LOOPER.

LOOPER has traditionally been conducted using hexamethylenediamine (HMDA) linkers between the 8-position of adenine and the modification. This has largely been due to the commercial availability of amine-modified phosphoramidites used for automated solid-phase DNA synthesis. While these linkers have been successfully used in raising aptamers derived from LOOPER generated libraries, the length and flexibility of HMDA could negatively impact the thermodynamics of binding. To address this issue, the compatibility of shorter linkers in LOOPER has been explored (Guo et al, 2019). Using an ANNNN anticodon library as a model, various linkers were examined. Very short linkers, such as methyleneamine (RCH2NH-) polymerized efficiently and with good fidelity (92%), albeit suffered from very high codon bias, making this linker unsuitable for selections. Ethylene diamine (EDA) linkers provided a good middle ground, allowing LOOPER with a range of modifications (including acid, base, polar, and hydrophobic) in good yield (67-77%), good codon bias, and good fidelities (92-97%). Importantly, it was found that the EDA linker could be directly incorporated during the solid-phase DNA synthesis deprotection step using commercially available 8-bromo 2-deoxyadenosine phosphoramidites (Guo et al, 2019). While no selections have been performed on EDA-linker LOOPER libraries, such selections would be instructive in assessing the impact of linker length on modified aptamer binding.

T3 DNA LIGASE IN LOOPER

While T4 DNA ligase has been successful as a leading ligase in LOOPER, it suffers from several shortcomings, including: *i*) LOOPER efficiency with anticodons less than five nucleotides in length is low, which results in modest density of modifications; and ii) modifications are mostly limited to position 8 of adenine, limiting the sequence space explored during evolutions. The use of T3 DNA ligase has partially addressed these issues. T3 DNA ligase was found to be more efficient than T4 and T7 DNA ligases at LOOPER with modified trinucleotides (Chen et al, 2018), with full-length products observed for up to 45-nt reading frames (15 codons). T3 DNA ligase was also effective at polymerizing unmodified NNN, NNNN, and NNNNN anticodons with yields of 60% (13 codons), 89% (10 codons), and 65% (8 codons), respectively (Lei et al, 2019). Similar to T4 DNA ligase, modifications were optimal on the 5'-end of the anticodon, and accommodation of various small polar and hydrophobic groups have been shown (Chen et al, 2018); larger groups have yet to be explored. It is important to note that since T3 DNA ligase can only tolerate small modifications in trinucleotide systems, custom modified phosphoramidites are required (Chen et al, 2018), albeit this may not be required for longer codon sets. Interestingly, T3 DNA ligase has been shown to only tolerate modification via the 5-position of cytosine and thymine, thus trinucleotide codon sets have been restricted to a library size of 32 (NNY, where Y = C or T). This codon set was converted into an anticodon set with eight modifications, whereby each modification was represented by four anticodons (Figure 4b). This codon/anticodon set has been successfully used for in vitro selections of aptamers.

Using Sanger sequencing on a single template, it was qualitatively shown that T3 DNA ligase has good fidelity with a modified 32-membered anticodon library (Chen et al, 2018); using duplex DNA sequencing, T3 DNA ligase was

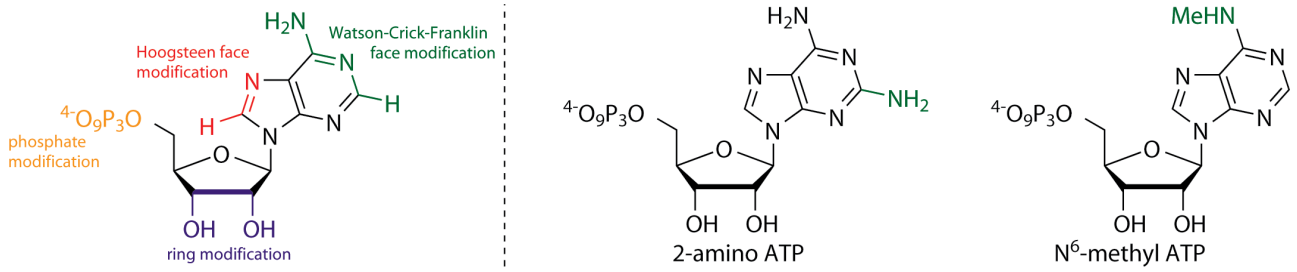

Figure 5. ATP modifications sites tested in T4 DNA Ligase mediated LOOPER. Of the 17 derivatives screened, 2-amino ATP and N^6-methyl ATP provided the most promising results.

later shown to have 97% fidelity with either unmodified or modified YNN anticodons (Lei et al, 2019). T3 DNA ligase can handle larger anticodons sets, such as modified YNNN with 92% fidelity; however, it struggles with pentamer anticodon sets, exhibiting only 74% fidelity with modified YNNNN, which is too low for *in vitro* selection.

IN VITRO EVOLUTION OF LOOPER-DERIVED APTAMERS

In an effort to mimic the surface of proteins for molecular recognition, the LOOPER method enables a sequence-defined display of functional groups along a library of ssDNA at high densities. Combining the LOOPER process with Systematic Evolution of Ligands by EXponential enrichment (SELEX) (Figure 6a) provides access to high quality functionalized aptamers with potential improvements of affinity and selectivity against molecular targets. Provided that fidelity, codon bias, and polymerization yield of LOOPER are well optimized, porting LOOPER-derived libraries into SELEX has been straightforward. Upon synthesis of the LOOPER aptamer library, *in vitro* evolution begins with selection against a target of choice followed by elution and amplification of the output library. Minor differences in LOOPER-SELEX cycles are largely related to the nature of highly modified DNA, which requires polymerases with larger active sites, such as family B polymerases (*e.g.*, KOD DNA polymerase), to amplify during PCR steps. Thus far, three protein targets have been successfully selected against using LOOPER-derived functionalized oligonucleotide libraries, which have provided insights into the impact of dense and diverse modifications on the evolution of aptamers.

Successful LOOPER-SELEX evolutions

The first successful LOOPER-SELEX aptamer evolution was demonstrated using a 256-membered ANNNN anticodon library encoding 16 unique modifications (Figure 4a), generated using T4 DNA ligase (Kong et al, 2017). Selections were performed against human α-Thrombin, which after six rounds resulted in convergence of the library onto a C-rich consensus sequence within the eight-codon reading frame; the highest frequency sequence, TBL1, represented 1.1% of the total output library (Figure 6b). Further to this, there was strong functional group homology observed from anticodons 3-6 within the reading frame, namely a cyclopentyl group at position 3, a carboxylic acid at positions 4 and 6, and a phenyl ring at position 5. This region within the reading frame was under strong evolutionary pressure during SELEX. While TBL1 was a C-rich sequence, its corresponding template was a G-rich sequence that was found to form a stable G-quadruplex. Since G-quadruplexes are known to bind thrombin, the affinity of the TBL1 template for thrombin was measured. Surprisingly the template bound to thrombin with a K_D of 350nM. Even more surprising was that TBL1 also bound to thrombin, and with much tighter affinity (K_D = 1.6nM). This represented the first known instance where both the genotype and phenotype achieved the same function during molecular evolution.

The modifications displayed on TBL1 were shown to be essential for binding (Kong et al, 2020). Deletion of all modifications fully ablated binding, as did deletions within the loop region of its hairpin structure. Furthermore, single modification deletions resulted in approximately 10-fold loss of binding affinity. Beyond this, the modifications were shown to be critical to the thermal stability of the folded structure of TBL1. TBL1 has a melting temperature of 85°C, while TBL1 without the modification has a melting temperature of between 50-55°C. This suggests that the modifications are integral to the folding of TBL1, which was supported by molecular modelling studies (Kong et al, 2020). TBL1 shares functional group similarities with known peptide-based inhibitors, such as hirugen and the FDA-approved drug bivalirudin – both are rich in acidic and hydrophobic residues similar to TBL1. These peptide inhibitors are known to bind exosite I on thrombin suggesting a potential similar mode of action for TBL1. Indeed, competitive binding assays demonstrated that TBL1 binds exosite I without interacting with nearby exosite II (Kong et al 2020). TBL1 exhibits considerably tighter binding compared to unmodified DNA aptamers that target exosite I of thrombin, such as the original thrombin-binding aptamer HD1 (Bock et al, 1992), which exhibits a K_D of 20 nM (Smirnov, 2021). TBL1 was also shown to inhibit thrombin activity in serum assays with an IC_{50} of 23nM. As the first LOOPER-derived aptamer, TBL1 highlights the importance of functional group diversity as it represents the first known DNA aptamer for thrombin that deviates from a G-quadruplex motif.

More recently, T3 DNA ligase has been used in LOOPER-SELEX with a 32-membered YNN anticodon library encoding eight modifications (Figure 4b). This library was used to generate aptamers against Proprotein convertase subtilisin/kexin type 9 serine protease (PCSK9) and interleukin-6 (IL-6) (Chen et al, 2018). After 9 rounds of selection, aptamer PCSK9-A5 was isolated, demonstrating a K_D of 98nM (Figure 6b). This shows markedly higher affinity against PCSK9 than a recently evolved unmodified DNA aptamer, known as AP-1, which had a K_D of 294 nM (Sattari et al, 2020). Deletion analysis showed that three residues were important for binding, namely phenol at position 9 isobutyl at position 11, and cyclopentyl at position 12. PCSK9-A5 was further reselected for another 6 rounds of SELEX to yield PCSK9-evo5, which exhibited a K_D of 3nM against PCSK9 in its truncated form. Interestingly, this aptamer showed no predicted secondary structure, again highlighting that the dense modifications installed using LOOPER can lead to non-canonical folded structures beyond Watson-Crick-Franklin base-pairing. T3 DNA ligase-mediated LOOPER-SELEX was also performed against IL-6, which after 7 rounds of selection, generated aptamer IL6-A7 that exhibited high affinity for IL6 with a K_D = 12nM (Figure 6b). This affinity was only marginally tighter than previously reported unmodified DNA aptamers for IL6, such as aptamer 12L, which was isolated after 5 rounds of selection against IL6 with a K_D of 17 nM (Spiridonova et al, 2016). IL6-A7 also contained two critical isobutyl groups at positions 14 and 15, which when deleted ablated binding. In a recent follow-up study, anticodon libraries that were unfunctionalized, charged, polar, nonpolar, or fully functionalized were used during selection against PCSK9 and IL-6 (Lichtor et al, 2019). The selection results demonstrated that modified aptamer libraries con-

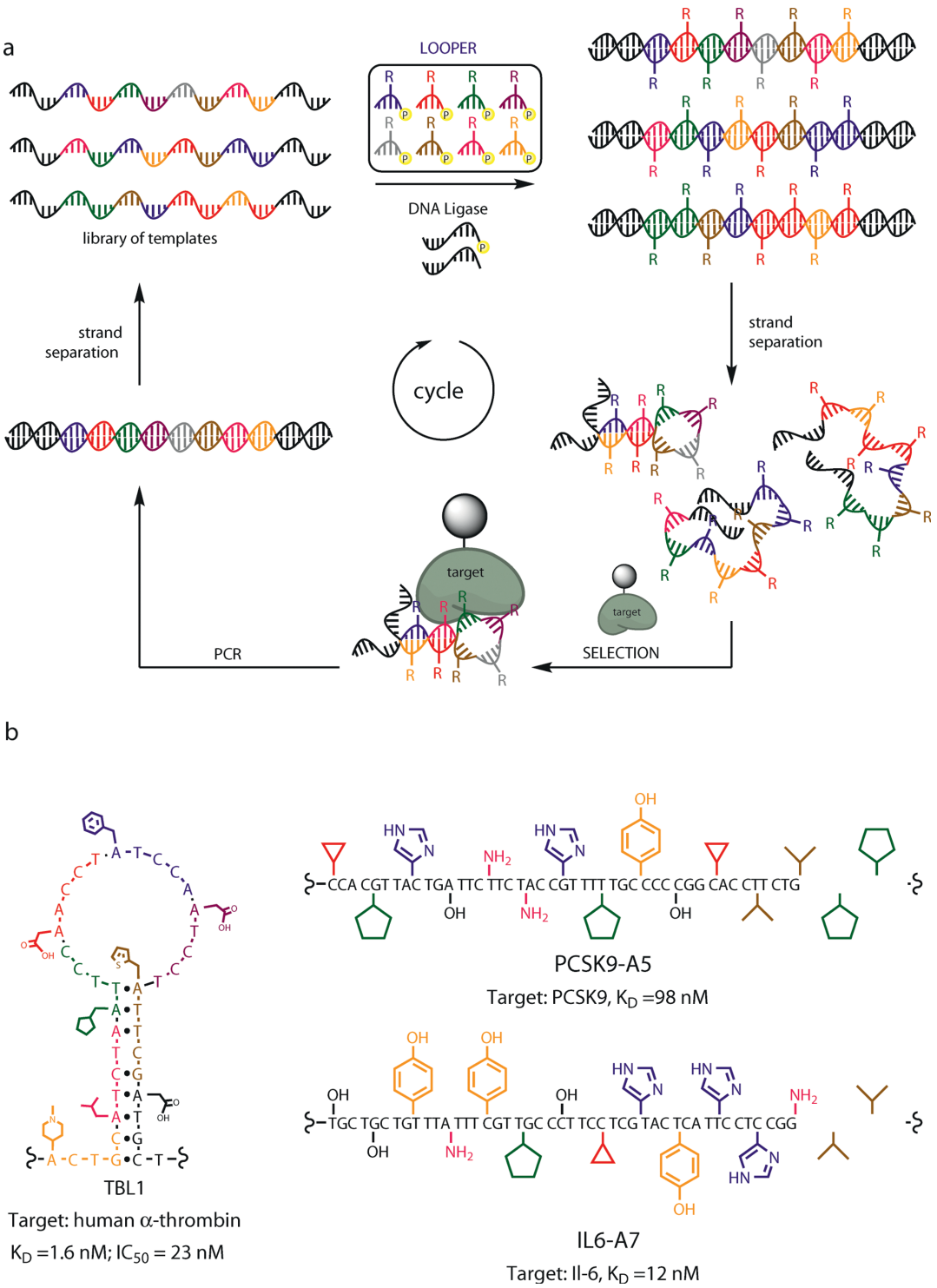

Figure 6. Evolution of sequence-defined diversely functionalized DNA aptamers. a) LOOPER-SELEX cycle used to evolve modified DNA aptamers against molecular targets. b) Aptamers evolved using LOOPER-SELEX including their binding and inhibition constants.

taining non-polar modifications converged more quickly and outperformed libraries lacking non-polar modifications, providing guidelines for the development of future LOOPER-derived aptamer libraries.

CONCLUSIONS AND OUTLOOK

While still in its early stages of development, LOOPER has demonstrated its ability to synthesize diversely modified oligonucleotide libraries that can be readily selected to generate aptamers against molecular targets. The diverse modifications have enabled the discovery of aptamers that break with traditional binding motifs and engage in non-canonical folding, allowing nucleic acids to bridge the functional divide toward protein-like properties. Notwithstanding, several challenges remain with LOOPER to facilitate its widespread use in the development of aptamers. The first challenge is that LOOPER currently does not

explore full sequence coverage with either T3 or T4 DNA ligase-based systems. This is due to the ligase preferences for modifications on the anticodon. Expanding T4 DNA ligase modified anticodon sets beyond ANNNN and T3 DNA ligase modified anticodon sets beyond YNN is expected to improve modified aptamer evolution outcomes. Second, the synthesis of LOOPER-derived aptamers is synthetically challenging on large scale. While LOOPER can be performed on small scale to evaluate binding of putative aptamers, performing LOOPER on large scale is not feasible due to the cost of the ligase involved. Solid-phase approaches that can generate LOOPER-derived aptamers without recourse to extensive custom phosphoramidite synthesis would significantly improve the utility of LOOPER-derived aptamers, while enabling deletion and minimizations, as well as structural studies by crystallography. Lastly, due to the longer process involved in LOOPER-SELEX compared with the traditional SELEX process, advances in high-efficiency selections (Le at al, 2021) that are compatible with LOOPER-derived libraries are needed. Indeed, new selection technologies, such as ideal-filter capillary electrophoresis (Le et al, 2019), demonstrates immense promise in achieving single-round aptamer selections.

Beyond these three major challenges, controlled evaluations are still needed that compare unmodified aptamer evolutions against LOOPER-derived aptamer evolutions. Such evaluations will provide a critical look at the scale of impact LOOPER-derived modifications provide to aptamer fitness during evolution, such as affinity and selectivity of binding. Furthermore, since LOOPER has the potential to generate nucleic acid polymers comprising other unnatural nucleoside derivatives, continued exploration into the modification preferences for ligases will instruct the design of new anticodon sets, as will results from selections against a diverse set of molecular targets, including proteins and small molecules. With the ability to incorporate a large repertoire of chemical functionality, selections of more complex functional nucleic acids, including those with enhanced selectivity or catalytic activity, are expected to yield interesting insights into the impact of expanded chemical diversity on the evolutionary fitness landscape of nucleic acid polymers.

COMPETING INTERESTS

None declared.

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ABBREVIATIONS

EDA: ethylene**di**amine
HMDA: hexa**m**ethylene**di**amine
IL-6: inter**l**eukin-**6**
K_D: Dissociation equilibrium constant
KOD: Thermococcus **kod**akaraensis
LOOPER: Ligase-catalyzed **O**lig**o**nucleotide **P**olym**er**ization
PCSK9: Proprotein **c**onvertase **s**ubtilisin/**k**exin type **9** serine protease
SELEX: Systematic **e**volution of **l**igands by **ex**ponential enrichment
SPR: Surface **p**lasmon **r**esonance
TBL1: Thrombin-**b**inding **L**OOPER aptamer 1

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