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Threomer Inhibition of S1–ACE2 Binding

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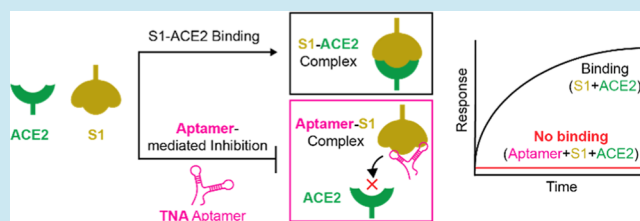
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ABSTRACT: The interaction between the receptor binding domain (RBD) of the SARS-CoV-2 spike protein (S1) and human ACE2 receptor is essential for viral entry into host epithelial cells and a key target for drug discovery. Here, we describe the evolution of a threomer, a base-modified version of threose nucleic acid (TNA), that binds to the S1 protein and inhibits its interaction with ACE2. The aptamer was isolated by in vitro selection using a DNA display strategy that linked each TNA molecule to its encoding double-stranded DNA template. Following iterative cycles of selection and amplification, lead candidates were identified by parallelized screening of individual variants in hydrogel particles. The top-performing hit exhibits low nanomolar affinity to the S1 protein and inhibits formation of the S1–ACE2 complex. Together, this work establishes threomers as an emerging platform for inhibiting protein–protein interactions in therapeutic targets.

KEYWORDS: threose nucleic acid (TNA), threomer, base modifications, aptamers, S1–ACE2 inhibition, SARS-CoV-2



INTRODUCTION

Since its discovery in 1990, in vitro selection, also known as SELEX, has been used to generate thousands of aptamers, which are nucleic acid molecules that mimic antibodies by folding into complex three-dimensional structures that can recognize molecular targets with high affinity and specificity.^{1–3} The ability of aptamers to bind and inhibit clinically relevant targets with antibody-like activity has generated considerable interest in their development as therapeutic agents for drug discovery.^{4–6} However, most aptamers are composed of natural RNA or DNA,⁷ which have limited therapeutic utility due to their susceptibility to nuclease degradation.⁸ While the path to approval from the US Food and Drug Administration (FDA) has been achieved through the use of post-SELEX modifications,^{9,10} structural changes of this type are often difficult to implement and come with a risk for loss of activity.¹¹ Artificial genetic polymers, also known as xeno-nucleic acids or XNAs,^{12,13} provide a possible solution to this problem by utilizing backbone chemistries that are not recognized by the enzymes that degrade DNA and RNA.¹⁴ This approach, though challenging in other areas, such as chemical synthesis, bypasses the need for backbone-stabilizing modifications once the selection is complete.¹⁵

Focusing on the unnatural genetic polymer α -L-threofuranosyl nucleic acid (TNA, Figure 1a),¹⁶ we have previously demonstrated that XNA aptamers with affinity for small molecules and proteins can be evolved from random sequence libraries.^{17–20} This body of work, which was performed alongside similar studies by other groups using 1',5'-anhydroxitol nucleic acid (HNA)^{21,22} and 2'-fluoroarabino nucleic acid (FANA),^{23,24} demonstrated that the concepts of heredity and evolution could be extended to genetic polymers that are distinct from those found in nature.²⁵ In an effort to improve the

binding properties of XNA aptamers, we sought to emulate nature by augmenting our libraries beyond their standard base chemistries with diversity-enhancing functional groups.²⁶ Uniformly modified libraries, prepared from chemically synthesized C-5 modified TNA pyrimidine triphosphates (Figure 1b),²⁷ yielded a new class of aptamers, termed threomers, that functioned with higher affinity binding and slower off-rate kinetics.^{28,29} These findings expanded the chemical space of evolvable non-natural genetic polymers to include functional groups that enhance target binding affinity by mimicking the paratope surface of antibody–antigen complexes.³⁰

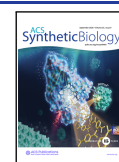
Here, we investigate the potential for threomers bearing tryptamine (Trp) side chains at the C5 position of uracil bases (Figure 1b) to inhibit a therapeutically relevant protein–protein interaction and evaluate whether XNAs can serve as a functional alternative to conventional nucleic acid aptamers. Using a competitive in vitro selection strategy, we isolated threomers targeting the receptor binding domain (RBD) of the SARS-CoV-2 spike glycoprotein subunit 1 (S1), a clinically important protein responsible for viral entry through host cell recognition. Through mid-throughput secondary screening, we identified candidate threomers with strong affinity for the S1 protein. Competitive binding assays demonstrate that a representative threomer binds to S1 and inhibits its interaction with angiotensin-converting enzyme 2 (ACE2), indicating that TNA

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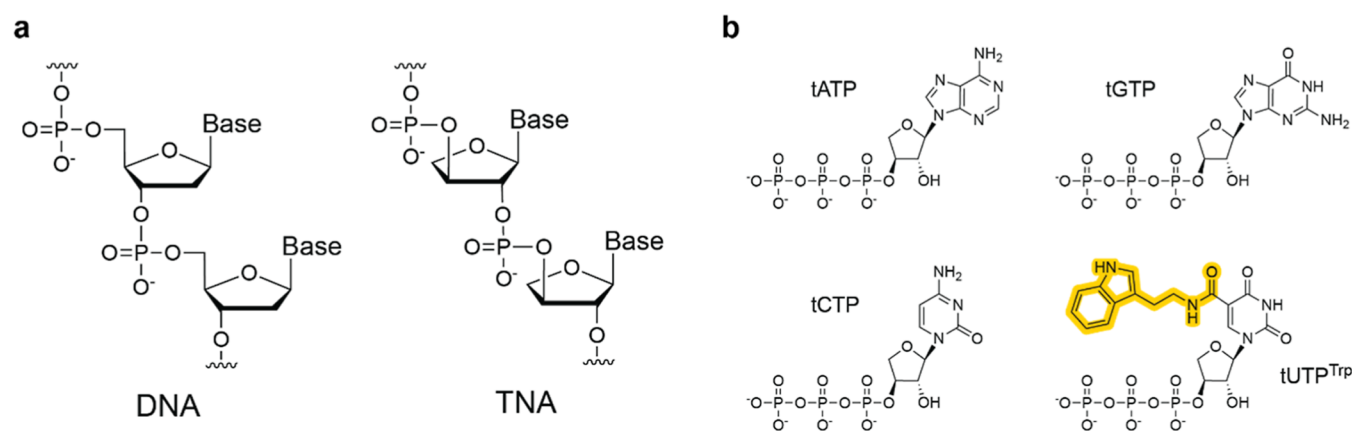


Figure 1. TNA structure. (a) Structural comparison of DNA and TNA. (b) Chemical structures of the TNA triphosphates used in this study. The tryptamine side chain on tUTP is highlighted in yellow.

aptamers can disrupt the binding interaction of a biologically relevant protein–protein interface.

RESULTS AND DISCUSSION

We designed a selection strategy to favor the enrichment of threomers with affinity to the RBD of the S1 protein of SARS-CoV-2. For each round of selection, a library of DNA-tagged threomers was prepared by ligating a DNA stem-loop structure onto a single-stranded DNA library (Figure 2a, Table S1).³¹ The pool of self-priming templates was then extended using a laboratory-evolved TNA polymerase known as 10-92 that functions with a rate of 1 nts^{−1} and 99% fidelity³² and chemically synthesized TNA nucleoside triphosphates^{33,34} to copy the unpaired DNA templates into TNA, yielding a pool of chimeric TNA–DNA hairpins. To minimize dependence of aptamer function on the fixed-sequence PCR primer binding site (PBS) downstream of the random region, we used a high-affinity blocking primer containing four locked nucleic acid residues (LNA) at each terminus to pause extension. Standard primer extension reactions performed in the absence and presence of the blocking strand show strong chain termination at the junction between the random region and PBS (Figure 3). Next, the TNA portion of the DNA–TNA duplex was displaced in a separate primer extension step by extending a DNA primer annealed to the loop region of the hairpin with DNA using Bst DNA polymerase, an enzyme with strong strand displacement activity.³⁵ The product of strand displacement is a library of TNA molecules, each physically linked to their encoding dsDNA template (Figure 2a).

For each round of selection, the library of DNA-tagged threomers was passed through a negative selection step to remove sequences with affinity to the Ni-NTA beads and a positive selection step for binding to a His-tagged version of the glycosylated S1 protein (Figure 2b and Table S2). The selection buffer contained salmon sperm DNA and bovine serum albumin, which were used as generic DNA and protein competitors. Threomers that bound to the S1 protein in solution were partitioned away from the unbound material by passing the mixture over Ni-NTA beads to capture the bound threomer–protein complexes on a solid support. Following several wash steps, including stringent washing with urea and dextran sulfate, free ACE2 protein was added to competitively elute threomers that bound the RBD of the S1 protein.

Threomers recovered in the eluant were amplified in a two-step polymerase chain reaction (PCR) using primers complementary to the PBS sites on the DNA tag. The pool was first amplified by droplet PCR to minimize amplification bias.³⁶ Molecules isolated from the droplets were amplified in a bulk PCR using a PEG-modified DNA primer to allow size separation of the DNA strands by denaturing polyacrylamide gel electrophoresis (PAGE).²⁰ Following PAGE purification and electroelution, the single-stranded, PEGylated DNA template was recovered and used as the starting point for the next round of in vitro selection and amplification.

We performed four rounds of selection starting from the output of a previous screen that sampled 10¹³ unique TNA molecules for affinity to the RBD of S1.²⁹ The library contains a central random region of 30 unbiased nucleotide (nt) positions flanked on either side with fixed-sequence positions for primer binding during PCR amplification. The TNA library was uniformly derivatized at each uracil position with a C-5 amide-conjugated tryptamine side chain,²⁷ which has been shown in previous studies to increase the proportion of functional aptamers in random sequence libraries relative to unmodified libraries,^{29,37} an observation consistent with its abundance on the paratope surface of protein–antibody co-crystal structures.³⁰ Library synthesis was achieved by primer extension using a TNA triphosphate mixture containing C-5 Trp-modified α -L-threofuranosyl uracil nucleoside triphosphate (Figure 1b) in place of the thymidine residue.²⁷ Because the wash steps used in the selection protocol contained denaturants, the selection was performed using the full S1 protein rather than the RBD, which proved less stable in our hands than the S1 protein.

DNA molecules recovered in the final round of the selection were subjected to high-throughput sequencing, and 96-representative members were evaluated for binding to the S1 protein using a hydrogel aptamer particle display strategy (Figure 4).³⁸ Aptamer hydrogel particles were prepared by polymerase-mediated extension of a DNA primer cross-linked into the gel matrix of a polyacrylamide hydrogel shell encapsulating a magnetic particle. This approach is compatible with a standard 96-well screening format using 96 DNA templates to encode the desired TNA sequences in separate wells of a 96-well plate. Following TNA synthesis, the templates were removed by washing, and the resulting hydrogel particles containing the threomers were resuspended in binding buffer and folded by heating and cooling the plate. The TNA aptamer hydrogel

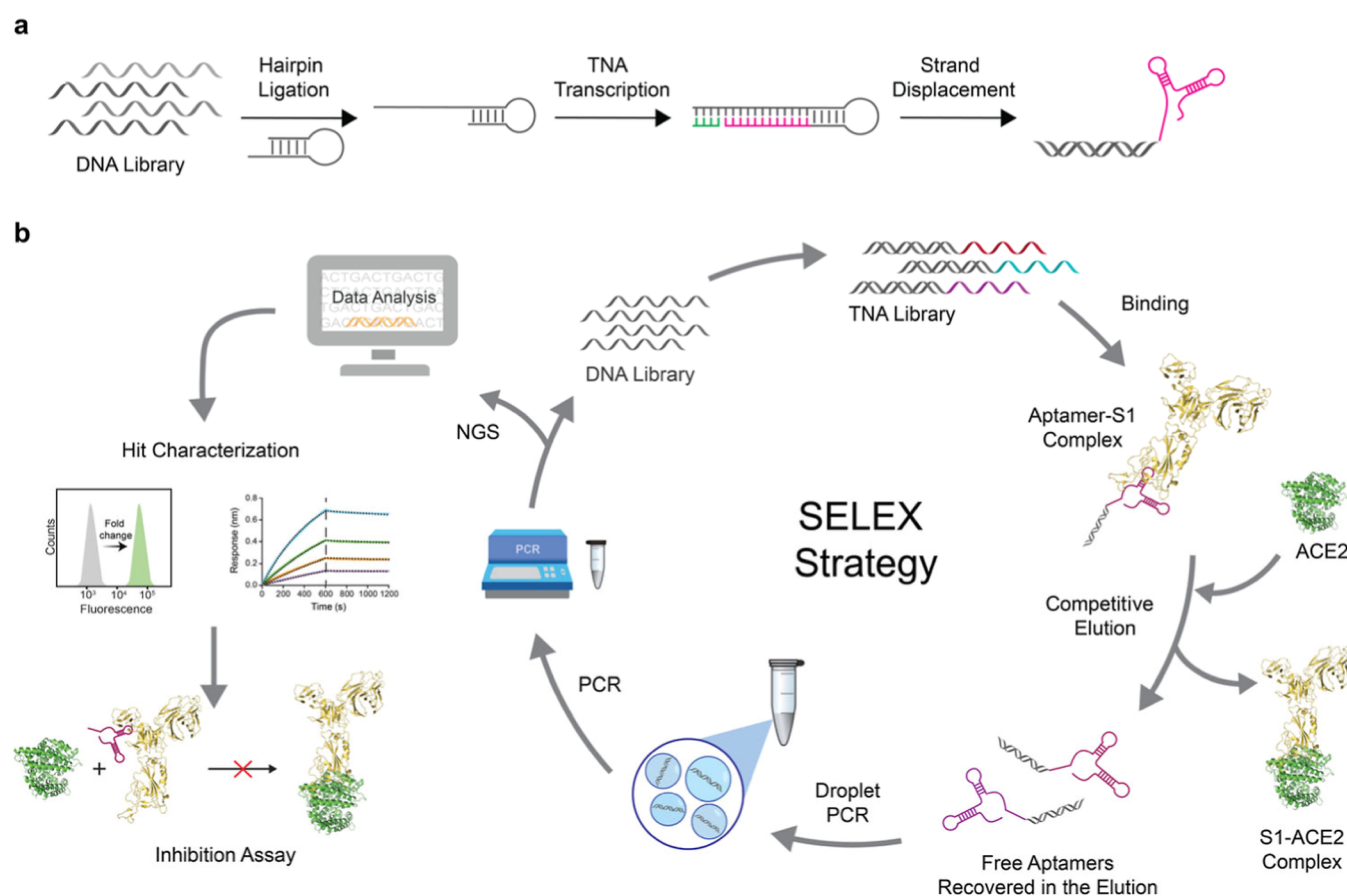


Figure 2. Selection strategy. (a) Schematic depiction of TNA library preparation. A DNA display was used to establish a genotype–phenotype relationship by covalently linking each TNA molecule to its encoding dsDNA template. (b) Aptamer selection. The TNA library is incubated with the His-tagged S1 protein, and bound molecules are separated from the unbound pool by affinity capture on Ni-NTA beads. Bound TNA molecules are recovered by incubating the beads with an excess of the S1-binding ACE2 protein. The encoding dsDNA is amplified by droplet and standard PCR, and the enriched pool of DNA templates is used to regenerate the TNA library for another round of selection. After five rounds of selection, representative members isolated from the pool were sequenced and characterized for S1 binding and inhibition.

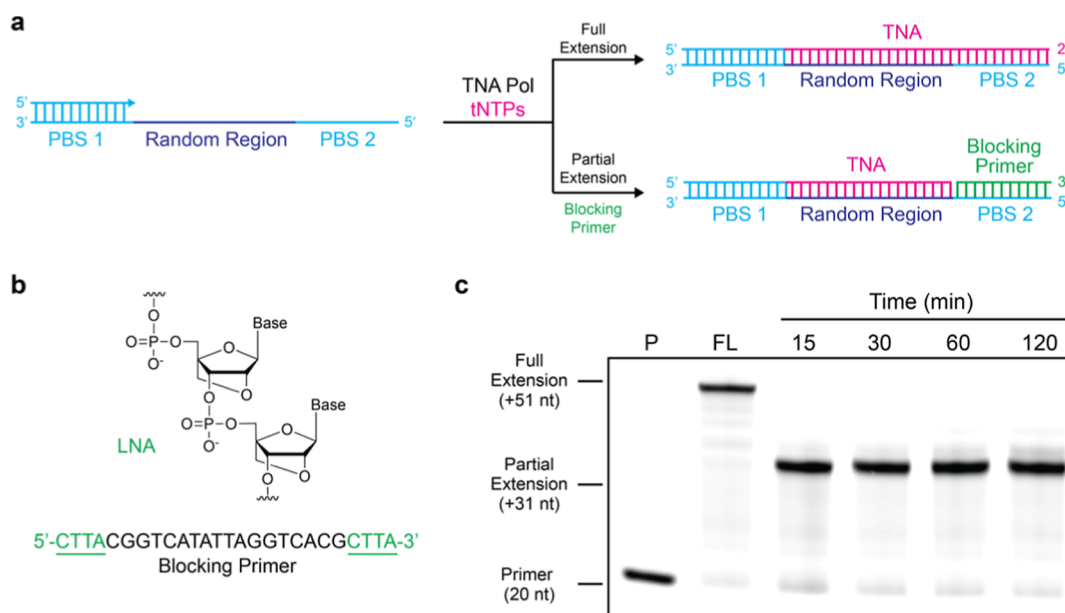


Figure 3. Blocking strategy for TNA library synthesis. (a) Cartoon illustration of TNA synthesis in the absence or presence of a blocking primer. (b) Sequence of the blocking primer with 4 LNA residues at both termini. (c) Time course of library synthesis in the presence of the blocking primer to inhibit TNA synthesis across from PBS 2. Reactions were analyzed by denaturing PAGE. Abbreviations: P: primer and FL: full-length product.

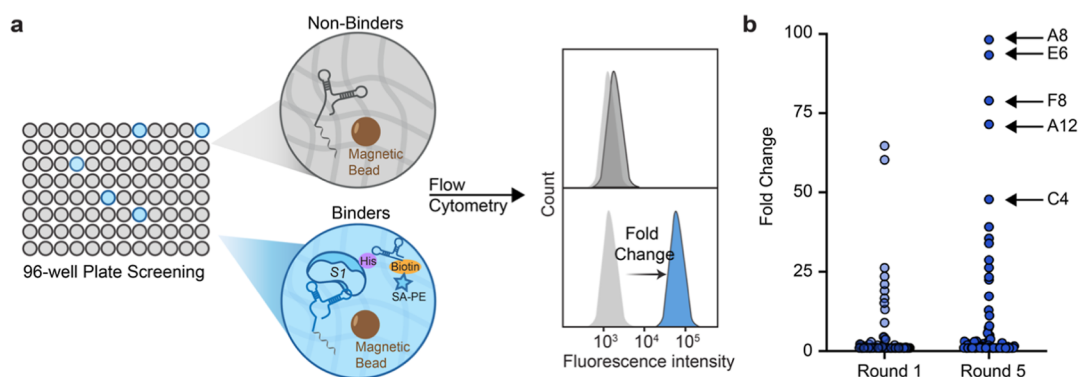


Figure 4. Parallelized aptamer screening. (a) Cartoon representation of a protein-binding assay performed in a hydrogel particle format using flow cytometry to identify hits. TNA aptamers bound to a His-tagged target protein fluoresce when incubated with a biotinylated anti-His aptamer and phycoerythrin-labeled streptavidin. (b) Flow cytometry analysis of TNA aptamers isolated from the first and fifth rounds of selection. Top hits are annotated with arrows. $n = 96$ unique sequences for rounds 1 and 5.

particles were then evaluated for binding to a His-tagged version of the S1 protein using an ELISA-type assay that is compatible with flow cytometry (Figure 4a). In this assay, TNA aptamers with affinity to the S1 protein exhibit a positive shift in fluorescence relative to a primer-only control when incubated with a biotinylated version of an anti-His tag aptamer and phycoerythrin-labeled streptavidin (SA).³⁹ This approach accelerates the pace of secondary screening by eliminating the need to individually purify each TNA sequence by denaturing PAGE. It also benefits from a reduced scale of TNA synthesis relative to the larger scales required for PAGE purification.

Flow cytometry analysis of 96 randomly chosen sequences from rounds 1 and 5 revealed a clear enrichment of S1-binding threomers in the final pool. We note that round 1 corresponds to sequences derived from our previously reported screen, which was the starting point for the current selection.²⁹ As shown in Figure 4b, sequences isolated from round 5 display an overall increase in the proportion of higher affinity binders relative to those from round 1, indicating that the selection strategy successfully enriched the pool for threomers with improved recognition of the S1 protein. Interestingly, 14 of the 96 sequences evaluated in the secondary screen contain a TTT motif, where T represents the base-modified tU^{TP} nucleotide. However, only 3 of these molecules (A8, E6, and F8) have activity in the particle display assay, indicating that the TTT motif is not essential for binding.

To quantify the binding activity of the top-performing hits identified by flow cytometry, we first established an enzymatic workflow to synthesize and purify the TNA aptamers at a scale suitable for biochemical characterization (Figure 5a). Threomers A8, E6, F8, A12, and C4 were prepared in 1–2 mL reaction volumes using the 10-92 TNA polymerase to extend a 5' biotinylated DNA primer annealed to a DNA template carrying a 3' overhang.³² The resulting full-length products were separated from the longer DNA template strand by denaturing PAGE, followed by electroelution, desalting, and quantification by UV absorbance. This approach enabled the efficient production of 5' biotinylated threomers for downstream binding affinity measurements.

Next, we measured the binding kinetics of the Trp-modified threomers by biolayer interferometry (BLI). The 5' biotinylated threomers were immobilized on SA-coated biosensor tips and evaluated for binding to the S1 protein. Background-subtracted sensorgrams collected across a range of concentrations reveal

clear, concentration-dependent association and dissociation profiles for each threomer (Figures 5b and S1). Global fitting of the kinetic data to a 1:1 binding model yielded equilibrium dissociation constants (K_D) spanning a narrow range of 1.3–5.4 nM (Table S3), indicating that the five threomers selected for characterization bind S1 with affinities comparable to that of high-affinity antibodies.⁴⁰ Close examination of the binding curves (Figures 5b and S1) reveals that the threomers exhibit strong binding responses with rapid association kinetics and slow dissociation phases (k_{off} values in the range of 2×10^{-4} to $8 \times 10^{-5} \text{ s}^{-1}$), which is consistent with the formation of stable aptamer–protein complexes. The slow off-rates observed for the selected threomers are comparable to previously discovered peptide-based affinity reagents developed against S1^{41,42} but less active than a de novo designed miniprotein binder.⁴³

Having established that the selected threomers bind the S1 protein with low nanomolar affinity, we next asked whether these molecules could inhibit the binding interaction between S1 and ACE2. To perform this analysis, we needed to generate a representative threomer on a larger scale to ensure sufficient aptamer material for quantitative inhibition studies. We decided the best approach for larger-scale threomer production was solid-phase synthesis using TNA phosphoramidites (Figure 6a) prepared by chemical synthesis.³³ Because all five threomers showed comparable binding affinity (Figures 5b and S1; Table S3), E6 was selected as a candidate for inhibition. After chemical synthesis and purification, the binding affinity of E6 was verified by BLI analysis, which yielded a K_D of 1.9 nM along with association and dissociation profiles that closely match the enzymatically synthesized version of this sequence (Figure S2 and Table S4).

The ability of the E6 aptamer to inhibit S1 binding to the ACE2 protein was evaluated using a concentration-dependent competition assay. Increasing concentrations of E6 bound to the S1 protein resulted in a progressive decrease in S1–ACE2 binding, yielding a sigmoidal dose–response curve characteristic of binding inhibition (Figure 6b, Table S5, and Figure S3). Fitting the data to a standard binding isotherm afforded an IC_{50} value of 15.2 nM, demonstrating that E6 effectively disrupts the S1–ACE2 interaction at low nanomolar concentrations. Importantly, the potency of E6 closely tracks with its equilibrium binding affinity, indicating that its high-affinity target engagement closely translates into the functional disruption of a protein–protein interface.

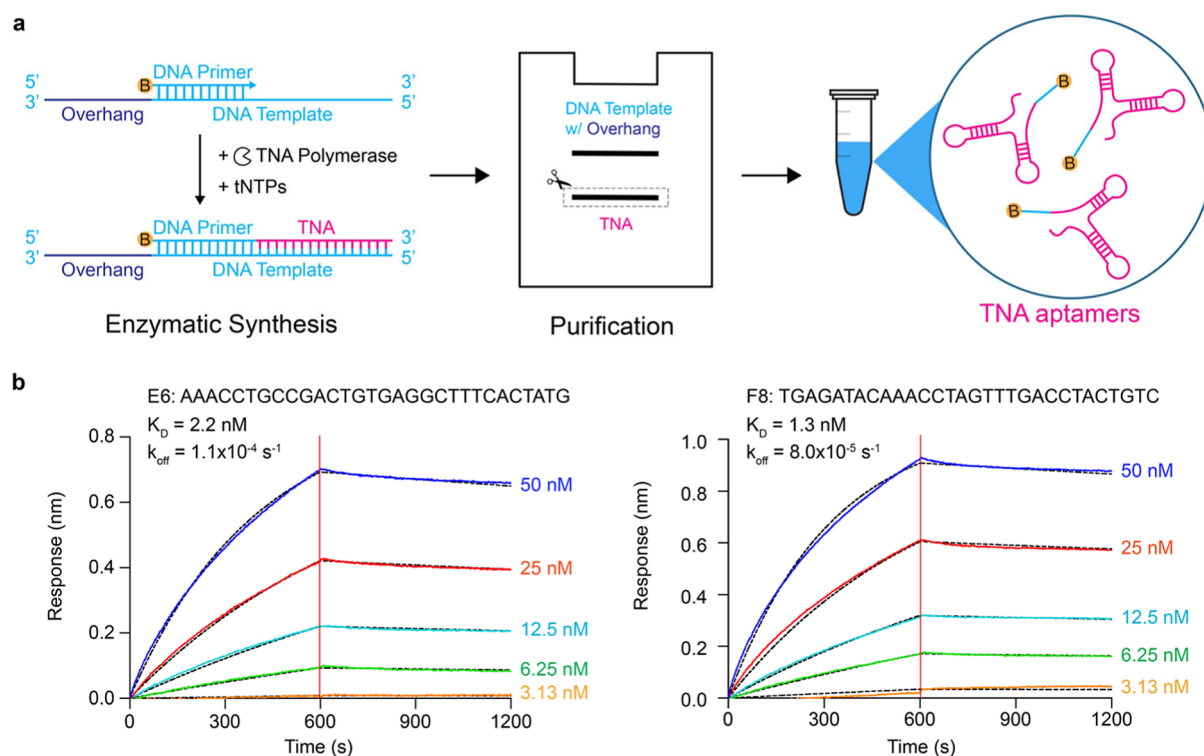


Figure 5. Enzymatic synthesis and characterization. (a) Enzymatic synthesis and purification of top-performing aptamers identified by flow cytometry. TNA aptamers were enzymatically generated by primer extension, purified by denaturing PAGE, electroeluted, and UV-quantified. (b) Kinetic binding analysis of Trp-modified TNA aptamers. The TNA sequence is provided with T denoting the position of modified U residues. Background-subtracted sensorgrams collected by biolayer interferometry of E6 and F8. The red line indicates the transition between the association and dissociation phases. Global fits are shown as dashed black lines. Abbreviation: B, biotin.

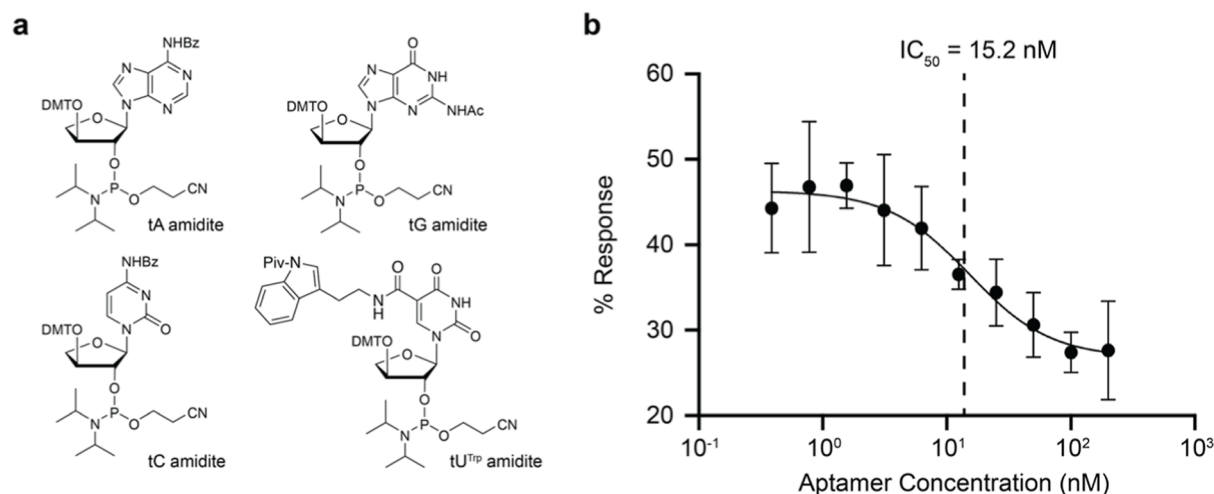


Figure 6. Inhibition of S1 binding to ACE2 by a TNA aptamer. (a) Chemical structure of TNA phosphoramidite monomers used to prepare the Trp-modified E6 TNA aptamer by solid-phase synthesis. (b) Inhibition curve. The IC_{50} curve was calculated by measuring changes in S1–ACE2 binding across increasing concentrations of E6. Data presented as the mean standard deviation ($n = 3$).

By showing that E6 can inhibit formation of the S1–ACE2 complex, this study extends threomers beyond their initial demonstration as high-affinity protein-binding reagents to functional modulators of biologically important protein interfaces. This distinction is important because inhibition of protein–protein interactions remains a challenging objective in aptamer therapeutics, often requiring precise recognition of large, complex surfaces rather than simpler ligand-binding pockets. In the present study, this challenge was addressed

by targeting the receptor binding domain of S1 through a competitive elution strategy in which ACE2 was used to selectively displace threomers bound at or near the ACE2-binding region of S1. The recovery of E6 from this selection and its subsequent ability to inhibit S1–ACE2 binding support the idea that cognate binding partners can be used as functional competitors to enrich aptamers that recognize therapeutically relevant epitopes. More broadly, this strategy provides a general framework for evolving threomers and other aptamers that not

only bind disease-associated proteins but also interfere directly with protein–protein interactions that drive biological function. Such properties, coupled with the favorable toxicity profile of TNA in mice, warrant continued investigation of TNA as a polymer for therapeutic oligonucleotides.⁴⁴

The high-affinity binding observed among the selected threomers reinforces the conclusion that the shorter backbone architecture found in TNA, relative to that in DNA and RNA, does not preclude the formation of ligand-binding structures. This observation is notable because previous biophysical studies have shown that TNA adopts a more conformationally constrained backbone than natural DNA and RNA, a property that could, in principle, limit its ability to fold into the types of compact three-dimensional structures required for ligand binding.^{45,46} Despite this concern, the current results add to a growing body of evidence demonstrating that TNA retains sufficient conformational plasticity to generate the types of tertiary structures required for selective target binding. These findings suggest that backbone rigidity alone is not inherently incompatible with evolvability and that alternative nucleic acid architectures can support sophisticated levels of molecular recognition even though substantial differences may exist in their backbone chemistry.

Although the current study establishes a proof-of-concept demonstration that threomers can function as antagonists by blocking the formation of a protein–protein interface, several important questions remain for future investigation. These include structural studies to define the molecular basis of target recognition and determine whether E6 inhibits S1–ACE2 complex formation through direct competition at the receptor binding site or through an allosteric mechanism. In addition, while strong binding affinity and inhibitory activity were established in vitro, further studies are needed to evaluate threomer activity in physiologically relevant cellular and in vivo systems. In this regard, the inherent nuclease resistance and enhanced acid stability of TNA may provide important advantages over conventional DNA aptamers by improving biological stability and persistence under therapeutic conditions.^{47,48}

In summary, we demonstrate that a TNA aptamer can bind the SARS-CoV-2 S1 protein and inhibit formation of the S1–ACE2 complex with low nanomolar potency. These findings expand the functional scope of synthetic genetic polymers beyond molecular recognition alone and establish threomers as a promising platform for the development of therapeutic aptamers capable of modulating biologically important protein interactions.

■ ASSOCIATED CONTENT

Data Availability Statement

All data are available upon reasonable request.

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssynbio.6c00516>.

Materials and Methods; Table S1: DNA oligonucleotides; Table S2: selection conditions; Table S3: BLI kinetic analysis for enzymatically synthesized threomers; Table S4: BLI kinetic analysis for solid-phase synthesized threomer E6; Table S5: BLI kinetic analysis for the threomer E6 inhibition study; Figure S1: BLI analysis of top-performing threomers; Figure S2: BLI analysis of

threomer E6 prepared by solid-phase synthesis; and Figure S3: BLI analysis of E6-mediated inhibition of S1 binding to ACE2 (PDF)

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Author Contributions

[†]ALC and JL contributed equally. ALC and JC designed the study. ALC, JL, EL, and NK performed the experiments. All authors participated in manuscript writing.

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Notes

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