

Fundamental studies for small molecule aptamer selection using capture-SELEX

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

The work presented in this thesis is the result of work performed by the authors and other scientific collaborators. Contributions from each scientist and the resulting publications are listed below.

The work in Chapter 2 has been published as follows: Yuzhe Ding, and Juewen Liu. "Pushing adenosine and ATP SELEX for DNA aptamers with nanomolar affinity." *Journal of the American Chemical Society* 145.13 (2023): 7540-7547. All of the experiments were done by Yuzhe Ding. The manuscript was written by Yuzhe Ding and Juewen Liu.

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For Chapter 4, some of ThT binding assay were done by Yicheng Heng. All other experiments were performed by Yuzhe Ding. Ka-Ying Wong contributed to the paper's structural planning and assisted with the preparation of some figures. The manuscript was written by Yuzhe Ding and Juewen Liu.

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Abstract

DNA aptamers for small molecules hold transformative promise in biosensing, diagnostics, and therapeutics, yet their in vitro evolution has been hampered by incomplete knowledge of the parameters that drive efficient enrichment. In recent years, the development of library-immobilization based method, so called capture-SELEX, has generated over 100 high-quality DNA aptamers for various types of small molecules. Importantly, capture-SELEX allows systematic investigation of fundamental problems in the selection of aptamers. This thesis studies the capture-SELEX platform by dissecting thermodynamic, kinetic, and methodological variables to accelerate the discovery of high-affinity DNA aptamers.

Using adenosine/ATP as targets for selection has repeatedly produced the same guanine-rich aptamer motif that was first reported by the Szostak group in 1995. This aptamer has been considered as the adenosine/ATP aptamer by the field. First, by gradually increasing the selection stringency on classical targets (adenosine and ATP), we selected two new aptamers with $K_d \approx 230$ nM, 35-fold tighter than that of the classical aptamer sequence. This was achieved through gradual reduction of target concentration from 5 mM to the low-micromolar range. The evolution of the sequence abundance cross different rounds was traced by deep sequencing, and the reason for the previous repeated report of the classical sequence was attributed to its short 12-nucleotide conserved binding regions, whereas the two new aptamers have approximately 16 conserved nucleotides. This study highlights the importance of using low target concentration in order to enrich high affinity aptamers.

During aptamer selection, using a lower target concentration tends to favor the enrichment of higher affinity binders, raising the question of whether a practical lower limit exists. Next, we performed three capture-SELEX campaigns using 5 μ M, 500 nM, and 50 nM guanine as the target, respectively, to investigate it. Both the 5 μ M and 500 nM selections successfully enriched the same guanine aptamer-requiring eight rounds at 5 μ M guanine versus 17 rounds at 500 nM guanine. However, the 50 nM selection failed to yield any aptamers. The highest affinity and most enriched aptamer from these selections displayed a K_d of 200 nM, indicating that if the target concentration is much lower than K_d can lead to failed selections. Mutation analysis further revealed a critical cytosine in the guanine binding pocket: substituting this cytosine with a thymine switched selectivity from guanine to adenine. A similar specificity switching was previously seen in the natural guanine riboswitches. These findings define a lower limit for target concentration in capture-SELEX and offer a practical guidance for selecting target levels to isolate high-affinity aptamers.

Selection of high-affinity aptamers underpins all downstream applications, yet most protocols emphasize thermodynamic factors-such as target concentration-while overlooking binding kinetics. Third, we performed a library-immobilization selection against ampicillin to dissect these influences. Under typical gravity-flow conditions (1-2 min interaction), a low-affinity aptamer ($K_d = 12.7 \mu\text{M}$) dominated the enriched pool. In contrast, extending the incubation time to 10 min enriched a higher affinity sequence ($K_d = 1.8 \mu\text{M}$), differing by only three nucleotides from the weaker K_d aptamer. Systematic comparison of library immobilization efficiency, release fraction, and release kinetics confirmed that dissociation rate from the capture duplex was the primary determinant of the selection outcome. We observed the same kinetic bias in parallel adenosine selections, demonstrating the generality of this effect. Based on these findings, we recommend combining low target concentrations with extended incubation time to favor the enrichment of high-affinity aptamers. This study not only yields a robust, high affinity and selective ampicillin aptamer but also highlights a critical interplay between thermodynamics and kinetics during in vitro aptamer selection.

Since 1990, numerous aptamer-selection techniques have been developed, yet quantitative comparisons of their enrichment efficiencies remain scarce. Finally, we evaluated three library-immobilization SELEX methods, capture-SELEX, GO-SELEX, and gold-SELEX, using a spiked library containing DNA aptamers with varying affinities for adenosine. Using $100 \mu\text{M}$ adenosine as target, all three methods showed that $<1\%$ of the library was released by adenosine as revealed by qPCR, with gold-SELEX showing virtually no DNA elution. Deep sequencing of three model aptamers (Ade1301, Ade1304, and the classical adenosine aptamer) revealed 30-50-fold enrichment in capture-SELEX, whereas GO-SELEX and gold-SELEX both yielded enrichment factors below 1, indicating a lack of aptamer enrichment. Blocking the primer-binding regions improved GO-SELEX enrichment to $\sim 14\%$ but still fell far short of capture-SELEX's performance. Finally, we compared nonspecific versus target-induced release and elucidated why capture-SELEX's structural-switching mechanism offers superior aptamer enrichment. Overall, capture-SELEX is a markedly more efficient strategy for isolating high-affinity aptamers.

Collectively, this work establishes a quantitative framework for capture-SELEX-balancing target concentration, kinetic control, and partitioning strategy-to reliably isolate nanomolar-class DNA aptamers for small molecules.

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Dedication

I would like to dedicate this thesis to my family for their encouragement and support.

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Chapter 1 Introduction

1.1 Nucleic acids

Nucleic acids, which include both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), are polymers made up of repeating nucleotide units. They play crucial roles in the storage, transmission, and expression of genetic information through their highly organized molecular structures. Beyond their biological functions, nucleic acids found potential applications in diverse fields such as chemistry, physics, materials science, energy, and computer science.¹⁻⁴ Compared to RNA, DNA is more attractive for applications outside cells due to its much higher stability and lower cost of synthesis.⁵ This wide-ranging utility stems from DNA's multifunctional properties—including its abilities in molecular recognition, catalysis, and the programmable construction of nanostructures—which all originate from its unique chemical architecture.

1.1.1 Nucleic acids structure

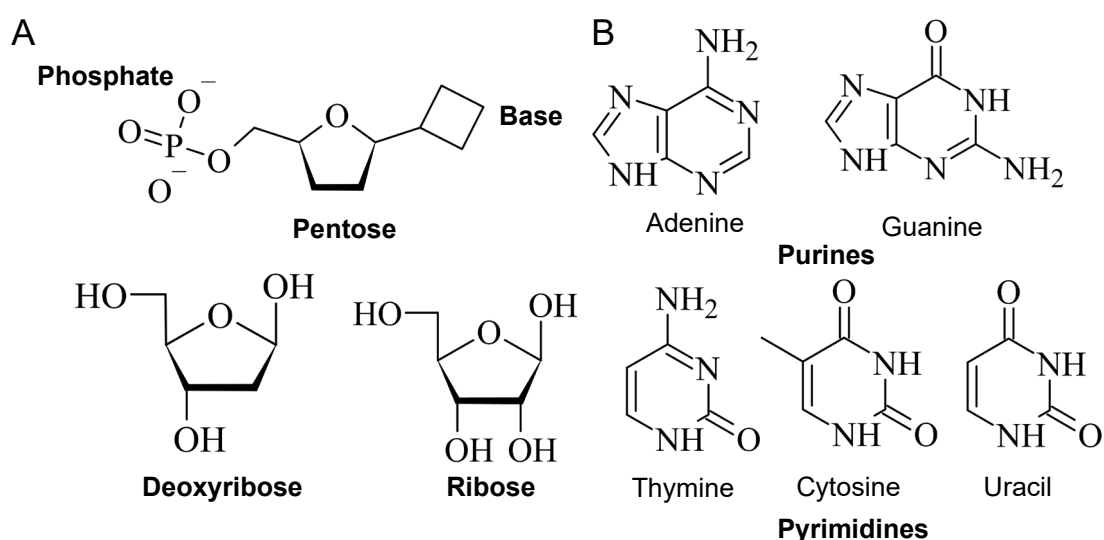


Figure 1.1. (A) Schematic representation of nucleic acid structure, highlighting nucleotide components: pentose sugar (deoxyribose in DNA or ribose in RNA), phosphate group, and nucleobases. (B) The major purine and pyrimidine bases of nucleic acids.

Nucleic acids are polymers composed of repeating nucleotide units. Each nucleotide comprises three essential components: a pentose sugar—deoxyribose in DNA or ribose in RNA—a phosphate group that links successive nucleotides via phosphodiester bonds, and a nucleobase (Figure 1.1A). The nucleobase could be categorized as purine (adenine and guanine) and pyrimidines (cytosine, with thymine in DNA or uracil in RNA) (Figure 1.1B). The formation of phosphodiester bonds between the 5' and 3' carbons of adjacent sugars impart a distinct structural

polarity to the polymer (Figure 1.2).⁶ In addition, the nucleobases establish specific hydrogen-bonding interactions—two hydrogen bonds in A-T (or A-U) pairing and three in C-G pairing—that not only stabilize the canonical double helix of DNA but also enable single-stranded nucleic acids to fold into complex secondary and tertiary structures.

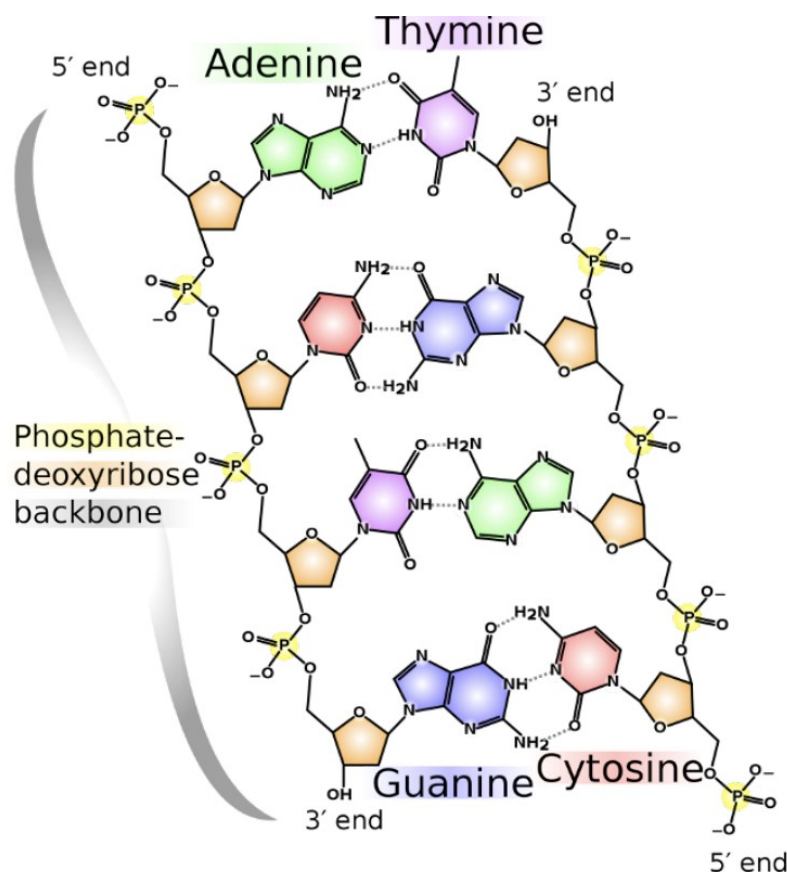


Figure 1.2. Chemical structure of duplex DNA. Adapted from Ref. ⁶. Copyright 2019 Springer Nature.

The identity and arrangement of nucleobases encode genetic information while also conferring significant structural versatility. In DNA, the absence of the ribose 2'-hydroxyl group, combined with the presence of a methyl group on thymine, enhances chemical stability and favors long-term information storage.⁷ Conversely, the 2'-hydroxyl group in RNA contributes to its increased structural lability, thereby facilitating rapid conformational changes.⁷ The sequence-specific organization of nucleobases drives a self-assembly process that dictates secondary structure formation. Canonical Watson-Crick base pairing forms helical regions, while non-canonical interactions—such as Hoogsteen bonding seen in G-quadruplex structures, wobble base pairs like G-U in RNA, and even base triplets—add further complexity to the molecular architecture (Figure 1.3).⁸⁻¹¹ Moreover, base stacking interactions, resulting from overlapping π -orbitals between

adjacent aromatic rings, contribute significantly to duplex stability.¹² Electrostatic and solvation effects also play a crucial role; counterions such as Mg^{2+} and Na^+ help shield the negatively charged phosphate groups, thereby promoting compact folding.¹³ These structural features emerge from a delicate balance between favorable enthalpic contributions from hydrogen bonding and stacking interactions, and the entropic penalty associated with reduced conformational freedom.

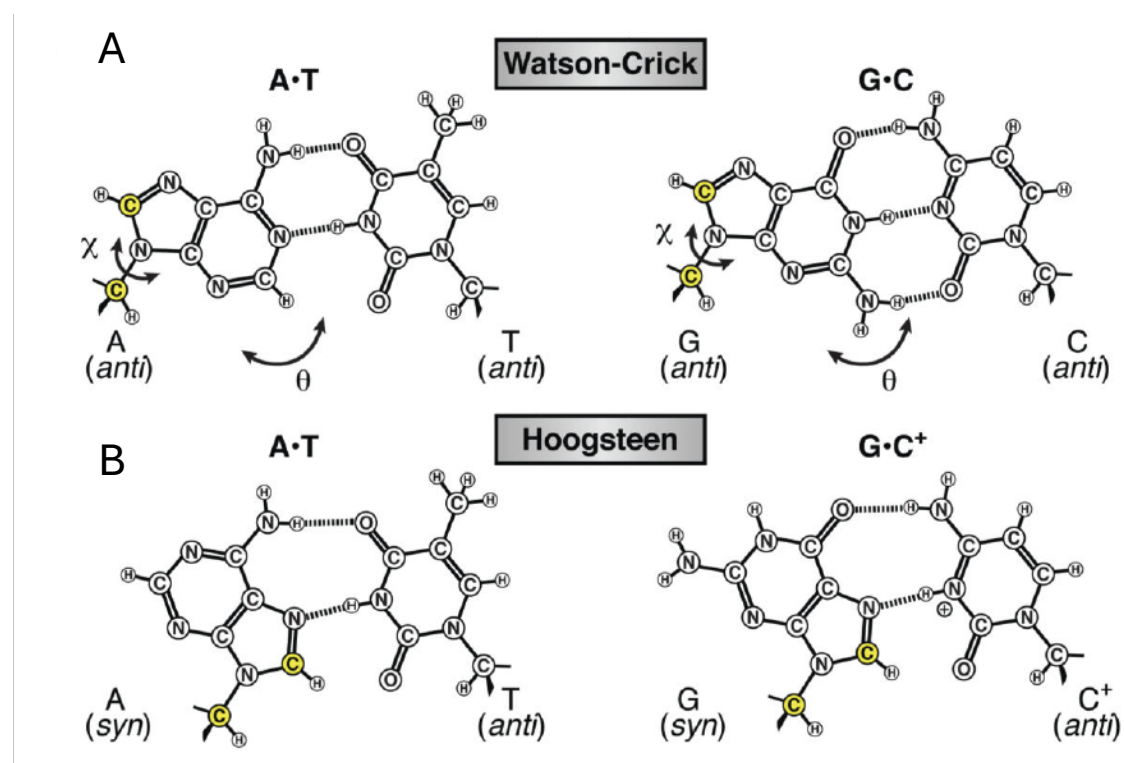


Figure 1.3. (A) The most common base pair found in RNA and DNA helices defined by Watson and Crick. (B) The structure of Hoogsteen base pair. Adapted from Ref. ¹¹. Copyright 2011 Nature.

Collectively, these molecular principles underpin a diverse range of nucleic acid topologies, including the B-form helix of DNA, A-form RNA duplexes, the left-handed Z-DNA, RNA pseudoknots, and G-quadruplexes stabilized by guanine tetrads. Notably, the structural plasticity of single-stranded nucleic acids facilitates the formation of ligand-binding pockets, catalytic centers, and dynamic molecular switches—features that are extensively exploited in aptamer engineering.¹⁴

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1.2 Aptamers

1.2.1 Brief overview of aptamers

Aptamers are single-stranded DNA or RNA oligonucleotides that fold into specific three-dimensional structures, enabling them to bind molecular targets with high affinity and specificity. They are typically selected through the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) process. Aptamers can recognize a diverse array of targets, including proteins, small molecules, metal ions, viruses, bacteria, cells, and amino acids.¹⁶ This versatility allows for widespread applications across various fields, such as environmental detection, in vivo regulation, drug delivery, and food safety.¹⁷

1.2.2 History of aptamers

Aptamer research has evolved remarkably since its inception in 1990. In that year, Tuerk and Gold pioneered the in vitro selection of RNA sequences capable of binding T4 DNA polymerase, thereby introducing the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) process and demonstrating the first instance of a protein-binding aptamer.¹⁸ In the same year, Ellington and Szostak independently isolated RNA aptamers that bind organic dyes which is the first examples of small molecule-targeting aptamers and introduced the term “aptamer,” derived from the Latin *aptus* (fit) and *meros* (part).¹⁹ Building on these foundational studies, Bock and colleagues in 1992 successfully selected a DNA aptamer that could bind and inhibit human thrombin, while Ellington and Szostak also reported a DNA aptamer capable of binding dyes around the same time.^{20, 21} In 1997, the Gold group extended the application of aptamer technology by using human red blood cells to select a DNA aptamer that specifically binds the transferrin receptor monomer (CD71).²² This trend continued in 1999 when Homann and Goringner employed live cells to isolate RNA aptamers that target African trypanosomes.²³ The clinical potential of aptamers was further underscored by the 2004 FDA approval of pegaptanib (Macugen), an RNA aptamer designed to bind the VEGF-165 isoform and inhibit VEGF-mediated angiogenesis, thereby reducing aberrant blood vessel growth in the retina.^{14, 24} In 2023, the FDA approved a second aptamer-based therapeutic, avacincaptad pegol. This PEGylated RNA aptamer targets complement protein C5, preventing its cleavage into biologically active fragments. Like pegaptanib, it is indicated for the treatment of age-related macular degeneration.²⁵

1.2.3 Mechanism of aptamer-target binding

Aptamers achieve high specificity and affinity for their molecular targets through an adaptive structural recognition mechanism. This binding is governed by sequence-dependent

folding and target-induced conformational changes, which together enable the formation of unique tertiary architectures that create complementary binding interfaces.^{16, 26} A variety of non-covalent interactions—including electrostatic forces, hydrogen bonding, π - π stacking, van der Waals forces, and hydrophobic effects—contribute to the overall stability of these complexes.¹⁶

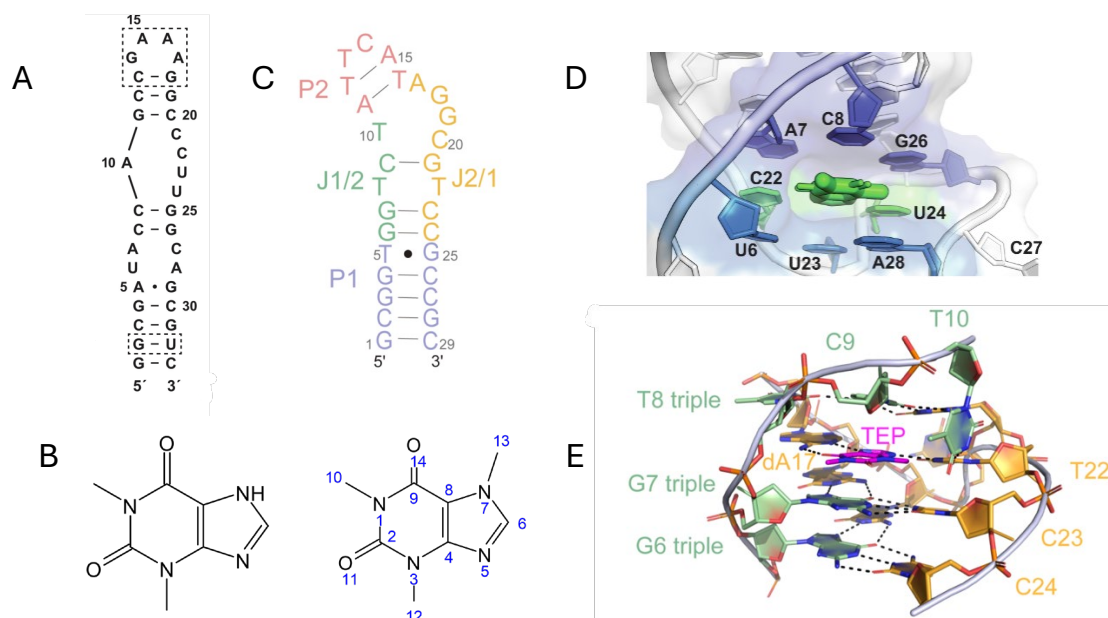


Figure 1.4. (A) Predicted secondary structure of the RNA theophylline-binding aptamer. (B) Chemical structures of theophylline and caffeine, differing only by a methyl group at N7. (C) Predicted secondary structure of the DNA theophylline-binding aptamer, showing the key base-paired stem and ligand-binding pocket. (D) The schematic of the RNA aptamer–theophylline complex from X-ray crystallography, with base triples that sandwich the ligand.²⁷ (E) X-ray crystallographic view of the DNA aptamer–theophylline complex, illustrating hydrogen bonds from A17 and T22 and π - π stacking interactions that stabilize the ligand within the binding site. Adapted from Ref. ^{27, 28}. Copy right 2022 PNAS and 2025 Angewandte Chemie.

A compelling example of this specificity and affinity can be observed in aptamers selected for theophylline. In 1994, Jenison et al reported an RNA aptamer for theophylline (Figure 1.4A) and its selectivity for theophylline over caffeine exceeded 10,000-fold, although these two molecules are structurally similar which have caffeine possessing an additional methyl group at the N7 position that theophylline lacks (Figure 1.4B).²⁹ In 2023, Huang and Liu reported a DNA aptamer for theophylline (Figure 1.4C) which binds its target with an affinity of approximately 0.5 μ M, over 250,000-fold higher than its affinity for caffeine.³⁰ Importantly, the 3D structures of both RNA and DNA aptamers have been solved. The RNA aptamer’s structure was solved using x-ray crystal and it binds theophylline via forms a base triple with C22 (two hydrogen bonds) and U24

(one hydrogen bond), which stacks between two other base triples (A7-C8-G26 and U6-U23-A28) (Figure 1.4D).²⁷ High-resolution X-ray crystallography has also elucidated the structure of a DNA aptamer bound to theophylline (Figure 1.4E).²⁸ In this complex, the theophylline O6 and N9 atoms each accept a hydrogen bond from the N6 of A17 and the N3 of T22, respectively, while the theophylline N7 donates a hydrogen bond to the N1 of A17. The purine ring of theophylline lies in a coplanar between the purine ring of A17 and the pyrimidine ring of T22, forming stabilizing π - π stacking interactions that reinforce the binding interface.

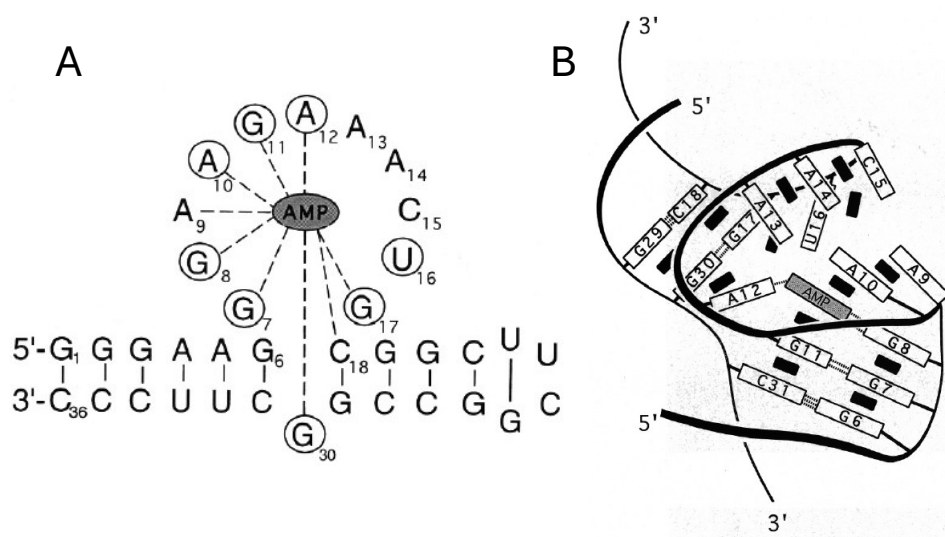


Figure 1.5. (A) The sequence and proposed secondary structure of RNA based ATP-binding aptamer. (B) The schematic of NMR-derived structure of an RNA aptamer bound to AMP.³¹ The conserved 11-base loop adopts a ζ -shaped conformation, enclosing AMP within a binding pocket stabilized by hydrogen bonds and π - π stacking with adjacent bases. Adapted from Ref. ³¹. Copyright 1996 *RNA*.

Another important aptamer with rich structural information is that for ATP. In 1993, the Szostak group reported an RNA aptamer for ATP.³² Dieckmann et al.³¹ used NMR to solve an ATP-binding RNA aptamer, revealing an 11-base internal loop flanked by a bulged G and duplex regions (Figure 1.5A). AMP binding triggers this loop to adopt a compact, ζ -shaped fold, enclosing the ligand in a pocket where multiple hydrogen bonds and π - π stacking interactions cement the complex (Figure 1.5B).

Early models of aptamer–ligand recognition emphasized on polar contacts and electrostatic complementarity, with hydrophilic functional groups seen as the primary drivers of binding and hydrophobic moieties largely considered detrimental. For example, Davis et al. (2013) described aptamer–target interactions as dominated by shape complementarity, hydrogen bonding, and

charge–charge contacts, noting that purely hydrophobic interactions—absent π -stacking—play only a minor role.³³ Similarly, Rohloff et al. (2014) reported that early SELEX libraries incorporating a hydrophobic pentynyl-dU modification yielded only micromolar-affinity binders, whereas subsequent inclusion of positively charged (hydrophilic) modifications produced aptamers with substantially improved affinities.³⁴ However, Stojanovic et al.³⁵ (2023) challenged this paradigm by systematically comparing functional-group contributions (Figure 1.6). The authors showed that aromatic and alkyl groups enhance affinity via hydrophobic contacts: for example, a phenylethylamine (PHEA) aptamer improves has a K_d of 7.2 μ M, which was much better compared to that for methylamine (12.3 mM), while the introduction of an isobutylamine moiety also tightens binding to 330 μ M (Figure 1.6A and B). In both cases, the increased hydrophobic surface reduces local water contact and entropy loss and engages in van der Waals interactions with methyl groups on thymine or adenine within the binding pocket.

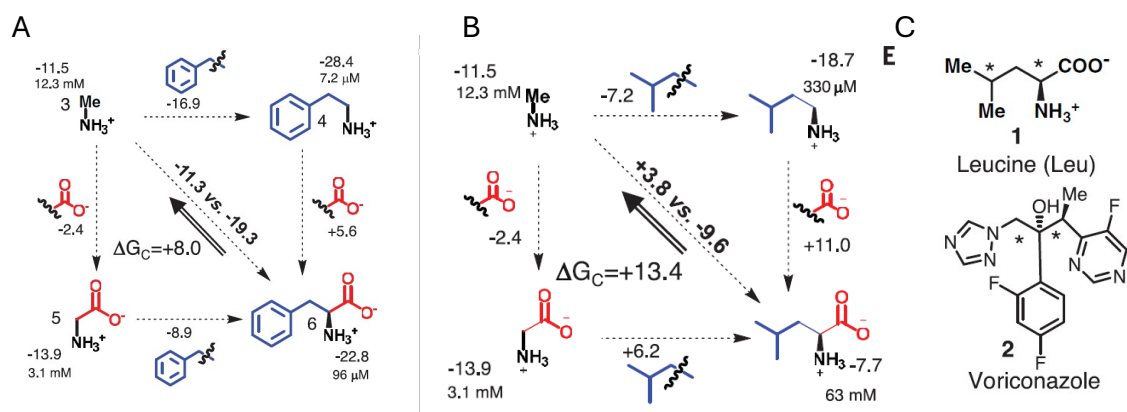


Figure 1.6. (A). Cooperativity is assessed by double functional group replacement cycles. (B). Double-functional group replacement cycle, methylamine to leucine. (C) The structure of leucine and voriconazole. Adapted from Ref. ³⁵. Copy right 2023 Science.

Conversely, steric hindrance can impair binding. For example, in leucine, the spatial proximity of its isobutyl side chain to the α -carboxyl group restricts hydrophobic contacts, elevating K_d . Likewise, the bulky, propeller-like conformation of voriconazole limits effective interface packing (Figure 1.6C).

Specificity—the multiplicity of target functional groups engaged—has traditionally been equated with higher affinity.³⁶ However, comprehensive analyses challenge this assumption. Xiao and co-workers³⁷ examined thermodynamic data from 317 aptamers, evaluating roughly 6,000 aptamer–ligand pairs by isothermal titration calorimetry (ITC). They found no simple relationship between affinity and specificity (Figure 1.7A). Instead, specificity was more closely linked to the

magnitudes of enthalpic and entropic contributions: aptamers displaying highly exothermic binding ($\Delta H_{\text{bind}} < -35$ kcal/mol) coupled with large entropy penalties ($T\Delta S_{\text{bind}} < -25$ kcal/mol) exhibited the greatest specificity (Figure 1.7B).

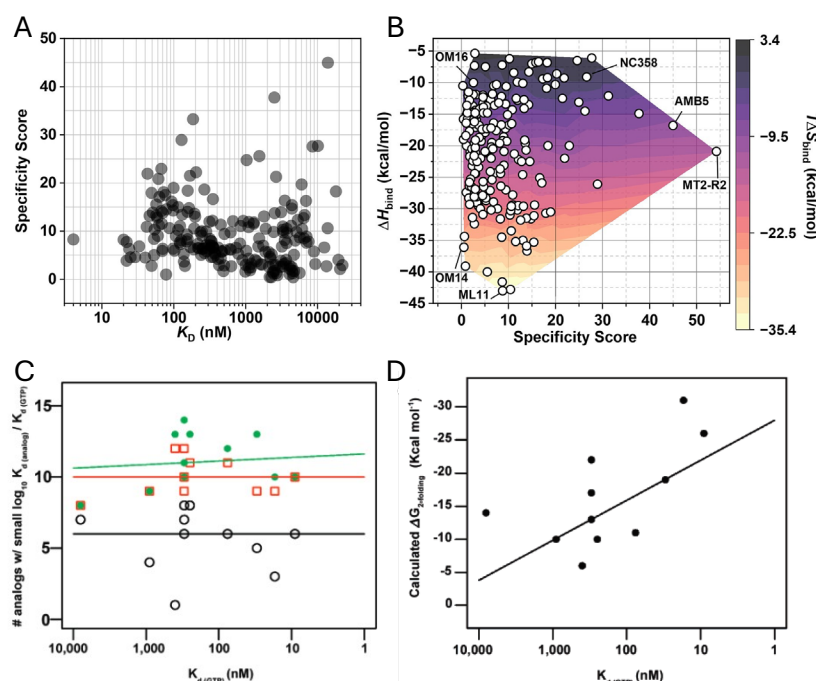


Figure 1.7. Relationship between aptamer specificity and binding thermodynamics. (A) Plot of binding affinity versus specificity score for ~6,000 aptamer–ligand pairs analyzed by Xiao’s group.³⁷ (B) Contour map of specificity score as a function of binding enthalpy (ΔH_{bind}) and entropy ($T\Delta S_{\text{bind}}$), with color indicating specificity.³⁷ (C) Specificity versus affinity for 11 GTP-binding RNA aptamers from the Szostak group. The number of GTP analogs for which $\log K_d(\text{Analog})/K_d(\text{GTP}) < N$ is plotted against K_d (GTP), using three thresholds: $N=1$ (open black circles), $N=2$ (open red squares), and $N=3$ (solid green circles). This demonstrates that higher affinity does not guarantee greater specificity.³⁶ (D) Correlation between the free energy of secondary-structure formation ($\Delta G_{2^\circ}^{\circ}(\text{folding})$), computed by RNAeval, and $K_d(\text{GTP})$ (in nM).³⁶ A significant relationship ($P < 0.02$) indicates that aptamer scaffold stability, rather than additional intermolecular contacts, underlies higher affinity. Adapted from Ref.^{36, 37}. Copy right 2006 Journal of the American Chemical Society and 2025 Nucleic Acids Research.

Similarly, Szostak’s group³⁶ measured the ability of 11 GTP-binding RNA aptamers to discriminate among 16 GTP analogs in competitive assays. They scored each aptamer for specificity and confirmed that tighter binding did not necessarily imply better discrimination (Figure 1.7C). Instead, they observed that apparent affinity for GTP correlated significantly ($P < 0.02$) with the

intrinsic stability of the aptamer's secondary structure—quantified as the free energy of folding, ΔG_2° —rather than with additional intermolecular contacts to the ligand (Figure 7D). This suggests that enhancing aptamer affinity may depend more on optimizing the scaffold's structural stability than simply increasing the number of target interactions.

These examples underscore how adaptive folding, combined with diverse non-covalent interactions, endows aptamers with both the strong binding and fine selectivity required for advanced applications in biosensing, in vivo regulation, and targeted delivery.

1.2.4 Comparison of DNA aptamers, RNA aptamers, and Antibodies

Antibodies represent a major competitor to nucleic acid aptamers in molecular recognition applications. Both modalities rely on non-covalent binding interactions such as hydrogen bonding, electrostatic forces, and π - π stacking to engage their targets; however, several key differences have significant practical implications.¹⁶ Although antibodies often achieve lower limits of detection and their larger size can simplify immobilization on biosensors and assay platforms, they also present notable drawbacks. Antibodies tend to be considerably more expensive, exhibit substantial batch-to-batch variability, and suffer from lower chemical stability.³⁸ Their fragility further complicates regeneration and reuse, as the conditions required for dissociating the target without denaturing the antibody are often harsh. Moreover, the development of antibodies against non-immunogenic targets, such as small molecules or metal ions, is challenging due to the complex hapten design and extended development timelines.¹⁶

Aptamers, in contrast, are broadly classified into DNA-based and RNA-based types. RNA aptamers are typically selected via the SELEX process and also occur naturally as riboswitches, where they regulate gene expression by monitoring intracellular ligand concentrations.^{39, 40} Owing to the inherent flexibility of RNA, these aptamers can adopt more diverse and intricate three-dimensional structures, which can translate into enhanced target-binding potential. For instance, RNA aptamers have demonstrated low micromolar binding affinities for ATP while maintaining high specificity between ATP and adenosine.⁴¹ On the other hand, early DNA aptamers often displayed moderate binding affinities—for example, a classical ATP-binding DNA aptamer selected in the mid-1990s exhibited an affinity on the order of 30 μ M.⁴² With improvements in SELEX technology, more recent DNA aptamers have achieved binding affinities in the low nanomolar range for small molecules.⁴³⁻⁴⁵ Additionally, DNA aptamers generally offer superior chemical stability and greater resistance to degradation compared to their RNA counterparts, making them particularly attractive for applications in complex assay environments.⁴⁶ The Table 1.1 compares the advantages and disadvantages of RNA aptamers, DNA aptamers and antibodies.

Table 1.1. Advantages and disadvantages of RNA aptamers, DNA aptamers and antibodies

Category	DNA Aptamers	RNA Aptamers	Antibodies
Advantages	• High chemical stability • Low production cost • Rapid development cycle • Minimal batch-to-batch variability • Broad target compatibility • Easy to modify • Customizable assay conditions • High binding affinity to targets • Commercially available in portable biosensor formats	• Cost-effective • Rapid development cycle • Minimal batch-to-batch variability • Broad target compatibility • Easy to modify • Customizable assay conditions • High binding affinity to targets • Commercially available in portable biosensor formats	• Exceptionally high binding affinity • Widely integrated into commercial portable biosensors due to robust performance and ease of immobilization on assay platforms
Disadvantages	• Generally lower binding affinity than antibodies • Lack of established portable biosensor formats for some applications	• Lower chemical stability, making them more susceptible to degradation in complex environments • Lack of commercial portable biosensors in some cases	• High production costs • Significant batch-to-batch variability • Limited applicability for non-immunogenic targets such as small molecules, metal ions, or toxic compounds

In summary, while antibodies offer certain advantages in sensitivity and ease of integration into various assay formats, aptamers provide compelling benefits in terms of cost-effectiveness, stability, and versatility. Their ability to be regenerated and optimized through selection processes makes them especially valuable for targeting non-immunogenic molecules, ultimately positioning aptamers as a robust complement—or even an alternative—to conventional antibody-based recognition systems.

1.3 SELEX procedures

SELEX (Systematic Evolution of Ligands by EXponential Enrichment) refers to an in vitro selection technique for isolating nucleic acid aptamers that adopt defined secondary and tertiary structures to bind a specific target. First described by Tuerk and Gold and Ellington and Szostak in 1990, SELEX begins with a highly diverse pool of single-stranded oligonucleotides, which is

incubated with a target of interest.^{18, 19} During this incubation, sequences with appreciable affinities form complexes with the target, while non-binders remain free in solution. A critical partitioning step determines the sequences that are retained for further amplification. For the selection of aptamers for small molecules, the major separation methods relied on the immobilization of either the target or the library to facilitate the separation.

Ideally, only high-affinity aptamers are collected, but in practice a significant fraction of non-specific sequences are also carried forward. To enrich true aptamers, SELEX is performed iteratively: after each round, the eluted pool is amplified by PCR, converted back into single strands (and, for RNA selections, reverse-transcribed to RNA), and then re-challenged with the target under increasingly stringent conditions. The degree of enrichment achieved in each cycle depends directly on the stringency and efficiency of the separation method which is a topic explored in detail below.

Once sufficient enrichment is achieved, the final pool is characterized by sequencing (via Sanger or high-throughput platforms) to identify aptamer candidates. These candidates are then synthesized and subjected to detailed binding assays to confirm affinity and specificity.

1.3.1 Target immobilization SELEX

Target-immobilized SELEX, one of the earliest and most widely used methods for screening aptamers, is applicable to a diverse range of target types including small molecules, proteins, polypeptides, and cells.⁴⁷ Figure 1.8 illustrates a scheme of a target-immobilization method for screening aptamers.⁴⁸ In this approach, a target is conjugated to a solid - phase carrier either directly or via a linker (using, for example, covalent chemical methods like EDC/NHS or a biotin modification to facilitate biotin-streptavidin binding). Figure 1.9A and 1.9B show the chemistry for the immobilization of ATP⁴² and theophylline,⁴⁹ respectively; both yielded the classical aptamers that have been used as model systems for aptamer research. After incubating the target-conjugated carrier with the oligonucleotide library, sequences that bind to the target remain attached to the carrier while non-binding sequences are washed away. The bound aptamers can then be eluted using methods such as by adding competitive ligands, heating, EDTA-mediated removal of magnesium ions, or alkaline conditions.

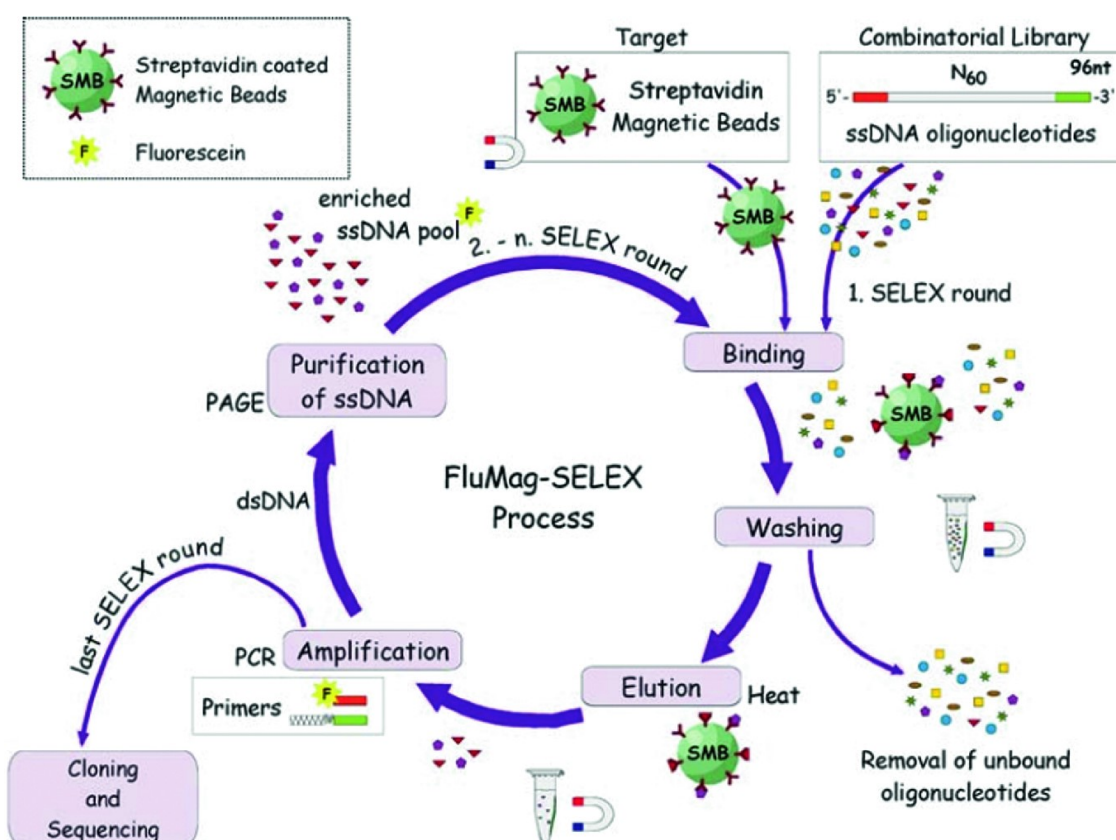


Figure 1.8. The schematic of target immobilization SELEX method. Adapted from Ref. ⁴⁸. Copyright 2021 Aptamers for Medical Applications: From Diagnosis to Therapeutics.

While many classical aptamers have been obtained using the target immobilization method, this method also has several challenges. The first problem is that not all target molecules can be immobilized, and trying to immobilize some very small molecules may lead to the enrichment of sequences that cannot bind to free target molecules. More importantly, because the target must be conjugated via one of its functional groups, the resulting aptamers might not recognize the native target if binding is directed toward the modified group, potentially reducing both specificity and binding affinity. Figure 1.9C shows the chemistry of immobilization of ethanolamine,⁵⁰ where its amine group is reacted. Compared to the free ethanolamine molecule, immobilization exposes only a single hydroxyl group; therefore, it is hard to imagine that the selected aptamer can bind to free ethanolamine. Indeed, when we used homogeneous assays, binding could not be confirmed.⁵¹ Similarly, Figure 1.9D shows the conjugation product intended for the selection of aptamers for arsenite.⁵² Compared to free arsenite, this structure has a benzyl ring connected directly to arsenic, which is chemically very different and binding of the reported aptamer to arsenite could not be confirmed either.⁵³

This issue is less problematic for larger molecules with multiple functional groups (such as cells and proteins) but can be critical for small molecules with limited functional sites. Additionally, non-specific adsorption of either the library or the target to the solid support can prolong the SELEX process or even lead to its failure. For example, solid - phase carriers like metal nanomaterials may non-specifically bind DNA, resulting in higher background signals. Similarly, targets such as cells and proteins may non-specifically absorb DNA, particularly when electrostatic interactions occur between negatively charged DNA and positively charged surfaces.

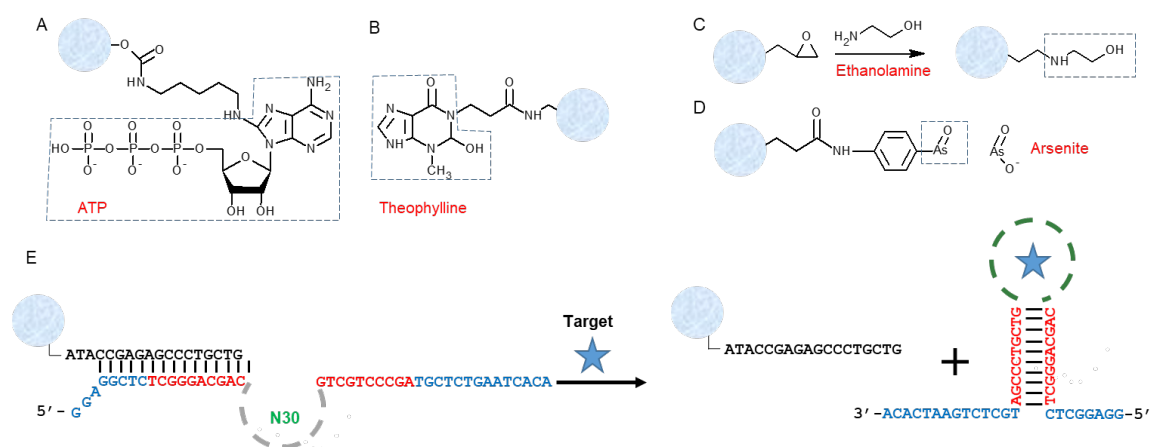


Figure 1.9. (A–D) Representative chemistries for covalently linking small molecules to a solid support for target-immobilized SELEX: (A) ATP, (B) theophylline, (C) ethanolamine, and (D) arsenite. Each molecule requires specific functional groups to enable conjugation while preserving relevant binding epitopes. (E) Schematic of the capture-SELEX approach, a library-immobilization strategy that allows the use of unmodified, free target molecules, thereby avoiding potential interference from surface tethering.

1.3.2 Library-immobilization SELEX methods

To address the limitations of target-immobilization SELEX methods, library immobilization methods have also been developed. These methods can be classified into two types. The first type is also called capture-SELEX, which immobilizes DNA libraries via hybridization. The second type immobilizes DNA libraries by adsorption on various nanomaterials.

1.3.2.1 Capture-SELEX

Capture-SELEX is a specialized library-immobilization SELEX method designed to accelerate the discovery of high-affinity DNA aptamers against small-molecule targets. This method was based on the strand-displacement idea demonstrated by Nutiu and Li.⁵⁴ Rajendran and

Ellington used this system for the selection of molecular beacons,⁵⁵ and Nutiu and Li first used it for selecting aptamers for small molecule targets.⁵⁶ The library sequence design was improved by the Stojanovic group, who incorporated a base-paired region in the two primer binding regions (Figure 1.9E).⁵⁷ Instead of immobilizing the target, the entire oligonucleotide library is anchored to a solid support via a biotinylated capture strand hybridized to streptavidin-coated beads. Each library member features a central random region, typically 30-40 nucleotides long, flanked by primer-binding sites that form a pre-engineered 10-base-pair stem-loop. Figure 1.10 illustrates the scheme of capture-SELEX method for screening aptamer.⁵⁸ Upon addition of the target, those aptamers that undergo a binding-induced conformational change are released from their capture strands and diffuse into the supernatant, while non-binding sequences remain immobilized on the beads. The collected sequences are PCR amplified to generate a new library for the next round of selection. After about 10 to 20 rounds of selection, the library is highly enriched and sequenced.

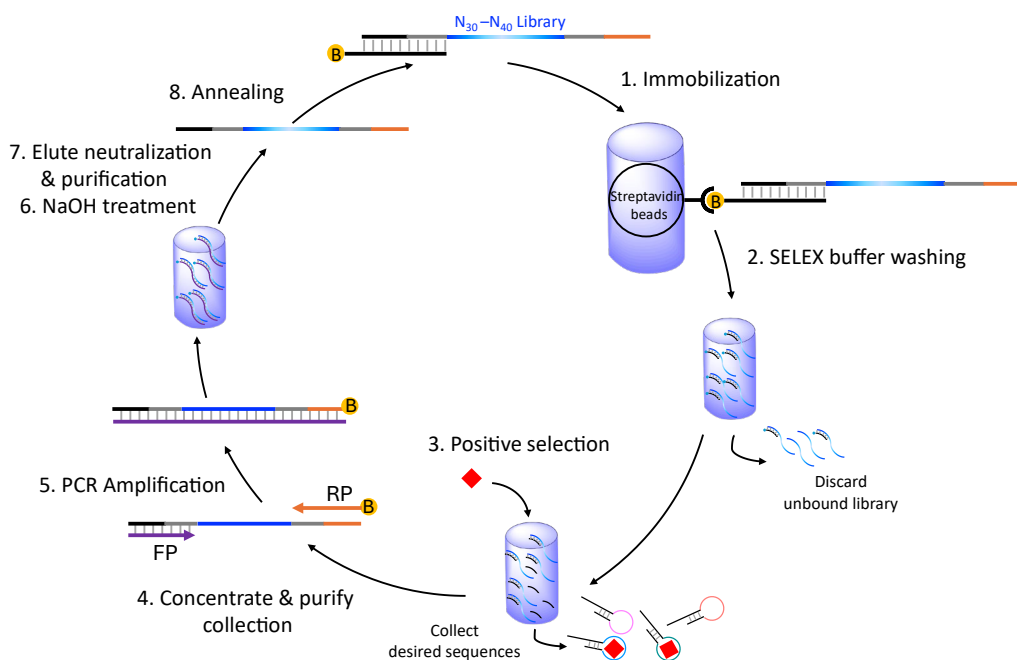


Figure 1.10. The schematic of capture-SELEX method. Adapted from Ref. ⁵⁸. Copy right 2023 Journal of the American Chemical Society.

Following immobilization, more than 99% of the library strands are bound through a 15-base-pair duplex, and stringent washing (twelve cycles) removes virtually all non-specifically adhered sequences, only about 0.13% elutes in the absence of target.⁵⁹ When the small-molecule ligand is introduced, a subset of library strands undergoes a conformational switch: the designed hairpin forms around the random region, drawing it away from five residual base pairs of the capture

duplex, which can no longer maintain hybridization. This destabilization triggers the selective dissociation of target-bound aptamers, which diffuse into solution for recovery and amplification. In our prior study, a single round of Capture-SELEX enriched adenosine-binding sequences by 30–50-fold, as confirmed by quantitative PCR and deep sequencing.⁵⁹

Because the target remains free in solution, native ligand epitopes are preserved, avoiding the masking effects often encountered when tethering small molecules. Capture-SELEX has proven effective for over 100 target molecules, including caffeine,⁶⁰ theophylline⁶¹, and its structural-switching mechanism readily translates into signal-on biosensors and conformation-dependent therapeutic systems. By combining homogeneous target presentation with solid-phase partitioning rigor, Capture-SELEX stands out as a versatile and powerful platform for small-molecule aptamer selection.

1.3.3 Comparison of aptamers from capture SELEX and target immobilized SELEX

In principle, different selection methods should result in similar aptamers. This was indeed observed in some cases. For example, the classical ATP aptamer was first reported using the target immobilization method in 1995, and it was re-discovered by the library immobilization by the Li group in 2005 and then by the Liu group in 2023.^{42, 58, 62} However, for many other targets, very different outcomes have been observed.

Figure 1.11 compares aptamers selected for the same small-molecule targets—oxytetracycline,^{45, 63} estradiol,^{64, 65} and chloramphenicol^{66, 67} by using either target-immobilization or library-immobilization approaches. Aptamers derived from target-immobilization SELEX tend to be unusually long (70-100 nucleotides), largely because the initial library lacks predefined secondary structure. As a result, these aptamers often resist rational truncation and display poorly defined folding motifs, making their biochemical and structural characterization difficult and hindering the design of streamlined biosensors. In contrast, the library-immobilization method begins with a pre-structured pool, which not only yields shorter sequences but also facilitates systematic truncation and reliable secondary-structure mapping.

Moreover, the choice of binding-affinity assay markedly influences reported K_d values: techniques such as isothermal titration calorimetry (ITC) and DNA strand-displacement reactions generally provide high accuracy⁶⁸, whereas gold-nanoparticle aggregation assays⁶⁹ and target-immobilization-based measurements can be less dependable—particularly in the absence of rigorously designed mutant or non-binding controls. Comparative analyses demonstrate that aptamers isolated via capture-SELEX achieve binding affinities on par with—or even superior to—

those obtained through traditional target-immobilization SELEX. Crucially, the capture-SELEX has been used to isolate aptamers against over 100 small molecules,⁷⁰⁻⁷⁶ representing a significant advance in the field by enabling the rapid discovery of concise, high-quality DNA aptamers.

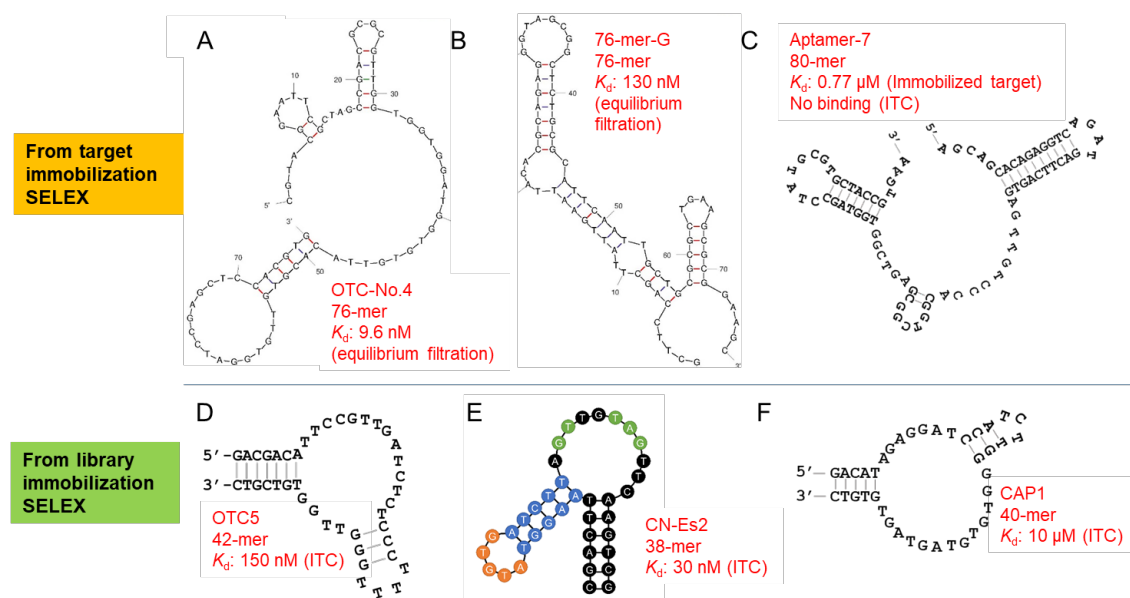


Figure 1.11. Secondary structures of aptamers selected for (A, D) oxytetracycline (OTC), (B, E) estradiol, and (C, F) chloramphenicol using two different SELEX strategies: (A-C) Target-immobilization SELEX and (D-F) library-immobilization (capture-SELEX) methods. The predicted secondary structures, aptamer lengths, dissociation constants (K_d), and corresponding measurement techniques are listed. Library-immobilization typically yields shorter sequences with more defined structures and improved potential for rational biosensor design.

1.3.4 Library adsorption-based methods for the selection of small molecule binding aptamers

Adsorption-based SELEX methods exploit the affinity between single-stranded DNA and certain nanomaterials to partition target–aptamer complexes from nonbinding sequences. In one workflow, a random library is first incubated with a target, and unbound strands are then captured by nanoparticles. Alternatively, the library may be pre-adsorbed onto nanoparticles and target binding induces aptamer desorption. Two prominent implementations of this strategy are GO-SELEX and gold-SELEX.

GO-SELEX leverages the strong π – π and hydrophobic interactions between unstructured ssDNA and graphene oxide (GO), a derivative of graphene bearing epoxy, carboxyl, and hydroxyl groups on its basal plane.^{77, 78} In the absence of a target, library strands adsorb readily onto the GO

surface; however, upon binding their cognate target (for example, the Nampt protein, as first demonstrated by Park et al.), these aptamer candidates undergo a conformational change that reduces their affinity to GO and allows them to remain in solution.⁷⁹ Subsequent removal of the GO pellet enriches for target-bound sequences. Figure 1.12 illustrate the scheme of GO-SELEX.⁸⁰ GO-SELEX has since been applied to a wide array of macromolecular and small-molecule targets, providing a convenient, solid-support-free selection strategy.

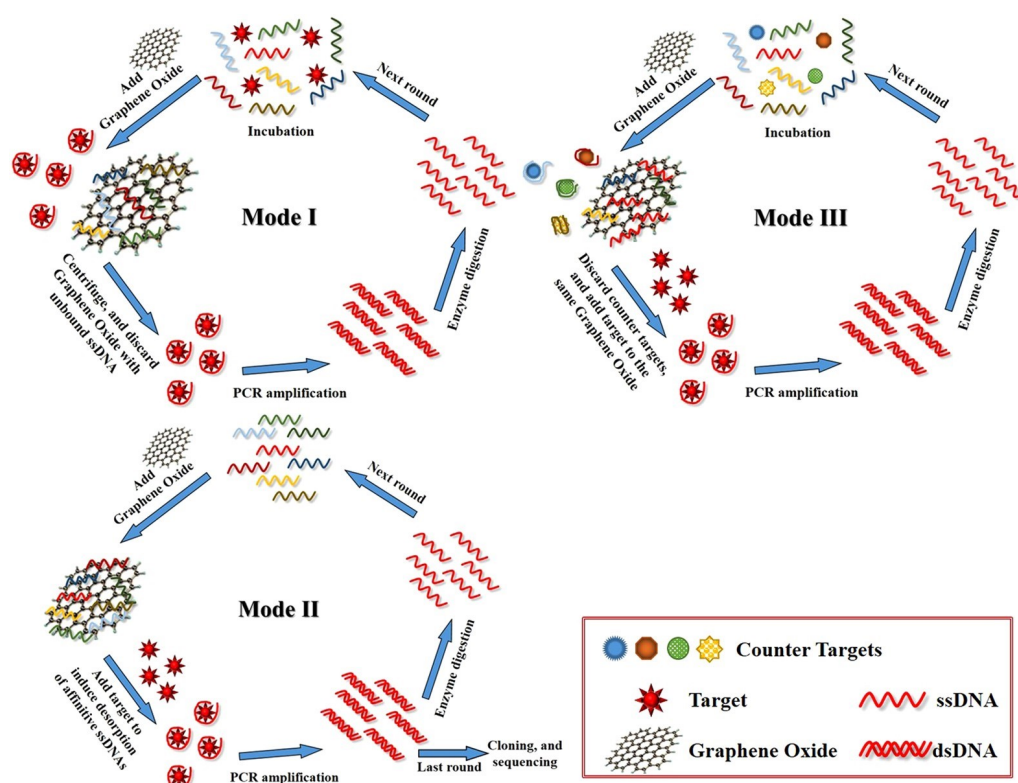


Figure 1.12. Schematic illustration of three models of GO-SELEX. Adapted from Ref. ⁸⁰. Copyright 2016 Scientific Reports.

Gold-SELEX uses citrate-stabilized gold nanoparticles (AuNPs) to achieve a similar end. All four DNA bases can coordinate with AuNP surfaces—adenine most strongly and thymine least—through their keto and imino moieties (Figure 1.13).⁷⁸ The strength of adsorption is quite high, exceeding 100 kJ/mol for each base in vacuum. Although both DNA and citrate-AuNPs carry negative charges, these interactions can be tuned by adjusting ionic strength and pH to promote adsorption of free library strands. Target-bound aptamers, in contrast, adopt folded structures that hinder AuNP binding. Upon salt-induced aggregation of unbound AuNP-DNA complexes, a visible color shift occurs (from red to purple/blue), allowing facile discrimination of bound versus unbound sequences directly in the selection buffer (Figure 13B).⁸¹

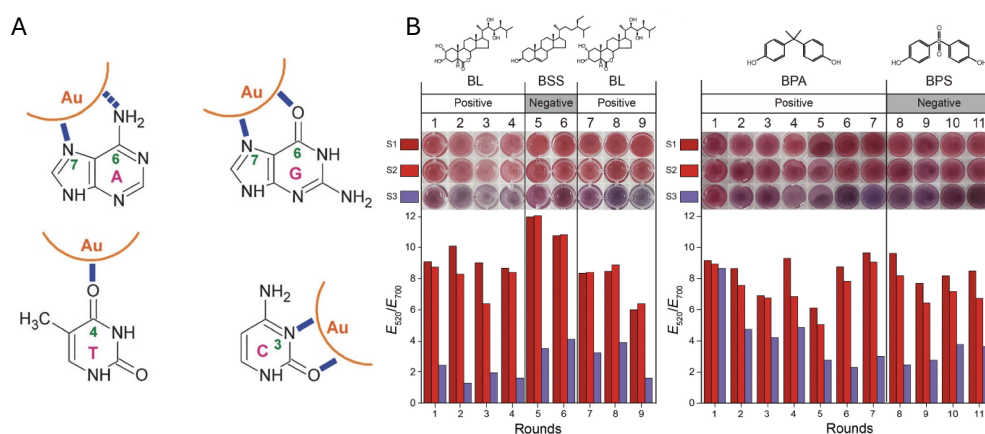
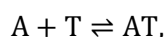


Figure 1.13. (A) Schematic illustration of DNA base adsorption onto the gold nanoparticle (GNP) surface, highlighting base-specific affinities. (B) GNP-SELEX workflow used by the Kim group to isolate ssDNA aptamers targeting butylated hydroxytoluene (BL) or bisphenol A (BPA). Visual color changes in microplate wells and quantitative extinction ratio values (E_{520}/E_{700}) are shown across SELEX rounds, indicating progressive aptamer enrichment. Adapted from Ref. ⁸¹. Copy right 2021 Biosensors and Bioelectronics.

Despite the increasing use of GO-SELEX and gold-SELEX for aptamer discovery, fundamental questions about their separation mechanisms remain. For an aptamer binding to its target, the aptamer must adopt a defined secondary structure, yet strong nanoparticle-DNA adsorption (as with gold nanoparticles) may prevent target-induced desorption in the “pre-adsorb” workflow or displace target–aptamer complexes in the “removing-free-library” approach. Conversely, if the adsorption of DNA is too weak, the separation efficiency may be compromised. In addition, pre-adsorption of DNA can perturb the secondary structures of DNA sequences, potentially impairing target recognition. To understand the aptamer enrichment efficiency of different library immobilization methods, we rigorously evaluated both GO- and gold-based methods and directly compared their performance against capture-SELEX for small-molecule aptamer selection.

1.4 Factors influencing aptamer properties during SELEX

Since non-immobilized target molecules are used in capture-SELEX, it provides an ideal platform for systematic investigation of aptamer selection. Several factors influence the ultimate binding affinity of aptamers enriched by SELEX, and these can be understood in terms of both thermodynamics and kinetics. Thermodynamically, the key parameter is the change in Gibbs free energy (ΔG) associated with the binding equilibrium



where the dissociation constant $K_d = \frac{[A][T]}{[AT]}$. The standard free-energy change ΔG^0 is related to K_d by

$$\Delta G^0 = RT \ln K_d,$$

so high-affinity aptamers (low K_d) necessarily correspond to more negative ΔG^0 . Therefore, the high-affinity aptamers could release out easier than low-affinity aptamers under similar target concentration.

Kinetically, the rates of association (k_{on}) and dissociation (k_{off}) also shape selection outcomes: fast on-rates improve the capture of rare, high-affinity sequences during brief target incubations, while slow off-rates help retain bound aptamers through stringent washing steps. Together, these thermodynamic and kinetic parameters define the window of selection stringency by adjusting pH, salt, target concentration, incubation time, and buffer structure, one can bias SELEX toward isolating small-molecule aptamers with better binding affinity.

1.4.1 Effects of target concentration

Stringency during SELEX exerts a powerful influence on the enrichment of high-affinity aptamers. At very high target concentrations ($[T] \gg K_d$), nearly all aptamer sequences, regardless of affinity, will bind to target molecules (thus be released from the capture strand), masking the difference in binding strength. Conversely, under limiting target conditions, only the tightest binders can compete effectively, driving their preferential retention and amplification.

Mathematical modeling by Soh and Atzberger⁸² confirms that lowering the target concentration enhances the theoretical enrichment efficiency of high-affinity aptamers (HAAs) (Figure 1.14A, B). However, these models also show that background binding (BG) imposes a practical limit: as nonspecific adsorption rises, the optimal target concentration must increase to overcome noise, which in turn reduces HAA enrichment (Figure 1.14C). Based on these insights, they recommend beginning SELEX with low target levels and incrementally raising them in tandem with improvements in library affinity, while employing techniques that minimize BG. Aita and co-workers⁸³ expand on this strategy by advocating a stepwise reduction of target concentration throughout the selection process through mathematical modeling calculation, thus continuously challenging the evolving library to yield progressively tighter binders. When dynamic adjustment is impractical, they suggest fixing the target concentration near the anticipated K_d of the best aptamers; although this approach may slightly diminish the overall enrichment efficiency, it still

effectively isolates high-affinity sequences. Together, these modeling studies affirm that low target concentrations can help select for superior binders, yet they also highlight the nuanced balance required between stringency and background noise. As a result, the optimal use of target concentration remains an area of active debate, underscoring the need for empirical validation tailored to each SELEX system.

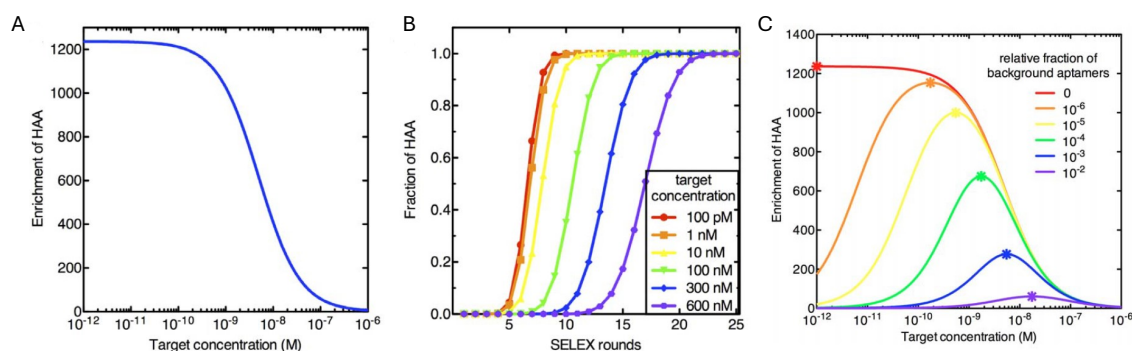


Figure 1.14. (A) Enrichment of high-affinity aptamers (HAAs) as a function of target concentration. As the target concentration decreases from 1 μM to 1 pM across six orders of magnitude, HAA enrichment increases significantly from 6.6 to 1240. (B) Fraction of HAAs in the selected pool over successive SELEX rounds, shown for different target concentrations. Lower target concentrations accelerate the enrichment of HAAs, supporting the theoretical advantage of high-stringency selection conditions. (C) Enrichment of HAA during first rounds of selection is shown as a function of target concentration at different background levels. Adapted from Ref. ⁸². Copy right 2012 PLOS ONE.

In 2022, Xiao and co-workers⁸⁴ investigated how target concentration influences aptamer enrichment in a Capture-SELEX campaign against the small molecule flunixin. In their first experiment (Trial 1), they began with 1 mM flunixin and gradually reduced the concentration to 20 μM over successive rounds (Figure 1.15A). The resulting pooled library exhibited only a modest affinity ($K_d \approx 251 \mu\text{M}$; Figure 1.15B, black trace), and individual aptamers isolated from this pool bound flunixin with K_d values in the 4-27 μM range. Concluding that high initial target levels had favored enrichment of low-affinity sequences, they repeated the experiment, this time starting at 600 μM and stepping down to 10 μM named as Trial 2. The second-round pool showed improved binding ($K_d \approx 77 \mu\text{M}$; Figure 1.15B, blue trace), and one aptamer (FX4) achieved a sub-micromolar K_d of 0.57 μM although its slow binding kinetics (~ 20 min to reach equilibrium in ITC) limited its practical utility; most sequences remained in the micromolar affinity range (2.7-735 μM). Further lowering the target concentration from 200 μM to 100 μM in round 5 did not materially improve the pool's affinity (Figure 1.15B, green trace), indicating that simply reducing target levels was

insufficient (Trial 3). Finally, they adopted a high-stringency protocol, commencing at 100 μM and stepping down to 10 μM named as Trial 4 (Figure 1.15C). Under these conditions, the eluted pool achieved a K_d of 35 μM (Figure 1.15B, red trace), and two aptamers, FX1 and FX3, were isolated with nanomolar affinities (0.28 μM and 0.34 μM , respectively). These results demonstrate that a low initial target concentration, combined with a step-down selection strategy, effectively suppresses the carry-over of low-affinity binders and enriches high-affinity aptamers.

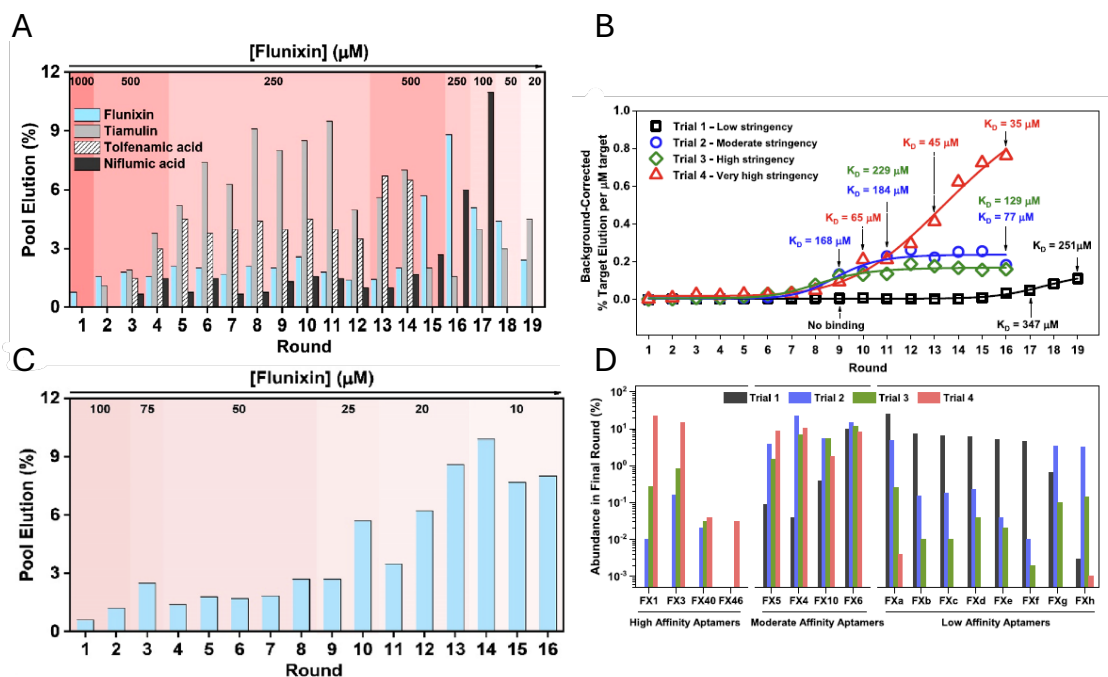


Figure 1.15. (A) Percentage of the library pool eluted by flunixin in each round of SELEX Trial 1, using a step-down concentration range from 1 mM to 20 μM . (B) Background-corrected percentage of the pool eluted per molar concentration of flunixin applied in each round. Dissociation constants (K_d) of selected pools, determined via gel elution assays, are indicated for specific rounds. (C) Percentage of the pool eluted by flunixin in each round of SELEX Trial 4, which employed a more stringent target concentration range from 100 μM to 10 μM . (D) Relative abundances of representative high-, moderate-, and low-affinity aptamer sequences in the final rounds of each SELEX trial, illustrating the impact of selection stringency on aptamer enrichment dynamics. Adapted from Ref. ⁸⁴. Copyright 2022 Journal of the American Chemical Society.

Although the Xiao group demonstrated that a stringent “step-down” selection environment can yield higher-affinity aptamers, their data also raise important questions about how best to choose and apply target concentrations. Deep sequencing of their flunixin SELEX pools (Figure 1.15D) shows that high-affinity clones became highly enriched in Trials 2, 3, and 4, but even in

Trial 2 (which lacked a strong stringency), these top binders were already present at appreciable levels. Moderate-affinity sequences, by contrast, remained abundant through Trial 4, and in Trial 1 (the least stringent), high-affinity clones were virtually undetected.

First, how should one select the initial and subsequent target concentrations? In Xiao's protocol, beginning at 1 mM and then dropping to 20 μ M yielded no high binding affinity aptamers which potentially because only two rounds were run below 100 μ M. It is unclear if additional rounds at 20 μ M (or lower) were performed, whether nanomolar-affinity aptamers can eventually dominate. Pinpointing this "sweet spot" is therefore critical: only by ensuring sufficient low-target cycles can we determine whether high starting concentrations are truly detrimental or simply masked by too few stringent rounds.

Second, might it be feasible to initiate SELEX entirely at low target concentrations and still overcome background noise? If so, what is the practical lower bound of [target] that still permits effective selection of high-affinity aptamers? Establishing such a threshold could suppress the carry-over of moderate-affinity sequences, simplify downstream analysis, and accelerate the isolation of nanomolar-class aptamers.

Answering these questions will reveal whether a gradual reduction in target concentration is truly essential or whether a single, well-chosen low concentration can streamline the SELEX workflow without sacrificing the recovery of the highest-affinity aptamers. In the following sections, we explore these issues through both modeling and experimental validation.

1.4.2 Effects of SELEX buffer

The composition of the SELEX buffer exerts a profound influence on aptamer enrichment by modulating both nucleic-acid folding and aptamer–target interactions. Two of the most critical parameters are the identity and concentration of cations, particularly K^+ , Mg^{2+} , and Na^+ , and the buffer pH.

1.4.2.1 Effects of cations on aptamer folding and binding

Potassium ions stabilize G-quadruplex motifs by coordinating between guanine tetrads, which can interfere with PCR amplification if carried over; accordingly, potassium-free PCR buffers are often recommended to prevent biases toward G-rich sequences.^{85, 86} Magnesium ions play a dual role: they shield the negatively charged phosphate backbone, promoting compact folding of the aptamer, and they can act as bridging ligands between phosphoryl groups on the oligonucleotide and polar functional groups on the target.⁸⁷ In the fluoride riboswitches, for

example, Mg^{2+} coordinates directly with both the RNA and the ligand to form the active binding pocket.⁸⁸ Sodium ions, although monovalent, can also weaken electrostatic repulsion and in certain cases serve as functional surrogates for magnesium. A uric-acid aptamer selected under Na^+ -rich conditions lost binding when K^+ replaced Na^+ , suggesting that Na^+ contributed directly to target recognition.⁸⁹ Subsequent selections confirmed that Mg^{2+} was required for high-affinity uric-acid binding, underscoring the need to tailor divalent-cation concentrations for each target.⁹⁰ Conversely, some aptamers, such as those for guanine and ampicillin, display similar affinities across a broad range of Na^+ and Mg^{2+} concentrations, indicating that not all selections are equally sensitive to cations.

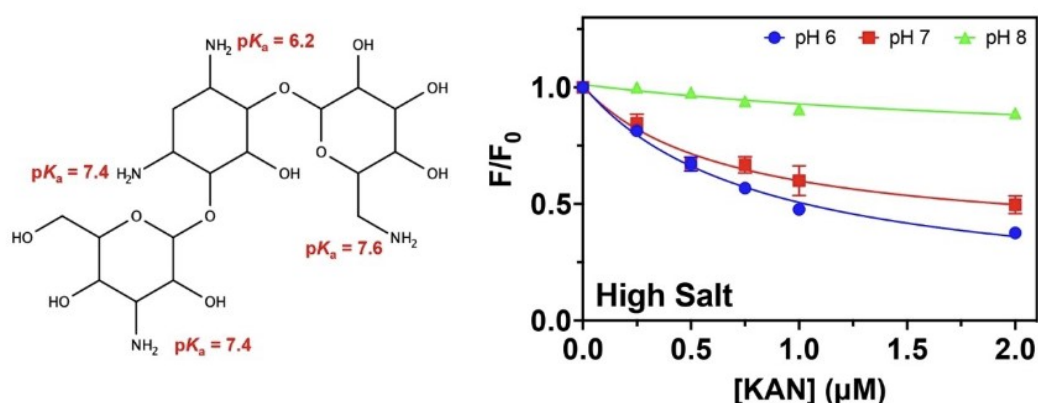


Figure 1.16. (A) Chemical structure of kanamycin A, highlighting its four amino groups and their respective pK_a values, which influence its protonation state across physiological pH ranges. (B) Fluorescence titration curves of kanamycin A (KAN) binding to 1 μM KAN6-1 aptamer in the presence of 1 μM Thioflavin T (ThT), measured under three different pH conditions. The results show strong pH dependence of aptamer-target interaction, with enhanced binding observed at lower pH due to increased positive charge on the target. Adapted from Ref. ⁹¹. Copyright 2025 Analysis & Sensing.

1.4.2.2 pH-Dependent target charge

Within the typical SELEX window (pH 6-8), unmodified DNA maintains its helical structure, but the protonation state of target functional groups can shift significantly. Kanamycin A⁹¹, for instance, contains multiple amino groups with pK_a values near 7; at pH 6 it is highly protonated and positively charged, promoting strong electrostatic attraction to the negatively charged DNA backbone (Figure 1.16). Parallel selections at pH 6 versus pH 8 revealed markedly

better enrichment of kanamycin-binding sequences under acidic conditions, yielding a 320 nM aptamer whose binding was strongly pH-dependent.

1.4.2.3 Effects of buffer molecules

Even nominally “inert” buffer components can inadvertently become selection targets. We previously demonstrated that Tris, a ubiquitous amine-based buffering agent, can be directly bound by DNA aptamers isolating a Tris-specific sequence with K_d values of 43 μ M (ITC) and 2.9 mM (fluorescence).⁹² By contrast, HEPES yielded no aptamer under identical conditions, suggesting that structural features of buffers (e.g., secondary amine versus sulfonate moieties) determine their propensity to interfere. Accordingly, buffer selection (Tris vs. MOPS, MES, CAPS, PIPES, etc.) should be carefully considered: structurally similar buffers may compete with or distract the evolving pool, leading to off-target enrichments.

In summary, SELEX buffer conditions, cation type and concentration, pH, and even the choice of buffering molecule, must be optimized for each aptamer-target pair to ensure robust folding, faithful target presentation, and minimal off-target selection.

1.4.3 Library length and design

A typical library design employed for SELEX consists of pools of single-stranded oligonucleotides containing both randomized sequence regions and defined primer binding regions. Typically, each oligonucleotide includes flanking consensus sequences that serve as primer binding regions for PCR amplification, along with a central random sequence region capable of adopting diverse secondary structures to facilitate target recognition. The length of the random region, often denoted as N , determines the total number of possible sequences in the library, given by 4^N . In principle, longer random regions enable the formation of more complex structural motifs, potentially enhancing binding specificity and affinity. Notably, Szostak successfully utilized a 220-nucleotide library to select a class I ligase, demonstrating the potential of extended sequence pools in functional RNA selection.⁹³

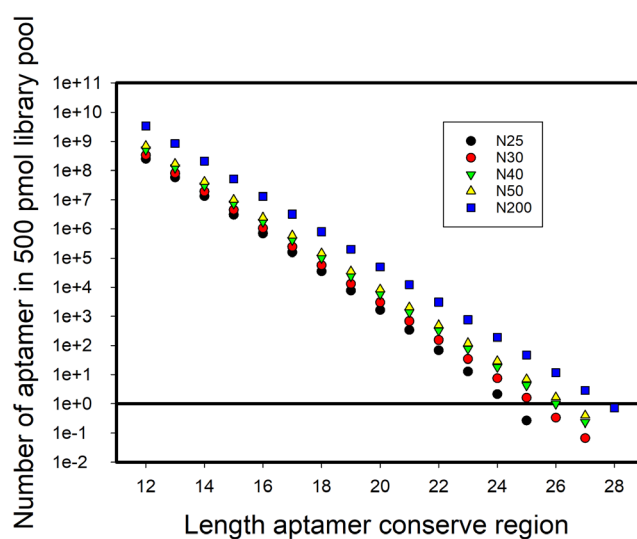


Figure 1.17. Relationship between the length of the aptamer conserved region and the number of such sequences present in a 500 pmol initial library. As the length of the random region increases, longer conserved regions become statistically more likely to occur within the pool. However, beyond a certain point, further increasing the random region length yields diminishing returns, as the overall sequence space becomes too large to be fully represented in the fixed library size limiting the practical gain in conserved region length within SELEX pools.

However, longer random regions are not always advantageous in SELEX. Excessively long sequences increase the risk of misfolding and aggregation, which may obscure binding sites and reduce functional aptamer recovery.⁹⁴ Additionally, longer sequences pose challenges for PCR amplification due to secondary structure formation and polymerase efficiency.⁹⁵ Moreover, as N increases, the theoretical sequence space grows exponentially, making it impossible for a practical library size to cover a meaningful fraction of all variants. In typical SELEX experiments, the initial library concentration ranges from 100 to 1000 pmol. For instance, a 1000 pmol library, equivalent to approximately 6×10^{14} molecules, can theoretically cover all possible sequences for a 24-nucleotide random region, as 4^{24} is approximately 2.8×10^{14} . In contrast, a 50-mer random region generates a sequence space of 4^{50} (approximately 1.3×10^{30}), far exceeding practical coverage. Achieving full sequence representation for a 50-mer would require an unfeasible quantity of DNA, estimated at roughly 2 million moles (or $\sim 6 \times 10^5$ kg of single-stranded DNA).⁹⁴ However, complete sequence coverage is not necessary for aptamer selection, as only a fraction of sequences within the library actively participate in target binding.

In the very first papers of the aptamer field, Ellington and Szostak wrote in the abstract that an aptamer sequence is present in approximately 10^{10} random sequences.²¹ With a simple

calculation, the authors observed that most aptamers they obtained have a conserved region of approximately 16 nucleotides. This length is also consistent with many of other reported aptamers. To quantify the relationship between aptamer sequence requirements and library length, a simple model can be designed. In a typical capture-SELEX experiment, the initial library has a size of approximately 500 pmol. Defining X as the number of conserved nucleotides required for target binding and N as the total random region length, the number of aptamer candidates in the initial library pool can be estimated using the equation:

$$Number = 3.01 * 10^{14} * (X - N + 1) * \left(\frac{1}{4}\right)^N$$

Figure 1.17 illustrates the relationship between X and the number of aptamers in a 500 pmol library under varying N values, as calculated using the equation. The data indicate that the conserved region length within an aptamer is constrained to approximately 25 nucleotides for N values between 25 and 40. Even at $N=200$, the maximum conserved region remains below 28 nucleotides. Consequently, most SELEX library designs employ a random region length of 30-40 nucleotides, optimizing the balance between sequence complexity and practical feasibility in aptamer selection. To address the limitations imposed by conserved region length, fixed-structure libraries have been developed as an alternative approach to enhance aptamer functionality.

In 2012, the Stojanovic⁹⁶ group employed a three-way junction structure for library design, using a random region of only 8 nucleotides to select aptamers for four different targets (Figure 1.18A). Although they successfully isolated aptamers for these targets after several rounds of selection, the binding affinities of the resulting aptamers were suboptimal. For example, using estradiol as a target, the authors isolated an aptamer with a K_d of approximately 1 μ M.⁶⁵ The secondary structure of this aptamer is shown in Figure 1.18B and it faithfully followed the scaffold structure of the initial library design. When Niu et al used a fully random library with an N30 region to do a selection, a few much higher affinity aptamers were obtained and one of them is shown in Figure 1.18C.⁶⁵ This aptamer has a K_d of 30 nM and its structure is very different from the three-way junction structure. Therefore, this pre-defined junction-based design, which also resembles a three-way junction, benefits from the incorporation of fixed DNA sequences that increase the fraction of the total sequence diversity represented in a practical library. However, the rigid nature of such fixed structures inherently limits the diversity of aptamer conformations and, consequently, their functional properties.

In 2016, Stojanovic⁵⁷ introduced a stem-loop structured library that incorporated additional base pairs to stabilize the aptamer's structure which is the major structure for capture-SELEX

(Figure 1.9E). Compared to the junction-based library, the stem-loop design offers a higher diversity of aptamer structures, with the stem region often playing a critical role in target binding. Typically, the random region in these stem-loop libraries spans 30-40 nucleotides, and by incorporating an extra 6 base pairs for binding interactions, this design further increases the fraction of total sequence diversity that can be practically represented. Moreover, aptamers derived from stem-loop structured libraries can be directly integrated into strand-displacement fluorescence sensors by labeling both the aptamer and a complementary strand with a fluorophore-quencher pair, which also aids in overcoming Debye length limitations in sensing applications.⁷¹ Consequently, this stem-loop structured library has become widely adopted for small molecule aptamer selection.

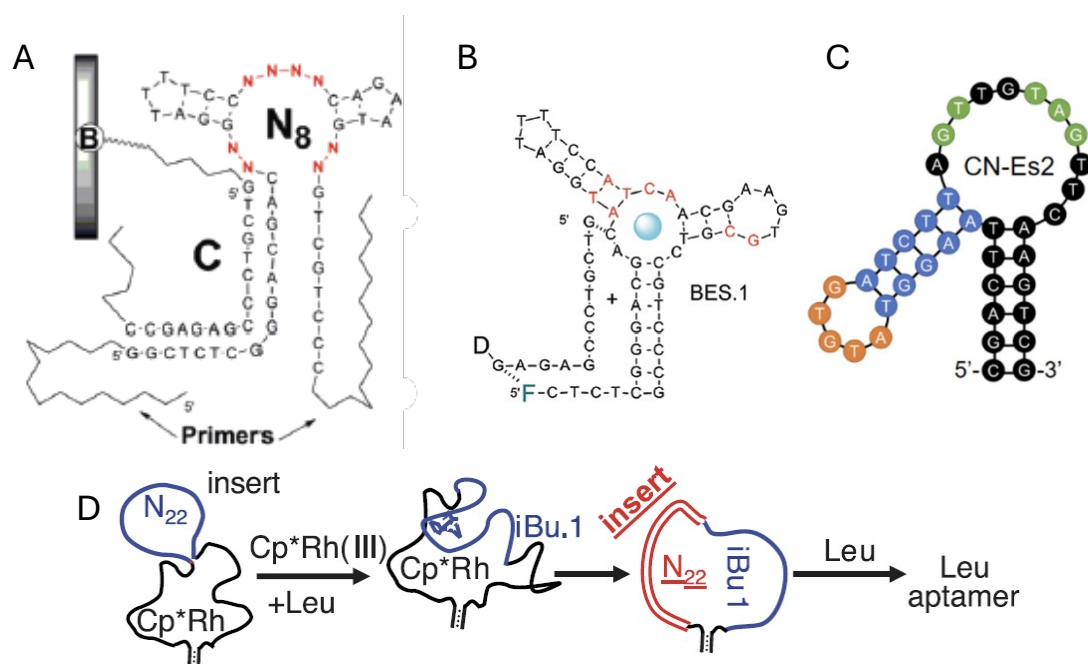


Figure 1.18. (A) Schematic representation of a three-way junction structure based on an N8 random region library design.⁹⁶ The secondary structure of estradiol aptamer selected by (B) three-way junction structure⁹⁶ and (C) fully random library.⁶⁵ (D) Stepwise aptamer selection strategy employed by the Stojanovic group³⁵: starting with a Cp*Rh(III)-binding aptamer, an N22 random region was inserted to generate a hybrid library for identifying the iBu.1 sequence, which contains key motifs for recognizing the isobutyl (iBu) group. A second N22 library (shown in red) was then introduced to apply selection pressure on the 2-aminoethanoate moiety, ultimately yielding aptamers specific for leucine. Adapted from Ref. ^{35, 65, 96}. Copy right 2012 Journal of the American Chemical Society, 2022 Environmental Science & Technology, and 2023 Science.

In 2025, the Li group⁹⁷ introduced a novel library design based on the partial randomization of an existing aptamer. In Li's approach, the previously selected MSAS2 aptamer, which targets a

SARS-CoV-2 viral protein, was used as a template, and a mutation rate of 24% was introduced at each nucleotide site within a 40-nucleotide random sequence for further selection against mutant virus targets. The original MSAS2 aptamer was selected from a stem-loop structured library. This strategy generated an initial library pool containing approximately 2.4×10^{14} molecules. With a 24% mutation rate, it is expected that introducing mutations at 19 specific sites will yield a mutant variant that occurs at least once within the pool.

Compared to the original target aptamer selection, this mutation library sped up the selection process for targets with similar structures, which is an advantage given that mutant viruses often share significant similarity with the original virus. In contrast, a fully random 40-nucleotide library is expected to yield only about 3.4 sequences that incorporate a 25-nucleotide consistent conserved region. Although mutation-based libraries can accelerate aptamer discovery for large complex targets such as proteins or whole cells where each aptamer engages only a subset of surface epitopes, they are less suitable for small molecule selections. For proteins or cells, their large and complex structures offer multiple potential binding sites, and aptamers typically bind to only a portion of the target surface.^{98, 99} In contrast, small molecules are fully encapsulated within the aptamer's binding pocket; even a single functional-group difference (for example, the extra N7 methyl in caffeine versus theophylline) can dramatically alter binding affinity (Figure 1.4). As a result, the mutation strategy may not effectively explore the structural diversity needed to generate high-affinity aptamers for small molecules.

In 2023, to overcome the limited sequence capacity inherent to long conserved regions in traditional library pools, the Stojanovic group introduced a splice method for library design.³⁵ Initially, they attempted to select leucine-binding aptamers using a random 36-nucleotide library; however, the SELEX process failed under these conditions. To circumvent this issue, they divided the leucine molecule into two fragments, namely isobutyl and 2 aminoethanoate, and employed an N22 random library to first select an aptamer that specifically binds the isobutyl fragment (Figure 1.18D).

Given the relatively simple structure of isobutyl, the researchers combined the N22 library with an aptamer known to bind CpRh(III) to create a composite library for selecting targets comprising both isobutyl and CpRh(III). Sequence analysis of the resulting candidates yielded an aptamer capable of binding isobutyl. This isobutyl-binding aptamer was then inserted into the N22 library as a fixed motif, and a combinatorial library approach was employed to re-select for a leucine-binding aptamer. A similar splice method was later adopted by the Xiao group. They fixed the conserved region of an existing morphine aptamer, appended an additional random region, and

performed a re-selection, termed Motif-SELEX, for morphine binding. This approach enhanced the binding affinity of the re-selected aptamer from 12.6 μM to 1-2 μM .

1.4.4 Kinetic influence

Kinetic factors play a crucial but often overlooked role in determining which aptamers are enriched during SELEX. Although many groups engineer “slow-off” aptamers (for example, by base modification or elevated selection temperatures) to enhance practical sensor performance, the impact of kinetics on overall SELEX outcomes has received comparatively little attention. Since the dissociation constant K_d equals to $k_{\text{off}}/k_{\text{on}}$, both the association rate (k_{on}) and dissociation rate (k_{off}) directly influence affinity.

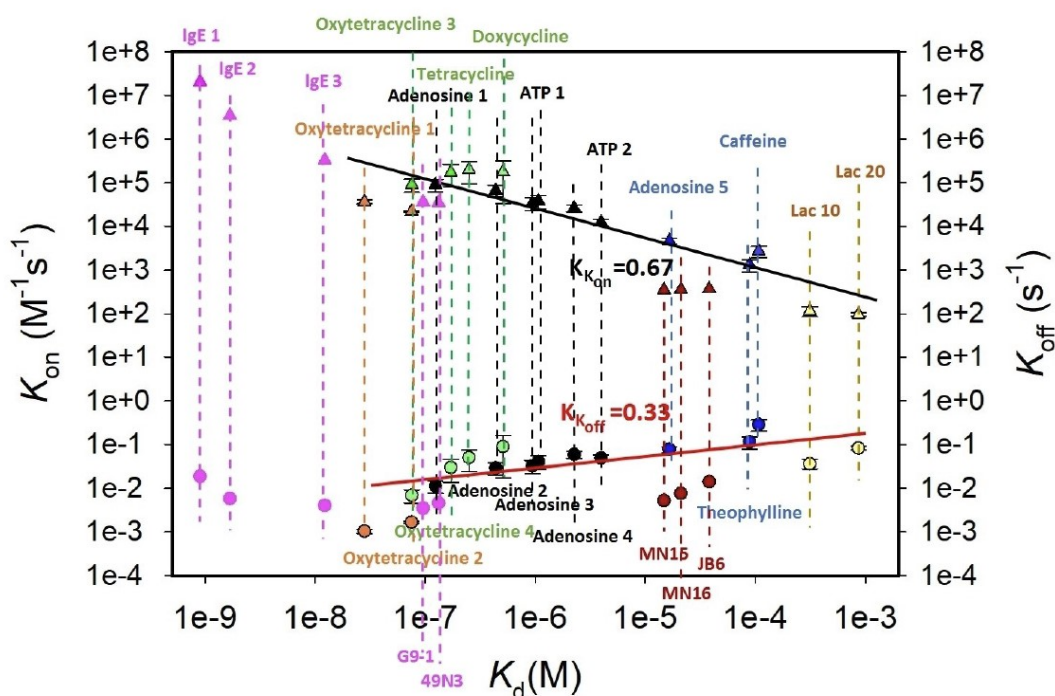


Figure 1.19. Plot of log-transformed association rate constants $\lg(k_{\text{on}})$ and dissociation rate constants $\lg(k_{\text{off}})$ versus log-transformed dissociation constants $\lg(K_d)$ for various aptamers. Both axes are presented on a logarithmic scale.¹⁰⁰ The data illustrate the relative contributions of k_{on} and to k_{off} overall binding affinity. The triangle is the data of k_{on} and circle is the data of the k_{off} . Adapted from Ref. ¹⁰⁰. Copy right 2024 ChemBioChem.

From our kinetic ITC study of small-molecule DNA aptamers, we found near-linear correlations of $\lg(k_{\text{on}})$ or $\lg(k_{\text{off}})$ and $\lg(K_d)$, with slopes of 0.67 and 0.33, respectively (Figure 1.19).¹⁰⁰ Over a K_d range spanning 28 nM to 864 μM , k_{on} dropped from 2×10^7 to $96 \text{ M}^{-1}\text{s}^{-1}$, while k_{off} rose from 1.03×10^{-3} to 0.012 s^{-1} . This indicates that high-affinity aptamers both bind their

targets more rapidly and release more slowly than weak binders. However, because even the weakest aptamers exhibit measurable off-rates, simply adjusting reaction times in conventional SELEX has limited discriminatory power: all sequences will eventually bind or dissociate given enough time.

In contrast, capture-SELEX inherently leverages toehold-mediated strand displacement (TMSD) kinetics to sharpen selection (Figure 1.20A).^{59, 101} In this three-step process, (1) the target-bound aptamer nucleates on its complementary “toehold” (the capture strand), (2) branch migration replaces base pairs in the capture duplex, and (3) the original capture strand fully dissociates, releasing the aptamer. The overall displacement rate

$$k_{(\beta^m, \beta_m)} = \frac{k_{r(\beta^m)} k_f k_b}{k_{r(\gamma^n)} k_{r(\beta^m)} + k_{r(\gamma^n)} k_b + k_{r(\beta^m)} k_b}$$

depends on the hybridization rate k_f , the toehold dissociation rates $k_{r(\gamma^n)}$ and $k_{r(\beta^m)}$, and the branch-migration rate k_b . From the three-step model system, the better binding affinity aptamers have the advantage during the first step toehold bind and all of aptamer should similarly releasing speed during the last step dissociation. However, the second step branch migration may be influenced by the structure of the aptamer.

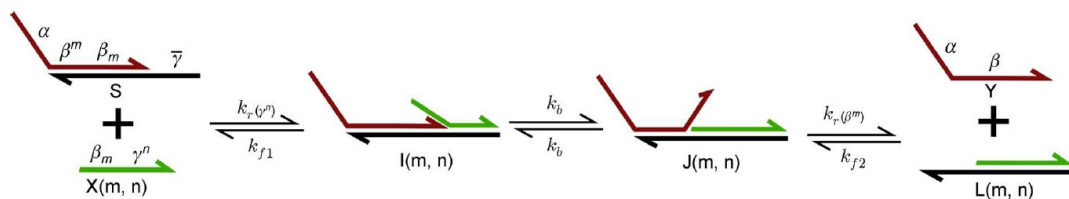


Figure 1.20. Mechanism of toehold exchange reaction. Adapted from Ref. ¹⁰¹. Copy right 2017 Quantitative Biology.

Empirical evidence supports this model. For example, a lactate aptamer with a free-binding rate of 357 min⁻¹ at 50 mM target produced a strand-displacement signal rate of only 1.3 min⁻¹, 274-fold slower, highlighting branch-migration as the rate-limiting step. Moreover, higher target concentrations shorten the half-time for displacement ($t_{1/2} \approx 0.06$ s at 10 mM versus 6 s at 1 mM), underscoring the interplay between thermodynamics and kinetics in dictating which aptamers are released.

Taken together, these insights suggest that by tuning incubation times, wash durations, and target concentration, one can bias SELEX, especially Capture-SELEX, toward aptamers that exhibit both rapid association and efficient strand displacement. In the following sections, we will explore

how kinetic optimization can be systematically applied to enrich high -affinity aptamers more effectively.

1.5 Scope of the thesis

Overall, the field of aptamer selection has been advancing dramatically since 1990, and many fundamental insights have already been obtained. Nevertheless, many important questions remained such as the optimal target concentration range for aptamer selection, the effect of target incubation time and the comparison of different library immobilization methods. Capture-SELEX has offered a useful platform to study these questions systematically, and the report of over 100 aptamers for different targets using this method has underscored its reliability. This thesis aims to use capture-SELEX to address the following problems.

Chapter 2: Target concentration is probably the most important factor in aptamer selection. We explore how target concentration influences aptamer enrichment by applying capture-SELEX to adenosine and ATP. By correlating target concentration with binding affinity of most enriched aptamers and conserved-region lengths of the aptamers, we tried to provide an example to define the “sweet-spot” concentration regimes for isolating nanomolar affinity aptamers. This work has provided new nanomolar affinity aptamers for adenosine and has improved an aptamer that was used by hundreds of groups since 1995.

Chapter 3: Following the studies in the Chapter 2, using low target concentration favors the enrichment of higher affinity aptamers. This viewpoint is also shared by other aptamer selection work. Then, a natural question is if there is a lower limit of target concentration, and if using too low of target concentration can lead to drastically increased selection cycles and even failed selections. To address these questions, using guanine as a model, we establish the practical lower limit of target concentration in Capture-SELEX. If the target concentration is comparable with the K_d of the aptamer, the enrichment of aptamer took more than doubled rounds. When the target concentration was about 5-fold lower than the K_d of the aptamer, we failed to enrich aptamers even after 17 rounds of selection. In this chapter, we determined how low one can push target levels before high-affinity binders fail to elute and related the target concentration to the K_d of aptamers.

Chapter 4: Aside from target concentration, another important parameter is the time of incubation. This incubation time was not previously controlled for capture-SELEX since most labs rely on gravity flow taking approximately 2 min to complete. Target solutions are always under a flow condition and thus some aptamers that release slowly from the capture strand may not be collected. We demonstrate that strand-displacement kinetics—specifically, dissociation rates from

the capture duplex—can sometimes override thermodynamic affinity during selection. Through varying the incubation time for the selection of aptamers for ampicillin and adenosine, we discussed conditions that favor the enrichment of thermodynamically strongest aptamers by mitigating kinetic bias.

Chapter 5: This thesis is based on hybridization-based library immobilization, which relies on a subtle energy difference between hybridization and aptamer binding to its target. This is not the only library immobilization method. For example, many nanomaterials have been used for library immobilization based on adsorption, and the most popular material for this purpose is graphene oxide (GO-SELEX). In addition, gold nanoparticles were also reported for this purpose (gold-SELEX). In this chapter, we quantitatively compared capture-SELEX with GO-SELEX and gold-SELEX—using a spiked aptamer library and deep sequencing. By measuring DNA release, enrichment factors, and sequence fidelity, we reveal much higher enrichment of aptamer in capture-SELEX, whereas the other two adsorption-based methods failed to show obvious enrichment. This study calls for a careful validation of selection methods using well-established targets first before using new methods for new targets.

Together, this thesis uses capture SELEX as a platform to advance understanding of how selection parameters such as concentration, kinetics and partitioning strategy influence the enrichment of high-performance aptamers for small molecule targets.

Chapter 2 Pushing adenosine and ATP SELEX for DNA aptamers with nanomolar affinity

The results presented in this chapter have been published as:

Ding, Yuzhe, and Juewen Liu. "Pushing adenosine and ATP SELEX for DNA aptamers with nanomolar affinity." *Journal of the American Chemical Society* 145.13 (2023): 7540-7547.

2.1 Introduction

In the aptamer field,¹⁰²⁻¹⁰⁴ the adenosine and ATP binding aptamers are among the most studied and used not only in biochemistry but also for analytical, biomedical and nanotechnology applications.¹⁰⁵⁻¹¹⁰ In 1993, Sassanfar and Szostak isolated an ATP-binding RNA aptamer ($K_d \sim 0.7 \mu\text{M}$), which can also bind adenosine.¹¹¹ In 1995, using the same ATP column, Huizenga and Szostak reported a DNA aptamer that can bind adenosine and ATP ($K_d \sim 6 \mu\text{M}$ adenosine).⁴² Later, NMR studies showed that the RNA aptamer binds one target molecule,^{31, 112, 113} whereas the DNA aptamer binds two.¹¹⁴ Despite this difference in binding stoichiometry, these two aptamers have some similarities in the way of target binding in terms of non-canonical G·A (the A being the target molecule) base pairing and its stacking with G·G pairs.

Over the last two decades, ATP has been used as a target by many groups to test new aptamer selection methods.¹⁰⁵ A very popular method is to immobilize nucleic acid libraries and use free target molecules for selections.^{55, 56, 71, 76, 115, 116} For example, Nutiu and Li used the library immobilization method, but obtained the exact same classical DNA aptamer.⁵⁶ The other reported aptamers were much less studied or used likely due to their lower affinities, longer sequences, less well-defined structures or containing modified nucleotides.^{41, 117-119} All reported DNA aptamers have K_d values higher than $6 \mu\text{M}$ for adenosine and ATP.

Interestingly, no one used adenosine as a target for selection and all the previous work used ATP.^{41, 42, 56, 111, 117-119} The interest in ATP stems from its biological importance. Aside from its role as the major energy currency in cells, ATP is also a biomarker for cancer and other diseases.¹²⁰ Being a major signaling nucleoside, adenosine is a highly important molecule as well with functions such as the regulation of sleep and stress.^{121, 122} Without the highly negatively charged triphosphate group, adenosine selections may result in new aptamers with stronger affinities.

In this work, we respectively used adenosine and ATP as targets to select new DNA aptamers using the library immobilization method.^{56, 71, 76} While we also observed the classical aptamer in both selections, new aptamers with higher binding affinities emerged when we pushed

the selections with increased stringency by lowering target concentrations. The growth of new aptamers also suppressed the classical aptamer.

2.2 Methods

2.2.1 Chemicals

The DNA samples used in this study were purchased from Integrated DNA Technologies (Coralville, IA, USA, see Table S1 for sequences). Streptavidin agarose resin was purchased from Fisher Scientific. AMP (adenosine monophosphate), guanine, uridine, cytidine, sodium chloride, and magnesium chloride were obtained from Amerseco (Framingham, MA, USA). ATP (adenosine triphosphate), ADP (adenosine diphosphate), thymidine, sodium hydroxide, hydrochloric acid, tris(hydroxymethyl)aminomethane (Tris), and Amicon Ultra-0.5 centrifugal filter unit (3k and 10k molecular weight cut off) were purchased from Millipore-Sigma (Oakville, ON, Canada). Micro bio-spin chromatography columns and SsoFast EvaGreen supermix were from Bio-Rad. dNTP mix, Taq DNA polymerase with ThermoPol buffer, and the low molecular weight DNA ladder were purchased from New England Biolabs (Ipswich, MA, USA). All of the buffers and solutions were prepared with Milli-Q water. SELEX buffer: 50 mM Tris, pH 7.6, 500 mM NaCl, 20 mM MgCl₂.

2.2.2 SELEX

The library design (Table 2.1) and selection method were based on previous reports of the Stojanovic group⁵⁷ with some modifications.¹²³ We used the same method and library for both selections. For each round, the single-stranded library was annealed by heating with five times excess of biotinylated capture strand in boiling water for 1 min followed by gradual cooling to room temperature over 30 min in the SELEX buffer. Meanwhile, 288 µL streptavidin agarose resin was loaded into a micro chromatography column and washed five times with 500 µL SELEX buffer. The annealed DNA was passed through the resin six to eight time for binding. After that, the resin was washed 12 times to remove unbound library. After washing, 750 µL of adenosine or ATP solution dissolved in the SELEX buffer was adding and the eluted DNA was collected using the gravity flow. The collected DNA was concentrated by a 3k molecular weight cut off filter and washed by Milli-Q water six times. The final volume of the purified DNA was adjusted to 60 µL using Milli-Q water. Biotinylated reverse prime was used to synthesize 3 mL PCR products, which were purified and concentrated to 250 µL with strand separation buffer (50 mM Tris, pH 7.6, 250 mM NaCl) by using a 10k molecular weight cut off filter. The purified PCR products were mixed with streptavidin agarose resin and washed ten times by the strand separation buffer. Then, 600 µL NaOH (0.2 M) was added into the column and incubated 40 min to elute ssDNA. After

neutralization, the concentration of the DNA was quantified by using a Spark microplate reader (Tecan) and it was used for the next round of selection.

Table 2.1. The DNA sequence used in this work

DNA names	Sequences (from 5' to 3')
N36 library	GGAGGCTCTCGGGACGAC-N36-GTCGTCCCGATCACTTGAATGGTCT
Reverse primer (RP)	AGACCATTCAAGTGATCGGGACG
Biot-Reverse primer	/5Biosg/ AGACCATTCAAGTGATCGGGACG
Biot-Column	GTCGTCCCGAGAGCCATA /3BioTEG/
Forward primer (FP)	GGAGGCTCTCGGGACGAC
Ade 1301	ACGACGTTTGCGATGAGAAACGTAATACATTGGTTTCGAAGGTCGT
Ade 1301b	ACGACGTTTGCGATGAGAAACGTATGGTTTCGAAGGTCGT
Ade 1304	ACGACACGGAGGCTTATGGTGTTTGCGCCGTAAATGGTCATGTCGT
Ade 1304b	ACGACACGGAGGCTTAGTTTGCTAAATGGTCATGTCGT
Classical DNA aptamer	ACCTGGGGGAGTATTGCGGAGGAAGGT
Ade 1301c	ACCACCTTTGCGATGAGAAACGTATGGTTTCGAAGGTCGT
Ade 1301d	ACGACGATTGCGATGAGAAACGTATGGTTTCGATGGTCGT
Ade 1301e	ACGACGTATGCGATGAGAAACGTATGGTTTCGTAGGTCGT
Ade 1301f	ACGACGTTTGCGATGAGAAACGTATGGTTTCAAAGGTCGT
Ade 1301g	ACGACGTTTGCGATGACAAACGTATGGTTTGGAAGGTCGT
Ade 1301h	ACGACGTTTGCGATGAGTAACGTATGGTTACGAAGGTCGT
Ade 1301i	ACGACGTTTGCGATGAGCGAAGGTCGT
ATP-CBA	CAAGCACCTTTTCTGCAGCGATTCTTGTTTAGAATCGTCGCCAACAGCCA
ATP-CBA truncated	CAAGCACCTTTTCTGCAGCGATTCTTGTTTAGAATCG
FAM-Ade1301b	/56-FAM/ CTCTCGACGACGTTTGCGATGAGAAACGTATGGTTTCGAAGGTCGTC
FAM-classical aptamer	/56-FAM/ CTCTCGACGACTGGGGGAGTATTGCGGAGGAAGTCGT
Quencher-strand	AGTCGTGAGAG /3IABkFQ/

Note: /5Biosg/ is biotinylation at the 5'-end, /3BioTEG/ is biotinylation at the 3'-end, FAM is carboxyfluorescein, and /3IABkFQ/ is Iowa Black FQ (IDT) quencher at the 3'-end.

2.2.3 Isothermal Titration Calorimetry (ITC)

All the ITC experiments were performed on a VP-ITC microcalorimeter instrument (MicroCal). Before each time experiment, the cell chamber and syringe were cleaned by Milli-Q water carefully. DNA aptamers and target molecules were dissolved in the SELEX buffer and then degassed for 5 min before experiment. 1.4 mL aptamer was loaded into the cell chamber and 300 μ L target was loaded into the syringe. The titration experiment were carried out at 25°C. Each time, 10 μ L target was injected except for the first time injection (2 μ L). The time between each injection was 360 s. The final thermodynamic values were obtained by fitting the titration curves to a one-site binding model by using the Origin software.

2.2.4 Sensor testing

The sensing experiments were performed in a 96-well microplate ($\lambda_{\text{Ex}} = 485$ nm; $\lambda_{\text{Em}} = 525$ nm) using a microplate reader (Tecan Spark). 1 μ M FAM-labeled Ade1301b aptamer was annealed with 2 μ M quencher-labeled DNA in the SELEX buffer. The final sensor contained 20 nM FAM-labeled strand in 100 μ L volume. A small volume of concentrated target molecule was added to trigger sensor response. For detection in serum, human AB serum was diluted into the SELEX buffer to reach a final of 10%, and 100 μ M of various target molecules were individually tested.

2.3 Results and discussion

2.3.1 Aptamer selections using adenosine and ATP as targets.

To select aptamers, we immobilized a DNA library containing a 36-nucleotide random region (N_{36}) by hybridization it to a biotinylated DNA attached to streptavidin-coated beads.^{61, 123} The initial library contained 500 pmol ($\sim 3 \times 10^{14}$) random DNA sequences. When adenosine or ATP was added, aptamer sequences were released, collected, and amplified by PCR. For the adenosine selection, the adenosine concentration was gradually decreased from 5 mM to 1 μ M over a total of 13 rounds. The PCR products from round 3, 6, 8, 9, 10, 11, and 13 were deep sequenced. A total of 30073 sequences were obtained from the round 13 library. We analyzed the top ten most abundant sequences, and they can be separated in two families (Figure 2.1A). The sequences in family 1 (represented by Ade1301) have two conserved regions marked in red. We used these conserved regions to search for the entire library, and family 1 represented 40.4% of the round 13 sequences. The family 2 sequences (represented by Ade1304) also have two conserved regions (blue/purple nucleotides in Figure 2.1A), and they represented 3.8% of the round 13 sequences (Figure 2.1C).

We then searched the two families from the other rounds and found that the family 1 sequences gradually increased over the selection rounds (Figure 2.1C, red bars). The family 2 sequences also increased but were overall much less compared to family 1 (Figure 2.1C, blue bars). We also searched for the classical aptamer sequence (containing GGGGA and GGAGGA) in the libraries. Its abundant peaked at round 6 (Figure 2.1C, black bars), after which it gradually dropped to only ~0.5% in round 13. This result can be explained by the low affinity of this classical aptamer ($K_d \sim 6 \mu\text{M}$). When we dropped the adenosine concentration to below $10 \mu\text{M}$, it was suppressed.

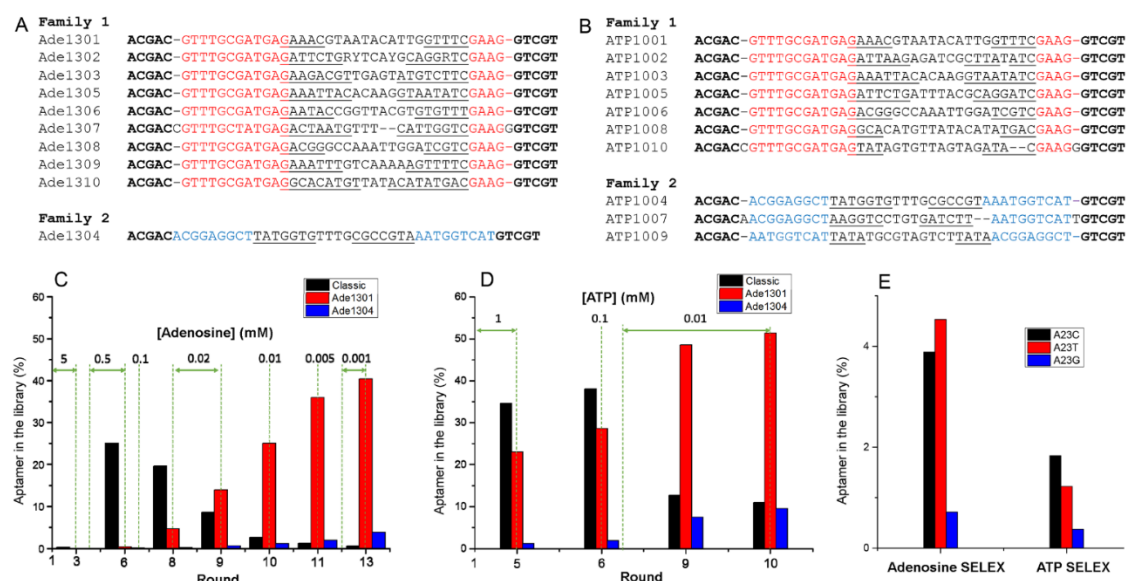


Figure 2.1. Alignment of the top 10 most abundant sequences in the final round of (A) the adenosine and (B) the ATP selections. The nucleotides marked in red and blue are the conserved regions of the aptamers. The N36 regions are in the middle, and we only kept five extra nucleotides on each end (in boldface) that can pair with each other. The abundance of the top sequences in different rounds of (C) the adenosine and (D) the ATP selections. (E) The abundance of a few mutated sequences at the 23rd position of the classical aptamer in round 6 of the two selections.

For the ATP selection, we started with 1 mM ATP and stopped at $10 \mu\text{M}$ over a total of ten rounds. We deep sequenced the PCR products from round 5, 6, 9, and 10. A total of 64769 sequences were obtained from the round 10 selection. Interestingly, the top ten most abundant sequences (named ATP1001 to ATP1010) also belong to the above two adenosine families, although relatively more family 2 sequences were present. For ATP round 10, family 1 accounted for 51.3% and family 2 reached 9.5% (Figure 2.1B and 2.1D). In the ATP selection, the abundance of the classical adenosine aptamer peaked in round 6 (38.0%) and decreased to 11.1% in round 10 (Figure 2.1D, black bars).

The two new aptamers have longer conserved regions (12+4=16 for Ade1301, 9+7=16 for Ade1304) compared to the classical aptamer (6+6=12). The longer the conserved sequences, the lower the abundance in the initial pool, which may explain the dominance of the classical aptamer in the early rounds. Recurrence of the same functional motifs was also observed in other selections such as repeated discovery of the 8-17 DNAzyme.^{124, 125} Our ATP and adenosine selections resulted in the same sequences (only differ by the relative abundance of the two families), suggesting that under our selection conditions, aptamer binding occurred mainly in the adenosine part. This is reasonable since the nucleobase can have more types of interactions with aptamers including hydrogen bonding and π - π stacking.^{31, 113, 114, 126}

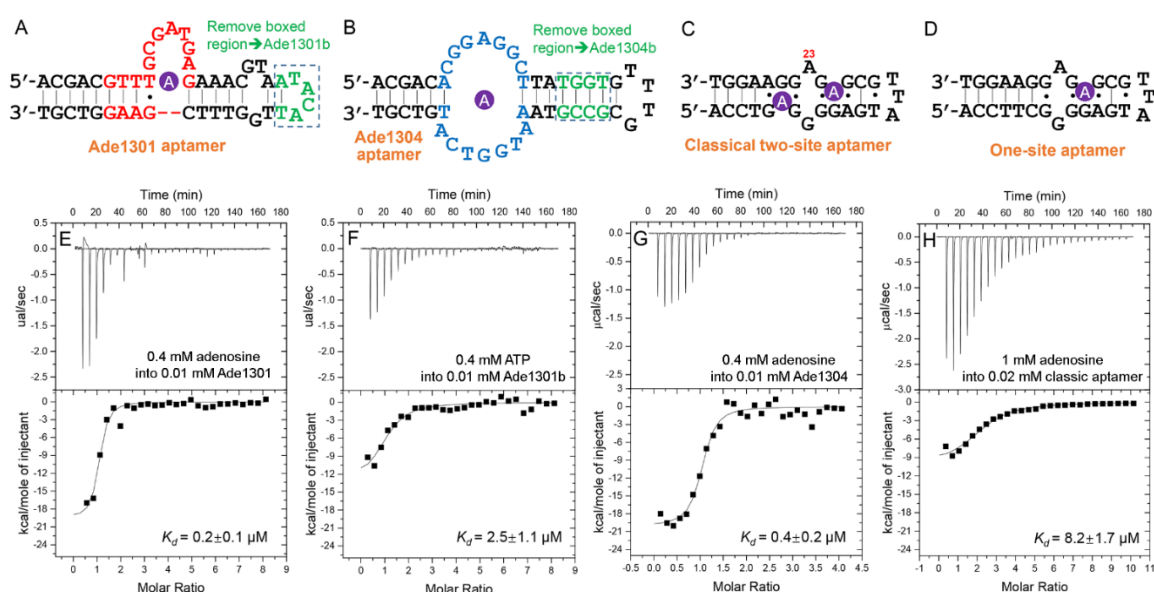


Figure 2.2. The secondary structure of (A) the Ade1301 (B) the Ade1304, (C) the classical DNA aptamer, and (D) a one-site classical aptamer. In (A, B), the red and green nucleotides are conserved, and the green nucleotides are truncated. The target molecules are represented by the purple A circles. In (C), the adenine in position 23 is marked and this number was based on the literature.¹²⁷ ITC analysis of aptamer binding by titrating (E) 400 μM adenosine into 10 μM Ade1301, (F) 400 μM ATP into 10 μM Ade1301b, (G) 400 μM adenosine into 10 μM Ade 1304Ade, and (H) 1 mM adenosine into 20 μM classical DNA aptamer. The buffer contained 50 mM Tris, pH 7.6, 500 mM NaCl, and 20 mM MgCl_2 .

By comparing the relative sequence abundance of the two selections at round 6, the ATP selection had more new aptamers, and this can be attributed to the use of lower ATP concentrations in the early rounds. For the selection by Huizenga and Szostak, a constant ATP concentration of 3 mM was used to yield the classical aptamer.⁴² For here, had we stopped at round 6 for the adenosine

selection (0.5 mM adenosine), we would have isolate the classical aptamer again as well. Although Nutiu and Li used a low target concentration of 0.1 mM ATP to finish their selections, they had two random regions (N₁₀ and N₂₀) linked by a constant region in their library design.⁵⁶ Such a design may disfavor the evolution of the new aptamers (e.g. the middle region needs to form a hairpin structure, *vide infra*). This may explain the dominance of the classical sequence in their selection.

2.3.2 New aptamers with nanomolar affinities.

For the adenosine selection, while over 40% of the round 13 sequences belong to family 1, the most abundant sequence Ade1301 had only 543 copies (1.8%) of the library. The Mfold¹²⁸ predicted secondary structure of Ade1301 is shown in Figure 2.2A, where the conserved nucleotides are colored in red, and the rest of the nucleotides from the random region can fold in a hairpin. We also checked the other top sequences and they also have such a hairpin structure (the stem region underlined, Figure 2.1A). Therefore, having a hairpin structure is important, although the sequence of the hairpin is less important. The secondary structure of Ade1304 is shown in Figure 2.2B, which also has two conserved regions colored in blue connected by a hairpin.

To measure the binding affinity of the selected aptamers, we used isothermal titration calorimetry (ITC).^{129, 130} We titrated 400 μ M adenosine into 10 μ M Ade1301 aptamer in the selection buffer. The ITC traces and integrated heat are shown in Figure 2.2E, and the exothermic binding can be seen from the downward spikes. The K_d value was fitted to be 350 ± 80 nM. To test the role of the hairpin, we deleted the nucleotides in the box and named this truncated sequence Ade1301b (Figure 2.2A). Ade1301b showed an even lower K_d of 230 nM (Figure 2.4A Table 2.2). Since Ade1301b is shorter, our subsequent studies focused on this truncated sequence. We then titrated Ade1301b with ATP (Figure 2.2F), wherein the heat release decreased nearly by half and the K_d was 2.5 μ M. This K_d value is also smaller than that of the classical aptamer for ATP.^{42, 131}

We then titrated adenosine into the Ade1304 aptamer, and a K_d of 230 ± 90 nM was obtained (Figure 2.2G). Since Ade1304 has a long stem, we deleted the boxed nucleotides in green and made a mutant named Ade1304b (Figure 2.2B), which had a similar K_d of 400 ± 160 nM (Figure 2.4B). When ATP was titrated into Ade1304b, the K_d was 2.3 μ M. For comparison, the classical DNA aptamer (Figure 2.2C) had a K_d of 8.2 μ M adenosine under the same buffer condition (Figure 2.2H), consistent with the literature.⁴² Therefore, the two new aptamers had ~35-fold higher affinity for adenosine than the classical aptamer.

The thermodynamic values from ITC are presented in Table 2.2. We first noticed that the binding stoichiometry was close to 1 for both Ade1301 and Ade1304. This is in sharp contrast to

the binding of two adenosine molecules by the classical aptamer (the last entry in Table 2.2). Their different sequences, affinities and stoichiometry indicated that we obtained two new aptamers. All the three aptamers can bind both adenosine and ATP. For the same aptamer, binding adenosine released more heat but had more entropy loss compared to binding ATP. The less entropy drop for ATP can be attributed to the phosphate chain still being flexible, also suggesting that binding took place mainly in the adenosine part.

2.3.3 Revisiting the classical aptamer: one-site aptamers and mutants from deep sequencing.

This is the first time that deep sequencing was done to generate nearly 10,000 classical aptamer sequences. The two previous selections only yielded less than 100 sequences.^{42, 56} We thus took this opportunity to answer some interesting questions regarding the classical aptamer. While the classical DNA aptamer is featured with two identical binding sites, sequences with just one binding site can also bind adenosine with a similar K_d .¹³¹⁻¹³³ An example of a previously characterized one-site binding aptamer is shown in Figure 2.2D, where the other binding site was removed by forming extra base pairs. Previous selections did not show any sequence with such a one-site structure, and the reason remained elusive.¹³² We then searched the round 6 sequences of the adenosine selection (Table 2.2). Indeed, we found one-site aptamers, although at a very low abundance of ~0.7% (312 out of 46239). One of the sequences from the search result was folded and it shows a one-site aptamer similar to that in Figure 2.2D (Figure 2.6C). Therefore, the previous selections missed the one-site sequences likely due to its low abundance, but such sequences can be evolved from selections. Since one-site aptamers had even less sequence requirements compared to the classical two-site aptamer, one would expect one-site aptamers to outnumber two-site ones. However, the opposite was observed. It might be that for a library with a long random region, having a very small binding region would pose more requirements for the rest of the sequences (e.g. need to form long base paired regions to consume the sequences).

We further used the sequences to analyze mutations. The A23 position in the classical aptamer (Figure 2.2C) can be mutated to other three nucleotides as seen in the original selection paper by Szostak.⁴² Xiao and coworkers separately modified A23 to thymine, guanine, and cytosine in the classical aptamer and proved the mutants can still bind ATP.¹²⁷ Interestingly, the A23T mutant led to better selectivity for adenosine over ATP. We then examined our round 6 adenosine selection library and indeed found all the three point mutations, although their abundance was lower than the classical sequence: A23C (3.9%), A23T (4.5%), and A23G (0.7%) (Figure 2.1E). Indeed, the A23T mutation had the highest abundance among the three, although they were all much lower

than the classical sequence (24.9%). We also searched the round 6 sequences of the ATP selection, in which the A23T mutant (1.2%) was less abundant than A23C (1.8%) (Figure 2.1E). This was also consistent with the expected selectivity. Therefore, using deep sequencing, we also gained more insights into the classical aptamer.

Table 2.2. Aptamer binding thermodynamic data from ITC.

Aptamer	Target	N	K_d (μ M)	ΔH (kcal/mol)	ΔS (cal/mol/K)
Ade1301b	Ade	0.73 ± 0.03	0.2 ± 0.1	-24.3 ± 1.6	-51.1
Ade1301b	ATP	1.1 ± 0.1	2.5 ± 1.1	-13.5 ± 2.2	-19.6
Ade1304b	Ade	1.00 ± 0.04	0.4 ± 0.2	-23.1 ± 1.2	-48.7
Ade1304b	ATP	1.04 ± 0.03	2.3 ± 0.4	-18.5 ± 0.8	-36.3
Classical	Ade	2.3 ± 0.1	8.2 ± 1.7	-10.2 ± 0.7	-11
Ade1301	adenosine	1.00 ± 0.04	0.4 ± 0.1	-21 ± 2	-36.2
Ade1304	adenosine	1.00 ± 0.03	0.2 ± 0.1	-20.1 ± 0.8	-37.1
Ade1301f	adenosine	1.05 ± 0.03	1.5 ± 0.3	-23 ± 1	-50.0
Ade1301g	adenosine	0.92 ± 0.05	1.1 ± 0.3	-27 ± 2	-62.6
Ade1301h	adenosine	0.92 ± 0.02	0.4 ± 0.1	-20.8 ± 0.6	-40.1

2.3.4 Secondary structure analysis.

Since the binding performance for adenosine and ATP was very similar for the two new aptamers selected in this work, and Ade1301 was the dominating sequence, we focused on it in this work. While Ade1301b appeared very different from the classical DNA aptamer, its secondary structure Ade1301b (Figure 2.3A) has some similarity to the classical RNA aptamer named ATP-40-1 (Figure 2.3C).¹¹¹ For example, the RNA aptamer has an unpaired guanine (G30) and the Ade1301 appeared to have a similar guanine (G32), if this guanine is not in the predicted G·T wobble pair in reality (Figure 2.3A). Mfold predicted base pairs all the way to this G·T wobble pair, including a G·G mismatch (G6·G35). To test the validity of this structure, we first made a G6C mutation (mutant named Ade1301c) to change the G·G mismatch to a C·G base pair. Interestingly, this change inhibited adenosine binding (Figure 2.3D), indicating that this GG is not a mismatched pair and thus the downstream base pairs may not be needed for aptamer binding. To test this hypothesis, we then swapped the next two A·T base pairs respectively (Ade1301d and 1301e) and

indeed neither showed binding (Figure 2.4C, D). We further made a G32A mutation in the Ade1301b aptamer (named Ade1301f), which still showed binding and its K_d value increased only 6-fold (Figure 2.3E). Therefore, this guanine can be mutated to an adenine, and it does not form a wobble pair with T9.

We then turned our attention to the hairpin. We swapped C31 and G17 to produce the Ade1301g mutant, and the K_d decreased only 5-fold (Figure 2.3F). We further swapped T30 and A18 (Ade1301h) and the K_d value was similar to Ade1301b (Figure 2.3G, Table 2.2). Therefore, C31 and G17 formed a base pair. Finally, we cut all DNA base in the blue box in Figure 2.3A to make Ade1301i, which abolished binding (Figure 2.3H). Therefore, the hairpin structure cannot be removed, although it can be shortened. The correct secondary structure is shown in Figure 2.3B, in which the two conserved regions are in two loops. These mutation studies also served as controls to confirm specific aptamer binding. We also noticed that for both the classical DNA and RNA aptamers and the two new aptamers reported here, their conserved regions are rich in purines.

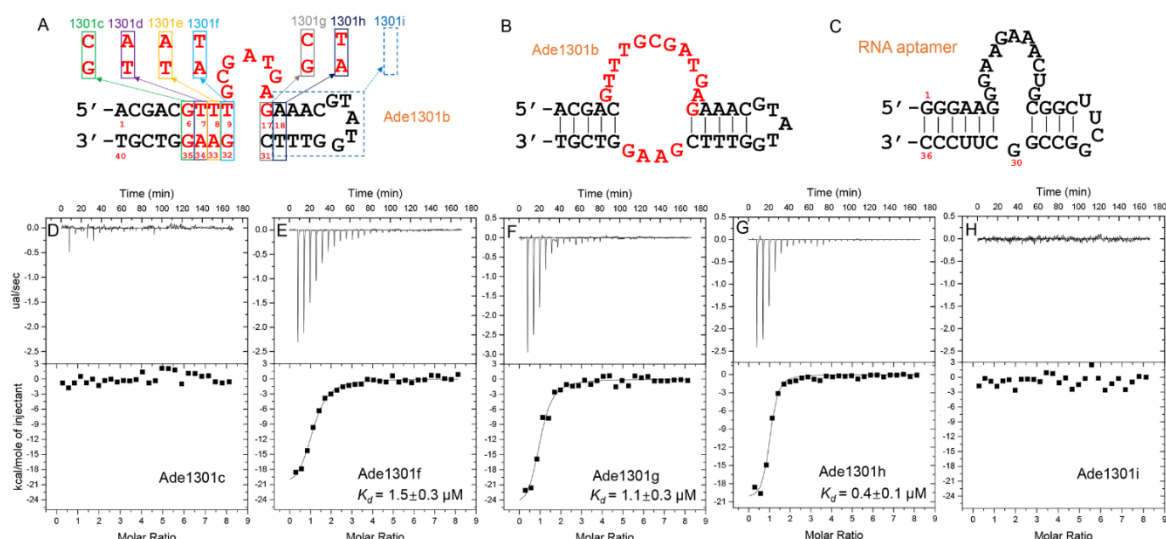


Figure 2.3. (A) The mutants tested to understand the secondary structure of Ade1301b. The secondary structure of (B) Ade1301b based on the mutation studies. (C) The secondary structure of the classical RNA aptamer. ITC analysis of aptamer binding by titrating 400 μM adenosine into 10 μM (D) Ade1301c, (E) Ade1301f, (F) Ade1301g, (G) Ade1301h, and (H) Ade1301i.

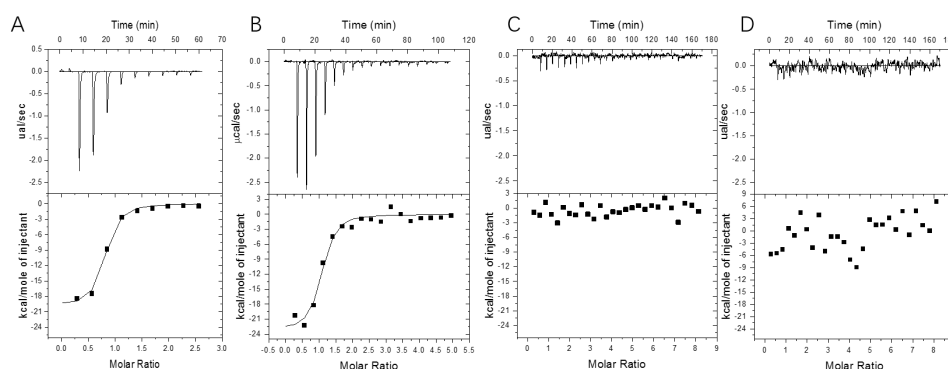


Figure 2.4. ITC analysis of aptamer binding by titrating 400 μM adenosine into 10 μM (A) Ade1301b, (B) Ade1304b, (C) Ade1301d, and (D) Ade1301e aptamers in the SELEX buffer.

2.3.5 Comparison with previously reported aptamers.

Table 2.3. The conserved regions used for searching one-site aptamers and their copy number in the round 6 of the adenosine selection.

Sequences used for searching	Copy number in the sequenced library
TCGGGGA and GGAGGA	34
TAGGGGA and GGAGTA	38
TTGGGGA and GGAGAA	69
TGGGGGA and GGAGCA	18
AGGGGGA and GGAGCT	36
ACGGGGA and GGAGGT	58
AAGGGGA and GGAGTT	19
ATGGGGA and GGAGAT	40
sum	312

ATP has been a popular target for aptamer selection. The only other comparable small molecular target is probably estradiol (selected for at least eight times).¹³⁴ Aside from what already been discussed, at least two additional DNA aptamer selections were performed, each used a different selection method.^{117, 118} We aligned their sequences with our new aptamers and the classical aptamer, and none of the conserved regions aligned (Figure 2.5). Their binding properties

are compared in Table 2.3. Most of the previous selection used mM ATP and they did not push for higher affinity aptamers. As a result, their K_d values were also high.

The S10 aptamer was isolated using 4 mM ATP after 14 rounds of selection, but it has almost no structural features (Figure 2.6).¹¹⁸ The reported K_d for this aptamer was very high (692 μ M), and we did not analyze it further. The other aptamer named ATP-CBA was reported by Qu et al. using flow cytometry,¹¹⁷ using a library design similar to that by Nutiu and Li.⁵⁶ The reported K_d was 26 μ M ATP using microscale thermophoresis. We titrated ATP into 10 μ M of this aptamer using ITC, and weak binding was observed (Figure 2.7 A,C). When we truncated the aptamer, binding disappeared (Figure 2.7 B,D). So, our observation was consistent with weak binding of ATP with a K_d of 26 μ M. The use of modified nucleotides such as threose nucleic acid for selecting ATP aptamers was also reported (K_d 22 μ M).¹¹⁹ Overall, Ade1301b and Ade1304b are new aptamers with higher binding affinities for adenosine and ATP than all previously reported DNA aptamer (Table 2.4). Their $\sim$ 200 nM K_d values are already among the lowest in purine binding aptamers obtained from SELEX (e.g., the RNA aptamer for theophylline (100 nM),⁴⁹ the RNA aptamer for ATP (700 nM),¹¹¹ and the DNA aptamer for theophylline (500 nM),⁶¹ caffeine (2 μ M)¹²³ and uric acid (1 μ M)¹³⁵).

S10	--GTAGTAAGAACTAAAGTAAAAAAAAA-----TTAAAGTAGCCACGCTT--	44
ATP-CBA	----CAAGCACCCCTTTTCTGCAGCGATTCTTGTTTAGAATCGTCGCCAACAGCCA	51
YD1304	ACGACACGGAGGCTTATGGTGTTCGCGCGTAAATGGTCATGTCGT-----	46
YD1301	ACGACGTTTGCGATGAGAAACGTAATACATTGGTTTCGAAGGTCG-----	45
Classical	---ACCTGGGGG-----AGTATTGCGGAGGAAGGT-----	27

Figure 2.5. Alignment of different ATP aptamers reported in the literature with the two new aptamers selected in this work with the help of Clustal Omega. The alignment was poor indicating that these aptamers are not related.

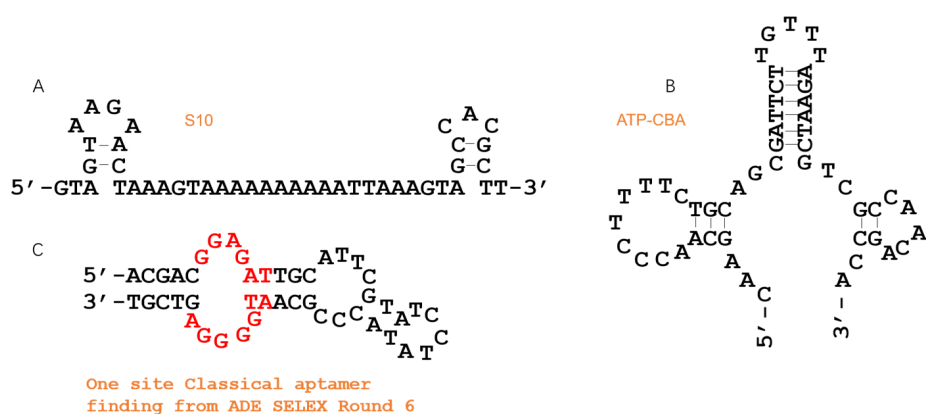


Figure 2.6. The secondary structure of (A) the S10 aptamer, (B) the ATP-CBA aptamer, and (C) a one-site classical aptamer predicted by Mfold from this selection.

Table 2.4. The ATP aptamers reported in previous publications.

Aptamer names	[ATP] used in the selections	Round of selection	Random region length	K_d for ATP	Final aptamer length	Year	Ref.
Classical	3 mM	8	72	31 μ M	27	1995	^{42, 136} ¹³⁶
Classical	0.1 mM	16	10+20	31 μ M	88	2004	⁶²
S10	4 mM	14	44	0.7 mM	44	2010	¹³⁷
ATP-CBA	round 1-2, 8 mM round 3, 4 mM	3	10+20	26.4 μ M	51	2018	¹¹⁷
Ade 1301b	1 mM to 10 μ M	10	36	2.5 μ M	40	2022	this work
Ade 1304b	1 mM to 10 μ M	10	36	2.3 μ M	38	2022	this work

2.3.6 Fluorescent sensing of adenosine and ATP.

With higher affinity aptamers, we then developed a fluorescent biosensor using the strand-displacement assay.⁵⁴ We extended the Ade1301b aptamer by a few nucleotides and labeled it with a FAM fluorophore (Figure 2.8A). This strand was annealed with a short quencher-labeled strand at a 1:2 ratio to mask the fluorescence. When the aptamer binds adenosine, the binding hairpin structure is formed and the quencher-labeled strand is released leading to increased fluorescence.

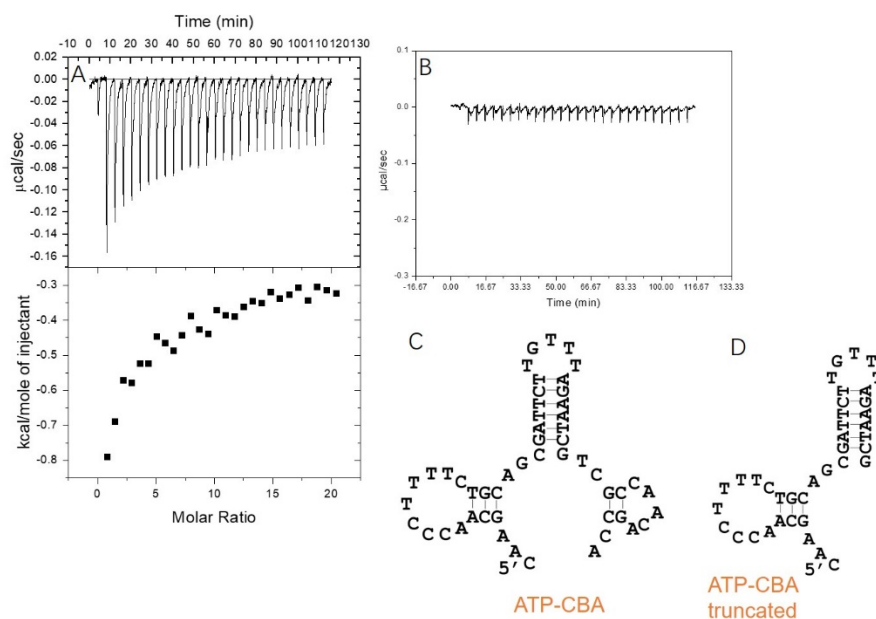


Figure 2.7. ITC analysis of aptamer binding by titrating 1 mM adenosine into 10 μ M (A) ATP-CBA and (B) ATP-CBA truncated aptamers. The secondary structure of (C) ATP-CBA and (D) ATP-CBA truncated predicted by Mfold.

To test the sensitivity, different concentrations of adenosine ranging from 0 nM to 500 μ M were added to the sensor. Upon adding adenosine, the fluorescence increased immediately (Figure 2.8B). The higher the adenosine concentration, the stronger the fluorescence increased (Figure 2.8C). The saturated fluorescence was 2.8-fold higher compared to the background. Based on the relative fluorescence increase (F/F_0) at 10 min, a calibration curve was plotted and an apparent K_d of 19 μ M adenosine was fitted. This K_d was 83-fold higher than the K_d value obtained from ITC (0.23 μ M), and it can be attributed to the competition from the quencher-labeled strand.⁵⁴ The limit of detection (LOD) was calculated to be 1.1 μ M based on the signal higher than three times of the standard deviation of background variation.

We further compared the performance of the sensors based on Ade1301b and the classical aptamer using the same design. At the low adenosine concentration region of below 20 μ M, the

Ade1301b sensor showed a higher sensitivity than classical aptamer. The slope of the Ade1301b sensor is 7-fold higher (Figure 2.8D), and the LOD of Ade1301b was 13.3-fold lower than the classical aptamer based sensor (14.6 μM). The LOD of our current classical aptamer sensor was also similar to that observed by Nutiu and Li ($\sim 25 \mu\text{M}$ ATP),⁵⁴ although the positions of the quencher-labeled strand was slightly different.

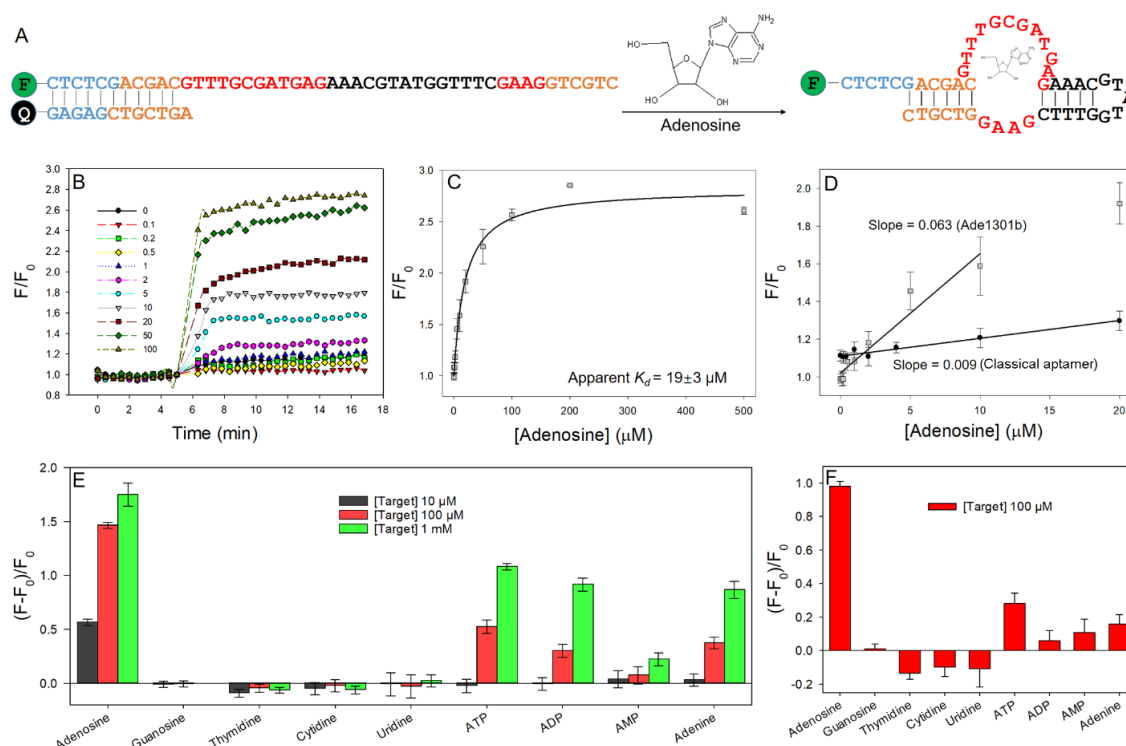


Figure 2.8. (A) A schematic illustration of the fluorescent sensor for the detection of adenosine. (B) Sensor signaling kinetics in the presence of different concentrations of adenosine in the SELEX buffer. Adenosine was added at 5 min from 0 to 100 μM . (C) Fluorescence intensity increase as a function of adenosine concentration. (D) The linear response at low adenosine concentrations of the Ade1301b and classical aptamer based sensors. (E) The selectivity of the Ade1301b sensor tested at three concentrations. The error bars indicate standard deviation from three independent samples. (F) The Ade1301b sensor selectivity in 10% serum.

For the selectivity test, different concentrations (10 μM , 100 μM , and 1 mM) various nucleosides and nucleotides were added to the Ade1301b sensor, and the relative fluorescence increase $(F-F_0)/F_0$ is shown in Figure 2.8E. The Ade1301b aptamer showed great specificity for adenosine compared to the other nucleosides including guanosine, thymidine, cytidine, and uridine. While it is not surprising that all the adenosine nucleotides showed fluorescence enhancement, it was interesting to note that the binding decreased following the order of ATP > ADP > AMP. This

response is similar to an RNA aptamer obtained after extensive negative selection.⁴¹ It appears that the increasing number of phosphate units helped aptamer binding likely via Mg^{2+} mediated bridging.^{138, 139} Finally, the response of adenine was also lower than adenosine, suggesting that the pentose participated in binding. This is also similar to the classical aptamer.⁴²

We also tested the selectivity in a 10% human blood serum sample added 100 μM of various nucleosides and nucleotides to the sensor (Figure 2.8F). Due to the raised background fluorescence from serum, the fold of fluorescence increased all decreased compared to that in buffer. Nevertheless, the high selectivity was still retained, indicating that the aptamer can function in biological sample matrix.

2.4 Conclusions

In this work, by pushing target concentrations to the low micromolar range, we discovered new and higher affinity DNA aptamers for adenosine and ATP. Their affinities for adenosine were in par with the best in vitro selected aptamers binding to purine derivatives. We have also unified the understanding of aptamer sequence evolution for these targets. Previous ATP selections stopped at a high target concentration and thus missed the higher affinity sequences. This research indicates the importance of choosing target concentrations in aptamer selections.^{140, 141} Considering the broad interest in adenosine/ATP detection, the new aptamers will outperform the previous aptamers in biosensors and other applications. They will give another 30-fold of room for engineering related smart DNA structures and switches compared to the classical aptamer.¹⁴²⁻¹⁴⁴ Using the deep sequencing data, statistical analysis was performed to gain insights into the classical aptamer in terms of the number of binding sites and mutations related to selectivity. Since the new aptamers had similar binding affinity and stoichiometry to the RNA aptamers, these new aptamers provide another example to compare RNA and DNA aptamers for binding.

Chapter 3 Exploring the lower limit of target concentration in Capture-SELEX using guanine as a model target

The results presented in this chapter have been published as:

Ding, Yuzhe, Ziyu Zhang, and Juewen Liu. "Exploring the Lower Limit of Target Concentration in Capture-SELEX Using Guanine as a Model Target." *ChemBioChem* 25.23 (2024): e202400570.

3.1 Introduction

The concentration of target molecules during the SELEX process significantly influences the affinity of final enriched sequences.^{58, 141} Previous SELEX methodologies typically started with a high target concentration, which was gradually decreased during the course of SELEX to enrich high affinity aptamers. However, this approach may result in a greater number of selection rounds. Prolonged exposure of a library to high target concentrations may lead to the dominance of suboptimal aptamers. Our group has previously demonstrated the selective enrichment of high and low affinity aptamers using adenosine/ATP as targets, where the classical adenosine aptamer with $\sim 7 \mu\text{M}$ K_d dominated the library initially, being replaced by higher affinity aptamers ($\sim 200 \text{ nM}$ K_d) in the later rounds.⁵⁸ Later, we proposed the direct use of a low target concentration allowing the enrichment of multiple high affinity aptamers in the sequence space for different targets.⁷³ The Xiao group also showed the enrichment of higher affinity aptamers using a lower target concentration.¹⁴¹ Starting with a low target concentration could bypass the enrichment of low-affinity aptamers and decrease the number of selection rounds. However, an excessively low concentration of target may impede the success of the selection. In this work, we aimed to understand the lower limit of target concentration that is practical for the enrichment of high affinity aptamers.

Guanine, one of the nucleobases of DNA, plays a pivotal role across biological, medical, and biotechnological domains. Guanine is a biomarker for the diagnosis of critical health conditions such as cardiovascular diseases, aging, inherited diseases and cancer.¹⁴⁵⁻¹⁴⁷ The Yokoyama group previously reported an RNA aptamer with a K_d of $1.8 \mu\text{M}$ guanine. The Breaker group discovered two guanine riboswitches, exhibiting K_d values of 5 nM and 8.3 nM , respectively. Our group previously identified a guanine DNA aptamer using a multiplexed SELEX containing a mixture of guanine, adenine, xanthine and hypoxanthine, in which $5 \mu\text{M}$ of guanine was used.⁷³ The selected R10G2 aptamer has a K_d of 140 nM guanine, 28-fold higher than that of the best guanine riboswitch. Therefore, we postulated that guanine would be an appropriate target to test the effect of whether high affinity DNA aptamers can be selected using lower target concentrations.

Since the R10G2 aptamer already has a K_d of 140 nM, which was isolated using 5 μ M guanine (in a mixture with other targets), in this work, we used 5 μ M, 500 nM, and 50 nM guanine as targets for three separate selections. The R10G2 aptamer appeared again in the two higher concentration selections, albeit the 50 nM selection failed to enrich aptamers. Furthermore, we studied the binding mechanism of this aptamer to guanine, and found some similarity with the guanine riboswitches.

3.2 Materials and methods

3.2.1 Chemicals

Table 3.1. The DNA sequences used in this work

DNA names	Sequences (from 5' to 3')
N30 library	GGAGGCTCTCGGGACGAC-N30- GTCGTCCCGATGCTCTGAATCACAG
Reverse primer for N30 library	CTGTGATTCAAGAGCATCGGGACG
Biot-Reverse primer for N30 library	/5Biosg/ CTGTGATTCAAGAGCATCGGGACG
Biot-Column	GTCGTCCCGAGAGCCATA /3BioTEG/
Forward primer (FP)	GGAGGCTCTCGGGACGAC
R10G2	GACGACACCAACCGGTATTTCTGGGTCTGTAATGGATGTCGTC
5000G1	GACGACGGGTCTGTAATGGATTGTTCTCAACCAACTGTCGTC
A36T7	GACGACTCCAACCGGTATTTCTGGGTCTGTAATGGAAGTCGTC
Δ A35	GACGACACCAACCGGTATTTCTGGGTCTGTAATGGTGTGTCGTC
Δ G7A8	GACGACAGTCTGTAATGGATTGTTCTCAACCAACTGTCGTC
C8T	GACGACATCAACCGGTATTTCTGGGTCTGTAATGGATGTCGTC
C9T	GACGACACTAACCGGTATTTCTGGGTCTGTAATGGATGTCGTC
C12T	GACGACACCAATCGGTATTTCTGGGTCTGTAATGGATGTCGTC
C26T	GACGACACCAACCGGTATTTCTGGGTTTGTATGGATGTCGTC

Note: /5Biosg/ is biotinylation at the 5'-end and /3BioTEG/ is biotinylation at the 3'-end.

The DNA samples used in this study were purchased from Integrated DNA Technologies (Coralville, IA, USA, see Table 3.1 for sequences). Streptavidin agarose resin was purchased from

Fisher Scientific. Guanine, sodium chloride, and magnesium chloride were obtained from Amerseco (Framingham, MA, USA). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was obtained from Bio Basic (Toronto, ON, Canada). Sodium hydroxide, thioflavin T (ThT), and Amicon Ultra-0.5 centrifugal filter unit (3k and 10k molecular weight cut off) were from Millipore-Sigma (Oakville, ON, Canada). Micro bio-spin chromatography columns and SsoFast EvaGreen supermix were from Bio-Rad. dNTP mix, Taq DNA polymerase with ThermoPol buffer were from New England Biolabs (Ipswich, MA, USA). All of the buffers and solutions were prepared with Milli-Q water.

3.2.2 Aptamer selection

The detailed method of the capture-SELEX method was the same as our previous reports.^{59, 60} The SELEX buffer for guanine: 10 mM HEPES pH 7.3, 150 mM NaCl, and 2 mM MgCl₂. Different concentrations of guanine were diluted in the SELEX buffer. The round 11 PCR products from the 5 μ M guanine selection pool were sequenced at McMaster University, and the round 17 PCR products from the 500 nM and 50 nM guanine selection pools were sequenced at University of Guelph.

3.2.3 ITC

The ITC experiments were conducted using MicroCal iTC200. Both aptamers and targets were dissolved in test buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 2mM MgCl₂) and degassed by sonication for 5 min before the start of the experiment. The cell chamber was loaded with 205 μ L aptamer solution (10 μ M), while the syringe contained 40.5 μ L of target solution. All titration experiments were conducted at a temperature of 25°C. The obtained titration curves were fitted using a one-site binding model by Origin software.

3.2.4 ThT fluorescence spectroscopy

In the ThT binding assays experiments, the R10G2 aptamer and ThT were dissolved to final concentrations of 100 nM and 2 μ M, respectively, in the assay buffer consisting of 100 mM HEPES pH 7.5, 150 mM NaCl, and 2 mM MgCl₂, to a final volume of 500 μ L. A target was titrated until the change in fluorescence intensity plateaued. Fluorescence spectroscopy experiments were conducted using the Horiba Fluoromax 4 fluorometer, with excitation at 420 nm and emission at 490 nm. The temperature was maintained at 20°C using a water bath. Titrations commenced once stable background fluorescence was established.¹⁴⁸ Each experiment was repeated three times. The

K_d value was fitted using $y = y_0 + aK_d/(K_d + x)$, where x is the concentration of the titrated target, and a is the maximal signal change upon saturated binding.¹⁴⁹

In the metal-dependent experiments, the Na^+ concentration was varied between 0 mM, 150 mM, and 500 mM, with the substitution of the HEPES buffer by Tris to avoid sodium ions in the buffer. To test the effect of Mg^{2+} , 0 mM, 2 mM, and 10 mM Mg^{2+} was used along with 100 mM HEPES pH 7.5. In tests with 0 mM MgCl_2 , 1 mM EDTA also was included to chelate potential divalent cations in the water. All solutions were prepared using Milli-Q water.

3.3 Results and discussion

3.3.1 Aptamer selection using three concentrations of guanine

To select guanine binding aptamers, we employed the capture-SELEX method as previously reported.^{57, 59, 150} Briefly, a DNA library containing a 30-nucleotide random region (N30) was immobilized onto streptavidin-coated beads by hybridizing it with a biotinylated DNA. After washing the beads with buffer to remove free DNA, guanine was introduced, and the DNA sequences released from the beads were collected and amplified by PCR. A total of three guanine concentrations were used in three parallel selections. We conducted 11 rounds of selection for the 5 μM guanine selection, whereas 17 rounds were performed for each of the 500 nM and 50 nM guanine selections.

To monitor the overall SELEX process, we utilized qPCR to measure the cycle difference between the solutions eluted with buffer alone and with the same buffer but containing guanine (Figure 1A, bars). For the 5 μM guanine selection experiment, the cycle difference consistently exceeded one starting from round 5. At round 8, the difference already reached five cycles, suggesting that the DNA eluted by 5 μM guanine was 2^5 -fold (32-fold) higher compared to that non-specifically released by buffer wash. This cycle difference remained constant over the next few rounds of selections. Thus, we stopped at round 11 for this selection. The PCR products from rounds 5, 6, 8, and 11 were subjected to deep sequencing. Out of a total of 36,555 sequences from the round 11 library, we analyzed the top 5 most abundant sequences, which collectively occupied nearly 99% of the library (Figure 1B). The first sequence named 5000G1 (“5000” denoting 5000 nM) dominated the library. The first four sequences belong to the same family, which featured two conserved regions highlighted in red and blue, respectively. We also analyzed the sequencing data from the other rounds, and observed a gradual increase in the abundance of this family from round 5 to round 8, reaching 91.1% as early as round 8 (Figure 1A, red dots). Even though 5 μM is typically considered to be a very low starting concentration for small molecule targets, and few

aptamer selection experiments started with such a low target concentration,^{73, 141} we were able to enrich a single aptamer to 91.1% of the library in just eight rounds. Therefore, for a target, whose high affinity aptamers are available, it is feasible to use a low concentration. From this, we wanted to determine how low we could push the guanine concentration and if we could obtain an even higher affinity aptamer.

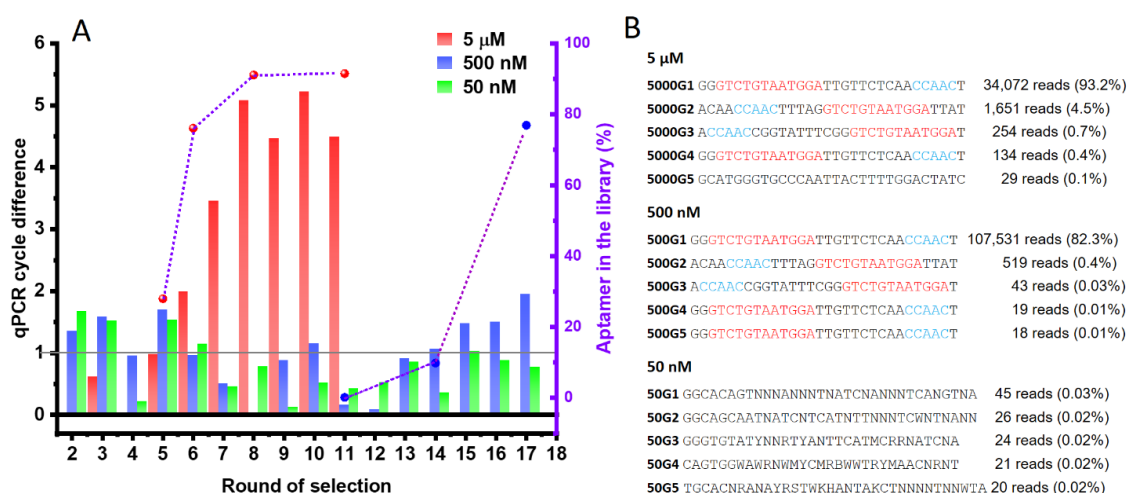


Figure 3.1. (A) The left axis: the difference of qPCR cycles between the buffer-eluted and target-eluted DNA. The number of PCR cycles was calculated based on 50% of the total fluorescence increase. The right axis: the percentage of the 5000G1 sequence in different rounds of the three parallel selections. The dots represent the percentage of the top aptamer in the library for the respective round, with the 5000G1 sequence in red, and the 500G1 sequence in blue. (B) Sequence alignment of the N30 region of the top 5 most abundant sequences in the final round of the 5000 nM, 500 nM, and 50 nM guanine selections. The nucleotides marked in red and blue are the conserved regions of the aptamers.

For the 500 nM guanine selection, the cycle difference consistently exceeded one cycle after round 14 (Figure 3.1A, blue bars). Although the cycle difference exhibited a gradual increase, the maximum difference was only 2 cycles at round 17. The PCR products from round 17 were deep sequenced, resulting in a total of 130,708 sequences. The top 5 most abundant sequences were grouped into a single family that shared the same conserved regions as those from the round 11 of the 5 μ M guanine pool (Figure 3.1B). Utilizing these conserved regions to search the entire library pool revealed that this family represented 76.9% of the round 17 sequences of the 500 nM selection (Figure 3.1A, blue dots), similar to the abundance observed in round 6 of the 5 μ M guanine pool. Not surprisingly, these two rounds also had a similar qPCR difference. Therefore, 500 nM guanine

was also a feasible target concentration, although more selection rounds are needed to achieve the same level of aptamer enrichment (17 rounds versus 6 rounds).

Finally, for the 50 nM guanine selection, the qPCR cycle difference did not consistently exceed one cycle during the entire 17 rounds (Figure 3.1A, green bars). Deep sequencing of the round 17 library yielded a total of 133,024 sequences, with only 13,104 reads assembled into 5,237 groups, analyzed by Geneious software. The top five sequences comprised only a total of 136 reads, which appeared to be unrelated (Figure 3.1B). This outcome suggested that the library remained highly diverse, and the enrichment of aptamers was unsuccessful with such a low target concentration. Therefore, 500 nM was the lowest feasible guanine concentration.

3.3.2 Aptamer binding affinities

Since we've previously obtained a guanine aptamer, named R10G2, in a mixed target selection, we then compared the sequences of 5000G1 and R10G2.⁷³ Interestingly, they shared the same conserved regions, albeit circularly permuted (Figure 3.2A, marked in red). Circular permutation of conserved regions is a common feature of small molecule binding aptamers, attributable to the variable orientations of the target molecule.^{49, 151}

Further binding analysis using isothermal titration calorimetry (ITC) was conducted for these two aptamers. The titration traces and integrated heat are presented in Figure 3.2B, with the thermodynamic values provided in Table 3.2. The K_d value for the 5000G1 aptamer was determined to be 268 ± 68 nM, similar to that of R10G2 (221 ± 34 nM). Both aptamers exhibited a binding stoichiometry close to one, and their thermodynamic values were comparable. Combining the data from sequence comparison and ITC, we concluded that 5000G1 and R10G2 belong to the same family of aptamers. Given that their K_d is around 200 nM, it is reasonable that it was selected using 500 nM guanine but not 50 nM guanine. Therefore, an implication is that by using the N30 library and capture-SELEX method, this is the best aptamer one can achieve.

Table 3.2. The ITC thermodynamic data for R10G2 and 5000G1.

Aptamer	Target	Stoichiometry (N)	K_d (nM)	ΔH (kcal/mol)	ΔS (cal/mol/deg)
R10G2	guanine	0.90 ± 0.01	221 ± 34	-36.9 ± 0.7	-93.5
5000G1	guanine	0.80 ± 0.02	268 ± 68	-41 ± 2	-108

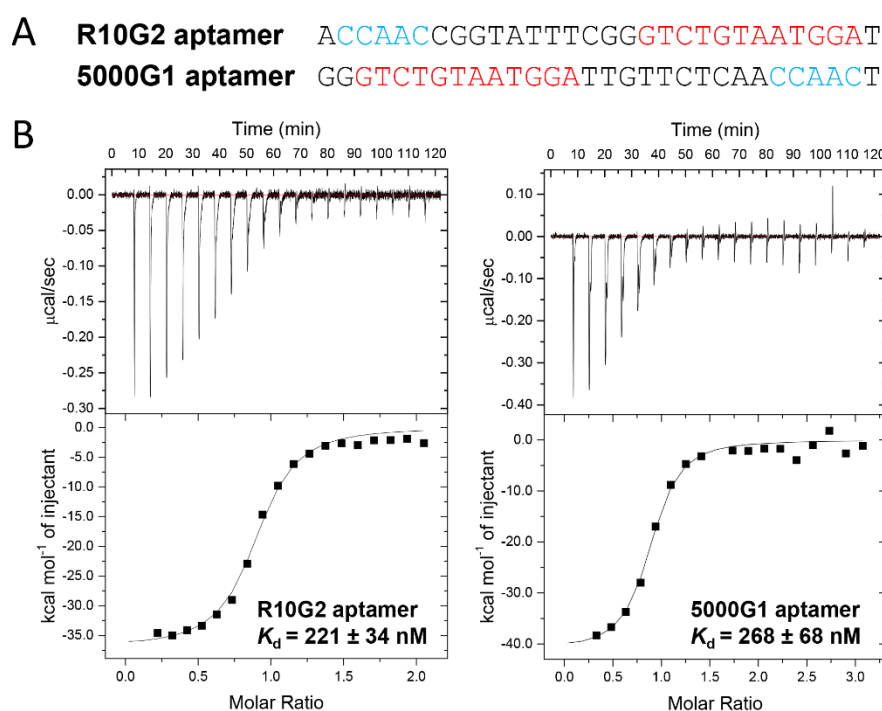


Figure 3.2. (A) The primary sequence comparison between the R10G2 and 5000G1 aptamers. The red and blue conserved regions are the same for these two sequences. (B) ITC traces and integrated heat of titrating 100 μM guanine into 10 μM R10G2 aptamer and titrating 150 μM guanine into 10 μM 5000G1 aptamer.

3.3.3 Lower limit of guanine concentration

Since the K_d of these guanine aptamers is around 200 nM, we can easily understand that 5 μM guanine (25-fold higher than K_d) was sufficient for rapid enrichment of aptamers. So, for the capture-SELEX method, 5 μM guanine is a good target concentration (better than 500 nM) and the aptamer was isolated within eight rounds. Since there are 17 conserved nucleotides in this aptamer (*vide infra*), we can estimate that its initial abundance is approximately $(1/4)^{17} = 5.8 \times 10^{-11}$, or out of 1.7×10^{10} sequences, there is one aptamer. This is consistent with the estimation made by Ellington and Szostak.¹⁹ By round 5 for the 5 μM selection, the abundance of this sequence already reached 27.9%. If we assume the same enrichment factor in the first five rounds, each round enriched this aptamer by 86-fold. In contrast, the enrichment factor was only 6.9-fold for the 500 nM selection (9.8% aptamer in round 14). The reason that 500 nM never released too much DNA can be attributed to its close to the aptamer K_d . When an aptamer is hybridized to another DNA (the biotinylated capture strand in this case), the apparent K_d can be a few folds to over 100-fold higher than the

intrinsic K_d of the free aptamer.^{54, 151-153} Since 500 nM guanine only released a small fraction of the library, the apparent K_d in the aptamer selection system is observably higher than 500 nM.

For a given target, it is hard to predict the intrinsic K_d as well as the difference between the intrinsic K_d and the apparent K_d of the resulting aptamer due to the capture strand. Thus, we do not want to make a quantitative discussion based on this particular example. In general, we recommend using a target concentration higher than the predicted intrinsic K_d , but it should not be too high either. So then, is there a higher concentration limit? We reasoned that the answer relies on the presence, or lack thereof, of aptamers with different affinities or not. Using the adenosine aptamers as an example, there are at least two types of aptamers: the lower affinity classical aptamer (K_d 7 μ M),^{42, 132} and the high affinity aptamers (K_d 200 nM).⁵⁸ This 35-fold difference in K_d was sufficient to favor the low affinity aptamer when 1 mM to 5 mM target was used, since the low affinity aptamer has a shorter conserved region and thus higher initial abundance. 1 mM adenosine was sufficient to fully release both types of aptamers. So, for a target with just one type of aptamer, a higher target concentration is better. In contrast, for targets with different types of aptamers with sufficient gaps in affinities, the concentration of target should be lower than the K_d of the next type of aptamer. Theophylline is an example of target with aptamers with a range of different binding affinities,^{61, 154, 155} and using a lower target concentration is likely to be more favorable for enriching high affinity aptamers for it.

When we used 50 nM guanine, we did not obtain an even lower affinity aptamer. Whether there exists a DNA aptamer for guanine with a 5 nM K_d remains unknown (note that guanine riboswitch can reach 5 nM K_d).¹⁵⁶ At least, using the N30 library and the capture-SELEX method, we can confidently say that the 200 nM K_d aptamer is the best one can achieve. 200 nM is 40-fold higher than 5 nM; since 500 nM target can enrich the 200 nM K_d aptamer, we should be able to enrich an aptamer with 5 nM K_d if it does indeed exist. The guanine riboswitch has a small binding loop containing only 9-nucleotides. However, it has two side loops that form a pseudoknot leading to a higher order structure that can ‘wrap’ around guanine. The entire binding sequence contains 82-nt, which was longer than our 30-nucleotide random region or 42-mer final aptamer.¹⁵⁷ Therefore, for our current condition, 500 nM is the lower limit of the target concentration.

It might be true that if a longer library was used and a 5 nM aptamer can be enriched, then for that condition 50 nM or 500 nM guanine might be an optimal condition. Since our library amount was 100 pmol dispersed in a volume of approximately 600 μ L, the DNA concentration was approximately 160 nM. So, if the target concentration is too low, even assuming that one target molecule can release one DNA strand, the fraction of released DNA might still be lower than

randomly dissociated DNA. Thus, there should be an even lower limit, regardless of the aptamer affinity. We previously calculated that the non-specifically released DNA was 0.13% (or 0.2 nM) in our system.⁵⁹ So, the target likely cannot be much lower than this concentration, even for aptamers with low pM affinity. Practically, the majority of reported DNA aptamers for small molecules have K_d higher than 1 nM.

3.3.4 Secondary structure analysis

Since the 5000G1 and R10G1 aptamers belong to the same family and exhibit similar secondary structures predicted by Mfold (Figure 3.3A and B),¹²⁸ we combined these two aptamers to study their structures. Both aptamers predicted an extra adenine (A35 in R10G2, A20 in 5000G1) inserted in duplex regions. We first deleted A35 from the R10G1 sequence (the mutant named Δ A35). A binding assay was performed using ThT fluorescence spectroscopy. ThT serves as a fluorescent marker with a high affinity for DNA, especially sequences abundant in guanine (G-rich DNA), which triggers a noticeable increase in fluorescence.¹⁵⁸ When a target molecule binds, it may displace some ThT molecules and cause a reduction in fluorescence intensity.^{70, 154} Therefore, the resulting K_d values measured by the ThT assay are apparent K_d . When we titrated guanine into Δ A35, the change in ThT fluorescence was very moderate (Figure 3.3C, blue line), indicating that A35 is important for guanine binding. Thus the three base pairs next to it in the conserved region is likely to be opened in the binding structure. This hypothesis is further supported by the fact that these three base pairs have fixed base contents. For a duplex region for structural purposes, the base content should be variable as long as base pairing interactions are maintained. To study the other side of A35, we then reversed the A7-T36 base pair to T7-A36 (mutant named A36T7). The K_d of this mutant was not significantly different from that of the R10G2 aptamer (Figure 3.3C, green line), indicating that these two nucleotides should form a base pair and not participate in binding with guanine.

We then analyzed the other end of the aptamer, where an extra T (T36) and two extra guanines (G7G8) are in the middle of the duplex region (Figure 3.3B) of the 5000G1 aptamer. Thus, we replaced the two guanines by an adenine to form a base pair with T36 to make a long duplex region (this mutant named Δ G7A8). However, the mutant did not bind guanine (Figure 3.3C, purple line). Therefore, G9C35 and T10A34 should not be paired in the aptamer binding state, which is also supported by the fact that these bases are in the conserved region. Based on the above mutation experiments, the correct secondary structure of R10G2 is illustrated in Figure 3.3D, where the two conserved regions are linked by two duplex regions. 5000G1 should share a similar secondary

structure as well (Figure 3.4). Since these two aptamers were essentially the same, our subsequent studies focused on the R10G2 aptamer since it was published first.

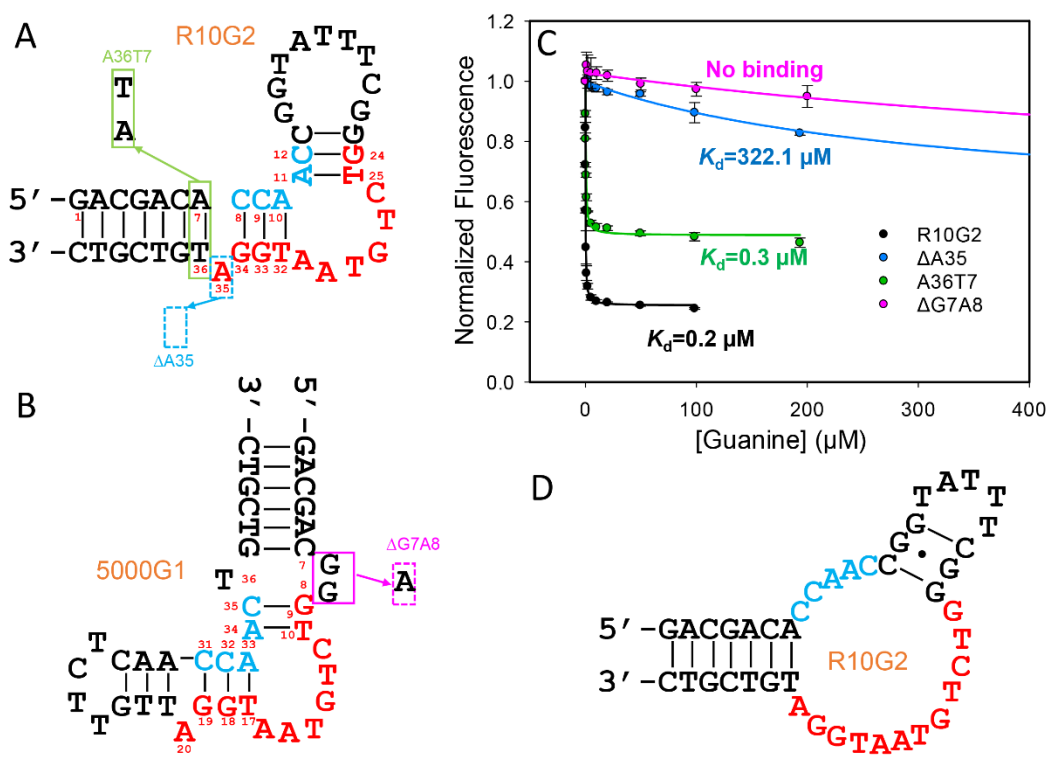


Figure 3.3. The secondary structures of (A) the R10G2 aptamer and (B) the 5000G1 aptamer, predicted by Mfold. (C) The ThT fluorescence titration curves of guanine for a few aptamer mutants. (D) The secondary structure of the R10G2 aptamer based on mutation studies.

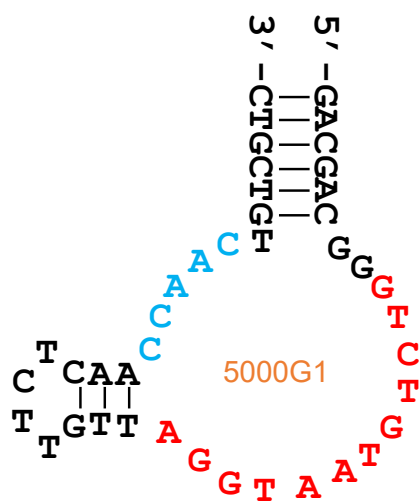


Figure 3.4. The secondary structure of 5000G1 based on the mutation studies.

3.3.5 A cytosine-to-thymine mutation yielding an adenine aptamer.

For the guanine-I riboswitches, three uracils form a pocket through binding with N3, N7, and N9 in the guanine target, and N1, N2, and O6 of the target guanine form base pairs with a cytosine.¹⁵⁷ After changing the cytosine to a uracil, the riboswitch showed good binding affinity and specificity with adenine.¹⁵⁹ This phenomenon is also known in the 8-oxoguanine riboswitch, where the riboswitch transfers specificity to 8-oxoadenine after changing one critical cytosine to a uracil.¹⁵⁷

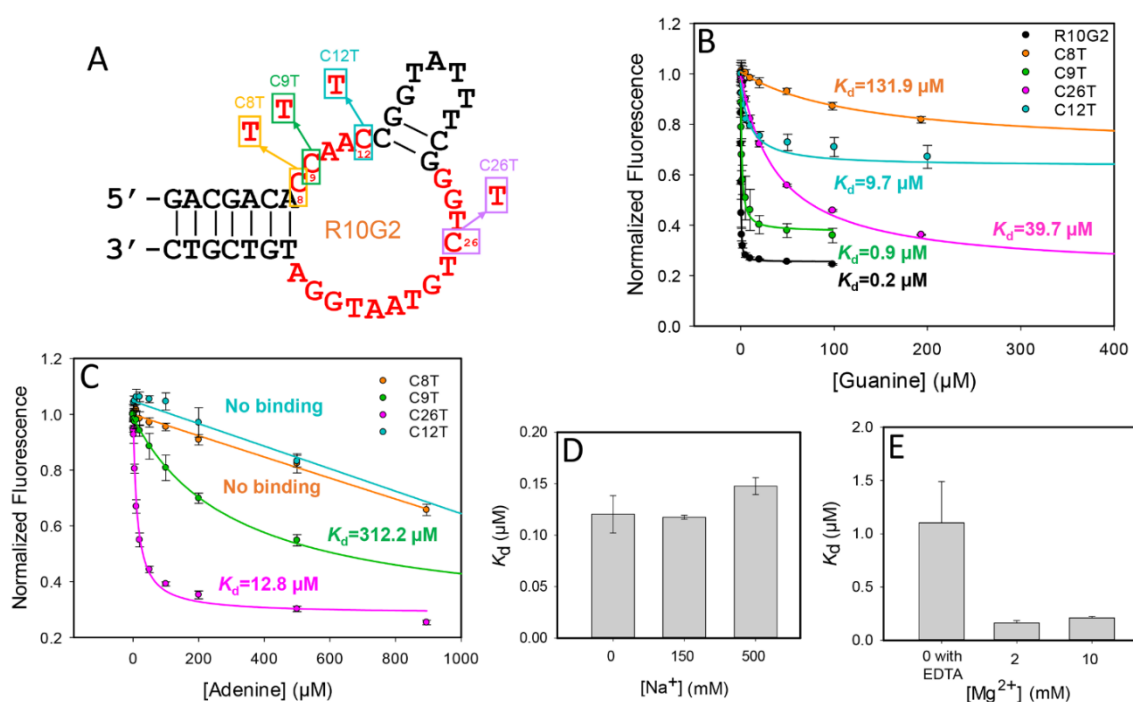


Figure 3.5. (A) Mutation of cytosines to thymine to study the binding specificity of the R10G2 aptamer. A total of four mutants were tested. The ThT fluorescence titration curve of (B) guanine and (C) adenine to mutated aptamers. (D) The K_d of R10G2 and guanine in different sodium ion concentrations. All the samples contained 2 mM Mg^{2+} . For this experiment, we used Tris buffer to replace HEPES buffer to minimize the background sodium content in the system. (E) The K_d of R10G2 and guanine in different magnesium ion concentrations. All of the samples contained 150 mM NaCl. For the Mg^{2+} -free sample, 1 mM EDTA was added to mask all divalent cations in the system.

We suspected that our guanine aptamer might bind guanine using a similar mechanism, and there may be a critical cytosine playing a similar role. As shown in Figure 3.5A, R10G2 has four cytosines in the conserved region and we separately changed them to thymine (named C8T, C9T, C12T and C26T). After testing binding affinity with guanine, the C8T and C26T aptamers showed

much worse binding affinities than the parent R10G2 aptamer (Figure 3.5B), suggesting their importance for binding with guanine. We further used these four aptamers to bind with adenine and found that C26T has a binding affinity of 12.8 μM for adenine (Figure 3.5C). The mutated guanine-I riboswitch has a K_d of just 300 nM adenine, where its affinity for guanine decreased over 2000-fold. However, the C26T aptamer mutant still showed a binding affinity of 40 μM with guanine, decreasing 200-fold. This result showed that the binding between R10G2 and the guanine-1 riboswitch for guanine has some similarity, but the riboswitch had higher affinities overall for both targets.

3.3.6 Effect of salt

Next, we studied the effect of metal ions for the binding of the R10G2 aptamer. Our initial focus was to examine the impact of sodium ions (Figure 3.5D). When no additional sodium ions were introduced into the system, the observed K_d remained similar to that under SELEX buffer conditions. Subsequently, we increased the concentration of sodium ions from 150 mM to 500 mM, but this adjustment did not produce a significant alteration in the binding affinity. Hence, it appears that sodium ions do not exert a substantial influence on the binding interaction between R10G2 and guanine.

For magnesium ions, our findings were comparable to those for sodium ions (Figure 3.5E). Elevating the magnesium ion concentration from 2 mM to 10 mM did not yield a substantial change in binding affinity. However, there was a 5-fold decrease in binding affinity when magnesium ions were absent from the system, and the resulting K_d became close to 1 μM . A K_d of 1 μM is still an indication of a good aptamer. Therefore, it appears that magnesium ions do not play a pivotal role in the binding between R10G2 and guanine either.

3.3.7 Specificity for the R10G2 aptamer

We previously compared the binding of adenine, xanthine, hypoxanthine, and guanine for the R10G2 aptamer.⁷³ Herein, we further studied the binding with a few guanine analogues, including guanosine, GMP, and a few other nucleosides (Figure 3.6A). Overall, R10G2 is highly selective for guanine, and the next best target is guanosine, which has a K_d of ~ 60 μM (345-fold higher than that for guanine, Figure 3.6B). This result demonstrates the high specificity of the R10G2 aptamer for its target.

We then compared the selectivity of the Guanine-I riboswitch with our R10G2 aptamer. It can be seen that the R10G2 aptamer has better specificity for hypoxanthine and xanthine.¹⁵⁶ In contrast, R10G2 has worse specificity for adenine than Guanine-I (Figure 3.6C).

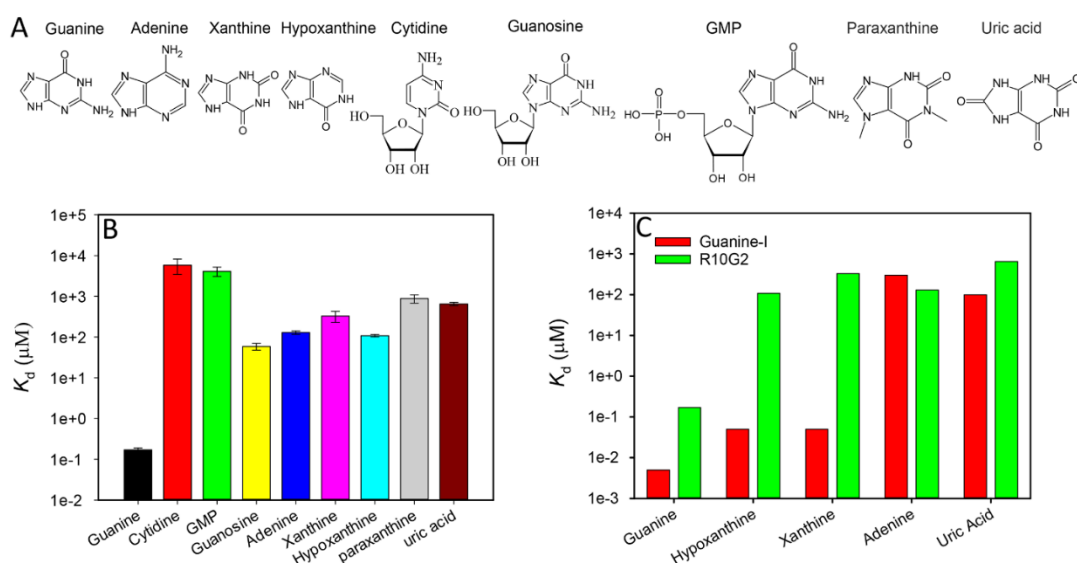


Figure 3.6. (A) Structures of some guanine analogs tested in this work. (B) The specificity of R10G2 for guanine analogs. (C) Specificity comparison between the Guanine-I riboswitch and R10G2 aptamer. The Guanine-I riboswitch data were from Ref.¹⁵⁶.

3.4 Conclusions

In this work, we used guanine as a model target to explore the lower limit of target molecules in aptamer selections. With the capture-SELEX method, we used three guanine concentrations from 50 nM to 5 μM to carry out three independent selections. Fast enrichment of aptamer was achieved with 5 μM guanine, even though 5 μM is already one of the lowest concentrations used for aptamer selection. Even 500 nM guanine still successfully generated aptamers, although it took more rounds of selection to achieve. 50 nM guanine failed to enrich any aptamers, which is rationalized by the highest affinity aptamer having a K_d of 200 nM. We discussed the optimal target concentration in the context of having different aptamers with a large difference in K_d (e.g. >30 fold). In the second part of the paper, we characterized the binding of this aptamer and showed its excellent selectivity for guanine. By mutating a single cytosine in the aptamer to a thymine, its affinity to guanine decreased by about 200-fold, and this mutant then showed binding to adenine, which is reminiscent of the guanine riboswitch. The DNA aptamer for guanine did not have a strong requirement on salt, which may make it useful as a model aptamer that can be used in many studies and sensing applications.

Chapter 4 Enriching thermodynamically best aptamers by addressing the kinetic aspect of the DNA strand-displacement reaction

Yuzhe Ding, Yicheng Heng, Ka-Ying Wong, Juewen Liu*

4.1 Introduction

In capture-SELEX, the concentration of target molecules is critical for enriching high affinity aptamers.^{58, 84, 100, 150} Typically, using a low target concentration can push for high affinity aptamers, although if the target concentration is as low as the K_d of aptamers, it will take more rounds of selection.¹⁶⁰ When the target concentration is extremely low (e.g. much lower than the K_d of the best aptamer), the selection may fail.¹⁶⁰ Target concentration represents the thermodynamic aspect of aptamer selection. The kinetic aspect, however, has yet to be explored. In a typical capture-SELEX, a target solution flows through the immobilized library over 1-2 min by gravity with little kinetic control.⁵⁷

Ampicillin, a widely used β -lactam antibiotic, can contribute to antimicrobial resistance through environmental and food residues.¹⁶¹ Thus, its monitoring is crucial for food safety, environmental, and biomedical applications.¹⁶² In 2012, Song et al reported a few aptamers for ampicillin obtained by covalent target immobilization.¹⁶³ However, a later study claimed that those aptamers were unable to bind.¹⁶⁴ In this work, we used ampicillin as a target for capture-SELEX. We discovered that a low affinity aptamer dominated the library due to a kinetic advantage, whereas high affinity aptamers failed to enrich due to a kinetic disadvantage. By allowing a longer incubation time to even the kinetic effect, high affinity aptamers were better enriched based on their thermodynamic affinity. To test the generality of this kinetic effect, another selection was also performed using adenosine as a model target. This work not only provides a high affinity aptamer for ampicillin but also offers key kinetic insights into aptamer selection.

4.2 Materials and methods

4.2.1 Chemicals

The DNA samples used in this study were purchased from Integrated DNA Technologies (Coralville, IA, USA) (see Table 4.1 for sequences). Streptavidin agarose resin was obtained from Thermo Scientific (IL, USA), while metronidazole and 6-aminopenicillanic acid were sourced from Aladdin (Shanghai, China). Sodium chloride and magnesium chloride were purchased from

Amerseco (Framingham, MA, USA). Ampicillin and penicillin G were obtained from Sigma-Aldrich (Burlington, MA, USA), and kanamycin A was supplied by Bio Basic Canada Inc (Markham, ON, Canada). Sodium hydroxide, aspirin, hydrochloric acid, and Amicon Ultra-0.5 centrifugal filter units (10 kDa molecular weight cutoff) were purchased from Millipore-Sigma (Oakville, ON, Canada). Micro bio-spin chromatography columns and SsoFast EvaGreen supermix were obtained from Bio-Rad (Hercules, CA, USA). dNTP mix, Taq DNA polymerase with ThermoPol buffer, and the low molecular weight DNA ladder were purchased from New England Biolabs (Ipswich, MA, USA). All buffers and solutions were prepared using Milli-Q water. The SELEX buffer for ampicillin consisted of 50 mM MES (pH 6.1), 500 mM NaCl, and 10 mM MgCl₂, while the SELEX buffer for adenosine contained 20 mM HEPES (pH 7.4), 150 mM NaCl, and 2 mM MgCl₂.

Table 4.1. The DNA sequence used in this work

DNA names	Sequences (from 5' to 3')
N30 library	GGAGGCTCTCGGGACGAC-N30- GTCGTCCCGATGCTCTGAATCACA
Reverse primer (RP)	CTGTGATTCAGAGCATCGGGACG
Biot-Reverse primer	/5Biosg/ CTGTGATTCAGAGCATCGGGACG
Biot-Column	GTCGTCCCGAGAGCCATA /3BioTEG/
Forward primer (FP)	GGAGGCTCTCGGGACGAC
AMP-H1	GACGACGCCGTCTCGTACTCAGCCGTCATCTTACTAGTCGTC
AMP-L2	GACGACGCCATCCCGTACCCAGCCGTCATCTTACTAGTCGTC
AMP-L3	GACGACGCCATCCCGTACTCTGCCGTCATCTTACTAGTCGTC
A10G	GACGACGCCGTCCCGTACCCAGCCGTCATCTTACTAGTCGTC
C13T	GACGACGCCATCTCGTACCCAGCCGTCATCTTACTAGTCGTC
C19T	GACGACGCCATCCCGTACTCAGCCGTCATCTTACTAGTCGT
G12A	ACGACGTTTGCAATGAGAAACGTATGGTTTCAAGGTCGT
FAMAMP6H-1	/56-FAM/ GGCTCTCGGGACGACGCCGTCTCGTACTCAGCCGTCATCTTACTAGTCGTCCCGA
FAMAMP6L-2	/56-FAM/ GGCTCTCGGGACGACGCCATCCCGTACCCAGCCGTCATCTTACTAGTCGTCCCGA

FAM-Ade1301	/56-FAM/ CTCTCGACGACGTTTTCGATGAGAAACGTATGGTTTC GAAGGTCGTC
FAM-classical aptamer	/56-FAM/ CTCTCGACGACTGGGGGAGTATTGCGGAGGAAGTCGT
Quencher-strand1	GTCGTCCCGAGAGCC/3IABkFQ/
Quencher-strand2	AGTCGTCGAGAG /3IABkFQ/

Note: /5Biosg/ is biotinylation at the 5'-end, /3BioTEG/ is biotinylation at the 3'-end, FAM is carboxyfluorescein, and /3IABkFQ/ is Iowa Black FQ (IDT) quencher at the 3'-end.

4.2.2 SELEX

The library design (Table 4.1) and selection method were based on previous reports of the Stojanovic group⁵⁷ with some modifications. For each round, the single-stranded library was annealed by heating it with a threefold excess of biotinylated capture strand at 95°C for 3 min, followed by gradual cooling to room temperature in SELEX buffer. Meanwhile, 200 µL of agarose resin without streptavidin modification was introduced into a micro chromatography column and washed five times with 500 µL of SELEX buffer. Next, 20 µL of streptavidin agarose resin was added to the single-stranded library tube and incubated for 10 min. The resin, after incubation, was then transferred to the column containing the non-streptavidin-modified agarose resin. The column was subsequently washed 12 times to remove unbound library. After washing, 750 µL of target solution (dissolved in SELEX buffer) was added. For SELEX1, SELEX2, and ADESELEX1, the eluted DNA was directly collected using gravity flow. In contrast, for SELEX3, SELEX4, and ADESELEX2, the target was incubated in the micro chromatography column for 10 min before being collected via gravity flow. The collected DNA was concentrated using a 10 kDa molecular weight cutoff filter and washed with Milli-Q water three to five times. The final volume of purified DNA was adjusted to 60 µL with Milli-Q water. A biotinylated reverse primer was used to synthesize 1 mL of PCR products, which were then incubated with 50 µL of streptavidin agarose resin for 20 min and washed ten times with a strand separation buffer (50 mM HEPES, pH 7.4, 150 mM NaCl). Subsequently, 600 µL of 0.2 M NaOH was added to the column and incubated for 10 min to elute the single-stranded DNA. After neutralization, the DNA was concentrated to 60 µL using a 10 kDa filter, and its concentration was measured with a Spark microplate reader (Tecan) before being used in the next round of selection. The target concentrations and incubation times for the various selections are summarized in Table 4.2.

Table 4.2. List target concentrations and incubation time used for different selection

SELEX	[Target]	Total round of SELEX	Additional incubation time
Ampicillin SELEX 1	[Ampicillin] (5 mM)	15	0
Ampicillin SELEX 2	[Ampicillin] (100 μ M)	16	0
Ampicillin SELEX 3	[Ampicillin] (5 mM)	15	10 min
Ampicillin SELEX 4	[Ampicillin] (100 μ M)	16	10 min
Adenosine SELEX 1	[Adenosine] (100 μ M)	11	0
Adenosine SELEX 2	[Adenosine] (100 μ M)	11	10 min

4.2.3 ITC

The ITC experiments were conducted using MicroCal iTC200. Both aptamers and targets were dissolved in their own SELEX buffer and degassed by sonication for 5 min before the start of the experiment. The cell chamber was loaded with 205 μ L aptamer solution, while the syringe contained 40.5 μ L of target solution. All titration experiments were conducted at a temperature of 25°C. The obtained titration curves were fitted using a one-site binding model by Origin software.

4.2.4 ThT fluorescence spectroscopy

In the ThT binding assays, the aptamer and ThT were prepared at final concentrations of 500 nM and 10 μ M, respectively, in their respective SELEX buffers, with a total volume of 500 μ L. Fluorescence measurements were performed on a Horiba Fluoromax 4 fluorometer, with excitation at 420 nm and emission at 490 nm. The temperature was maintained at 25°C using a water bath, and titrations began once a stable background fluorescence was confirmed.¹⁴⁸ Each experiment was performed in triplicate. The K_d value was fitted using $y = y_0 + aK_d/(K_d + x)$, where x represents the concentration of the titrated target and a is the maximal signal change upon saturation.¹⁴⁹

In the metal-dependent experiments, the Na⁺ concentration was varied among 0 mM, 150 mM, and 500 mM. To assess the effect of Mg²⁺, the system was supplemented with 0 mM, 2 mM, or 10 mM Mg²⁺. In experiments with 0 mM MgCl₂, 1 mM EDTA was added to chelate any residual divalent cations present in the water. All solutions were prepared using Milli-Q water.

4.3 Results and discussion

4.3.1 Ampicillin SELEX 1: A high target concentration

To select aptamers for ampicillin, we employed the library-immobilization capture-SELEX method.^{57, 60} Our DNA library has a 30-nt random region flanked by two constant regions for hybridization to PCR primers. A 10-base pair (bp) stem was designed within the two primer binding regions to facilitate the release of aptamer sequences upon target binding. The DNA library was immobilized via 15 base pairs to a biotinylated strand attached to streptavidin beads. When aptamer sequences bind to ampicillin and forms the 10-bp stem, the sequences are released from the capture strand (Figure 4.1A), collected and then PCR amplified to seed the next round of selection. The initial library consisted of 500 pmol ($\sim 3 \times 10^{14}$) random sequences, and a high ampicillin (5 mM) was used for 15 rounds of selection.

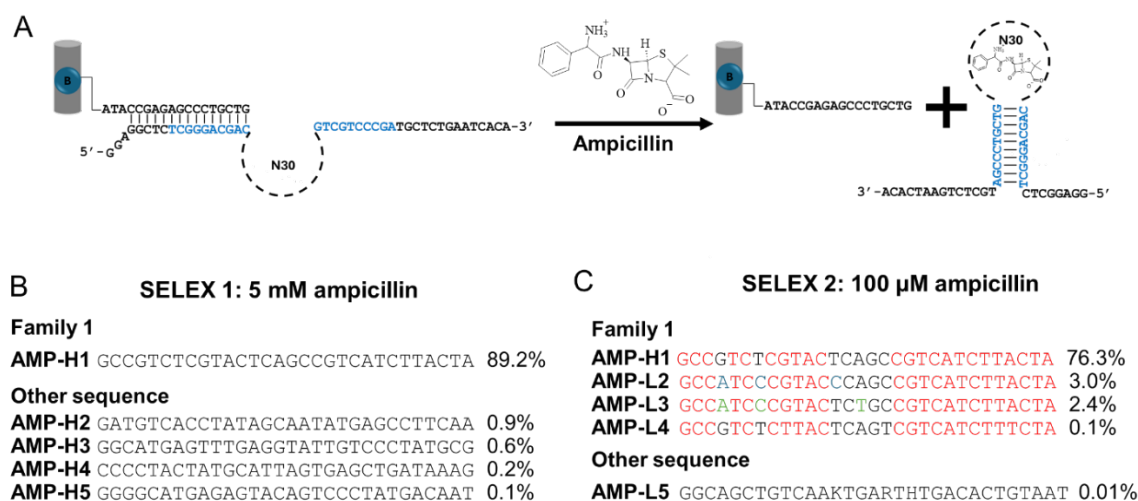


Figure 4.1. (A) Scheme of using capture-SELEX to select ampicillin aptamers. The alignment of the N30 region of the top 5 sequences in the final round of (B) SELEX 1 using 5 mM ampicillin, and (C) SELEX 2 using 100 μ M ampicillin.

The round 15 PCR products were deep-sequenced. Out of the 29,740 obtained sequences, the top sequence named AMP-H1 (H denotes for high ampicillin concentration) reached 89.2% (Figure 4.1B). All the other sequences were below 1%, and these sequences do not have an obvious relationship. To evaluate the binding affinity of AMP-H1, we performed isothermal titration calorimetry (ITC). A 1.5 mM ampicillin was titrated into a 40 μ M AMP-H1 in the selection buffer, and exothermic binding was observed (Figure 4.2A). The dissociation constant (K_d) was fitted to

be 12.7 μM . Given the structure of ampicillin with many potential hydrogen bonding and stacking interactions with DNA (Figure 4.1A), we thought that higher affinity aptamers might exist.

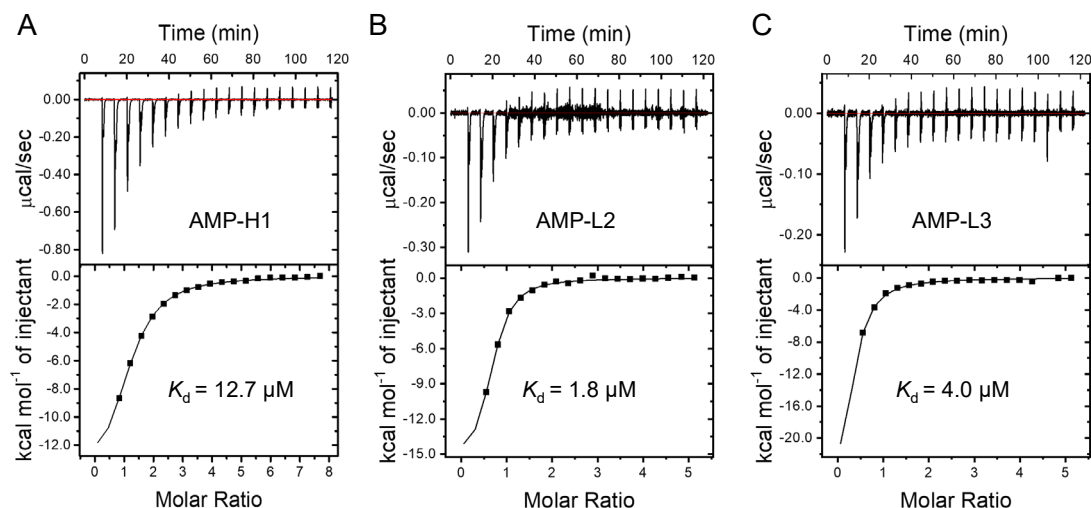


Figure 4.2. ITC traces and integrated heat profiles for titrating ampicillin to different aptamers: (A) 1.5 mM ampicillin with 40 μM AMP-H1 aptamer, (B) 0.5 mM ampicillin with 20 μM AMP-L2 aptamer, and (C) 0.5 mM ampicillin with 20 μM AMP-L3 aptamer.

4.3.2 Ampicillin SELEX 2: A low target concentration

To search for higher affinity aptamers, we did another selection using a lower ampicillin concentration of 100 μM . This way, we may disfavor the growth of low-affinity aptamers and enrich high affinity ones.^{58, 84} After 16 rounds (Figure 4.1C), however, the most enriched sequence was still AMP-H1 reaching 76.3% (Figure 4.1C). Nevertheless, the rest of the top five sequences were different from SELEX 1. The second (AMP-L2) and third (AMP-L3) aptamers ('L' denotes low ampicillin concentration) belong to the same family as AMP-H1, and they differ only by three nucleotides (Figure 4.1C, the colored nucleotides). Based on the predicted secondary structures, these differences occur in the loop regions (Figure 4.3A, Figure 4.4), and may affect aptamer binding. It is interesting that AMP-L2 and AMP-L3 were not enriched SELEX 1, and we suspected that they might have a higher affinity.

Indeed, ITC showed that AMP-L2 (K_d 1.8 μM) and AMP-L3 (K_d 4.0 μM) are both higher affinity aptamers (Figure 4.2B, C). Additionally, as a separate method to evaluate aptamer binding, we used thioflavin T (ThT) fluorescence spectroscopy.^{35, 165} ThT was first mixed with the aptamers and their nonspecific binding can increase the fluorescence of ThT. Upon aptamer binding to ampicillin, some ThT may be released resulting in decreased fluorescence. With the AMP-L2 and

AMP-L3 aptamers, an apparent K_d of 5.7 μM was achieved, much better than that of AMP-H1 (82 μM , Figure 4.3B). Therefore, while AMP-L2 is a better aptamer, it was poorly enriched in both selections.

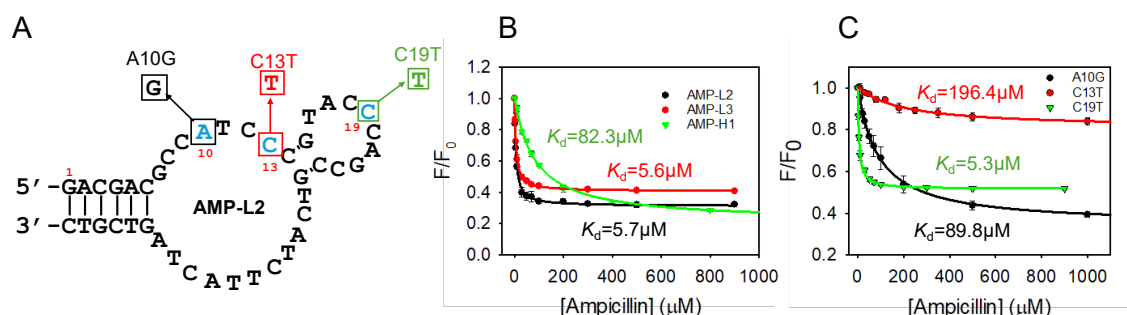


Figure 4.3. (A) The secondary structure of the AMP-L2 aptamer generated by mFold. (B) ThT fluorescence titration curves of ampicillin binding to three ampicillin aptamers. (C) ThT fluorescence titration curves of ampicillin binding to the three point mutants of AMP-L2.

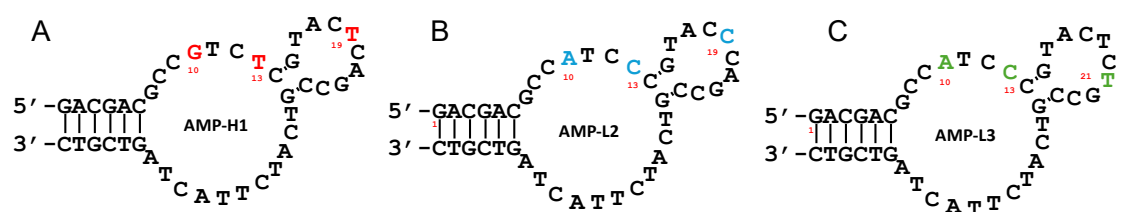


Figure 4.4. The secondary structure of the (A) AMP-H1, (B) AMP-L2, and (C) AMP-L3 aptamers generated by mFold. The differences are marked in colored nucleotides.

4.3.3 Evaluation of low abundance aptamers and systematic mutation studies

AMP-L2 and AMP-H1 differ in three positions: 10, 13 and 19 (Figure 4.1C, 4.3A). To understand which nucleotides are important for binding, we mutated each position separately based on AMP-L2 and evaluated binding using ThT fluorescence spectroscopy. When the adenine at position 10 of AMP-L2 was mutated to a guanine (A10G), the binding affinity decreased by 15-fold (K_d 89.8 μM , comparable to that of AMP-H1), suggesting this position is important. Similarly, the C13T mutant has a K_d of 196.4 μM , an even more pronounced decrease in affinity. However, the C19T mutant had a K_d of 5.3 μM , similar to the affinity of AMP-L2. Therefore, A10 and C13 play critical roles in enhancing AMP-L2's binding affinity. From the secondary structure, position 19 is in the small hairpin loop. In fact, AMP-L3 differ from AMP-H1 also in that loop, suggesting that this loop was not directly involved in target binding. The bigger loop containing A10 and C13 forms the binding pocket.

4.3.4 Why better aptamers were poorly enriched?

AMP-H1 and AMP-L2 provide an ideal model system to understand aptamer selection, since they have the same secondary structure but very different enrichment unexpected from their binding affinities. Capture-SELEX relies on the strand-displacement reaction.^{35, 166} For aptamers with almost the same secondary structure, one would expect that higher affinity sequences should be more enriched. This however is not the case for ampicillin. In contrast, the high affinity aptamers were strongly disfavored during SELEX. We searched the AMP-L2 sequence in the SELEX-1 library and its abundance was only 0.007% (13,000-fold lower than the low affinity AMP-H1 aptamer, Figure 4.5B). Even though for the low ampicillin concentration SELEX-2, AMP-L2 was only enriched to 3%, still much lower than AMP-H1 (76.3%). To explain the discrepancy between aptamer binding affinity and enrichment, we had a few hypotheses.

Hypothesis 1: difference in capture efficiency. Our first guess was that the AMP-L2 aptamer has a lower hybridization efficiency to the capture strand. To test this hypothesis, we designed fluorophore and quencher labeled DNA sequences for hybridization assays. The two ampicillin aptamers were extended by 9-nt on the 5'-end and labeled with a FAM fluorophore (mimicking the library strand). It was then hybridized to a 15-nt quencher-labeled complementary strand (mimicking the capture strand, Figure 4.5A). After annealing, the fluorescence intensity was measured upon gradually increasing the temperature (Figure 4.5C). Both AMP-H1 and AMP-L2 exhibited a similar melting temperature (T_m , defined as the maximum of the first derivative), approximately 78°C, indicating AMP-H1 and AMP-L2 have a comparable binding affinity to the capture strand.

To confirm this finding, varying concentrations of quencher-labeled strands (0–30 nM) were annealed with 2 nM FAM-labeled aptamers (Figure 4.5D). The calculated K_d values between the two strands were 5.7 ± 1.8 nM for AMP-H1 and 7.5 ± 2.8 nM for AMP-L2, confirming similar binding affinities to the capture strand. Therefore, the two aptamers should have a similar initial abundance on the beads, and this hypothesis cannot explain the difference in the final enrichment.

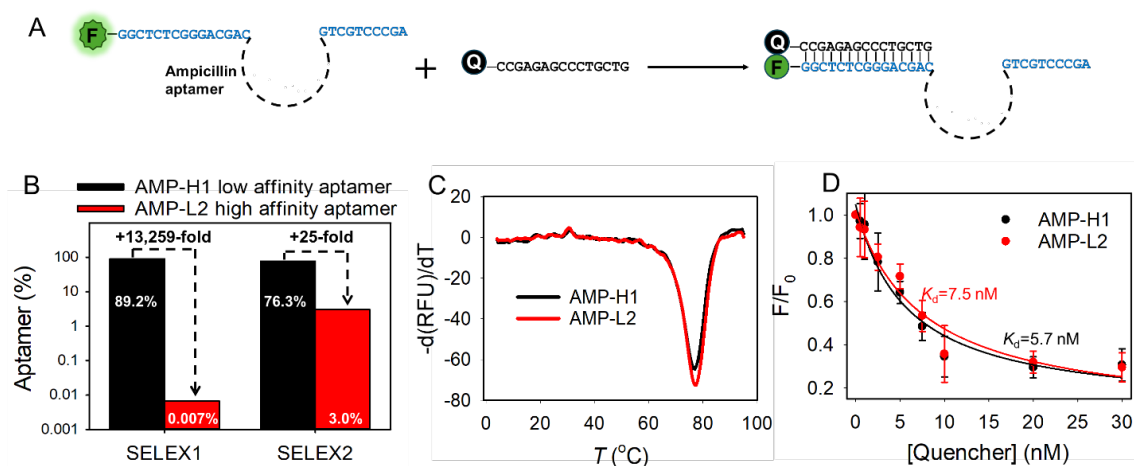


Figure 4.5. (A) Fluorescence assay of the binding between aptamers and the capture strand mimicking the library capture step in SELEX. (B) Percentage of the two aptamers in the two SELEX experiments. (C) First derivative of melting curves for FAM-labeled AMP-H1 and AMP-L2 hybridized with quencher-labeled capture strand. (D) K_d measurement for FAM-labeled aptamers with quencher-labeled capture strand.

Hypothesis 2: difference in the strand displacement efficiency. Since the capture efficiency was similar for these two aptamers, our next hypothesis was on the next step of the reaction: ampicillin-induced release of aptamers. An aptamer needs to overcome the hybridization to the capture strand to be released. To test this hypothesis, varying concentrations of ampicillin were added to the 20 nM hybridized FAM-aptamer/quencher-cDNA complex, and the fluorescence intensity was measured after 10 min reaction time (Figure 4.6A). The calculated apparent K_d values were 574 μM for AMP-H1 and 188 μM for AMP-L2. We called the obtained K_d here to be apparent K_d because of the presence of the capture strand acting as a competitor.^{35, 167, 168} Thus, the better aptamer, AMP-L2, was released more from the capture strand (Figure 4.6B). Therefore, AMP-L2 should have been enriched more than AMP-H1, yet it did not. This hypothesis cannot explain the results as well.

Hypothesis 3: difference in release kinetics. The above hypotheses were focused on the thermodynamic difference of the two aptamers. The remaining possibility is on the kinetic side. We then tested the release kinetics of AMP-H1 and AMP-L2 from the capture strand using the fluorophore/quencher system. After adding 5 mM ampicillin, the fluorescence increase was recorded immediately. The release rates were fitted using first-order kinetics. Interestingly, AMP-H1 was released 2.25-fold faster than AMP-L2 (Figure 4.6C). Up to approximately 4 min, AMP-H1 released more than AMP-L2, after which there was a crossover in their kinetic traces, and AMP-

L2 took over. So, we can divide this plot in two regions. In region one, the system was under kinetic control, whereas in region 2 was under thermodynamic control.

In our selections, 750 μL ampicillin solution flows through the library-loaded beads by gravity, which takes 1 to 2 min. Such a gravity flow method was used by most of our selections and also by other groups.^{57, 150} Therefore, the system may not in a thermodynamic equilibrium but under a kinetic influence. This difference in release speed may allow AMP-H1 to overcome its disadvantage in apparent K_d and achieve higher enrichment.

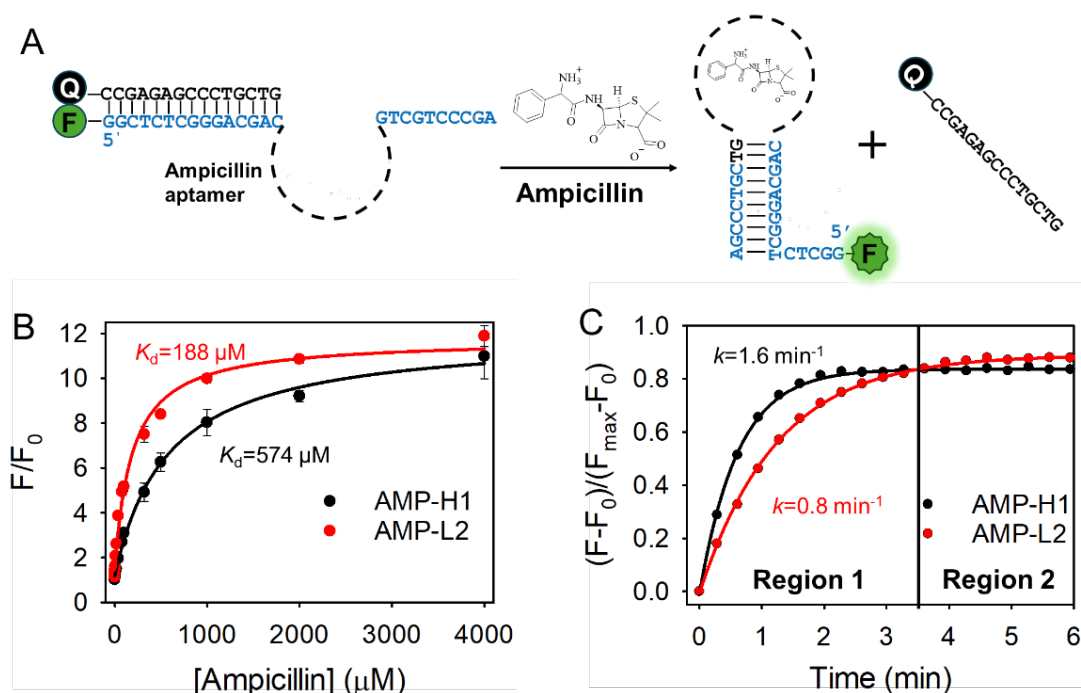


Figure 4.6. (A) Schematic of the fluorescence strand displacement reaction mimicking the aptamer release step in capture - SELEX. (B) Fluorescence intensity increase as a function of ampicillin concentration. The samples were incubated for 10 min after adding ampicillin before fluorescence measurement. (C) The kinetics of fluorescence increase after adding 5 mM ampicillin for the two aptamers, and the pseudo-first-order rate constant k values are shown.

4.3.5 SELEX 3 and 4: extended incubation to remove kinetic effects

After knowing a potential cause of enrichment of low affinity aptamers, we then repeated the ampicillin SELEX with an extended incubation time. The SELEX conditions remained the same, except that ampicillin was incubated with the beads for 10 min before elution by gravity flow (Figure 4.7A, B for the comparison of the two methods). To also evaluate the effect of target concentration, we again performed two SELEX using 5 mM and 100 μM ampicillin, respectively.

With 5 mM ampicillin (SELEX 3), after 15 rounds of selection, AMP-L2 indeed dominated the library (54.3%), whereas AMP-H1 dropped to 25.7% (Figure 4.7C, see Figure 4.8 for sequence alignment). Compared to the same selection without additional incubation (SELEX 1), the relative abundance of AMP-L2 increased over 7700-fold (Figure 4.9).

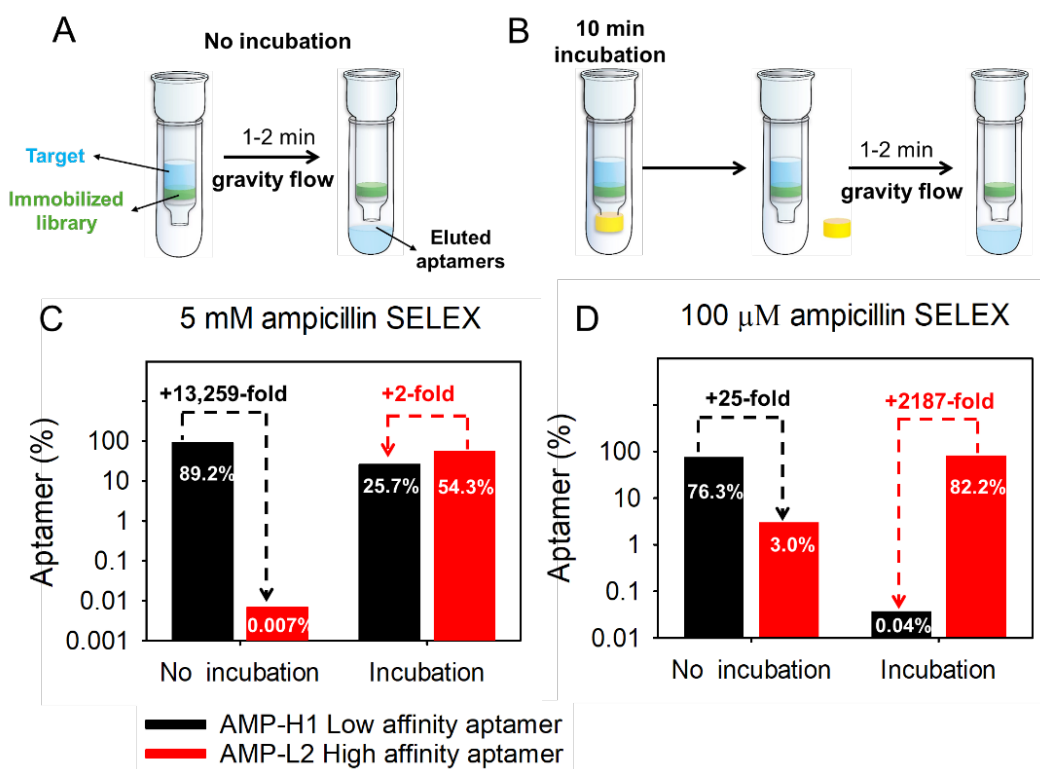


Figure 4.7. The schematic of incubate target solution with the immobilized library with (A) simple gravity flow and (B) 10 min incubation before elution. Percentage of the two aptamers in the (C) 5 mM ampicillin SELEX with and without 10 min incubation and (D) 100 μM ampicillin SELEX with and without 10 min incubation.

In SELEX 4, 100 μM ampicillin was used and results favored AMP-L2 even more. Out of 29,274 sequences, only 11 are AMP-H1, whereas AMP-L2 occupied 82.2% of the library (Figure 4.7D, 4.9). This flip in the relative abundance of the two aptamers strongly supports our hypothesis regarding the kinetic effects during SELEX.

The release kinetics of the two aptamers differed only by approximately 2.25-fold. Yet, the abundance of the two aptamers can differ by ~13,000-fold (in the 5 mM ampicillin selection Figure 4.7C). We attributed this to the power of exponential enrichment in SELEX. A relatively small difference was amplified in each round and compounded to the subsequent rounds. For example, $2.25^{12} = 13301$, or after 12 rounds, such a large difference can be achieved (15 or 16 rounds used

in our SELEX experiments). Thus, conditions that may only have a small influence in sequence enrichment may be highly important for the final sequence distribution.

A SELEX 3: 5 mM ampicillin with 10 min incubation			B SELEX 4: 100 μ M ampicillin with 10 min incubation		
AMP-L2	GCCATCCCGTACCCAGCCGTCATCTTACTA	69.1%	AMP-L2	GCCATCCCGTACCCAGCCGTCATCTTACTA	93.2%
AMP-H1	GCCGTCTCGTACTCAGCCGTCATCTTACTA	29.9%	AMP-L3	GCCATCCCGTACTCTGCCGTCATCTTACTA	5.7%
DAMP-H3	GCCATCYCTTACCCAGCTKTCATCTTACTA	0.1%	AMP-H1	GCCGTCTCGTACTCAGCCGTCATCTTACTA	0.1%
DAMP-H4	GCCATCCCGTACCCAGCCGTCATCTTACTA	0.04%	DAMP-L4	CNARGCGYGRRWGAAC TACTKCWGNCASGT	0.01%
DAMP-H5	GCCATCCCGTACCCAGCCGTCATCTTACTA	0.03%	DAMP-L5	GCCAGCCAGGACCCAGCAGGARATTACTA	0.01%

Figure 4.8. The ampicillin sequencing result for the two selections done with 10 min incubation (SELEX 3 and 4). Sequencing results for ampicillin selection showed a total yield of 38,591 sequences for the 5 mM ampicillin SELEX and 29,274 sequences for the 100 μ M ampicillin SELEX.

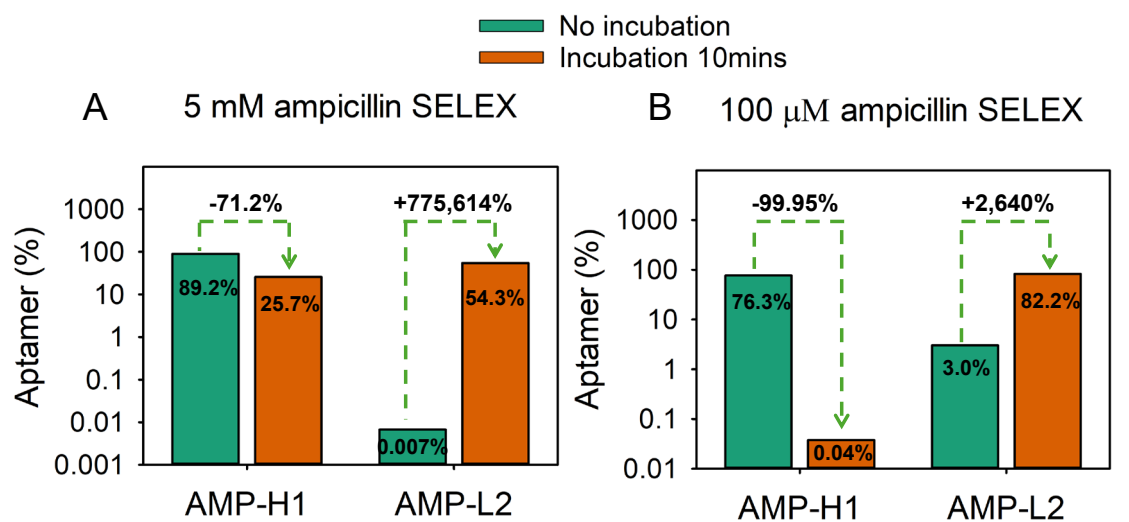


Figure 4.9. Distribution of analyzed sequences obtained from (A) 5 mM ampicillin SELEX and (B) 100 μ M ampicillin SELEX performed under incubation target and without incubation.

4.3.6 Revisiting adenosine aptamer selection also revealing a kinetic influence

Our study of ampicillin selections raised two pivotal questions. First, does the kinetic effect also occur in other aptamer selections? Second, would extending the incubation time always favor the enrichment of high-affinity aptamers? To address these questions, we used adenosine as a SELEX target, since this system has been well studied.^{42, 58, 127, 166}

For many years, the adenosine aptamer refers to the classical aptamer reported by the Szostak group.⁴² This 27-mer aptamer has a 12 - nucleotide conserved pocket that can simultaneously bind two adenosine molecules with a K_d of approximately 7 μ M (Figure 4.10A). By

gradually decreasing adenosine concentration, our group discovered a higher affinity aptamer named Ade1301 (Figure 4.10B), which has a 16 - nucleotide conserved region and binds one adenosine molecule with a K_d of 200 nM.⁵⁸ We previously attributed the dominance of the classical aptamer to their smaller number of conserved nucleotides, leading to a higher abundance in the initial library. Herein, we wanted to test if kinetics also played a role.

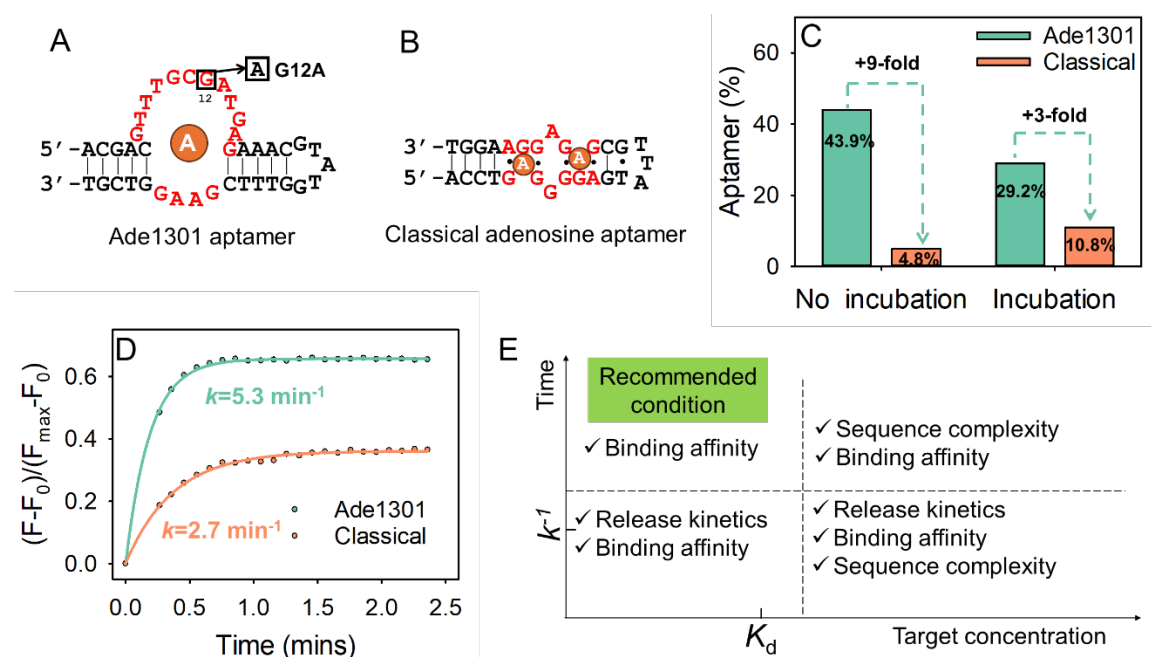


Figure 4.10. The secondary structures of the (A) Ade1301 and (B) classical adenosine aptamers. (C) Percentage of the Ade1301 and classic families in the 100 μ M ampicillin SELEX with and without 10 min incubation. (D) The kinetics of fluorescence increase after adding 100 μ M adenosine for the two aptamers, and the pseudo-first-order rate constant k values are shown. (E) The effect of target concentration and incubation time during SELEX. Here, K_d denotes the dissociation constant of the best aptamer, and k represents the release rate of the slowest-releasing aptamer. The low target concentration and long incubation time region is determined only by aptamer affinity and thus it is the recommended condition.

Using 100 μ M adenosine, we conducted aptamer selections without and with 10 min incubation for 11 rounds (Figure 4.11). In the pool without extra incubation, the most enriched sequences was Ade1301 (conserved region marked by red). We also discovered a single base mutant (see the G-to-A mutation point in Figure 4.10A). This mutant has a K_d of 700 nM based on ITC (Figure 4.12B), which is similar to that of Ade1301, and it can be counted in the Ade1301 family. The Ade1301 family collective occupied 43.9% of the library, whereas the classical aptamer was only 4.8% (Figure 4.10C).

ADESELEX 1: 100 μ M adenosine No incubation		ADESELEX 2: 100 μ M adenosine with 10 min incubation	
Family 1301		Family 1301	
ADE1	GAAGGAGGACTCCGTAAGGGTACTCGTTTGCATGAG 24.8%	ADE1	GAAGGAGGACTCCGTAAGGGTACTCGTTTGCATGAG 19.4%
ADE2	GAAGGAGGACTCCGTAAGGGTACTCGTTTGCATGAG 11.0%	ADE2	GAAGGAGGACTCCGTAAGGGTACTCGTTTGCATGAG 7.4%
ADE5	GAAGAACTCGGTATGGCGTAGTTTGTTCGATGAG 0.4%	ADE3	GAAGAACTCAGTATGGCATAGTTTGTTCGATGAG 1.0%
Other family		ADE4	GAAGAACTCAGCATGGCATAGTTTGTTCGATGAG 0.6%
ADE3	CGAGTTAGTGAGGCGGGTCTAGCTTCCA 1.8%	ADE5	GAAGAACTCTGTACGGCATAGTTTGTTCGATGAG 0.3%
ADE4	GGCGAGGGGCTTGTCCGATGGCGATGAGTA 0.5%		

Figure 4.11. The adenosine sequence result (A) without incubation and (B) after incubation 10 min. Sequencing results for 100 μ M adenosine selection showed a total yield of 40,966 sequences without incubation and 39,999 sequences with 10 min incubation.

We then repeated the selection but with 10 min incubation. The library was still dominated by the Ade1301 family, although its abundance dropped to 29.2%, while the classical aptamer increased to 10.8% (Figure 4.10C Figure 4.12A). Therefore, extending the incubation time during the adenosine selection did not favor the higher affinity Ade1301 aptamer, but favored the lower affinity classical aptamer. Nevertheless, the higher affinity Ade1301 aptamer still dominated the final library (Figure 4.11 Figure 4.12A).

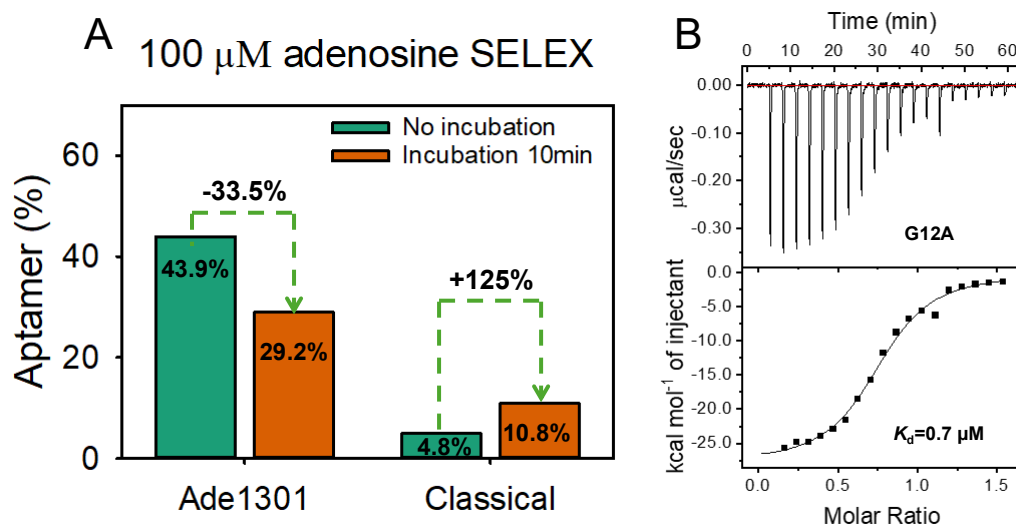


Figure 4.12. (A) Distribution of analyzed sequences obtained from 100 μ M adenosine SELEX performed under two conditions: without additional incubation and with 10 min incubation. Two sequences were analyzed: Ade1301 along with the G12A mutant and the classical adenosine aptamer. (B) ITC traces and integrated heat profiles for titrating 150 μ M adenosine to 20 μ M G12A mutant of the Ade1301 aptamer.

The above results suggested that Ade1301 may dissociate from the capture strand more rapidly than the classical aptamer. We then measured the release kinetics from the capture strand at 100 μ M adenosine using the fluorophore/quencher strand-displacement system. Fitting the release data to a first-order kinetic model, Ade1301 indeed reacted 2-fold faster than the classical aptamer (Figure 4.10D).

4.3.7 Recommended SELEX conditions

Capture - SELEX has been proven to be a robust method for selecting aptamers. Over time, the critical role of target concentration has been recognized. For example, Xiao and coworkers improved the binding affinity of a cocaine aptamer from 20 μ M to 22 nM by gradually reducing the target concentration.¹⁵⁰ Similarly, our group demonstrated that gradually decreasing adenosine/ATP concentrations yields higher-affinity aptamers.⁵⁸ The Xiao group also systematically investigated flunixin concentration confirming a lower target concentration leads to better aptamers.⁸⁴ In this work, we demonstrated that aside from target concentration, target incubation time can also have a huge influence on the outcome of aptamer selection. Some high affinity aptamers may suffer from a kinetic disadvantage, a factor that had not been recognized until now.

Combining current understandings, we summarize our understanding in Figure 7E, where the selection conditions are divided into four regions based on time and concentration. The two lines defining the four regions are drawn based on target concentration being higher than the K_d of the best aptamer and the incubation time longer than the inverse of the release rate constant of the slowest aptamer. The optimal condition for enriching high-affinity aptamers is achieved by using a low target concentration coupled with extended incubation time. Under this condition, kinetic effects are removed and the selected aptamers are based on their thermodynamic affinity, which was nicely demonstrated in the ampicillin selections. In the adenosine selections, the lower-affinity aptamer exhibits slower release kinetics. Therefore, extending the incubation time led to an increased fraction of the low-affinity aptamer. However, because the low target concentration, the adenosine selection remained dominated by Ade1301 regardless of incubation time. For a given target, it is difficult to predict the dissociation kinetics of its highest affinity aptamers. Thus, the most reliable strategy is to let thermodynamics dominate (a low target concentration) and a long incubation time.

If a high concentration is used along with a long incubation time, all aptamers are released regardless of their binding affinity. In this case, aptamers with a simple structure (fewer conserved nucleotides) are in favor since they have a higher initial abundance in the library. Such aptamers

are likely to be low affinity ones due to their limited binding to the target. We believe that the classical adenosine aptamer has such a structural advantage since it has only 12 conserved nucleotides (versus 16 for Ade1301).^{36, 58} For the two bottom regions with a short incubation time, the outcome is strongly affected by the dissociation kinetics and it is difficult to predict the outcome of the selection due to the involvement of many contributing factors.

4.3.8 Aptamer binding kinetics without and with a capture strand

The release kinetics discussed in this paper are all from the capture strand. A slow release from the capture strand does not mean slow binding for free aptamers. Free aptamers binding to small molecules often happens very fast.¹⁶⁹ Association (k_{on}) and dissociation (k_{off}) rate constants are typically determined in real time with label-free biosensors such as surface plasmon resonance (SPR)¹⁷⁰. In this study, we applied kinetic ITC to measure the kinetic constants of free AMP-H1 and AMP-L2 aptamers (Figure 4.13).^{100, 171} Notably, the k_{on} of AMP-L2 ($9400 \text{ M}^{-1}\text{s}^{-1}$) was larger than that of AMP-H1 ($1300 \text{ M}^{-1}\text{s}^{-1}$), indicating that free AMP-L2 binds to ampicillin more rapidly. Therefore, the slower release of AMP-L2 cannot be explained by its slower intrinsic binding (Figure 4.13B).

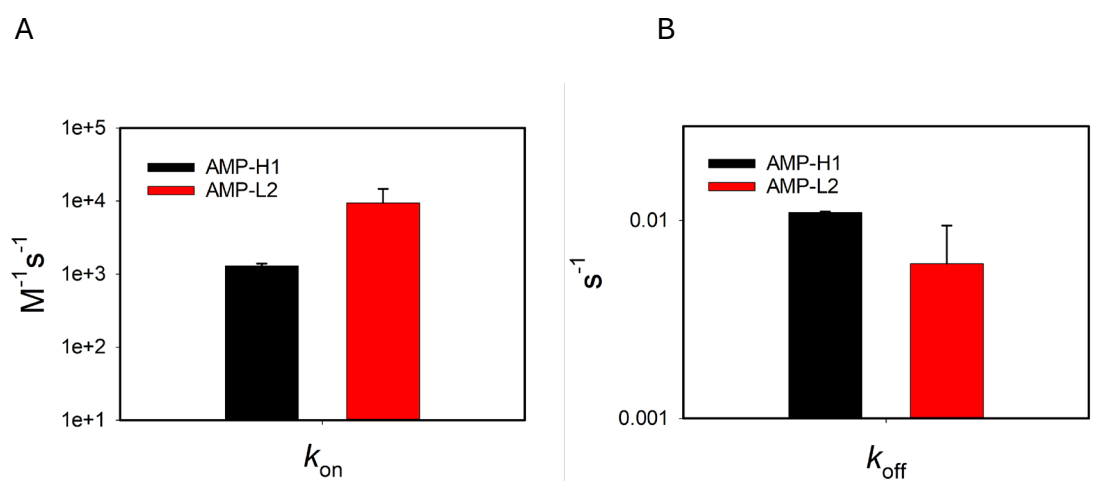


Figure 4.13. The (A) k_{on} and (B) k_{off} values of the two ampicillin aptamers from Affinimeter software using the Kinetic ITC method.

The more relevant kinetics to this study is the reaction in Figure 4.6C, where an aptamer is first hybridized to a capture strand. In this case, aptamer binding needs to overcome the capture strand and the kinetics is expected to be slower than that of free aptamer. For example, for the AMP-L2 aptamer (Figure 4.6C), the rate of its release from the capture strand is 0.8 min^{-1} or 0.013 s^{-1} , which is much slower than that of the free aptamer with 5 mM ampicillin: $9400 \text{ M}^{-1}\text{s}^{-1} \times 0.005 \text{ M}$

ampicillin = 47 s^{-1} (slower by 3615-fold). For capture-SELEX, the binding kinetics in the presence of the capture strand is more relevant.

4.3.9 Implication for biosensors

For biosensor applications, strand displacement reactions are commonly used.^{54, 153, 172, 173} In these cases, the release kinetics of aptamers from capture strands are crucial in determining the performance of aptamer-based biosensors. Slow-release aptamers exhibit prolonged dissociation time to reach equilibrium, which means a slower sensor response. Some other sensor designs do not involve hybridization to a cDNA or capture strand (e.g. aptamer beacons).¹⁷⁴ In these cases, the slow releasing kinetics discussed here is irrelevant as discussed above.

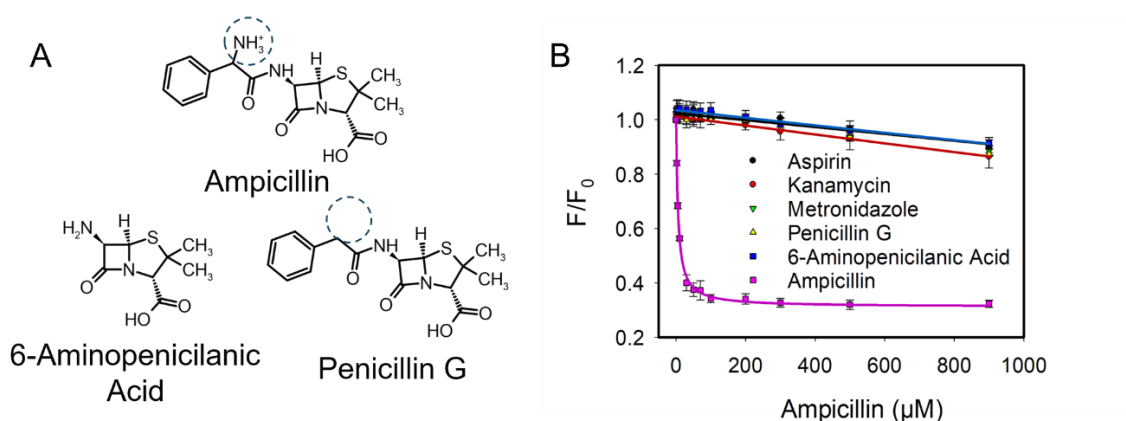


Figure 4.14. (A) Structures of some ampicillin analogs tested for selectivity. (B) The ThT fluorescence titration curve of the AMP-L2 aptamer for ampicillin analogs and other antibiotics and drugs.

4.3.10 The AMP-L2 aptamer is highly selective

In this work, we obtained a few aptamers for ampicillin. Among them, the AMP-L2 aptamer has the highest affinity. We then evaluated its selectivity using the ThT fluorescence assay. AMP-L2 exhibits high specificity for ampicillin, since other antibiotics including penicillin G, aspirin, kanamycin, and metronidazole did not show much fluorescence change (Figure 4.14B). Despite the structural similarity between ampicillin and penicillin G (differing only by a single amino group), AMP-L2 shows no affinity for penicillin G, indicating the critical role of the amino group in aptamer binding (Figure 4.14A). Furthermore, AMP-L2 does not bind to 6-aminopenicillanic acid, also suggesting that the left portion of the ampicillin molecule, which includes the benzene ring and amino group, is essential for aptamer binding (Figure 4.14A).

Finally, the influence of metal ions on AMP-L2 binding was studied. Increasing Mg^{2+} concentrations from 0 mM to 10 mM slightly enhanced the affinity, reducing the K_d from 16.8 μM to 5.7 μM (Figure 4.15). In contrast, varying Na^+ concentrations did not affect its binding (Figure 4.15). Thus, AMP-L2 is a robust aptamer capable of recognizing ampicillin across different buffer conditions.

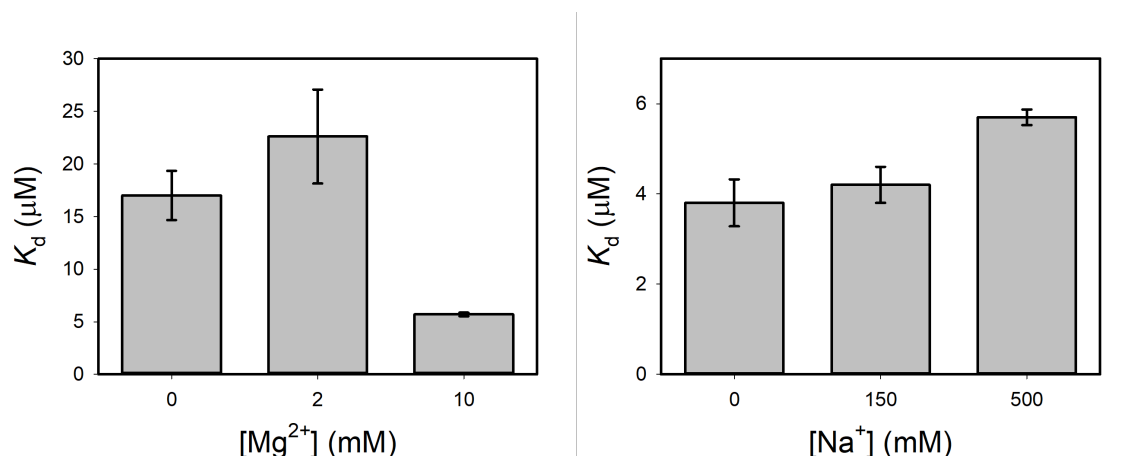


Figure 4.15. The K_d value of the AMP-L2 aptamer for ampicillin under three different (A) Mg^{2+} concentrations and (B) Na^+ concentrations measured using ThT fluorescence spectroscopy.

4.4 Conclusions

In this study, we started with the selection of aptamers for ampicillin and noticed a discrepancy between aptamer affinity and abundance. Some high affinity aptamers were poorly enriched. By dissecting the SELEX process from the library immobilization step to the target-induced aptamer release step, a strong kinetic effect was observed. Particularly, aptamer dissociation rates from the capture strand, critically influenced the SELEX outcome. By extending the incubation time, we successfully shifted enrichment from low affinity AMP-H1 (K_d 12.7 μM) to high affinity AMP-L2 (K_d 1.8 μM), achieving a >7,000-fold increase in AMP-L2 abundance. A strong kinetic effect was also observed for aptamer selections using adenosine, highlighting their broad applicability. Based on the results, we reasoned that the optimal condition for SELEX is a combination of low target concentration and long incubation time, which can remove kinetic effects and let thermodynamics dominate the reaction. We also discussed the implication in biosensor development. Finally, the AMP-L2 aptamer demonstrated high specificity for ampicillin, distinguishing it from structurally similar antibiotics like penicillin G.

Chapter 5 Quantitative comparison of Capture-SELEX, GO-SELEX and Gold-SELEX for enrichment of aptamers

The results presented in this chapter have been published as:

Ding, Yuzhe, and Juewen Liu. "Quantitative comparison of capture-SELEX, GO-SELEX, and Gold-SELEX for enrichment of aptamers." *Analytical Chemistry* 95.39 (2023): 14651-14658.

5.1 Introduction

With the use of nanomaterials for DNA-based biosensor development, especially the pioneering work of adsorbing DNA aptamers onto graphene oxide (GO),¹⁷⁵ many adsorption-based SELEX methods have been reported. Two representative methods are GO-SELEX,¹⁷⁶ and gold-SELEX.¹⁷⁷ In these methods, a DNA library is immobilized via adsorption onto a nanomaterial. The assumption was that target binding can result in the desorption of aptamer sequences from nanomaterials,¹⁷⁵ or can inhibit the adsorption of aptamers,¹⁷⁸ whereas non-binding sequences are adsorbed. These adsorption methods are popular since they are more cost-effective and simpler to perform compared to hybridization-based capture-SELEX.

Because the most important step in SELEX is to separate aptamers from non-aptamer sequences, a quantitative comparison of the separation efficiency in different methods is critical for the development of robust selection methods. We recently used capture-SELEX to obtain high affinity aptamers for adenosine and ATP, and the resulting libraries contained a few different aptamer sequences.⁵⁸ Herein, we spiked an enriched ATP selection library into a naïve library and systematically compared the separation efficiencies using quantitative PCR (qPCR) and deep sequencing. Based on our data, capture-SELEX allowed efficient aptamer enrichment, GO showed a much lower efficiency, while gold failed to enrich the spiked aptamer sequences.

5.2 Materials and methods

Materials. The DNA samples used in this study were from Integrated DNA Technologies (Coralville, IA, USA, see Table 5.1 for sequences). Streptavidin agarose resin was from Fisher Scientific. Graphene oxide (GO) flakes were from ACS Material (Pasadena, CA, USA) and they were dispersed in water with the assistance of ultrasound. H₂AuCl₄ was from Sigma-Aldrich (St. Louis, MO). Sodium citrate was from Mandel Scientific (Guelph, ON, Canada). Adenosine, sodium hydroxide, hydrochloric acid, tris(hydroxymethyl)aminomethane (Tris), and Amicon Ultra-0.5

centrifugal filter unit (3k and 10k molecular weight cut off) were from Millipore-Sigma (Oakville, ON, Canada). Micro bio-spin chromatography columns and SsoFast EvaGreen Supermix were from Bio-Rad. dNTP mix, and Taq DNA polymerase with ThermoPol buffer were from New England Biolabs (Ipswich, MA, USA). All of the buffers and solutions were prepared with Milli-Q water. The SELEX buffer contained 50 mM Tris, pH 7.6, 500 mM NaCl and 20 mM MgCl₂.

Table 5.1. The DNA sequence used in this work

DNA names	Sequences (from 5' to 3')
N36 library	GGAGGCTCTCGGGACGAC-N36-GTCGTCCCGATCACTTGAATGGTCT
Biot-Column	GTCGTCCCGAGAGCCATA /3BioTEG/
Forward primer (FP)	GGAGGCTCTCGGGACGAC
Reverse primer (RP)	AGACCATTCAAGTGATCGGGACG
P5-506	AATGATACGGCGACCACCGAGATCTACAACTGCATAAACTCTTCCCTACACGACGC TCTTCCGATCTAGACCATTCAAGTGATCGGGACG
P7-701	CAAGCAGAAGACGGCATAACGAGATTCGCCTTAGTGACTGGAGTTCAGACGTGTGCTCT T CCGATCTGGAGGCTCTCGGGACGAC
P7-702	CAAGCAGAAGACGGCATAACGAGATCTAGTACGGTGACTGGAGTTCAGACGTGTGCTC TTCCGATCTGGAGGCTCTCGGGACGAC
P7-703	CAAGCAGAAGACGGCATAACGAGATTCTGCCTGTGACTGGAGTTCAGACGTGTGCTCT T CCGATCTGGAGGCTCTCGGGACGAC

Note: /3BioTEG/ is biotinylation at the 3'-end, and FAM is carboxyfluorescein. The last four sequences are for DNA sequencing.

Capture-SELEX. The spiked library was prepared by mixing 1 pmol of the round 9 library from our previous ATP selection with 99 pmol of the naïve library.⁵⁸ We then used this spiked library to do a one-round selection. The 100 pmol of the library DNA was dissolved in 500 µL SELEX buffer and annealed with a five-fold excess of biotinylated capture strand by heating the sample in boiling water for 1 min followed by gradual cooling to room temperature over 30 min. Subsequently, 0.2

mL streptavidin-coated agarose beads were loaded into a micro chromatography column and washed five times using 500 μ L SELEX buffer each time. The annealed DNA was then passed through the resin eight times to facilitate binding. Subsequently, the resin was washed 12 times to remove unbound library, and the eluted solutions from the 11th and 12th wash were collected as a reference for qPCR. Following the washing steps, 750 μ L of a 100 μ M adenosine dissolved in the SELEX buffer was added, and the eluted DNA was collected using gravity flow.

Gold-SELEX. Gold nanoparticles (AuNPs) of 13 nm diameter (10 nM) were prepared by the citrate reduction method,¹⁷⁹ and were concentrated 5-fold following a previous study.¹⁷⁷ The spiked library dissolved in the SELEX buffer was immediately cooled on ice after heating for 5 min in boiling water. Subsequently, the library (3.5 μ M diluted in 28.6 μ L SELEX buffer) was incubated with the concentrated AuNPs (1 mL, final 14.3 mM Na⁺) for 30 min, and the resulting complex was centrifuged for 15 min and washed once by water to remove unbound library DNA. The target (100 μ M adenosine in the SELEX buffer) was then added followed by incubation for 1 h with gentle shaking on a vertical rotator. The supernatant was then collected after centrifugation for 15 min. After each centrifugation, the AuNPs were dispersed with a brief ultrasonication treatment.

GO-SELEX. The annealed library (100 pmol) was incubated with 100 μ M adenosine for 1 h in SELEX buffer, followed by incubation with 0.22 mg/mL GO for 1 h. The supernatant was then collected after centrifugation of the sample at 15000 rpm for 15 min.

Blocked GO-SELEX. The spiked library (100 pmol) was mixed with blocking DNA (the reverse primer and biotin capture strand, 200 pmol each) in SELEX buffer. The resulting mixture was annealed to ensure blocking. The subsequent steps were performed as outlined in the GO-SELEX described above.

qPCR test. For comparison purposes, all the samples, except for the capture-SELEX unbound library, were diluted to a final volume of 1 mL. The capture-SELEX unbound library was tested using a volume of 500 μ L. To prepare qPCR reactions, the following components were mixed in a 20 μ L system: 2 $\times$ SsoFast EvaGreen Supermix, 8 μ M forward primer, 8 μ M reverse primer, 6 μ L Milli-Q water and 2 μ L template.

DNA sequencing. The libraries obtained after the one-round selection were PCR amplified using a combination of P5-506 and one of the P7-701, P7-702, or P7-703 sequencing primers (Table 5.1). These primers were designed to include a unique index sequence for Illumina sequencing. The resulting PCR products were purified using small DNA fragment extraction kit (IBI Scientific). The concentration of the purified DNA was determined based on the absorbance at 260 nm (Tecan

Spark), and the sequencing was performed at McMaster University. The sequencing data were analyzed using Geneious Prime software.

5.3 Results and discussion

5.3.1 The three library-immobilization SELEX methods

Figure 5.1 illustrates the three library-immobilization SELEX methods studied in this work. In the capture-SELEX method (Figure 5.1A), the library was immobilized by hybridization to a biotinylated DNA attached to a streptavidin column via a 15-base pair region. After washing the unbound library, a target molecule (adenosine in this case) was added to elute aptamers. In library design, a 10-base pair (bp) stem is expected to form upon target binding.

Table 5.2. The conditions of some literature reported GO and gold SELEX methods. For GO-SELEX, we defined the method shown in Figure 5.1C to be method 2, and in Figure 5.1D to be method 1.

GO or Gold	Method 1 or 2	Volume	pH	[M ⁺] (mM)	[M ²⁺] (mM)	library quantity (pmol)	GO or AuNP Concentration	Ref.
GO	2	N/A	7.4	N/A	NA	200	1 mg	¹⁸⁰
GO	2	260 μ L	7.4	105	3	125	0.25 mg/mL	¹⁸¹
GO	2	800 μ L	7.6	105	3	1000	2 mg/mL	¹⁸²
GO	2	N/A	7.4	105	2	800	4 mg/mL	¹⁸³
GO	2	1 mL	7.5	150	2	100	5 mg/mL	¹⁸⁴
GO	1	N/A	7.4	160	2.5	2000	2 mg/mL	⁷⁷
Gold	-	2 mL	7.4	60	0	200	16.7 nM	¹⁸⁵
Gold	-	200 μ L	7.4	14	0	30	0.375 nM	⁸¹
Gold	-	500 μ L	7.5	75	10	N/A	60 nM	¹⁸⁶

For a fair comparison, the same library was used for the two adsorption-based SELEX methods. In gold-SELEX (Figure 5.1B), the library was incubated with AuNPs for 30 min and the resulting sample was centrifuged to remove unbound DNA. Adenosine was then incubated with the AuNP-library complex for 1 h. The supernatant containing the released sequences was collected.

Two GO-SELEX methods were reported in the literature. Method 1 just replaces AuNPs with GO as shown in Figure 5.1D. However, Wang and coworkers pointed out that this is not a reliable method,⁸⁰ and we were also concerned about nonspecific desorption of the DNA library.¹⁸⁷ In addition, when we searched the literature, most GO-SELEX papers (Table 5.2) used method 2 as shown in Figure 5.1C, where the library and target were mixed before adding GO. The assumption is that aptamer/target complexes are not adsorbed due to the formation of a rigid binding structure,¹⁸⁸ whereas non-aptamer sequences are adsorbed. For the development of biosensors, Fan et al. also articulated the advantage of this method.¹⁷⁸ Therefore, in this work, we used method 2 for GO-SELEX (Figure 5.1C).

It is interesting to note that no one used the method 2 in Figure 5.1C for AuNPs. In fact, the number of gold-SELEX papers was much lower than that of GO-SELEX. Based on our previous work to study aptamer-based label-free colorimetric biosensors, such a method is unlikely to work for most targets due to strong and fast adsorption of target molecules to AuNPs, which can inhibit DNA adsorption in a sequence-independent manner.^{69, 189}

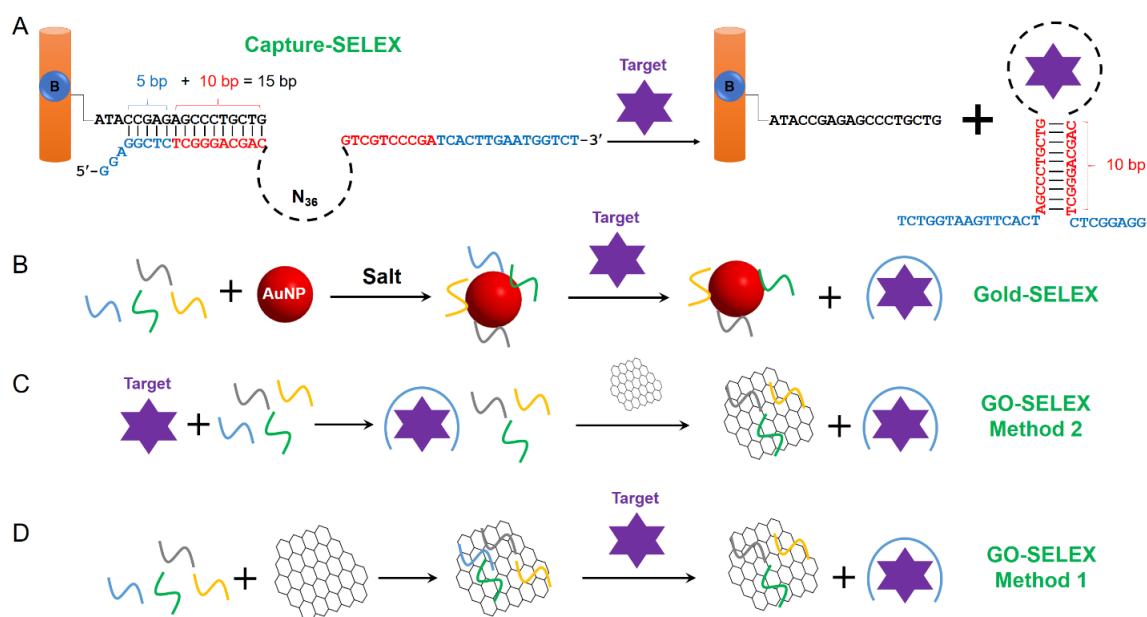


Figure 5.1. The three library-immobilization SELEX methods tested in this study: (A) capture-SELEX by hybridization, (B) gold-SELEX by adsorption on AuNPs, and (C) GO-SELEX by inhibited adsorption on GO (method 2). (D) Another GO-SELEX method (method 1), in which target binding is believed to induce aptamer desorption from GO, and this method was not tested in this work. The same library was used in all these studies, which contains an N₃₆ random region flanked by two constant regions that can form a 10-bp stem.

5.3.2 Aptamer separation efficiency measured by qPCR

To compare the aptamer selection efficiency of the three methods, we spiked 1% of the round 9 library from our previous ATP selection⁵⁸ into a naïve library and performed one round of selection by adding 100 μ M adenosine as a target. The partition of immobilized (mostly non-aptamer) and free DNA (potential aptamers) was first quantified using qPCR based on a standard curve (Figure 5.2). Figure 5.3A shows an example for the capture-SELEX method. The qPCR traces were normalized, and the PCR cycle number at a 50% fluorescence increase was compared. Each PCR cycle difference reflected a two-fold difference in the initial DNA concentration.^{190, 191}

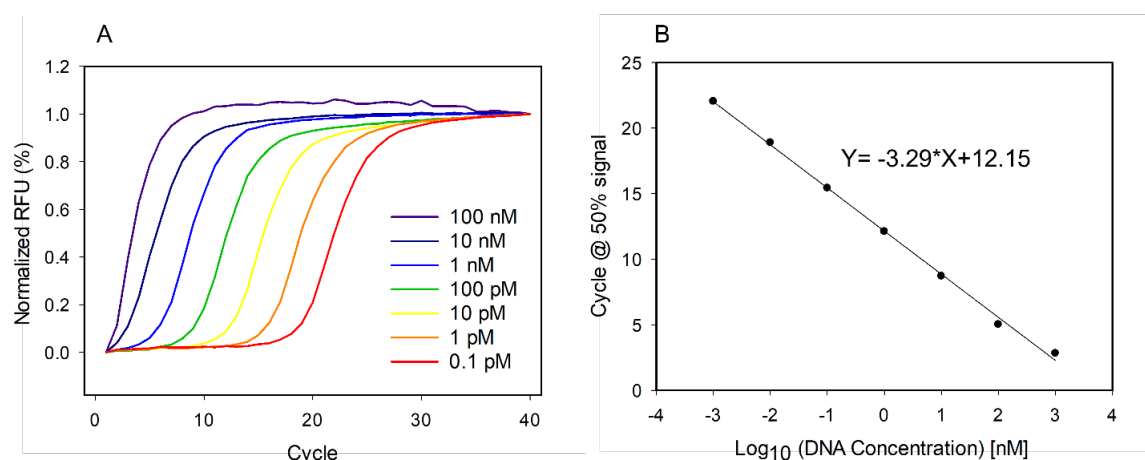


Figure 5.2. (A) Normalized qPCR traces of amplifying the DNA library at different initial concentrations. (B) The standard curve of different concentrations library in the qPCR based on the data in panel (A).

After immobilizing 100 pmol of the spiked library DNA via hybridization to streptavidin-coated agarose beads, the qPCR cycle of non-hybridized library DNA was 11.4 (corresponding to an absolute initial DNA quantity of 0.85 pmol), and thus, the fraction of hybridized library was $99.1\% \pm 0.4\%$. Such a high hybridization efficiency was attributed to the use of excess biotinylated complementary DNA (5:1). The immobilized library was then subjected to extensive washing by buffer to remove unbound or weakly bound DNA. To quantify such nonspecific release, we washed the beads 12 times with SELEX buffer (each time 500 μ L), and collected the last two washing eluents. They represented only $0.13\% \pm 0.01\%$ of the immobilized library (Figure 2B, blue bar for the capture-SELEX set). Therefore, at this point, the majority of the library sequences were stably hybridized to the DNA on the beads.

After adding 100 μ M adenosine, $0.41\% \pm 0.05\%$ of the library was eluted (Figure 5.3B red bar in the capture-SELEX set). Therefore, most of the library was still immobilized after adding

adenosine. This is reasonable since we only spiked 1% of the enriched library and the remaining 99% of the sequences were random and not expected to bind to adenosine. This extra 0.41% - 0.13% = 0.28% increase suggests selective aptamer enrichment, and at the beginning of the next round, $0.28\%/0.41\% = 68\%$ of the eluted library could be aptamers. In other words, aptamer abundance increased from below 1% to around 68%, based on qPCR measurements.

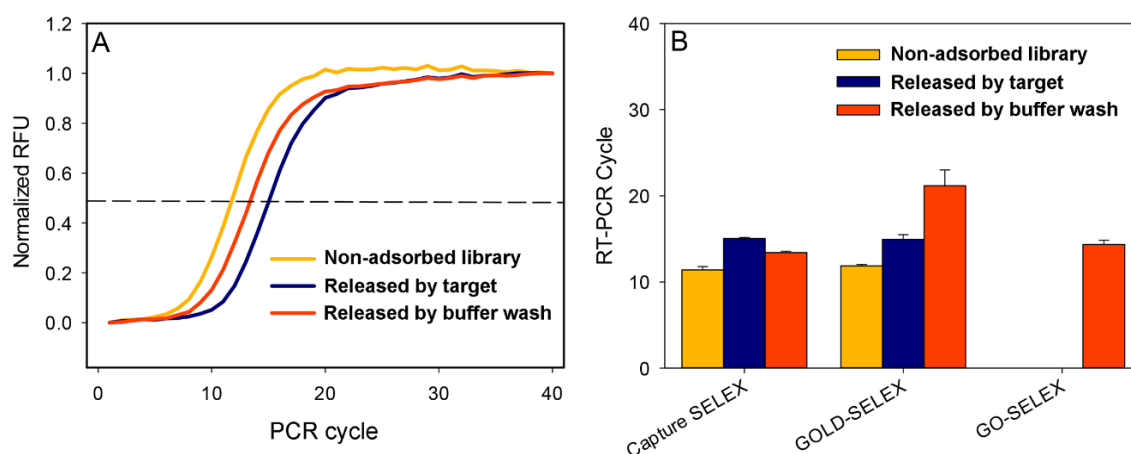


Figure 5.3. (A) Normalized qPCR fluorescence traces for the capture-SELEX sample. The target eluted trace has a lower PCR cycle number indicating it contained more initial DNA compared to the buffer wash sample. (B) The number of qPCR cycles for the three SELEX methods. The cycle is defined based on the 50% fluorescence increase and the error bar is from the standard deviation of three independent measurements. For the GO-SELEX experiment, since method 2 was used, it only has one bar.

We then did a similar measurement for the gold-SELEX system, and $1.2\% \pm 0.2\%$ of the library remained in the supernatant after adsorption by AuNPs (Figure 5.3, orange bar in the gold-SELEX set). Therefore, our 13 nm AuNPs (concentrated from 10 nM to 60 nM) absorbed most of the 100 nM library in the system (100 pmol library dissolved in 1 mL). Washing the library with water further decreased the DNA in the supernatant to $0.14\% \pm 0.06\%$ (Figure 5.3, blue bar). After 1 h of incubation with 100 μ M adenosine, only $0.003\% \pm 0.002\%$ of the library was eluted released (Figure 5.3 red bar). Thus, most of the library remained adsorbed by AuNPs. In the capture-SELEX experiment, the blue bar is higher than the orange bar, which is expected for aptamer enrichment. However, in the gold-SELEX, the blue bar is even lower. The very low amount of DNA released from the AuNPs suggests that most spiked aptamers were still adsorbed onto the AuNPs. This result can be explained by the strong adsorption of DNA by AuNPs,¹⁹² and adenosine could not compete with AuNPs for binding to DNA.

For GO-SELEX, we used a final of GO concentration of 221 $\mu\text{g/mL}$ to adsorb 100 pmol of the library in 1 mL in the presence of 100 μM adenosine. After 1 h incubation, non-adsorbed DNA was separated by centrifugation and quantified using qPCR. Only $0.22\% \pm 0.07\%$ of the library was in the supernatant, and the majority of the library was adsorbed onto GO (Figure 5.3, red bar). Since we used the inhibited adsorption method (method 2) for GO-SELEX, there were no buffer wash data. Nevertheless, since only 0.22% of the library was in the supernatant and we expected to have close to 1% aptamer in the library, most of the aptamer sequences in the library were adsorbed by GO, even in the presence of 100 μM adenosine.

For all three methods, the fraction of released library DNA in the presence of adenosine was quite low ($< 1\%$). AuNPs had the highest DNA adsorption affinity, resulting in the lowest level of non-adsorbed DNA. Since the aptamer content in the library was below 1%, had the released DNA been significantly more than 1%, and the majority of the released DNA would be non-aptamers, which would likely lead to failed selections. Therefore, the low fractions of DNA released in our experiments suggested a reasonable condition for SELEX.

5.3.3 Separation efficiency measured by deep sequencing

Although qPCR can measure the concentration of released DNA, it provides no information on the sequence distribution. We then used deep sequencing to evaluate the enrichment of three adenosine-binding aptamers: Ade1301, Ade1304, and the classical aptamer. The classical aptamer reported by Huizenga and Szostak has a K_d of approximately 6 μM adenosine,⁴² whereas the K_d of the Ade1301 and Ade1304 aptamers was approximately 200 nM.⁵⁸ We spiked 1% of the round 9 library from our previous ATP selection, which contained 48.5% Ade1301, 7.5% Ade1304, and 12.6% classical aptamers. We then sequenced the 1% spiked library, and the fractions of these aptamers were 0.7% Ade1301, 0.11% Ade1304, and 0.21% classical aptamers (Figure 5.4A, before SELEX), and the result was in agreement with the spiked amount.

We then sequenced the DNA in the supernatants for the three selection methods after adding 100 μM of adenosine. Approximately 42800 sequences were obtained for each sample, allowing statistical analysis. In the capture-SELEX method (Figure 5.4B, red bar), the Ade1301 aptamer was enriched 48.3-fold (from 0.7% to 35.9%). Similarly, the Ade1304 and classical aptamers were enriched 50.7-fold and 30.4-fold, respectively. The higher K_d value of the classical aptamer can explain its lower enrichment compared to the other two aptamers. The overall fold of enrichment was comparable to that estimated by qPCR (68-fold). After this round of selection, nearly half of the resulting library was aptamers (Figure 5.4A), indicating that capture-SELEX is an efficient method for aptamer selection.

In contrast, for gold-SELEX (Figure 5.4B, orange bar), the fractions of the Ade1301, Ade1304, and classical aptamers in the supernatant were only 0.3%, 0.08%, and 0.09%, respectively. Compared to the spiked library before adding adenosine, the abundance of the aptamers even decreased. This decrease is likely due to the aptamers being rich in purines, which have a relatively higher affinity to AuNPs.¹⁹³ Therefore, adenosine released very few DNA strands from the AuNPs, and the released DNA contained even fewer aptamer sequences than the initial library. Therefore, gold-SELEX cannot effectively enrich aptamers for adenosine.

For GO-SELEX (Figure 5.4B, yellow bar), the fractions of adenosine-released Ade1301, Ade1304, and classical aptamers were 0.8%, 0.08%, and 0.17%, respectively. The corresponding enrichment were 1.02, 0.82, and 0.82-fold (1-fold indicates no enrichment). Therefore, only Ade1301 was slightly enriched, while the overall aptamer content decreased. The decreased relative abundance of aptamers could also be explained by the purine-rich aptamer sequences.¹⁹⁴ Compared to typical aptamers used in biosensors, which are truncated shorter sequences,¹⁷⁵ the library sequence is much longer with two additional primer binding regions that can interact with the GO surface regardless of aptamer binding. This may explain the poor efficiency of the aptamer enrichment. The error rate of Illumina sequencing is between 0.1 and 1%,¹⁹⁵ and our entire library length is below 80-nt. In addition, we examined three different sequences and they all gave consistent results. Therefore, our results are statistically significant.

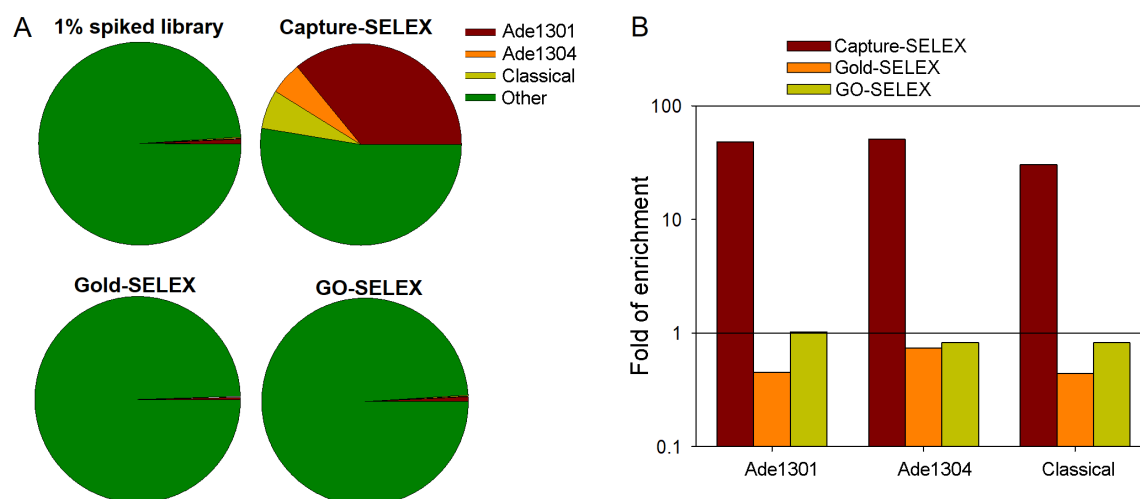


Figure 5.4. (A) Pie charts for the library compositions before and after the one-round selection using the 1% spiked library of the three SELEX methods. (B) The fold of aptamer enrichment after the one-round selection, where 1-fold means no enrichment of the aptamer sequence.

5.3.4 Blocked GO-SELEX

The poor enrichment of aptamers in the GO-SELEX was a bit surprising, since aptamer sensors based on the same principle have been repeatedly demonstrated by many labs.¹⁹⁶ We attributed the poor aptamer enrichment to the primer binding regions. The library has two constant primer binding regions, and its total length is approximately 40-nt. Despite the base paired region designed in our library, there were still 21 free nucleotides that could interact with GO.

To test this hypothesis, we blocked the primer binding regions by adding complementary DNA strands (Figure 5.5A). Because the adsorption of duplex DNA to GO is much weaker than the adsorption of single-stranded DNA,¹⁸⁷ we expected this system to have better enrichment. This blocked library was also subjected to one round of GO-SELEX. The DNA eluted collected in the presence of the target was $0.36\% \pm 0.19\%$ (Figure 5.6), which was compared with the non-blocked experiment. Deep sequencing revealed that the three aptamers reached 1.14, 1.11, and 1.14-fold increase, respectively, which were all higher than the non-blocked (Figure 5.5B). This result indicated that GO-SELEX could, in principle, work when the primer binding regions are blocked. Nevertheless, compared to the ~50-fold enrichment in the capture-SELEX, the blocked GO-SELEX enriched less than 20%, which was over 200-fold less efficient than capture-SELEX.

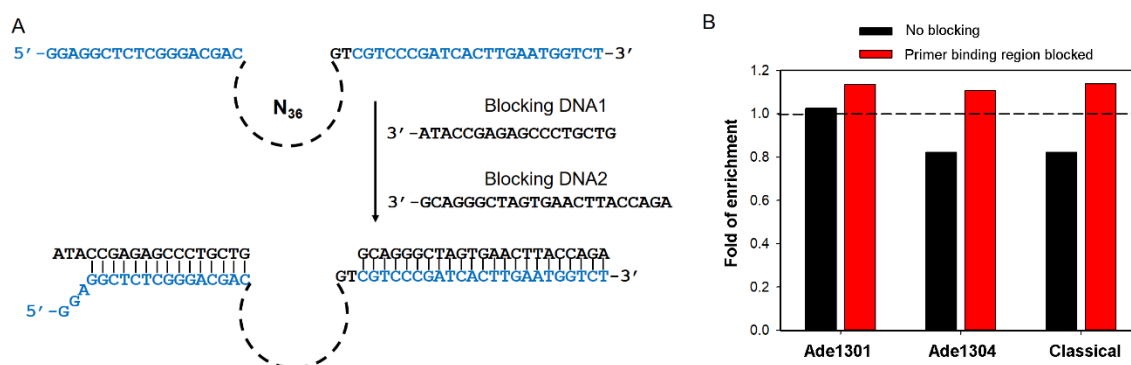


Figure 5.5. (A) A scheme of blocking the library by adding two complementary DNA strands to hybridize with the primer binding regions, which may decrease nonspecific adsorption to GO. (B) The fold of aptamer enrichment after the one-round blocked GO-SELEX, where 1-fold means no enrichment of aptamer sequences.

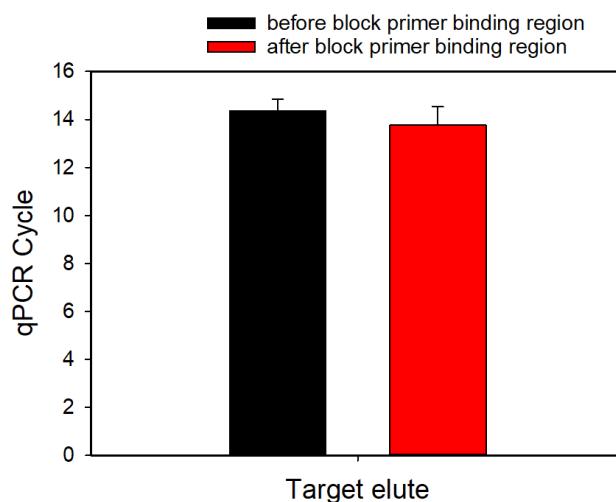


Figure 5.6. The number of target elute qPCR cycles for the GO-SELEX experiment. The cycle is defined based on the 50% fluorescence increase and the error bar is from the standard deviation of three independent measurements.

5.3.5 Thermodynamic aspect of library-immobilization SELEX

Many nanomaterials can adsorb DNA and have been used to develop aptamer-based biosensors.¹⁹⁷ Among them, GO was the first studied and the most used.^{175, 198} These sensing studies have inspired researchers to use the same strategy for aptamer selection, and the hope was to specifically release aptamer containing sequences from nanomaterials. In this work, we studied GO and AuNPs as two representative nanomaterials, which have different adsorption strengths for DNA.¹⁹² We also studied the hybridization-based capture-SELEX method. For any library-immobilized SELEX, the assumption is that aptamer/target binding is stronger than the force for aptamer immobilization, and adding target molecules can shift the equilibrium to aptamer/target binding. Herein, we discuss the energy aspects of each of the selection methods.

For the capture-SELEX method, the library was immobilized via a 15 bp duplex. After binding to a target, the aptamer formed a hairpin structure with a 10 bp stem (Figure 5.7A). Considering that a hairpin has a higher melting temperature than two separate DNA strands of the same sequence,¹⁹⁹ the energy difference that aptamer binding requires is less than that of 5 bp. Based on the success of structure-switching (or strand displacement) design of many aptamers,^{54, 200} aptamer binding can supply such energy. This aptamer release reaction is not dependent on the K_d of an aptamer, since even weakly binding aptamers can be released by using a sufficiently high target concentration (e.g., higher than the K_d of the aptamer). That's why aptamers with K_d values as high as 5 mM, such as the glucose aptamer, can still be isolated.⁷¹ Recently, the Stojanovic group

performed a systematic thermodynamic analysis of aptamer binding to target molecules containing different functional groups.⁷⁰ The ΔG of the displacement reaction (aptamer binding to a target molecule to displace the aptamer from the capture strand) was calculated to range from -7 to -40 kJ/mol for aptamers with K_d values ranging from low nM to mid-mM. Since target molecules can shift the equilibrium of aptamers but do not disrupt DNA hybridization (except when high concentrations of hydrogen bond disruptors, such as urea, or hydrophobic solvents are added), capture-SELEX showed good aptamer enrichment.

The above discussion is based on thermodynamics. Another note is that the 15 bp used to hybridize the library can be divided into two regions containing 5 and 10 bp, respectively (Figure 5.1A). Once an aptamer is bound to a target forming the 10 bp stem, the remaining 5 bp cannot stably hold the library strand, leading to its dissociation (Figure 5.7A). Therefore, this design lowers the kinetic barrier of the reaction. If aptamer binding needs to compete with the full 15 bp, the released of aptamer would be much lower, which was beautifully demonstrated by Nutiu and Li in their initial publication of structure-switching aptamers.⁵⁴ Therefore, a delicate balance between stable hybridization and aptamer release needs to be achieved.

GO can adsorb DNA with a range of affinities.^{201, 202} In particular, based on the excellent performance of GO for the detection of adenosine/ATP,¹⁹⁶ one would expect GO to be a good surface for enrichment of adenosine aptamers. In the case of GO-SELEX, the assumption was that aptamer/target complex cannot be adsorbed, but non-aptamer sequences are stably adsorbed. This assumption might be true for truncated aptamers, which were used in GO-based biosensors.¹⁷⁸ However, the library also contained primer binding regions that were of comparable length to the random region (Figure 5.7B). We have already demonstrated the adverse effect of the primer binding regions above.

For the gold-SELEX method, DNA adsorption was achieved via strong coordination interactions between DNA base and AuNPs, which is greater than 100 kJ/mol for each base.^{78, 203} The major interactions between an aptamer and target molecules are much weaker π - π stacking, electrostatic interactions and hydrogen bonding.²⁶ The energy of hydrogen bonding is around 25 to 40 kJ/mol and the energy of π - π stacking is even lower ($\sim$ 10 kJ/mol).²⁰⁴ Thus, the weaker binding affinity between aptamers and targets cannot compete with the stable adsorption of DNA to gold. This is why barely any aptamers were desorbed from AuNPs even for truncated aptamers.¹⁹² For the DNA library with primer binding regions, the desorption of DNA it is even more difficult.

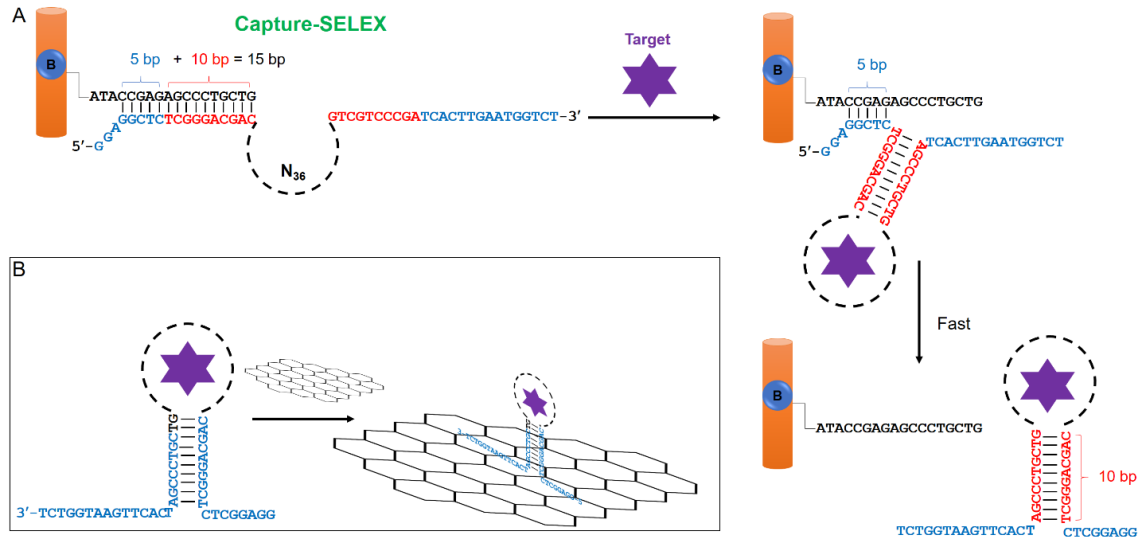


Figure 5.7. Schemes showing the immobilization of DNA in (A) capture-SELEX, and (B) GO-SELEX. In the capture-SELEX, the library was immobilized via a 15 bp region to the biotinylated capture strand. After binding with a target, the aptamer can form a hairpin structure with a 10-bp stem. In GO-SELEX, it is possible to have adsorption achieved via the primer binding regions even after binding to the target.

5.3.6 Enrichment factor and SELEX

Based on the above data, it was evident that different DNA immobilization methods resulted in significant differences in aptamer enrichment. Here, we mathematically describe the enrichment factor. Assuming the fraction of aptamer sequences in a library at round n is f_n , and at round $n+1$ is f_{n+1} , the enrichment factor is defined as f_{n+1}/f_n . After adding a target to the round n library, the round $n+1$ library is collected. Since more aptamers are expected to be released compared to non-aptamer sequences, we define a and b as the fraction of collected aptamers and non-aptamers, respectively. Therefore, in round $n+1$, the fraction of the aptamer is $f_{n+1} = \frac{af_n}{af_n + (1-f_n)b}$, and the enrichment factor is $f_{n+1}/f_n = \frac{a}{af_n + (1-f_n)b}$. It was estimated that for a naïve library, the chance of having aptamer sequences is about one in 10^{10} sequences ($f = 10^{-10}$).¹⁹ 10^{10} is roughly equal to 4^{17} , suggesting that a typical aptamer has around 17 conserved nucleotides, which is true for many reported aptamers.^{49, 58, 71} Therefore, for the initial rounds, $f_n \ll 1$, and $f_{n+1}/f_n \approx a/b$. Different target concentrations and different K_d values of aptamers can result in different enrichment factors. For the capture-SELEX using adenosine, the enrichment factor is around 50 for the $0.2 \mu\text{M}$ K_d aptamers using $100 \mu\text{M}$ adenosine. The enrichment factor of the classical aptamer is ~ 30 . We plotted the theoretical enrichment curves as a function of the number of selection rounds with a/b ratios of 1,

2, and 10, respectively (Figure 5.8). After 11 rounds of selection, the $a/b=2$ sample would only be enriched slightly above 1 ppm (expected to see only 1 copy of aptamer if sequencing 1 million DNA). In blocked GO-SELEX, a/b was less than 1.2 in our system, and after 10 rounds, aptamers would still blow 1 ppb, whereas the $a/b=10$ sample is enriched to nearly 100% aptamer after 9 rounds. So, more rounds of selections are needed to select aptamers with a poor aptamer enrichment factor. For $a/b=1$, aptamers are barely enriched, regardless of the round of selection.

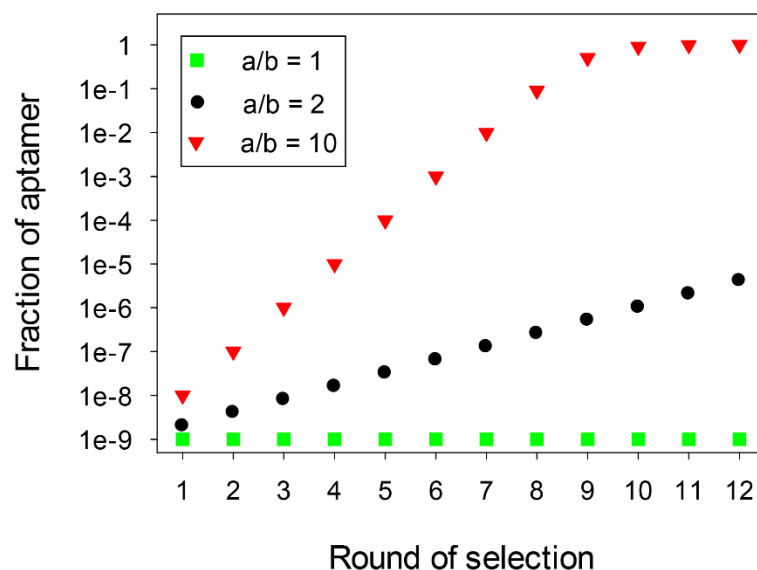


Figure 5.8. Fraction of aptamers during different rounds of selection with three a/b ratios, which describes the ratio of specific aptamer release over nonspecific DNA dissociation.

For the two SELEX methods based on library adsorption, the enrichment factor was close to 1 for GO, but lower than 1 for AuNPs. Therefore, for adenosine, the chance of enriching aptamers is very low for either GO-SELEX or gold-SELEX. GO and AuNPs are two representative nanomaterials. GO has a lower DNA adsorption strength compared to AuNPs. For even weaker adsorbing materials, one may run into the problem of having large b values, and the enriched DNA could be those with poor adsorption affinity instead of good aptamers. For stronger adsorbing materials, the target may hardly induce DNA desorption and result in very low a and b values. Based on our understanding of nanomaterials, it is unlikely that other nanomaterials can do better than GO, such as carbon nanotubes and iron oxide magnetic nanoparticles.²⁰⁵ This is because of two counter-acting factors. On one hand, it is desirable to have the library strongly adsorbed to the surface to avoid a high background release of DNA sequences. However, such strong adsorption can inhibit aptamer release. Surface adsorption is often nonspecific and each DNA base can

contribute to the adsorption. It is difficult to ask aptamer binding to overcome all the adsorption forces at the same time.²⁰⁶

The hybridization-based capture-SELEX method showed a high enrichment factor (a/b ratio). Importantly, it allows precise tuning of a and b values by simply changing the number of base pairs. For example, increasing the number of base pairs in the capture strand can decrease the b value.

In principle, both adsorption-based selection methods only considered target/aptamer binding and the adsorption of DNA, whereas the adsorption of target molecules is neglected. Therefore, in theory, our observations should be generalized to all small-molecule targets. In practice, however, the adsorption of the target molecules can affect the adsorption of DNA. In this study, we used adenosine as a model target molecule, which has strong interactions with both AuNPs²⁰⁷ and GO.¹⁹⁴ For gold-SELEX, the released DNA might be even less if a non-adsorbing or weakly adsorbing target is used.

For GO-SELEX without blocking the primer binding regions, the majority of the DNA library was adsorbed in the presence of adenosine. Therefore, adenosine binding to aptamers in the library did not prevent the adsorption of adenosine/aptamer complexes. The likelihood of success by using even weaker adsorbing molecules is thus also low. For stronger adsorbing targets (e.g. in an extreme case if proteins are used as a target for GO-SELEX), proteins may serve as a blocker to inhibit the adsorption of DNA, regardless of sequence,²⁰⁸ and selective enrichment of aptamer can hardly be achieved either.

5.4 Conclusions

Over the last few decades, numerous aptamer selection methods have been reported. However, a quantitative understanding in aptamer enrichment is lacking, which may be partially responsible for the report of some non-binding aptamers.^{209, 210} In this study, we compared aptamer enrichment factors of three library-immobilization SELEX methods: capture-SELEX, GO-SELEX, and gold-SELEX. Instead of performing full selections, we utilized a spiked library containing multiple DNA aptamers with varying affinities to adenosine. To evaluate aptamer enrichment, we employed qPCR and observed that all three methods exhibited minimal DNA release, with less than 1% of the DNA released in the presence of 100 μ M adenosine. Notably, the release of DNA from the AuNPs was particularly negligible. To further assess the enrichment of specific aptamers, namely Ade1301, Ade1304, and the classical aptamer, we employed deep sequencing. The results revealed that only the capture-SELEX method demonstrated significant enrichment, while the other

two methods exhibited enrichment factors even below 1. The GO-SELEX can be slightly improved by blocking the primer binding regions, but the enrichment factor was still over 200-fold lower compared to capture-SELEX. Our findings indicated that capture-SELEX is a more efficient approach for enriching aptamers. For a new SELEX method, using spiked library is a useful way to measure its enrichment factor and thus reliability of the method.

Chapter 6 Conclusions and future work

6.1 Conclusions

Aptamers offer a versatile and cost-effective approach for binding to small molecules, and high quality aptamers with strong binding affinity and high selectivity are required for their utility in biosensor design and therapeutic applications. With over 100 aptamers selected using the capture-SELEX method, the reliability of this method has been generally accepted in the field. At the same time, capture-SELEX allows for systematic study of aptamer selection conditions. In this thesis, we employed capture-SELEX to investigate how thermodynamic and kinetic parameters influence the enrichment of high-affinity aptamers.

In Chapter 2, adenosine and ATP were used as model targets to explore the impact of target concentration on aptamer affinity. The classical adenosine aptamer, selected by the Szostak lab in 1995 via the target immobilization method, exhibits a dissociation constant of $\sim 8 \mu\text{M}$ for adenosine and $\sim 30 \mu\text{M}$ for ATP. Using capture-SELEX, we rediscovered this classical sequence under high adenosine concentrations. However, by gradually reducing the concentration of adenosine from 5 mM to 1 μM over multiple rounds, two new sequences, Ade1301 and Ade1304, emerged and became dominant. These sequences bind adenosine with nanomolar K_d values and ATP with low-micromolar K_d values. This work demonstrates that starting SELEX at high target concentration followed by decreasing target concentration can yield high-affinity aptamers, provided sufficient rounds are performed under low-target, high-stringency conditions. This study implies that more efficient selections can be achieved by directly starting with a low target concentration, although it is difficult to know before hand what's a reasonably low target concentration.

A high concentration of target can induce the release both high and low affinity aptamers. It is likely that aptamers with a longer conserved binding region form higher affinity pockets with target molecules, although aptamers with a longer conserved region would have a lower initial abundance in the start library. Two scenarios can occur at this stage. First, if aptamers with short-conserved regions are lower-affinity aptamers, then under a stringent, low-target condition, they will dissociate less efficiently. Although these shorter aptamers are initially more abundant in the library, they will gradually decrease their abundance when a stringent condition is used. Second, if short aptamers are actually higher-affinity aptamers, they will dominate the pool regardless of whether the target concentration is decreased. This interplay among conserved-region length, target concentration, and stringency underpins the successful isolation of high affinity binders.

In Chapter 3, guanine was used as a model target to evaluate how different initial target concentrations affect aptamer enrichment. Three capture-SELEX campaigns were conducted with initial guanine concentrations of 5 μ M, 500 nM, and 50 nM, respectively. Both the 5 μ M and 500 nM selections converged on the same DNA aptamer ($K_d \approx 200$ nM) previously identified, whereas the 50 nM campaign failed to yield any guanine-binding sequences. Notably, the 5 μ M selection reached convergence more rapidly than the 500 nM selection, demonstrating that higher target concentrations can accelerate the SELEX process consistent with theoretical models of target-dependent enrichment kinetics.⁸³ Although 500 nM is approximately twice the K_d of the selected aptamer, binding in the capture-strand context would weaken the effective affinity relative to the affinity of the free aptamer, explaining why the 50 nM selection failed: the aptamer–capture duplex barrier likely outcompeted the target interaction/aptamer interactions at low target concentrations, preventing sufficient elution of aptamers.

These results also highlight the importance of avoiding distraction by lower-affinity cross-binding sequences. For example, multiple guanine aptamers with differing affinities exist. A xanthine aptamer we previously selected binds guanine in the low-micromolar range.⁷³ By starting at 5 μ M guanine, we enriched the highest-affinity guanine aptamer without capturing this weaker binder. However, even at very low target concentrations, we did not isolate a nanomolar-affinity DNA aptamer analogous to the well-known RNA guanine aptamer,¹⁵⁶ suggesting potential limitations of the capture-SELEX format, the requirement for a target-induced conformational switch to overcome the capture duplex barrier. Further reduction of background noise and optimization of selection stringency may be necessary to discover such ultra-tight binders in DNA libraries.

In Chapter 4, we explored the impact of both target concentration and kinetic effects on aptamer enrichment using ampicillin as the target. Two capture-SELEX experiments were conducted with initial ampicillin concentrations of 5 mM and 100 μ M, respectively. Both selections overwhelmingly enriched a lower-affinity sequence (AMP-H1, $K_d \approx 13$ μ M), even though a higher-affinity variant (AMP-L2, $K_d \approx 2$ μ M) exist, which differs by only three nucleotides from the low-affinity one. In our previous work, at high target concentrations, aptamers with shorter conserved regions tend to dominate simply because they are more abundant in the initial library. However, given that AMP-H1 and AMP-L2 differ by only three bases, AMP-H1 should not have a substantial abundance advantage in the starting pool.

Through detailed kinetic analysis, we discovered that AMP-L2 dissociates more slowly from the capture strand than AMP-H1, causing it to be under-represented despite its stronger target

binding. When we extended the incubation time, allowing equilibrium to be approached, the higher-affinity AMP-L2 consistently dominated in both high and low target selections. The same experiments with adenosine as a target was then performed. By extending the incubation time, classic adenosine aptamer showed that this kinetic bias is sequence-specific and not universal to all tight binders.

These findings highlight a critical interplay between target concentration, incubation duration, and capture-duplex kinetics. To preferentially enrich high-affinity aptamers, we recommend using a lower initial target concentration along with a longer incubation time. This approach mitigates kinetic bias against slow-releasing, high-affinity sequences and ensures that the strongest binders are effectively recovered.

The enrichment of a given aptamer species during SELEX can be described by the simple exponential relation:

$$C_n = C_0 * f^n$$

where:

C_n is the fractional abundance of an aptamer after n selection rounds,

C_0 is its initial fractional abundance in the starting library (determined by library design and conserved-region length),

f is the round-to-round enrichment factor (i.e., the fold-increase in relative abundance per cycle), and

n is the number of SELEX cycles performed.

The enrichment factor encapsulates all of the assay's stringency parameters—most notably the target concentration, ionic strength, incubation time, and buffer pH. By tuning these variables to maximize f for high-affinity sequences (while minimizing nonspecific carry-over), one can drive C_n rapidly toward unity for the best binders, even if C_0 is very small. Moreover, this framework explains how specificity can be tuned without altering the library itself. In traditional negative SELEX, a counter-selection step (using a “negative” target) selectively reduces f for cross-reactive or low-specificity sequences, shifting the enrichment in favor of highly specific aptamers. Likewise, one can exploit the f -dependence on buffer conditions—by adjusting pH, salt concentration, or other variables—to differentially modulate f between closely related aptamer variants, thereby enriching for those with superior specificity.

In Chapter 5, We directly compared three library-immobilization SELEX methods—capture-SELEX, GO-SELEX, and gold-SELEX—using a spiked library containing known adenosine aptamers of varying affinities. Rather than performing full iterative cycles, we performed one-round selection by incubating each library with 100 μM adenosine and quantified the released DNA by qPCR. All three methods liberated less than 1% of the pool, with gold-SELEX showing virtually no release. Deep sequencing revealed that only capture-SELEX drove meaningful enrichment of the high-affinity aptamers (Ade1301 and Ade1304) and the classical sequence; both GO-SELEX and gold-SELEX produced enrichment factors below unity meaning that these aptamers even decreased after this one-round selection. Even after blocking the primer-binding regions to reduce nonspecific adsorption, GO-SELEX lagged behind capture-SELEX by over 200-fold. These findings establish capture-SELEX as the superior strategy for rapidly and quantitatively enriching high-affinity aptamers and demonstrate that a spiked-library assay provides a convenient benchmark for assessing new SELEX technologies.

6.2 Original contributions

Based on the studies presented in this thesis, the following original contributions were made:

1. **Discovery of four novel high-affinity aptamers**

- Ade1301 and Ade1304 for selective adenosine recognition (nanomolar K_d)
- R10G2 for guanine (nanomolar K_d)
- AMP-L2 for ampicillin (low-micromolar K_d)

Each of these aptamers demonstrates strong target affinity and is immediately compatible with downstream biosensor development.

2. **Elucidation of target-concentration effects**

The influence of initial and step-down target concentrations on the enrichment of high-versus low-affinity aptamers was systematically evaluated, pinpointing the “sweet-spot” ranges that maximize the selection of nanomolar-class binders.

3. **Quantification of kinetic bias in Capture-SELEX**

Kinetic analyses and controlled incubations revealed that differential dissociation rates from the capture duplex can skew enrichment toward faster-releasing, lower-affinity sequences, and that extended incubation times mitigate this bias.

4. **Practical guidelines for high-affinity aptamer selection**

A set of actionable recommendations was developed—detailing optimal target concentration profiles and incubation times—to maximize enrichment factors and reliably isolate tight-binding aptamers across diverse small-molecule targets.

5. Mathematical modeling of SELEX dynamics

An exponential enrichment model ($C_n = C_0 * f^n$) was developed, integrating library design parameters (C_0), selection stringency (f), and cycle number (n) to predict aptamer abundance trajectories and provide a quantitative framework for SELEX protocol optimization.

6.3 Future work

The results presented in this thesis offer new insights into aptamer selection and improve the quality of aptamers available for biosensor development. Extension of current work can be carried out in the future:

1. **Elucidation of kinetic mechanisms by structural biology methods.** Although the three-nucleotide difference between AMP-L2 and AMP-H1 alters dissociation rates from the capture strand, the structural basis for this kinetic effect remains unclear. Future studies should employ high-resolution techniques, such as NMR spectroscopy or X-ray crystallography, to compare secondary and tertiary structures of these two aptamers. A detailed understanding of how specific base changes affect strand-displacement kinetics will enable more rational biosensor designs. Similarly, why Ade1304 and Ade1304 have different abundance and selectivity will need to be addressed by high-resolution structures as well.
2. **Dynamic target concentration strategies.** Rather than maintaining a fixed stringency throughout SELEX, one approach is to initiate selections at very low target concentrations to strongly favor high-affinity sequences, then raise the concentration to elute all bound aptamers from the beads. This two-stage protocol may enrich superior binders more efficiently and reduce the total number of SELEX rounds required.
3. **Extension to protein targets.** Capture-SELEX has been applied primarily to small molecules, while protein aptamer selection still relies largely on target-immobilization methods. A systematic comparison of capture-SELEX versus conventional target immobilization in protein SELEX would clarify the relative advantages of each approach and potentially enable rapid discovery of high-affinity protein aptamers without surface-tethering artifacts.

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Figure 1.2

Adsorptive Performance Mechanism of the DNA of Calf Thymus Gland (CTGDNA) on 3CR12 Stainless Steel as Corrosion Inhibitor in Acidic Medium

Author: Oluranti Agboola et al
Publication: Journal of Bio- and Tribo-Corrosion
Publisher: Springer Nature
Date: Apr 23, 2019
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Figure 1.3

Transient Hoogsteen base pairs in canonical duplex DNA

Author: Evgenia N. Nikolova et al
Publication: Nature
Publisher: Springer Nature
Date: Jan 26, 2011

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Publication: Science

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 Publication: Journal of the American Chemical Society
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