

Investigation into the Chiral Selectivity of DNA Aptamers for Essential Biomolecule Targets

by
David Shing-Hei Zia

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Chemistry

Waterloo, Ontario, Canada, 2024

© David Shing-Hei Zia 2024

Author's Declaration

I hereby declare that I am the sole author of this 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.

Abstract

In nature, chiral molecules are typically represented as a single enantiomer for most biomolecules. This aspect of homochirality is said to be connected to prehistoric RNA which led to the dominance of sugar and amino acids to exist exclusively as one chiral form. With the advent of geomatics technology becoming more prominent in therapeutics like biosensing and drug delivery, understanding a deeper aspect on nucleic acid chemistry can help with both improving efficiency and expanding applications. In this thesis, the focus will be on DNA, specifically aptamer technology and how their affinity to ligands can be influenced by chirality. The chiral biomolecules of both lactate and tryptophan are explored by conducting various selections for the different isomers. Both targets are important in clinical applications. Tryptophan is an essential biomolecule responsible for the production of neurological hormones in the body, while lactate is a unique biomolecule in that both of its enantiomers have distinct roles in the body.

In our lactate selection, even using only D-lactate as a target, high specificity aptamers for L-lactate were obtained. The aptamers showed capabilities of reaching K_D of 0.23 mM and a limit of detection (LOD) of 0.21 mM in blood serum. These concentrations nicely cover the physiological range of lactate (1-20 mM), which demonstrates its potential for therapeutics applications. Additionally, the aptamer also demonstrates a 5-fold enantioselectivity for L-lactate compared to D-lactate. From the evidence present of this experiment, it is likely that DNA aptamer exhibit a preference towards L-chirality for lactate.

For the selection with tryptophan, two separate experiments were conducted using racemic and homochiral solution of tryptophan as the selection targets. The obtained aptamers from these selections demonstrated high enantioselectivity for both L-tryptophan and D-tryptophan. One of the D-tryptophan aptamer exhibited a K_D of 11 μM and a 7-fold greater affinity compared to L-tryptophan. We can compare this affinity to that of aptamers specific to L-tryptophan reported in other studies, which displayed similar affinity and selectivity for the opposite enantiomer. Due to this result, we proposed that DNA's affinity for both enantiomers stems from the greater complexity and binding features presented in tryptophan's molecular structure.

By studying the sequences that were obtained from the selections, we observed two distinct cases of chiral bias in DNA for different biomolecules. We demonstrated how using a homochiral target solution can be applied to improve the selection of high affinity aptamers, as seen by the lactate study. Additionally, we demonstrated that highly selective DNA aptamers can also be obtained for both enantiomer of a target, as seen by the tryptophan study. Although the exact reason for the chiral preference in some targets remains uncertain, our findings suggests that variance in size may be a plausible reason to explain this phenomenon. Future studies should be taken to explore this case further by selecting other essential biomolecules that are similar in size to the two targets used. Exploring how DNA interacts with targets that have varying functional groups would help provide some more insight on the underlying mechanism of DNA's chiral binding.

Acknowledgments

First and foremost, I would like to express my heartfelt gratitude to my supervisor, Dr Juewen Liu, for providing me with continuous support and guidance throughout my research. I will always be sincerely grateful for his patience and the immense knowledge he provided, in which were invaluable to me during the completion of my degree. I would also like to thank Dr Thorsten Dieckmann and Dr Subha Kalyaanamoorthy, for agreeing to be on my advisory committee for my defence. I would like to thank the other members of my lab, especially Dr Po-Jung Jimmy Huang, Dr Yibo Liu, Yuzhu Ding and Yichen Zhao, for all the advice they offered me during the term. I am appreciative to them for offering their time to listen to my presentation and for all the suggestions they have provided me in my experiments as well. I thankful for the endless support from all the members of the Liu group who have made this experience an enjoyable and welcoming moment for me these past two years.

Table of Contents

Author's Declaration	ii
Abstract	iii
Acknowledgments	v
List of Figures	viii
List of Tables	ix
List of Abbreviations	x
Chapter 1 Introduction	1
1.0 <i>Chirality and its Importance in Biology</i>	1
1.1 <i>Chiral Discrimination and its Physical and Chemical Properties</i>	2
1.2 <i>Chirality Application in Drug Development, Biosensors and More</i>	4
1.3 <i>Chiral Selective Biosensing</i>	6
1.3.1 Various Detection Techniques for Chiral Molecules	6
1.3.2 Nucleic Acids and Aptamer-Based Biosensing for Chiral Selectivity	8
1.3.3 Characterization of Aptamer Binding by ITC	13
1.3.4 Detection Strategy for Aptamers	13
1.4 <i>Research Goals</i>	16
Chapter 2 Selection of D-Lactate Binding Aptamers	18
2.1 <i>Introduction</i>	18
2.2 <i>Results and Discussion</i>	20
2.2.1 Optimized Selection Strategies for D-lactate Aptamers	20
2.2.2 Analysis of the Newly Sequenced D-lactate Library	21
2.2.3 Determining Binding with Fluorescence Labeling	23
2.2.4 Determining Binding by ITC	27
2.2.5 Selectivity Test for New Aptamer	29
2.3 <i>Summary</i>	31
2.4 <i>Experimental Procedure</i>	32
2.4.1 Chemicals	32
2.4.3 FAM Displacement Assay	34
2.4.4 Isothermal Titration Calorimetry (ITC)	35
2.4.5 Selectivity Detection in Serum	35
Chapter 3 Selection of Tryptophan Binding Aptamers	36
3.1 <i>Introduction</i>	36
3.2 <i>Results and Discussion</i>	37
3.2.1 Selection with Racemic Tryptophan Target	37
3.2.2 Selection using D-Tryptophan	41

3.3	<i>Summary</i>	44
3.4	<i>Experimental Procedure</i>	45
3.4.1	Chemicals	45
3.4.2	SELEX Selection	46
3.4.3	Binding with ThT Assay	46
Chapter 4 Conclusions and Future Directions		48
References		51

List of Figures

Figure 1.1 Crystal structure of compounds alongside their enantiomeric counterpart	3
Figure 1.2 Illustration of DNA aptamer with enantioselectivity binding to thalidomide	10
Figure 1.3 Schematic diagram of structure switching and split aptamer fluorescence biosensors	14
Figure 1.4 Schematic design of the ThT assay for CAP target	16
Figure 2.1 Secondary structure and ITC binding results for L-lactate aptamer.....	20
Figure 2.2 Top families assembled from the 50 most abundant sequences.....	23
Figure 2.3 Schematic of the single strand displacement fluorescence assay titrated with lactate	25
Figure 2.4 Measuring the fluorescence quenching effect of FAM-LAC1103	27
Figure 2.5 ITC data for LAC1103 aptamers titrated with L-lactate	28
Figure 2.6 Selectivity assay for LAC1103 immersed in 10% blood serum with common biomolecules	31
Figure 3.1 Top families assembled from the 10 most abundant sequences for the racemic tryptophan selection	39
Figure 3.2 Detection of tryptophan concentration using ThT displacement assay for aptamers obtained from the racemic tryptophan selection.....	40
Figure 3.3. Top families assembled from the 8 most abundant sequences for the D-tryptophan selection	42
Figure 3.4 Detection of tryptophan concentration using ThT displacement assay for aptamers obtained from the D-tryptophan selection	44

List of Tables

Table 1.1 Different chiral targets with affinity towards DNA and RNA aptamers	11
Table 2.1 DNA sequences used for the lactate selection	33
Table 3.1 DNA sequences used for the tryptophan selection.....	46

List of Abbreviations

A	adenine
bp	base pair
C	cytosine
CE	capillary electrophoresis
CSP	chiral stationary phase
D	dextrorotatory
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dsDNA	double stranded deoxyribonucleic acid
FAM	fluorescein amidite
G	guanine
ΔH	enthalpy change
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high-performance liquid chromatography
ITC	isothermal titration calorimetry
K_D	dissociation constant
L	levorotatory
LC	liquid chromatography
LOD	limit of detection
n	ligand-binding stoichiometry
nt	nucleotides

PCR	polymerase chain reaction
RNA	ribonucleic acid
ΔS	entropy change
SELEX	systematic evolution of ligands by exponential enrichment
T	thymine

Chapter 1 Introduction

1.0 Chirality and its Importance in Biology

In chemistry and biology, chirality plays an important role in preserving physiological pathways in all living organisms. Introduced in 1894 by Sir William Thomson, chirality refers to the property of two molecules that are non-superimposable mirror images of one another.^[1,2] This concept falls under to the topic of stereochemistry, specifically for molecules that exist as stereoisomers. The term stereoisomer is characterized by two key parts of the term. The latter component, “isomers”, refers to two molecules that share the same molecular formula but possess something else that is different between them, whether constitutional, rotational, or conformational.^[3] The initial part, “stereo”, tells us that while the two molecules have the same order of atoms (connectivity), they have differences in their space and orientation, which are known as enantiomers. A common analogy of this is by imagining a person’s left and right hand, which although share identical features, are non-superimposable mirror images of each other.

In biology, chirality plays an integral role for all live things, as chiral molecules make up a significant part of all cellular structure. They are essential in affecting how molecules are recognized and are perceived which is a large enabler for numerous biological processes to function properly. For instance, the chirality of biomolecules can play a role in influencing the structure and function of protein and enzyme found within the human body.^[4] Many of these biomolecules will share the same chiral form in the sense of being in the same direction or configuration, in a phenomenon called homochirality. These molecules, such as sugar and

amino acids, exist almost exclusively in one form in nature, either “left-handed” or “right-handed” enantiomers.^[5]

We see this phenomenon of homochirality existing in both plants and animals. If we take glutamine for example, the most abundant amino acid that is used in the human body in protein synthesis, nature will always use the L-glutamine in its mechanism for life. Similarly, if we examine the other 19 essential amino acids present in all living organism, 18 of them will adopt the L-chiral form, with glycine being the sole exception due to its achiral structure.^[4,6] Another example of homochirality is observed with glucose, the breakdown product of starch, where the enzymes, glucose oxidase (GOx), would exclusively metabolize D-glucose but will remain inert when L-glucose is present.^[7,8] Additionally in DNA, the backbone is comprised exclusively of D-deoxyribose, highlighting another molecule that is always homochiral in nature.^[9]

1.1 Chiral Discrimination and its Physical and Chemical Properties

Determining the orientation of a molecule starts with locating its chiral center, which is a tetrahedral atom (usually carbon) attached to four different atoms or groups. Once the chiral center is located, Cahn-Ingold-Prelog rule is then used for determining the absolute configuration between isomers. By examining the atoms and groups attached to the chiral center, atoms with higher atomic number are assigned with higher priority with hydrogen ranking the lowest priority. Following the highest to lowest priority for the first three groups, “R” and “S” descriptors are used to denote clockwise direction and counter-clockwise direction respectively.^[3,10] Another form of identification is by using the relative configuration denotation

which are represented by dextrorotatory (D or +) and levorotatory (L or -). These identifiers establish the optical activity of a molecule, which indicates its ability to rotate plane-polarized light in a specific direction. The rotation of light is determined by the concentration and the three-dimensional structure of the chiral molecules. If the plane-polarized light rotates clockwise, it is said to be dextrorotatory. If the plane-polarized light rotates counter-clockwise, it is said to be levorotatory.^[11] For sugars, the optical rotations can be determined using Fischer projection, by locating if the amine or alcohol group is on either the left or right side, this will determine if the molecule is “L” or “D” respectively.^[10] Usually, D and L notation is used in a biological standard case when describing sugars or amino acids.

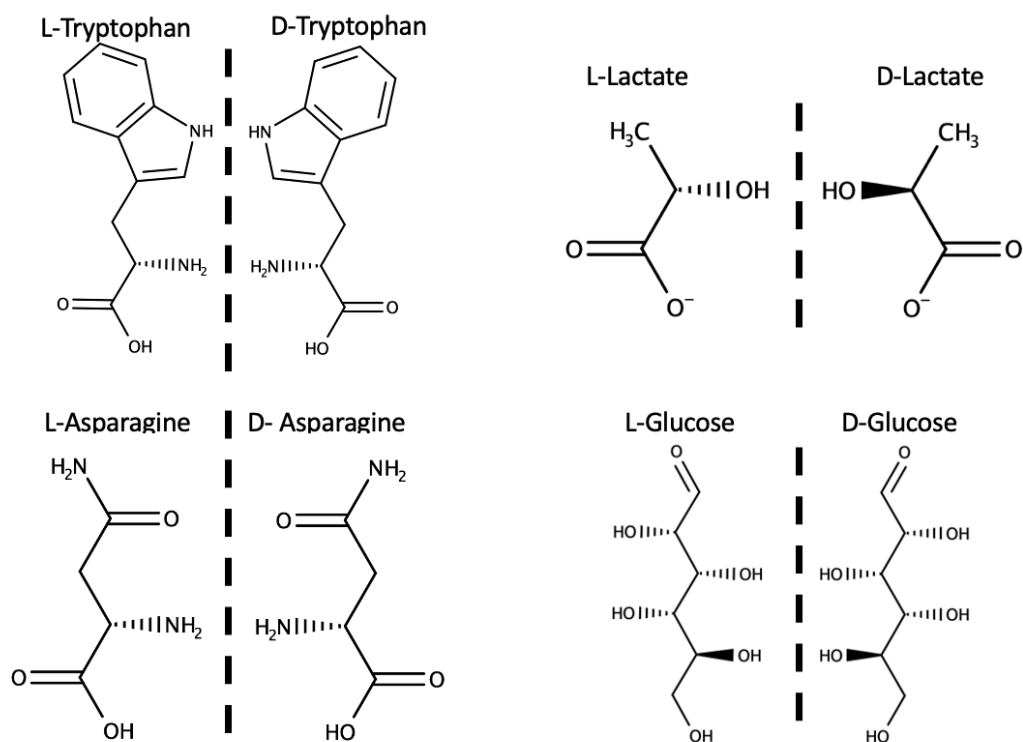


Figure 1.1. Crystal structure of compounds alongside their enantiomeric counterparts

The distinct spatial arrangement due to asymmetry between two enantiomers gives rise to two molecules that possess identical chemical properties. However, their presence in living systems can induce very different biological responses when interacting with other chiral molecules. In biology, the molecular structure is often closely related to its biological function. A prime example of how enantiomer can exhibit different functions is shown by the molecule limonene^[12] and carvone^[13]. Depending on the enantiomer that are present, two distinct odors can be produced as they interact differently to the receptors in the human nose.^[13–15] For instance, enantiomers of limonene can take on the smell of pine or oranges. Similarly, the enantiomers of carvone can produce odors of spearmint or caraway seeds. In more severe cases, irregular concentrations of the wrong enantiomer can lead to poisoning and disturb important pathway, thus showing the importance of stereochemistry in biology.^[14,16]

1.2 Chirality Application in Drug Development, Biosensors and More

From an analytical chemistry standpoint, methods for chiral discrimination plays a big role in enhancing our understanding of how molecules behave and interact. The uses of chiral molecules also have lots of application in both pharmaceuticals and therapeutics. Chirality can have a significant effect on drug efficacy, where one enantiomer could exhibit positive therapeutic effects while the other may exhibit adverse reaction in the same environment.^[16–18] Consequently due to this concern, there is a concentrated effort to bring only single enantiomers to the drug market. Since 1992, the FDA have implemented various regulations for the development of chiral drugs, with racemic mixtures being rarely approved.^[19]

Before these regulations by the FDA were put in place, the impact of enantiomers was not well understood. A famous example of the profound effects of chiral molecules is the molecule thalidomide, which was a racemic mixture originally marketed to treat morning sickness for pregnant women.^[20] However, while the R enantiomer had the desired therapeutic effect, the S enantiomer was found to be teratogenic, causing severe birth defects for over 10,000 babies.^[21,22] Another case is Clopidogrel, a medication that inhibits platelet aggregation. Its therapeutic effect comes from the active S enantiomer, whereas the inactive R enantiomer can induce convulsion at high concentrations.^[23] These major cases serve as a wakeup call that emphasises the importance of understanding and regulating chiral molecules in pharmaceuticals.

Chirality also plays a role in everyday chemical products. In the agriculture industry, many of the agrochemicals used are pure single-enantiomer molecules as different chirality may have different properties.^[24] For example, the chirality of dichlorprop and mecoprop, commonly used ingredients in herbicides, can determine the pesticide efficiency in weed killing.^[24,25] European governments have mandated the use of only the R (+) enantiomer as it activates fungicidal activity unlike the S enantiomer. Also, the R enantiomer degrades faster of the two, which reduces the number of pollutants brought into the ecosystem, resulting in a more environmentally safe product. This approach allows for greater chemical control, thus potentially resulting in reduced impact on wildlife and maintaining water quality when these chemicals enter the environment.

1.3 Chiral Selective Biosensing

1.3.1 Various Detection Techniques for Chiral Molecules

Over the years, various chirality sensing methods have been developed for the purpose of detecting and separating chiral molecules. Several common techniques used for chiral separation include liquid chromatography (LC), membrane separation, and capillary electrophoresis separation (CE).^[26–28] Each technique is highly effective at different aspects: CE is known for its speed, membrane separation for its simplicity, and LC for typically achieving the highest resolution separation. Among these methods, high-performance liquid chromatography (HPLC) with a chiral stationary phase (CSP) is the most well-known and widely used.^[29] The chiral stationary phase is responsible for chiral recognition and has wide variety of forms, making it suitable for analyzing various biomolecules. Ultimately the choice of method depends on the nature of the chiral compound and the resources that are available.

Compounds that can fit in chiral stationary phase (CSP) and can differentiate enantiomers apart are called chiral selectors. They have undergone many advancements throughout the decade and many high sensitivity separation applications have emerged for a wide range of compounds. One of the most standard chiral selectors used are polysaccharide derivatives, specifically cellulose-based derivatives, due to their commercial availability and proven record of being reliable in performing various chiral separation task.^[30–32] Various modes, including polar organic mode, reversed-phase mode, and normal mode, provides flexibility in designing experiments for separating compounds based on their polar properties.^[33] In addition to polysaccharides, other chiral selectors have also been explored to expand the range of chiral

separation techniques. A notable example among these are macrocyclic antibiotic CSPs, which are a set of chiral selectors with multiple groups to allow for impeccable selectivity to chiral molecules.^[34]

Enzymes are chiral selectors that can be found in nature and are known for their capability for high chiral selectivity. Due to their intrinsic ability for chiral recognition, they have inspired the idea of incorporating these proteins into various techniques to modify and enhance stereoselectivity. This is illustrated in Jun Haginaka's work, where enantiomer separation was performed using enzyme with HPLC. By incorporating various known enzymes to produce protein-based CSP, it allows for the detecting of a wide range of targets without much procedure alteration.^[35] Similarly, antibodies are also excellent chiral discriminators, which they can be tailor made to recognize virtually any molecule. With antibodies, they have been integrated into a wide range of chiral selecting applications such as immunosensors, electrochemical sensors and biosensors.^[36] However for both enzymes and antibodies, they face notable flaws in term of stability and reusability which limits their effectiveness after multiple uses. They are susceptible to denaturing and degeneration when under non-ideal environments which can be limiting in some clinical applications of chiral sensing.^[37]

Due to the directional nature that enantiomers interact with light, circular dichroism (CD) spectroscopy can be used to characterize the structure of chiral molecules. This technique measures a molecules ability to absorb circularly polarized light. The absorbed polarized light is not only influenced by the chirality of the molecule but can also impacted by the molecule's

secondary structural features.^[38] Since this CD spectroscopy is particularly sensitive in detecting the secondary structure of a molecule, it becomes especially useful in determining the conformational changes of compounds.^[39]

1.3.2 Nucleic Acids and Aptamer-Based Biosensing for Chiral Selectivity

An alternative to using enzymes and antibodies is by using aptamers. These signal-stranded oligonucleotide have a high affinity for specific targets and have been reported to have great enantioselectivity for chiral molecules.^[32,40] The intrinsic molecular recognition mechanism of RNA and DNA allows aptamers to select for a wide range of molecules, including small molecules, metal ions, proteins, lipids, and even cell surface receptors.^[41] What makes aptamers stand out is that they offer several advantages over other methods. First, unlike enzymes and protein variants, nucleic acids are highly stable, especially for DNA.^[37] Aptamers also have a wider range of transduction methods, including colorimetry, chemiluminometry, electrochemistry, fluorometry, and fluorescence anisotropy.^[42] Additionally, the cost for synthesizing and modifying these aptamers are much lower than that of antibodies and enzymes, as they don't require an animal host for production.^[41]

The process of obtaining an aptamer was first pioneered by Tuerk and Gold who devised a multi-round process named SELEX (Systematic Evolution of ligands by EXponential enrichment).^[43] In this process, potential aptamers were screened through a large random pool of oligonucleotide library to find the sequence that best binds to a target. SELEX can take different approaches depending on how the target and the library are presented in the

selection. In the first category, known as homogenous SELEX, both the target and the library are left in free solution to bind without interference.^[44] The bounded nucleic acids are then separated from the non-specific sequences which can be determined on size or charge. The other category is heterogenous SELEX, where either the target or the library is immobilized onto a solid support. Before the target and library are added together, a series of stringent buffer washes are applied to remove any weakly bound sequences that are left in the pool. Between the two heterogenous approaches, library-immobilization (also referred to as Capture SELEX) is better suited for small molecules, while target-immobilization works better with larger molecules. This is because small molecules have less surface area for binding when immobilized, which will lower the binding affinity of the target to the library.^[44] The DNA or RNA strands with strong binding affinity are isolated from the non-binding strands and then amplified using PCR to generate a large pool of single stranded aptamers, which are further used for future rounds of selection.^[44]

Many aptamers have been generated for all kinds of targets including amino acids, organic dyes, nucleotides, drugs, peptides, and proteins.^[45] These aptamers can range from 20 to 100 nucleotides in length, with longer aptamers typically be applied against targeted for larger biomolecules. The binding interactions rely on several non-covalent interaction, such as hydrogen bonding, van der Waals interaction, base stacking, and hydrophobic interactions.^[46] At physiological pH, DNA and RNA are strongly negatively charged due to their phosphate backbone which can induce electrostatic interaction with their targets as well.

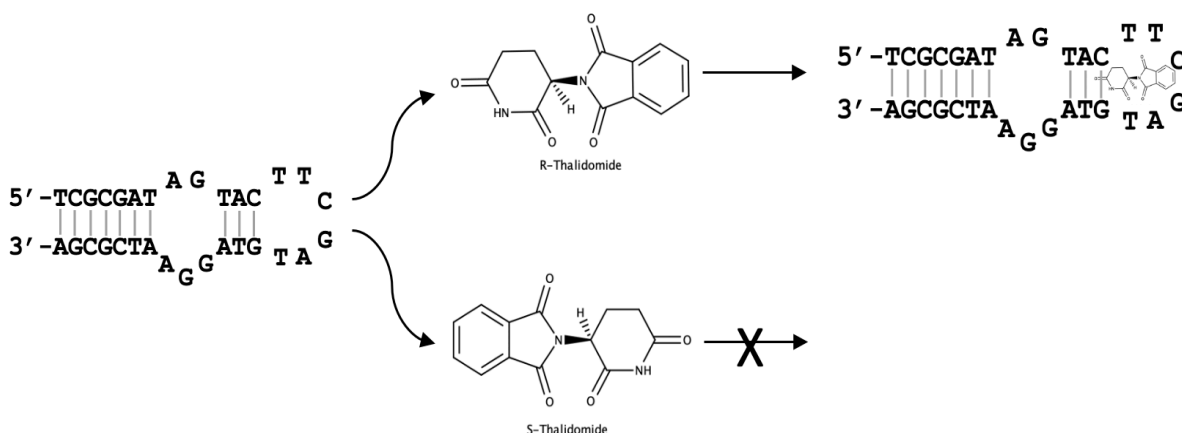


Figure 1.2. Illustrates a DNA aptamer with enantioselectivity binding to thalidomide based on design described in ref. 50. The scheme illustrates that (R)-thalidomide binds with the aptamer, while (S)-thalidomide does not. The secondary structure of the DNA aptamer is also shown.

Increasingly over the years, aptamers have been recognized to have significant prospective in chiral recognition. To demonstrate aptamers capability in distinguishing isomers apart, a paper by Eric Peyrin's group. reported the selection of aptamers for adenosine and tyrosinamide. In their experiment, they have successfully obtained enantioselectivity aptamer for D-adenosine and L-tyrosinamide (the natural isomer).^[47] Other cases of enantioselective aptamers include targets for lactate, drugs like thalidomide, oligopeptides such as vasopressin, and various other amino acids.^[48–51] All these aptamers demonstrate a range of selectivity, from minor differences of 8-fold to extreme highs of 10,000-fold or greater selectivity.^[49,52] A common trend that these aptamers have are that they show higher affinity for the “natural” isomer of the target molecule.

Table 1.1 Different chiral targets with affinity towards DNA and RNA aptamers

Target	Aptamer	K _D of target isomer	K _D of non-target isomer	Ref.
(D) Vasopressin	DNA (55 mer)	~1.1 μ M	Nd	[53]
(D) Adenosine	DNA (37 mer)	~6 μ M	Nd	[47]
(R) Thalidomide	DNA (31 mer)	~1.0 μ M	Nd	[50]
(L) Lactate	DNA (30 mer)	~430 μ M	Nd	[48]
(L) Tyrosinamide	DNA (49 mer)	~45 μ M	Nd	[47]
(L) Arginine	RNA (58 mer)	~330 nM	~4.0 mM	[54]

A paper by Thomas Carell et al. delves into the question of how RNA can influence the homochirality of amino acids in all living organisms.^[55] Their inference for why there is such a dominance for a particular chiral molecule, be it the L-amino acids or D-ribose, is due to partial preference found for these essential biomolecules all the way back to the primordial era. Their findings revealed a favourability for forming duplexes with the L-configured amino acids over D-configured amino acids from a select set. The experiment involved using RNA structures that include non-canonical nucleosides, such as 5-methylaminomethyluridine (mnm⁵U) and threonine-modified N⁶-carbamoyl adenosine (Thr⁶A), which were covalently attached to amino acids. The reaction involved two amino acids, one linked to a non-canonical mnm⁵U and the other linked to a m⁶aa⁶A, to form a complementary RNA strand that gives a hairpin structure. Subsequently, the reaction was heated to cleave the hairpin bond, releasing the Adenosine-based complementary strand, which was then annealed to introduce new amino acids, making

the peptide/strand more elongated. During this growing phase where complementary strands are coupled, RNA strands with L-chiral amino acids exhibited a higher yield among the combination of L-L pairings, L-D pairings, and D-L pairings. This is indicative that more favourable coupling occurs with homo-pairing of L-amino acids.

Both RNA and DNA have a natural handiness in the D-configuration. However, the reverse or “mirror-image” configurations can also be used in chiral selectivity. These mirror-image nucleic acids are called “Spiegelmer” which are made up of deoxyribose sugars that contains mirror-inverted chiral centers. These spiegelmers often will have similar affinity and specificity towards the mirror image of a target.^[56] In Heemstra experiment, their group illustrates this by demonstrating the simultaneous detection of opposite small-molecule enantiomers.^[57] Their group showcase the synthesis of spiegelmers using previously selected natural D-DNA aptamer sequences. Specifically, they used a previously reported aptamer selected for L-tryptanamide and synthesized a spiegelmer that binds to D-tryptanamide. The chiral binding affinity for the aptamer and spiegelmer to their respective target were 442 μM and 464 μM respectively. The close range between these binding affinities demonstrates that enantio-DNA ligands will bind to their reciprocal chiral targets. This approach of using mirrored sequences saves considerable time by skipping the need of finding a sequence for the other enantiomer. However, synthesizing a second L-DNA or L-RNA aptamer is expensive which can limit its application in research.^[58]

1.3.3 Characterization of Aptamer Binding by ITC

One of the gold standards for characterizing the binding between a ligand and a receptor is through Isothermal Titration Calorimetry (ITC). ITC is used to quantitatively determine the thermodynamic properties of a solution like the dissociation constant (K_D), enthalpy change (ΔH), entropy change (ΔS), and the ligand-binding stoichiometry (n). This is achieved by measuring the heat lost or absorbed during the gradually titration of the ligand to the receptor, which is done until all receptor binding sites are fully saturated. The benefit to using this technique is that it requires no labels, thus allowing for the binding to occur purely between the receptor and the ligand without external influence. They can also measure binding in a wide range of temperature, pH, and a flexible combination of salt concentrations for solution. In our lab, ITC is routinely used as a reliable method to verify the binding for our selected aptamers.^[51,59,60]

1.3.4 Detection Strategy for Aptamers

Various detection strategies have been developed with aptamers, with optical-based techniques being the most prevalent. These strategies typically take advantage of the significant conformational changes, such as folding and strand displacement, that occurs when the target binds with the aptamer. One method used in our lab involves modifying the aptamers with fluorescence tags. This can be achieved by hybridizing the aptamer with a fluorescence molecule, such as fluorescein (FAM), on one end while a complementary strand binds to the aptamer through non-covalent interactions. On this complementary strand, it is modified with a quencher, such as Black Hole Quenchers (BHQ), that can absorb the signal from the

fluorophore. Once the target is introduced, this will induce a strand displacement through structure switching mechanism causing the fluorescence signal to be re-emitted (Figure 1.3 A).^[61] In addition to structure switching aptamers, split-aptamers can also be designed to optically detect target molecules. The main difference with this method is that the aptamer sequence is pre-split into multiple segments, which will come together in the presence of the target, resulting in a signal change (Figure 1.3 B).^[62]

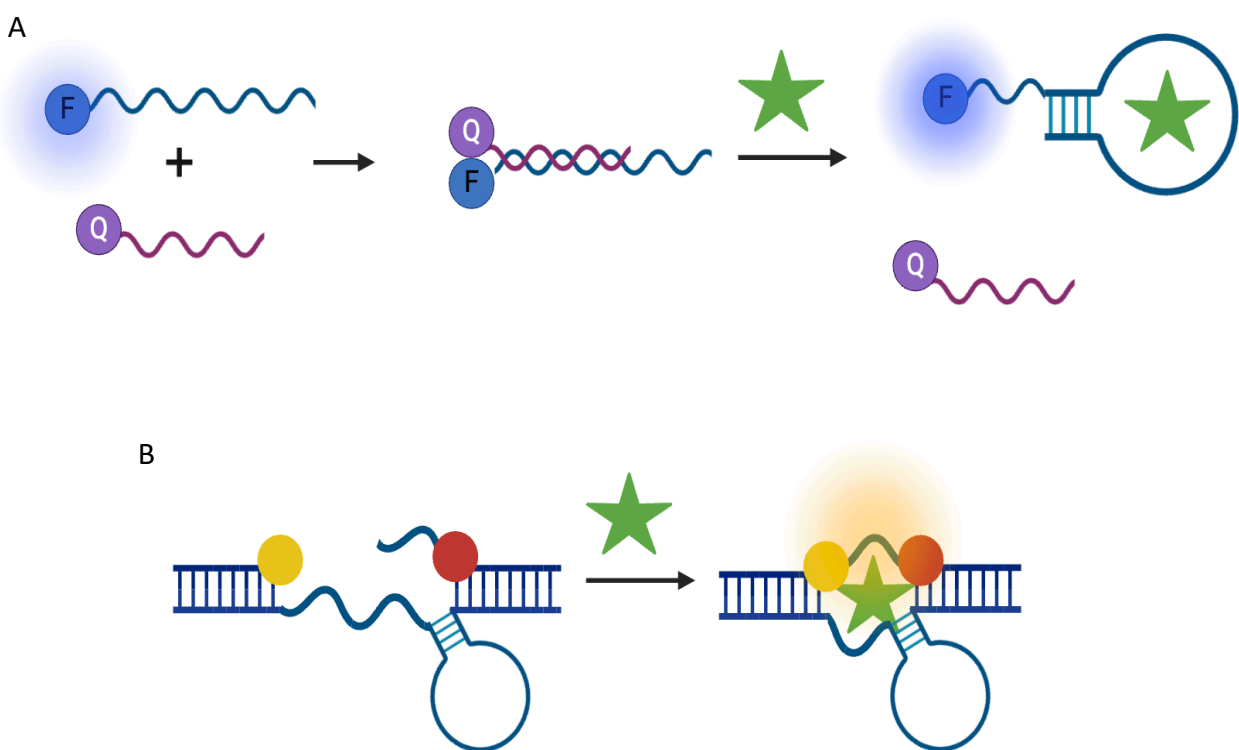


Figure 1.3. (A) Schematic diagram of a structure-switching fluorescence biosensor. Aptamer strands are functionalized with a fluorophore and bounded to a complementary quencher strand. In the presence of the target molecule, structure-switching releases the quencher strand and allows the fluorophore to re-emit signal. (B) Schematic diagram of a split aptamer fluorescence biosensor. In this design, the target binding induces the two aptamer domains to

assemble. This will bring the labels sites on each segment to come closer together and result in a change in fluorescence signal.

In addition, we have also employed ThT displacement-based assay, which is a label-free approach, to characterize aptamer binding affinity. Thioflavin T (ThT) is a fluorescence dye technique that accommodates many targets including DNA, RNA and proteins. With ThT, this molecule will experience enhanced fluorescence intensity when it comes in contact with a G-GGG unit that is found in DNA and RNA. The molecule achieves this by inducing the formation of a G-quadruplex structure within the nucleic acid, which works best with G-rich DNA/RNA sequences.^[63,64] This property can be used to measure when aptamer binds to the target. This type of sensing was illustrated by a work done previously by our lab, where ThT was used for label-free detection of the antibiotic Chloramphenicol (CAP). The results from ThT with the CAP aptamer was measured to be 4.2 μM which was similar to values verified by other methods.^[65]

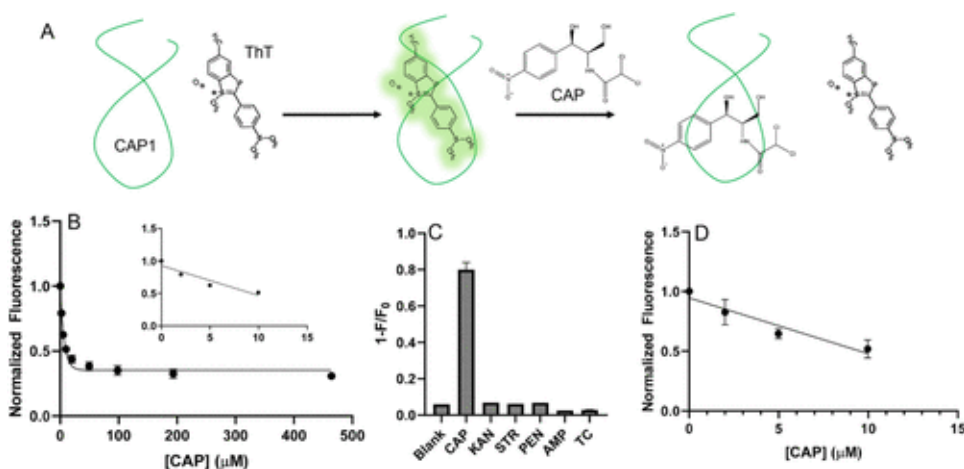


Figure 1.4. (A) Schematic of the ThT assay for CAP target. (B) Sensitivity of the aptamer measured through fluorescence intensity. (C) Selectivity of the aptamer compared to other antibiotics. (D) Detection of CAP in Lake Ontario water samples. (Figure was taken from ref. 65)

1.4 Research Goals

The selection of chiral molecules can often pose as a significant challenge due to their high molecular similarity between each isomer, especially for small molecules. Traditionally, the use of polysaccharides, enzymes, and antibodies have been the standard tools used for such selections. They can often provide reliable results but sometimes are limited by their targets. However, there is a continuous search for more approaches to not only widen the range of applications but also to offer better specificity and efficiency. Aptamers represent one of these promising methods. Though the limitations of aptamer selection capability are not fully understood, further investigation into these inherent properties should be encouraged. By expanding the number of methods for chiral recognition, more targets can be explored in greater effectiveness and versatility.

We hypothesize that DNA aptamers have a natural preference for a certain chirality of molecules. This thesis aims to investigate the natural preference using different targets of common biological molecules. While several papers have hypothesized the origin of chirality in certain molecules to be from RNA and demonstrate the inherent chirality of RNA, the relationship between chirality and DNA with “non-natural” enantiomers have not been extensively explored. In this thesis, I aim to explore the inherent chiral preference different

molecules have with aptamers. This is accomplished by conducting the selection of two different chiral essential biomolecules with DNA aptamers. We will be using the capture SELEX approach to isolate the high affinity sequences for the targets and determine any chiral biases from the enriched sequences. The goal is to determine if the data aligns with the trends observed with RNA, as discussed by many researchers previously. Additionally, we aim to explore more on the limitations that are prevalent in the aptamer selection for certain chiral molecules.

Chapter 2 Selection of D-Lactate Binding Aptamers

2.1 Introduction

The chirality of lactate holds significant importance in both biologically and clinically aspects.

Unlike many other chiral biomolecules that exist solely in one enantiomeric form in nature, both enantiomers of lactate, L-lactate and D-lactate, can be present and play distinct roles in the body depending on the physiological condition of a person. Therefore, being able to accurately differentiate the two enantiomers apart is an essential part of proper diagnosis and treatment of lactate-related health conditions.

Under physiological condition of pH 7.4, lactic acid will take the form of its conjugate base form, lactate. The enantiomer, L-Lactate, is a key metabolite found in all mammalian cells and plays a crucial role in metabolism and cellular respiration. When the body goes into anaerobic glycolysis, pyruvate is converted to L-lactate by the enzymes called lactate dehydrogenase (LDH).^[66,67] This conversion functions to not only regenerate nicotinamide adenine dinucleotide (NAD⁺), which are essential for ATP generation, but also to store and subsequently restore glucose molecules through gluconeogenesis when oxygen is no longer limited.^[68] These functions of L-lactate are vital for maintaining the energy production of the body.

Meanwhile, D-lactate is normally produced by strains of bacteria and are commonly found in fermentation process within the food and biotech industry.^[69,70] D-lactate has also been reported to be produced in small amounts by the human mitochondria found in the liver. However, certain diseases like short bowel syndrome, can lead to the overexpression of the

mitochondria, resulting in dangerous levels of D-lactate.^[71,72] Generally, D-lactate in the body is considered a harmful enantiomer and it is found in the body through food contamination or because of certain illnesses. In cases where D-lactate concentrations are at levels above the norm, this might be indicative of certain metabolic disorders such as poisoning or infections.^[66,73]

Generally, L-lactate concentration in the blood is around 0.5-2.2 mM but can rise up to 25 mM during extensive workouts.^[74] In contrast, D-lactate concentrations are usually in the micromolar range of 0.013-0.2 mM.^[75] When concentration of either lactate enantiomer exceeds 4 mM, blood pH begins to drop, leading to a condition known as lactic acidosis, which can lead to severe health issues or even death.^[73] Being able to accurately distinguish the cause of acidosis is important for proper diagnosis and treatment, and identifying which lactate isomer is elevated can significantly help narrow down the underlying cause.

As an important biomarker for the diagnosis of many diseases, many researchers are interested in the advancement of various forms of lactate detectors. Previously, our lab reported two L-lactate aptamers with K_d of 0.39 mM and 1.92 mM and selectivity ranging from 7 to 50-fold towards the target isomer.^[48] Our next goal was to find an aptamer with specificity towards D-lactate. Although D-lactate is not the predominant form found in the body, it still remains relevant in the detection of diseases when abnormalities are found. However, during our selection process with D-lactate, we discovered a new aptamer sequence that binds with higher affinity towards L-lactate than the previously reported L-lactate aptamer. This finding marked

the beginning of the hypothesis suggesting that DNA aptamers may exhibit intrinsic chiral preferential binding for certain molecules.

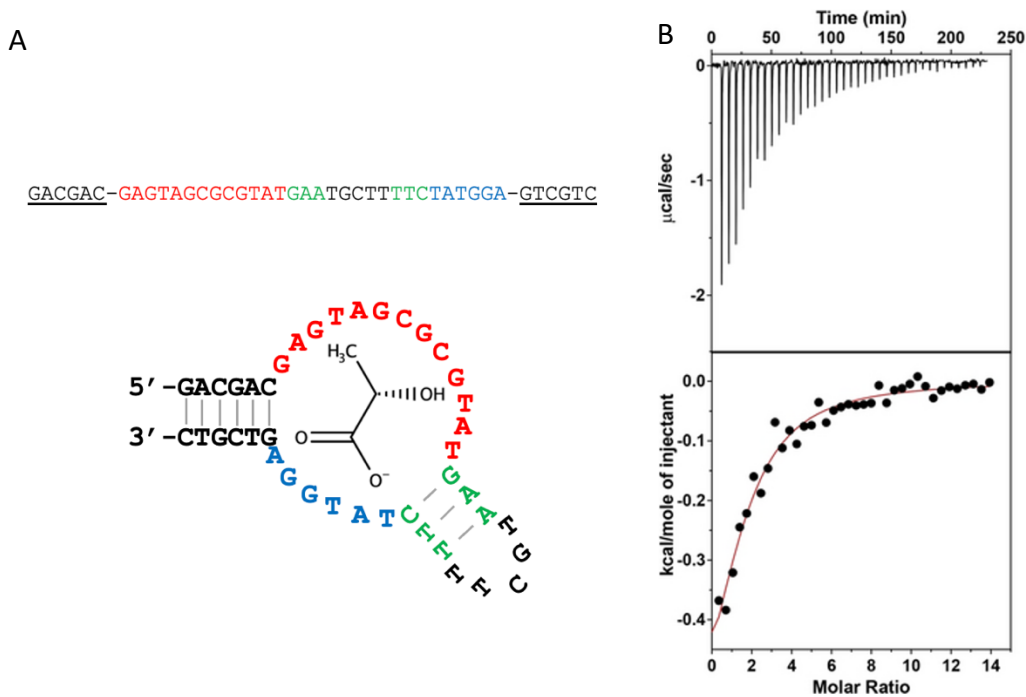


Figure 2.1. (A) Predicted secondary structure of the L-lactate aptamer from previous studies. (B) ITC binding curve for the L-lactate aptamer that is titrated with increasing concentration of L-lactate. (Figure was taken from ref. 48)

2.2 Results and Discussion

2.2.1 Optimized Selection Strategies for D-lactate Aptamers

Our original goal was to obtain a selective aptamer for the D-lactate enantiomer. To perform the selection of a DNA aptamer with D-lactate, a library-immobilized Capture-SELEX approach was utilized. This Capture-SELEX involves the immobilization of the DNA library onto a streptavidin agarose bead column and upon adding the addition of the target, the binding strands will be

released. These binding strands can be collected for further enrichment.^[76] Given that the previous aptamer selected for L-lactate showed an affinity of below 1 mM, we decided to perform the selection with an initial target concentration of 100 mM D-lactate.^[48] This concentration was chosen with the expectation that it would be enough to obtain aptamer strands that binds strongly with the target. The method of selection was under the same conditions as the selection used for L-lactate to limit the external influence that will affect the selection. The DNA library contains 30 random nucleotides, and a different primer sequence was used in this selection to avoid contamination from the L-lactate selection. As we hope to obtain aptamers capable of real human biosensing, we chose buffer conditions that closely mimic the pathological state of the human blood. The DNA amplification of each cycle was monitored by real time PCR to determine the enrichment progress of the library. After undergoing 11 rounds of selection, the PCR products were subjected for deep sequencing.

2.2.2 Analysis of the Newly Sequenced D-lactate Library

The sequenced data from round 11 were analyzed with the Geneious software and revealed four distinct families from the DNA sequences (Figure 2.1 A). These sequences were well converged as the top 50 sequences account for approximately 60% of the total sequence pool. The high degree of convergence is an indicator of a successful enrichment from our aptamer selection process. Any sequences that contained numerous degenerate nucleotide bases or were not the length of around 30-mer were considered too mutated for analysis and thus excluded from the evaluation.

The top two families share similar sequences, each containing the same conserved region but mirrored on opposite side of the sequence. This is a display of circular permutation which have demonstrated in the past that these two sequences would exhibit similar binding orientation with the target.^[77] Interestingly, these two families from the D-lactate selections were also top sequences found in the selection with L-lactate. This appearance of these family sequences can be rationalized based on previous observed affinity of these aptamers. The sequences were reported to have about 7-fold selectivity for L-lactate over D-lactate, indicating that it can still bind to the D-lactate isomer. Additionally, a third family was identified and was found in the L-lactate selection as well, but with significant lower percentage of abundance. This family of sequences was not initially investigated due to its low ranking, appearing outside the top 50 sequence for the previous L-lactate selection, specifically ranking 59th and 79th. However, in the current D-lactate selection it makes up 16.81% of the library pool which suggest a higher affinity for the target. A fourth minor family can also be found from a single sequence within the top 50 sequences, however the enrichment in the library pool barely reached 0.1% of the total sequences. Due to the low enrichment, its affinity for the target was questionable and this family was not focused on for further analysis.

The sequences were labelled by the round they were selected from and the ranking according to their abundances. For the new sequence, it was labelled LAC 1103, to represent the 11th round and 3rd most abundant sequence. Using Mfold, the structure for the top sequence of family 3 was predicted (Figure 2.1 B,C).^[78] The sequence contained an 18-nucleotide conserved region (shown in green and purple), with the 9th base thymine appearing 4 out of the 6

collected sequence. As this 9th base was flanked by a stem region, its relevance for target affinity was initially considered to be questionable. An 11-nt based stem region formed a hairpin structure, which was similar to the structures that were determined for the previous L-lactate aptamer (LAC1101 or L-LAC 1).

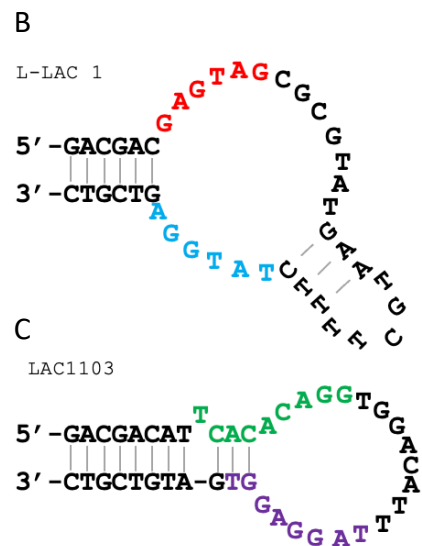


Figure 2.2. (A) Top families assembled from the 50 most abundant sequences from round 11, including appearances of sequences from the L-lactate selection. Secondary structure predictions for (B) LAC 1101 and (C) LAC 1103 were generated with Mfold.

2.2.3 Determining Binding with Fluorescence Labeling

between the fluorophore and the quencher. This in turn leads to an increased fluorescence signal, indicating greater affinity for the target. The previous L-lactate aptamer, L-LAC 1, was also measured as a control to compare the affinity of the two aptamers with the lactate targets. Using Hill's equation, the binding curves can be quantified to determine the aptamer's binding dissociation constant (K_D).

$$V = \frac{V_{max}[S]}{K_D + [S]} \quad (1)$$

Upon adding lactate to the aptamer sensor, a rapid increase of fluorescence signal was observed. When the curve was fitted (Figure 2.2 B,C), we observed a greater affinity of LAC1103 for L-lactate compared to D-lactate. The apparent K_D of LAC1103 was found to be 0.46 mM for L-lactate and 2.5 mM for D lactate, which indicates just over 5-folds higher sensitivity for the L-lactate isomer. Despite our selection using exclusively D-lactate as a target molecule, a new L-lactate orientated sequences emerged. For the control, L-LAC 1, the affinity for L-lactate was superior to that of D-lactate which corresponds to previous observations. It was noticed that the new aptamer was quicker to saturation when 5 mM of L-lactate was added compared to L-LAC 1 which could continue to increase in signal even when 25 mM of target was added. However, when comparing the affinity with the new aptamer, LAC1103 demonstrates stronger affinity for both enantiomer of lactate compared to L-LAC 1. This resulted in a smaller linear dynamic range for LAC1103. We suspect that using a lower target concentration of lactate was likely what contributed to the emergences of higher affinity aptamers. Since all of these aptamers were specific to L-lactate regardless of the chirality of target used, it suggest that DNA aptamers may have an intrinsic preference for binding to the L-lactate enantiomer. Additionally,

if DNA aptamers does have a selective bias towards L-lactate, our use of only D-lactate as a target could have pushed for more selective pressure and be another reason for instigating higher affinity aptamers for L-lactate.

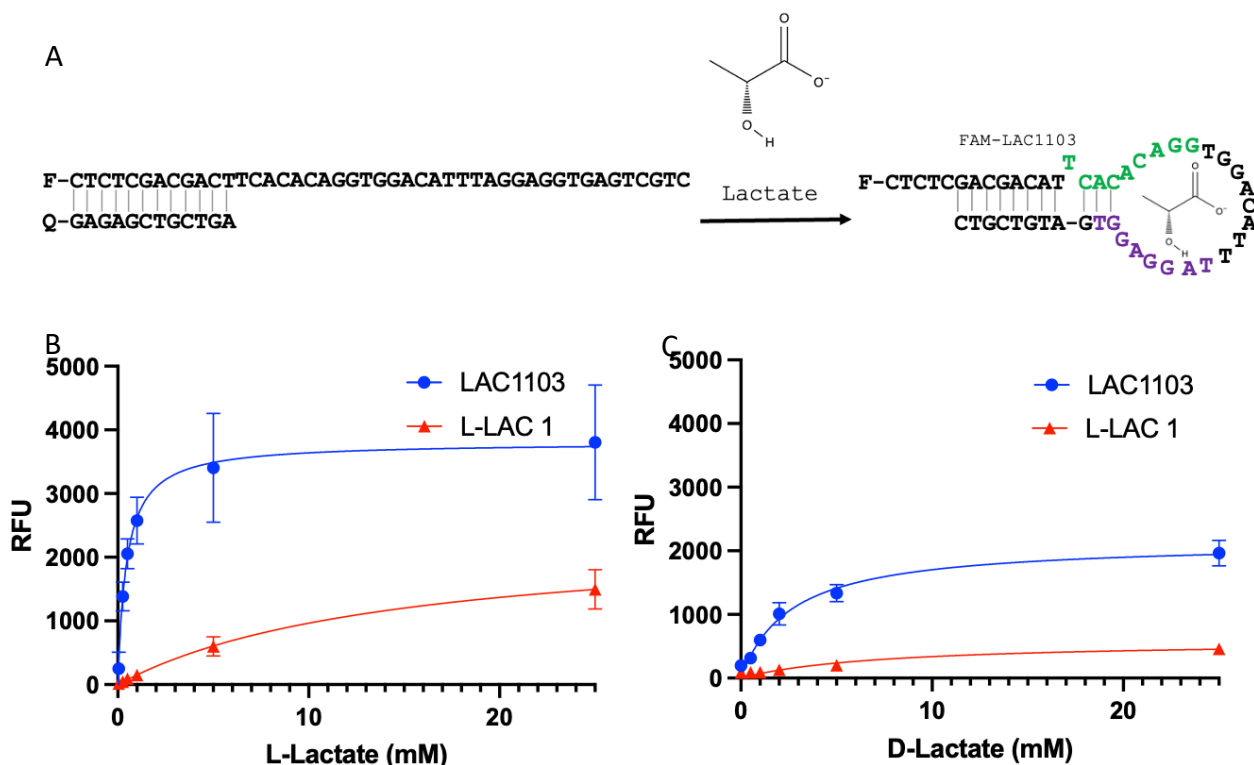


Figure 2.3. (A) Schematic of the single strand displacement fluorescence assay for lactate target. Fluorescence response of 20 nM of Lac1103 and L-LAC 1 with increasing concentration of L-lactate (B) and D-lactate (C).

To accurately measure the true K_D of the aptamer without the competing effects of the quencher strand, we followed the procedure based on Christopher Easley's work.^[79] This first involves determining the dissociation constant of the aptamer-quencher complex ($K_{D, \text{quencher}}$)

which is defined by using the concentration of the FAM aptamer ([F]), the concentration of the quencher strand ([Q]), and the concentration of the aptamer-quencher complex ([FQ]).

$$K_{D,quencher} = \frac{[F][Q]}{[FQ]} \quad (2)$$

We then defined the dissociation constant at equilibrium when the target lactate ($K_{D,apparent}$) is introduced with the concentration of the target lactate [L] and the aptamer-target complex [FL].

$$K_{D,apparent} = \frac{[FQ][L]}{[FL][Q]} \quad (3)$$

From the two measured equilibria, the true dissociation constant ($K_{D,true}$) can be determined.

$$K_D = K_{D,quencher} K_{D,apparent} = \frac{[F][Q]}{[FQ]} \frac{[FQ][L]}{[FL][Q]} = \frac{[F][L]}{[FL]} \quad (4)$$

The true K_D for the LAC1103 aptamer was calculated to be 0.23 mM which shows much tighter binding compared to the previous L-LAC 1 aptamer (0.4 mM).^[51] With this data, these two aptamers (LAC1103 and L-LAC 1) could be used to fit different clinical applications. As LAC1103 has a higher sensitive but a smaller linear dynamic range, it would be more suitable for monitoring patients with lactate acidosis (when lactate levels rise higher than 4 mM). While with L-LAC 1, its higher dynamic range makes it more suitable for monitoring lactate for more fitness-focused application like during exercising or other labour-intensive activities (when lactate levels rise to 25 mM).

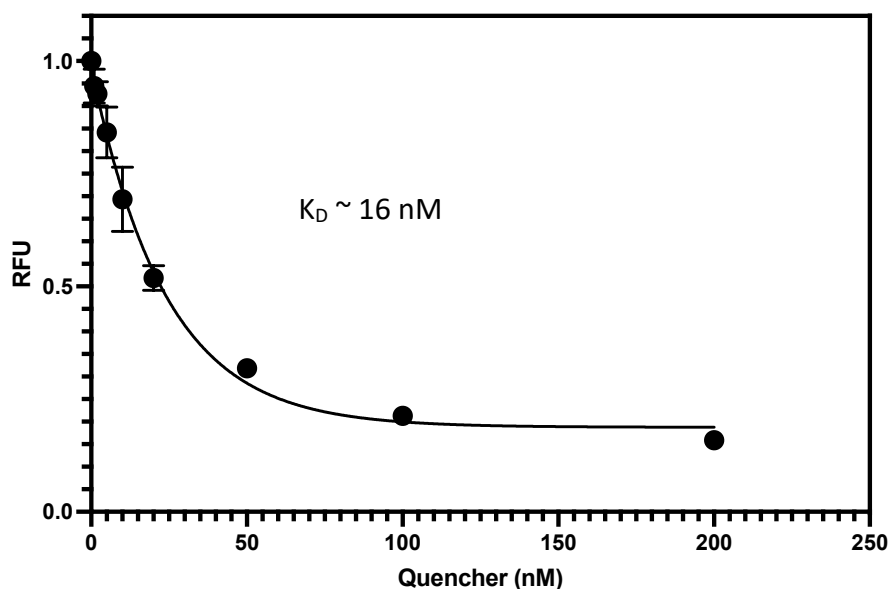
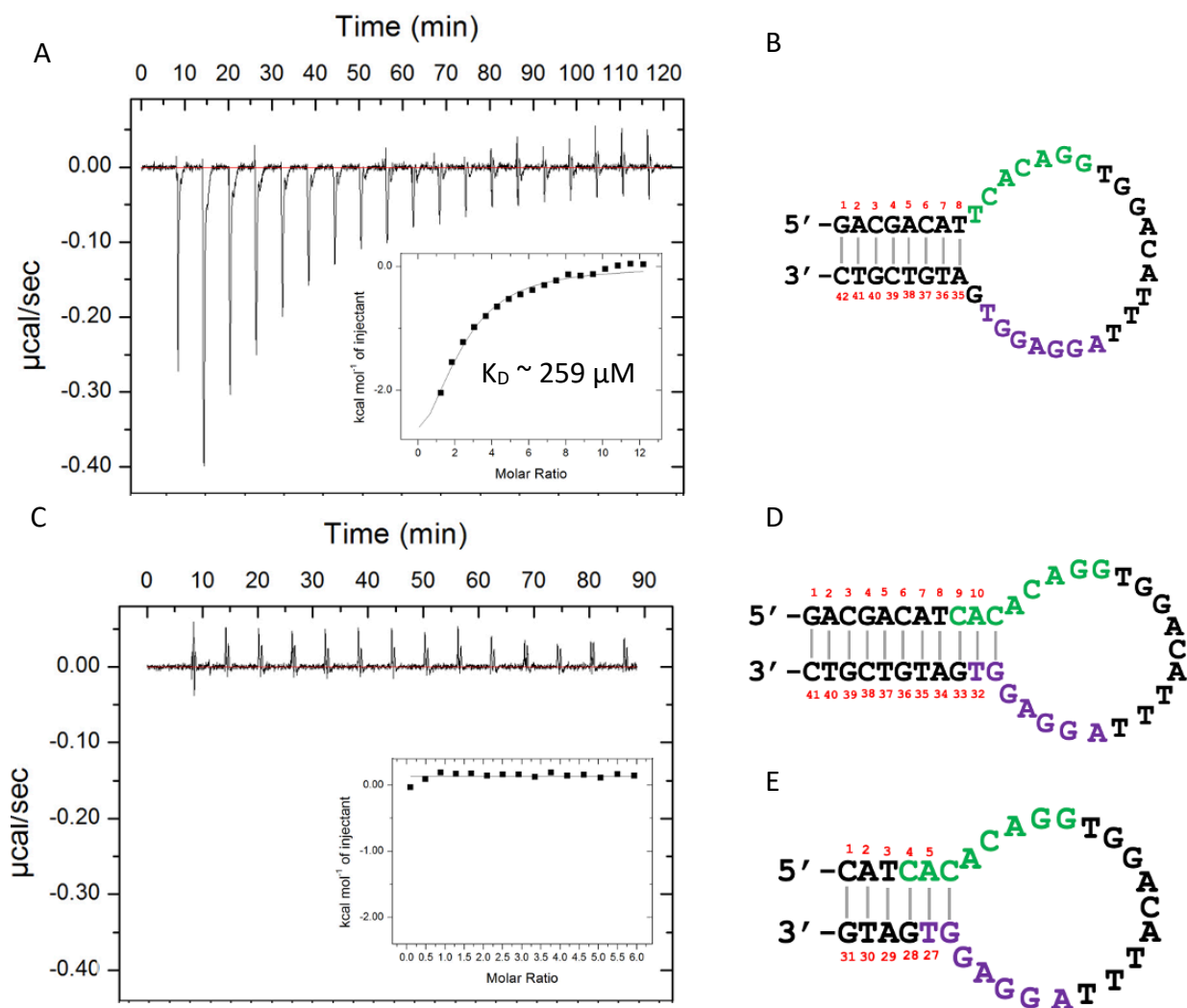


Figure 2.4. Measuring the fluorescence quenching effect of FAM-LAC1103 with increasing concentration of Quencher cDNA.

2.2.4 Determining Binding by ITC

From the fluorescence displacement assay experiment, we determined that the binding affinity of LAC1103 to be better with L-lactate. We further characterized the aptamer's affinity using isothermal titration calorimetry (ITC). The measurable heat change from the titration of lactate to the aptamer allowed us to obtain the thermodynamic properties of the aptamer binding process (Figure 2.3 A). For LAC1103, we obtained an exothermic profile with a K_D of 0.26 mM, closely matching the K_D obtained earlier for true dissociation constant. The enthalpy (ΔH) and entropy (ΔS) changes were -10.4 kcal/mol and ΔS was -18.3 kcal/mol, respectively. This differs from the L-LAC 1 aptamer, which had ΔH of -0.8 kcal/mol and ΔS of 3.8 kcal/mol. For our new aptamer, a larger negative enthalpy change was observed which indicates a tighter binding with

lactate. We also observe a negative entropy change which suggests that there is greater restrictive mobility for the aptamer-target complex, whereas the L-LAC 1 aptamer seems to be entropy-driven (positive ΔS). This is indicative that the two aptamers exhibit different binding mechanism in the presences of the ligand.



LAC1103 (B) and the modified LAC1103 (D,E) are shown. It is observed that when the T9 base was removed from the sequences, no heat change was detected by ITC.

We also conducted a mutation study where the original LAC1103 aptamer was modified by deleting the 9th Thymine base (T9). The initial predicted secondary structure of the aptamer suggest that the T9 base was located in the stem region, which lead us to believe it had no influence on the aptamer-target binding mechanism. However, when we measure the binding of the newly modified DNA without T9, no binding was observed through ITC (Figure 2.3 D). With this data, we realized that T9 base was critical for the aptamer-target binding, and we now believe that the actual secondary structure of the aptamer has a larger loop region than what was originally predicted (Figure 2.3 B).

2.2.5 Selectivity Test for New Aptamer

As we demonstrated that the new aptamer (LAC1103) is capable of higher sensitive and rapid detection of lactate, we proceeded next to monitor its capability for biosensor applications. By measuring the background signal of the fluorescence reading, the limit of detection (LOD) for LAC1103 was determined to be 0.21 mM ($3\sigma/slope$). We then performed a selectivity study by testing the aptamer against five biomolecules: oxalate, pyruvate, 3-hydroxybutyrate, acetate, and glycine (Figure 2.4). The sensor detection was tested in a buffer solution that was mixed with 10% blood serum. The biomolecules chosen were selected because they were common biomolecules found in the body and also because they share similar structural features to that of the lactate target. The general criteria for the selections focused on small molecules

containing a carboxyl functional group. For each target, the concentrations of the targets were analyzed at both 1 mM and 10 mM which covers the physiological conditions of most of the tested biomolecules.

Against pyruvate, 3-hydroxybutyrate, acetate and glycine, a slight response was detected at 10 mM concentration while essential no response was detected at 1 mM. In comparison to the lactate targets, the selectivity of lactate was approximately 4-folds larger than these 4 biomolecules at 10 mM. However, for the fifth biomolecules that was tested, oxalate, a high affinity was detected comparable to that of the lactate response to the aptamer. In fact, the affinity that the aptamer had for oxalate was even higher compared that of D-lactate. Based on this, inferences on the binding mechanism of LAC1103 can be deduced. From the structural similarity between oxalate and lactate, as both are small molecules which predominately consist of a carboxyl and a hydroxyl group, the LAC1103 aptamer likely has a strong favourable interaction with these functional groups. This hypothesis also coincides with the lack of response of the aptamer against pyruvate and acetate, which both do not have a hydroxyl group. Additionally, electrostatic attraction might contribute to stronger binding as a more negatively charged molecules like oxalate may promote better binding with the aptamer.

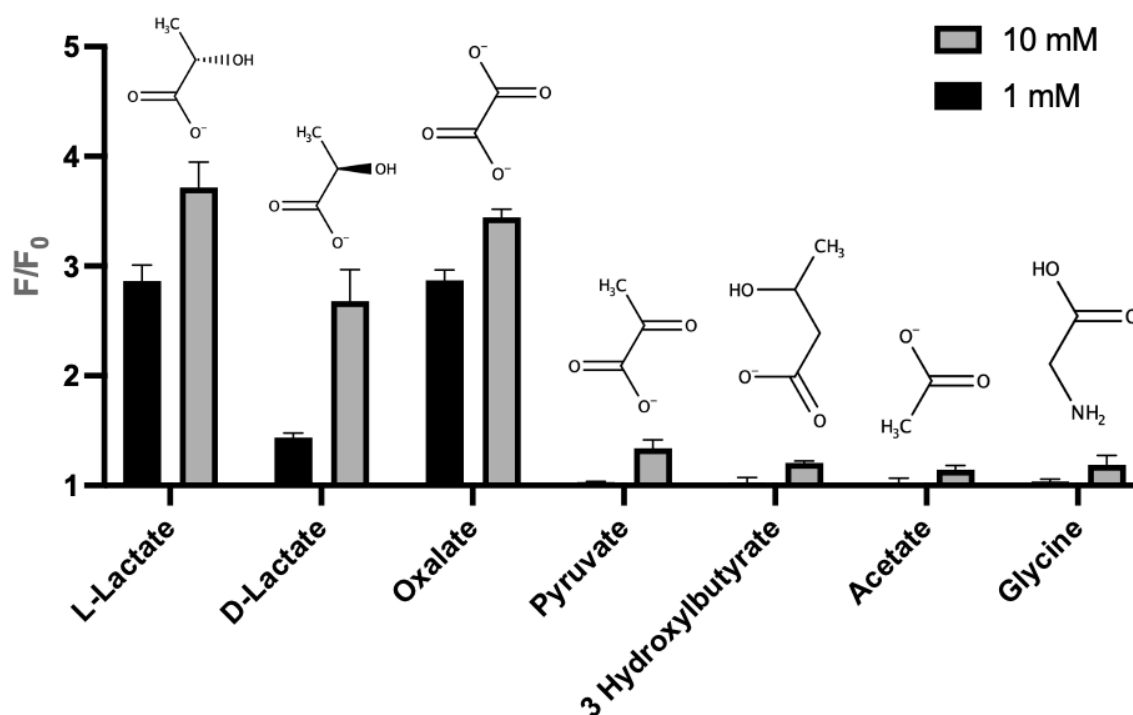


Figure 2.6. Selectivity Assay for LAC1103 immersed in 10% blood serum with common biomolecules that share similar structural feature with lactate.

2.3 Summary

In this chapter, we demonstrated that the selection of either L-lactate or D-lactate produced aptamers that are primarily selective towards the L-lactate isomer. This was shown from our selection using only D-lactate as a target, which upon completion, revealed DNA sequences that have higher affinity for L-lactate over D-lactate. This new selection also led to the emergence of a new aptamer family which displayed better binding towards lactate compared to our previously lactate aptamers. The new aptamer demonstrated better binding affinity for lactate, which could likely be due to the increased selective pressure of a lower concentration target

and also the use of a more challenging enantiomer target (D-lactate) that requires higher affinity aptamer for binding with the target to occur. Additionally, we demonstrated the aptamer's capability for lactate biosensing by designing a fluorescent biosensor for rapid detection. We tested a total of 5 other biomolecule ligands to illustrate the binding mechanism of the aptamer by observing the targets that it could and could not bind to. Our results show that the aptamer can recognize its target with high specificity, indicating its potential application in real world monitoring of lactate levels in the body.

2.4 Experimental Procedure

2.4.1 Chemicals

All the DNA sequences were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA) and are listed in Table 2.1. All chemicals which include sodium L-lactate, sodium D-lactate, oxalate, pyruvate, sodium 3-hydroxybutyrate, acetate, glycine, sodium hydroxide, hydrochloride acid, magnesium chloride, potassium chloride, and human blood serum were purchased from Sigma-Aldrich. The streptavidin agarose bead resin was purchased from Fisher Scientific (Ottawa, ON, Canada). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and its sodium salt form were purchased from Biobasic Inc. (Markham, ON, Canada). Taq DNA polymerase, Taq Reaction buffer and dNTPs solution mix were purchased from New England Biolabs (Ipswich, MA, USA). SsoFast EvaGreen supermix was purchased from Bio-Rad. Milli-Q water was used to prepare all solutions and buffers.

Table 2.1. DNA sequences used for the lactate selection.

DNA Names	Sequences 5' to 3'
FP n30	GGA GGC TCT CGG GAC GAC
RP n30	CTG TAA ATC CTA AAG GCG GGA CG
Biotin-RP n30	5Biosg/CTATAAATCCTAAAGGCGGGACG
Biotin-column	GTCGTCCCGAGAGCCATA/3BioTEG/
N30 Library	GGAGGCTCTCGGGACGAC-n30-GTCGTCCCGCCTTTAGGATTACAG
D-LAC1103	GACGACATTCACACAGGTGGACATTTAGGAGGTGATGTCGTC
D-LAC1103-6bp	CGACATTCACACAGGTGGACATTTAGGAGGTGATGTCG
FAM-Lac1103	56-FAM/CTCTCGACGACTTCACACAGGTGGACATTTAGGAGGTGAGTCGTC
Quencher	AGTCGTCTCGAGAG/3IABkFQ/

2.4.2 SELEX Selection

The aptamer selection used in this experiment was based on the previous published procedure designed for L-lactate with a few modifications.^[51] A selection (SELEX) buffer was prepared containing 500 mM NaCl, 10 mM MgCl₂, 10 mM KCl, 50 mM HEPES at a pH 7.4. For the initial round of selection, 500 pmol of DNA library was annealed with 5 times amount of biotin column capture strand in a 250 µL SELEX buffer for 3 minutes. After the first round, the size of DNA library used was lowered to 100 pmol. The annealed DNA was allowed to cool to room temperature and an additional 300 µL of SELEX buffer was added for a total of volume of 500 µL. Streptavidin agarose resin was then loaded into a micorscreen column filter and washed with 500 µL SELEX buffer 5 times. The annealed library was used to wash the microscreen column filter 6 times to ensure all the DNA is loaded in the bead resin. The filter is washed 12 times with 500 µL of SELEX buffer to remove all unbounded DNA strands. 750 µL of D-lactate solution was added to the filter and the elute with all the library strand bounded to the target was collected. 3K centrifuge filter was then used to concentrated the DNA elute and washed with 500 µL Milli-Q water 3 times to purify the DNA strands. The recovered DNA was diluted by

10-folds with Milli-Q water and amplified by PCR using biotin reverse primers. The enrichment of the DNA products was monitored by real-time PCR using ssoFast Evagreen super mix. Once the DNA was amplified, the dsDNA library was washed onto 200 μ L streptavidin agarose resin in a micocolumn filter for 6 times. The immobilized library was washed 10 times with SELEX buffer and then incubated for 30 minutes with 400 μ L 0.2 M NaOH to denature the double strand DNA product into single stranded DNA. After incubation, the elute was neutralized with 200 μ L of 0.2 M HCl and added to a 3K centrifuge filter to be washed 2 times with 450 μ L Milli-Q water and 1 time with 450 μ L SELEX buffer. The final eluted was concentrated to 60 μ L of DNA and the concentration of DNA product was measured by a microplate reader (Spark, Tecan). The determined concentration of the DNA product was then used for the subsequential rounds in selection. Once selection was completed, the DNA library was sent to McMaster for deep sequencing analysis using next generation sequencing (NGS) methods.

2.4.3 FAM Displacement Assay

The fluorescence measurements were conducted on a microplate reader (Infinite F200 Pro, Tecan) in a 96-well plate. The sensor stock solution was prepared with 1 μ M of FAM-labeled DNA annealed with 2 μ M of complementary quencher strand DNA. For a typical experiment, a final concentration 20 nM of sensor stock solution is used in 100 μ L SELEX buffer. For the FAM readings, an excitation wavelength at 485 nm and emission wavelength at 535 nm were used with a gain value of 50. The background fluorescence of the sensor was left to equilibrate prior to beginning the experiment. The initial fluorescence signal was measured for 5 minutes before

adding the target which was then left to incubate for 30 minutes while the kinetic signal was recorded.

2.4.4 Isothermal Titration Calorimetry (ITC)

The ITC measurements were performed in a MicroCal VP-ITC calorimeter. 2 mL of 100 μ M LAC1103 and 6 mM of L-lactate were prepared with SELEX buffer. Prior to loading the samples into the ITC instrument, the aptamer solution was annealed to 95°C for 3 minutes, followed by a slow cool to room temperature. The aptamer and L-lactate solution was then degassed for 5 minutes to remove all air bubble trapped in the liquid. The 1.5 mL of the aptamer solution was loaded into the cell chamber and 300 μ L of the L-lactate solution was loaded into the syringe. For the initial injection, 2 μ L of target is titrated over 4 seconds which was followed by 27 injections of 10 μ L target over 20 seconds. Each injections was spaced with 180 seconds between them and performed in 25°C temperature. Once the titration was completed, the thermodynamic data was analyzed using the Origin software.

2.4.5 Selectivity Detection in Serum

The stock solution was made by diluting the human blood serum by 10-folds with SELEX buffer to make a final concentration of 10% serum. The FAM sensor stock solution was added to the 10% serum solution to make a final sensor concentration of 20 nM. The back fluorescence signal was left to equilibrate prior to beginning the experiment. Once the signal has equilibrated, measurements were conducted following the procedure used for the FAM displacement assay.

Chapter 3 Selection of Tryptophan Binding Aptamers

3.1 Introduction

To further explore on whether there is an intrinsic chiral preference for DNA aptamers, we expanded our focus to the other essential biological molecules utilized by mammalian cells. For our study, the molecule we selected was tryptophan, one of the essential amino acids used in maintaining functions throughout the body. Compared to lactate, tryptophan is a relatively larger molecule, which contains indole side chain group. Due to tryptophan being an aromatic derivative, we anticipate that it would be easier to select a successful aptamer that can bind with this target.^[80]

Compared to other amino acids, L-tryptophan constitutes a relatively low concentration in the human body, only existing around 60-70 μM in healthy human blood serum.^[81] Like other essential amino acids, it serves as an important component in protein synthesis. However, the unique role for L-tryptophan is for the modulation of vital neurologically active compounds such as serotonin and melatonin which is facilitated by the kynurenine pathway. In the kynurenine pathway, the majority of tryptophan is catabolized in response to stress signaling hormones, which accounts approximately 99% of L-tryptophan not used in protein synthesis.^[82] The disruption of this pathway can lead to various neurological diseases and disorders, including Alzheimer's Disease, depression, schizophrenia, and various cancers.^[82,83] As such, monitoring the targets involved in the kynurenine pathway such as L-tryptophan is important for therapeutic development.

In contrast, D-tryptophan does not have a significant presence in normal mammalian cells, where it plays no known biological role in the human body. However, past studies have shown that D-amino acids, such as D-tryptophan, can be involved in accelerating cancer and tumors development.^[84] There is also some evidence suggesting a potential relationship between the uptake of D-amino acids and neurological diseases, but this area of research is still under investigation and remains a topic for debate.^[84]

A recent study done by the Stojanovic group reported the selection of various DNA aptamers that can bind to different small molecules.^[85] In their experiment, L-tryptophan was used to select an aptamer which reported to have a K_D of 1.2 μM . Continuing from our previous findings, a racemic mixture of tryptophan in new selection will be performed with the aim to further explore the chiral preference of DNA aptamers.

3.2 Results and Discussion

3.2.1 Selection with Racemic Tryptophan Target

Building on what was found from our lactate selection, we wanted to further investigate if chiral preference exists for other essential biomolecules necessary for mammals. We decided to perform another selection with a racemic mixture of tryptophan to test this hypothesis. By employing the same library-immobilized Capture-SELEX strategy used for the lactate selection, we started with 1 mM of racemic tryptophan as the target concentration for this selection. After 15 rounds of selection, the PCR products were sent out for deep sequencing. From the sequencing result, three major families were revealed among the top 10 sequences (Figure 3.1

A). The most enriched sequence, TRP 1501, makes up the first family which constitutes 23% of the total library and contains two conserved regions shown in red and blue. Using Mfold to predict the secondary structure, TRP 1501 contains an internal loop followed by a hairpin loop that is connected by a 5-nt helix sequence. This sequence shares similar conserved regions with sequence TRP 1503 which represents the second major family. This second family makes up 8% of the library and has conserved regions that are on opposite position from the TRP 1501. For the third family, it has the highest occupancy accounting for 28% of the library. 5 sequences among the top 10 sequences represent this family , which features two different conserved regions (shown in green and yellow) that are distinct to the other two families. From the Mfold predicted secondary structure, this family's structure features a multi-branched loop junction that splits off to two hairpin loops (Figure 3.1 C). The internal loop junction appears to be made up entirely of the conserved sequence which suggests that this loop has relevancy for target binding.

A

Sequence name		# Of Sequence	% of Sequence
TRP1501	GACGAC GATCAGTGTAACGA GTACTGAAAGT ATTGA GTCGTC	2957	23.57
TRP1502	GACGAC GGGTGTTA CAACACTGAATGCAAT CTC GTCGTC	1665	13.27
TRP1503	GACGAC GATTG AGCCGTGC GATCAGTG AAAA GA GTCGTC	1104	8.80
TRP1504	GACGAC GGGTTTA AGCATTAAACTGCAGTATG CTCA GTCGTC	749	5.97
TRP1505	GACGAC GGGTATA ACCGCGTTATACTGCGAAG CTCA GTCGTC	435	3.47
TRP1506	GACGAC GACCATTGCTTCCTGGGAAGAAGTGAAAGC GTCGTC	433	3.45
TRP1507	GACGAC GGGTGTT CAAAAACACTGCTATTTAG CTCA GTCGTC	407	3.24
TRP1508	GACGAC TCACGCAGAACTGACATTCAGTGGGTGGTT GTCGTC	312	2.49
TRP1509	GACGAC GGGGTTG AAAAACCGCGAGTACCATGA GTCA GTCGTC	283	2.26
TRP1510	GACGAC GAATCAACAACGTGAGTCGATAGACATGAA GTCGTC	257	2.05

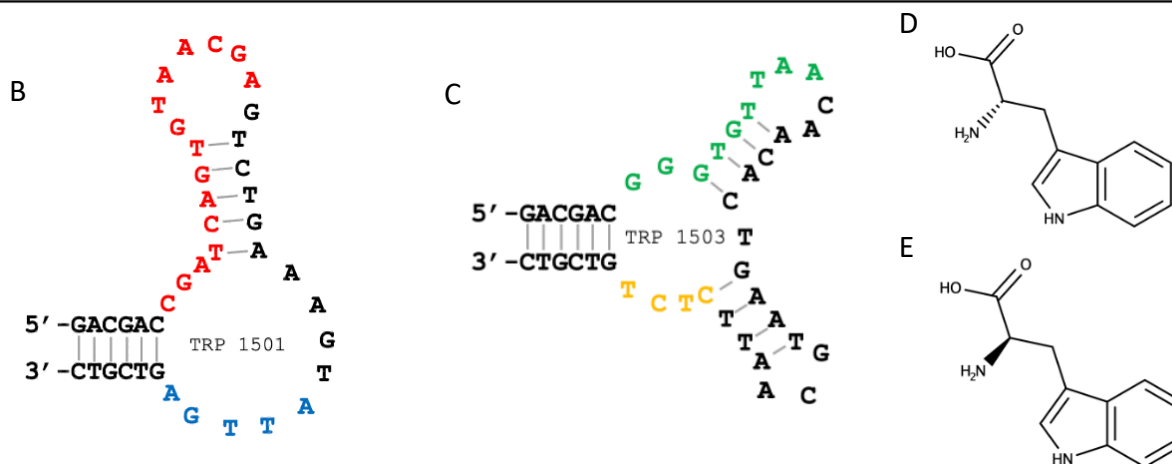


Figure 3.1. (A) Top families assembled from the 10 most abundant sequences from round 15 of the racemic tryptophan selection. Secondary structure predictions for (B) TRP 1501 and (C) TRP 1503 were generated with Mfold. Molecular structure of (D) L-tryptophan and (E) D-tryptophan

For further analysis, the sequences TRP 1501 and TRP 1503 were chosen to investigate their binding characteristics. To measure the affinity of these aptamers for the targets, we employed ThT, a label-free approach to provide details into their binding properties. Separate experiments using L and D tryptophan were conducted to compare the selectivity of both isomers with the aptamers. From the calibration curve for TRP 1501, relatively similar binding was observed between the two enantiomers with K_D of approximately 0.18 mM (Figure 3.2 A and B). With TRP

1503, a 4.3-fold higher affinity for L-tryptophan was observed compared to D-tryptophan with a K_D of 0.17 mM and 0.73 mM respectively. While selective bias was observed for L-tryptophan, the overall affinity for tryptophan was weak. As Stojanovic's group has demonstrated in their paper, higher affinity sequences for tryptophan can be achieved with DNA aptamers. Therefore, we decided to conduct a new selection in order to obtain aptamers with even higher affinity.

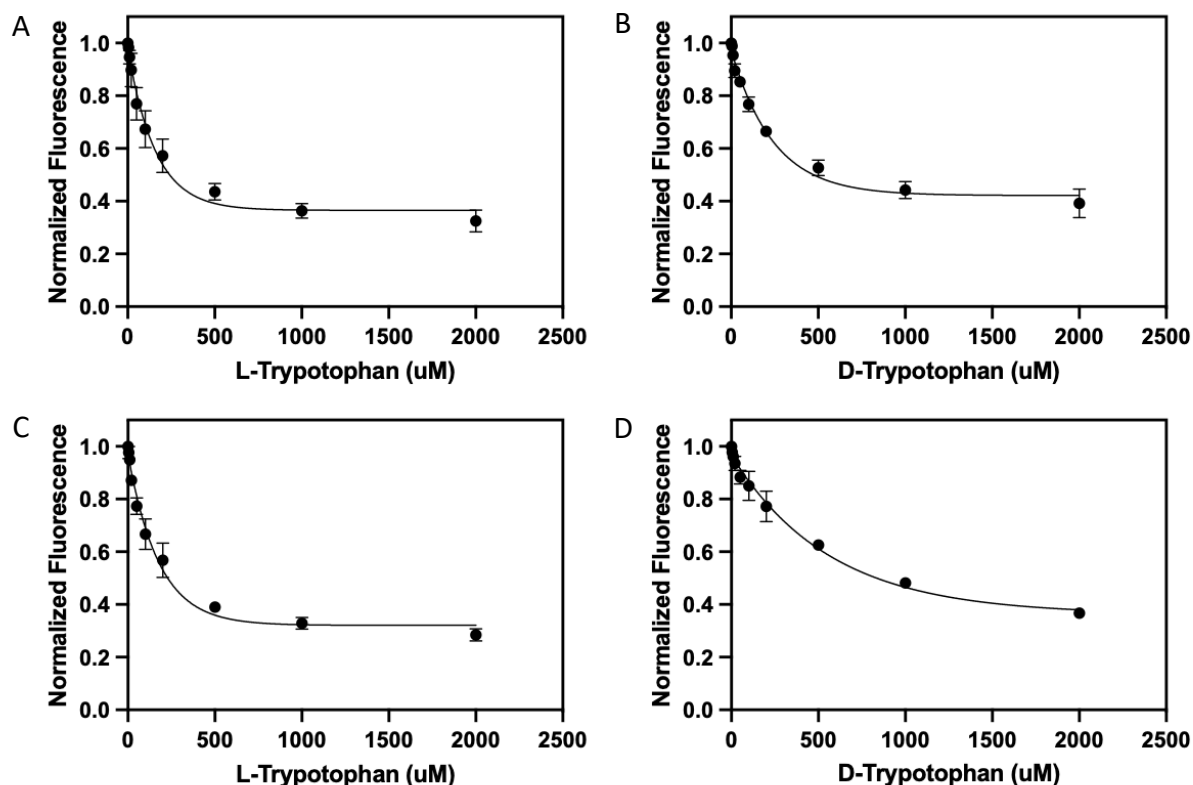


Figure 3.2. Detection of tryptophan concentration using ThT displacement assay. The figure illustrates the fluorescence changes detected for TRP 1501 with (A) L-tryptophan and (B) D-tryptophan at various concentrations. Fluorescence changes for TRP 1503 with (C) L-tryptophan and (D) D-tryptophan at various concentration are shown.

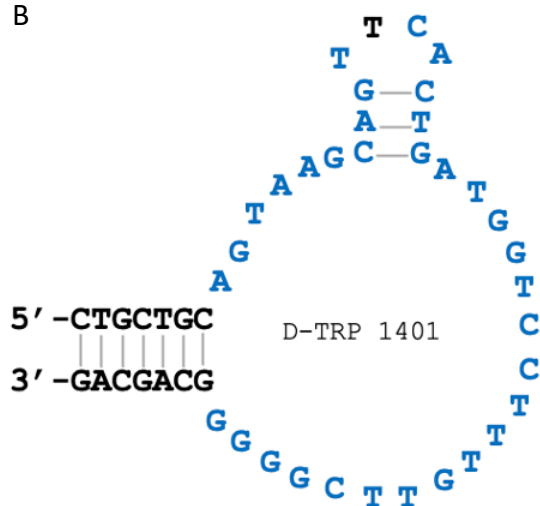
3.2.2 Selection using D-Tryptophan

In this new selection, we used a DNA library with a 36 random nucleotides domain. This was chosen in consideration to match the library used by Stojanovic's group, where a high affinity tryptophan aptamer was successfully obtained. The other change made to the selection was the use of only D-tryptophan as a binding target. Similar to the earlier selection with D-lactate in chapter 2, we hypothesized that the use of a harder selection target will cause for more selective pressure for higher affinity aptamers to emerge. With these changes, a total of 14 rounds of selection were completed before the last round was sent out for deep sequencing. From the sequencing result, high convergence was observed where the top 4 sequences contribute to 98% of the total library. The first family by far makes up the majority of the DNA pool, with two appearances out of the top 8 sequences representing 75.57% of the total library. The two sequences that comprise the first family share an identical 35-nt conserved region which is highlighted in blue in Figure 3.3. The predicted secondary structure reveals that this conserved region forms an internal loop connected to a small hairpin loop by a 3-nt helix (Figure 3.3 B). From this library, we have noted one other family with a strong sequence enrichment. For the sequence D-TRP 1402, it shares the same 7-nt region (marked in green) with TRP 1503 from the earlier racemic tryptophan selection discussed in chapter 3.2.1 (Figure 3.3 C). The emergences of these sequences likely indicate similar binding mechanism to the previously discovered aptamers. Beyond the D-TRP 1403, the other emerging sequences represent only a very small portion of the library pool (>3.87%) and we concluded that their binding affinity for tryptophan was considered questionable. In order to identify a new aptamer sequence, only D-TRP1401 from the first family was chosen for further analysis.

A

Sequence name		# Of Sequence	% of Sequence
D-TRP1401	GACGAC GGGGGCTTGTTTCCTGGTAGTCACTTGACGAATGAC GTCGTC	31086	56.97
D-TRP1402	GACGAC GATGCGAATGCATAGCGGAGCGTTTGTTTACAATA GTCGTC	10435	19.13
D-TRP1403	GACGAC GGGGGCTTGTTTCCTGGTAGTCACTTGACGAATGAC GTCGTC	10093	18.50
D-TRP1404	GACGAC GCGATACACCTCGTATCAGGGTCAGGGGTCCCCTCA GTCGTC	2109	3.87
D-TRP1405	GACGAC TACGTGCGGGGTTTATTCCTGGCATGATGCATGGA GTCGTC	254	0.47
D-TRP1406	GACGAC GCGATACACCTCGKACAGGGTCAGGGGTCCCCTCA GTCGTC	108	0.20
D-TRP1407	GACGAC GATCAGGTTTAAACCGAGTCTCAGGATTGAGATTGA GTCGTC	99	0.18
D-TRP1408	GACGAC AGCAATACCGGTGCTATCGTATACCGGATTGTTAT GTCGTC	77	0.14

B



C

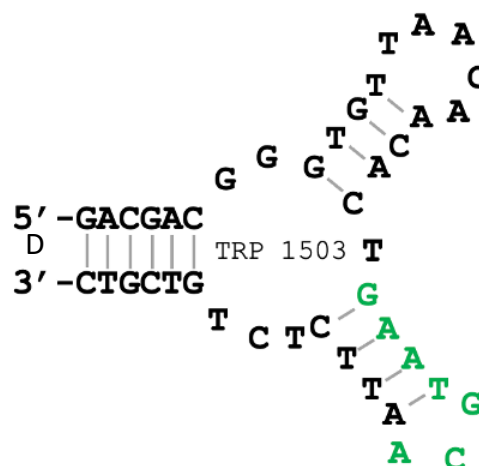


Figure 3.3. (A) Top families assembled from the 8 most abundant sequences from round 14 of the D-tryptophan selection. Secondary structure predictions for (B) D-TRP 1401 and (C) TRP 1503 was generated with Mfold.

ThT analysis was used again to measure the binding with tryptophan with the newly selected aptamer D-TRP 1401. Alongside our aptamer, we also ordered the aptamer sequence from Stojanovic's group to confirm and compare the binding with tryptophan. This aptamer was labeled as TRP-Milan. When testing the affinity for TRP-Milan, we reported a K_D of 6 μ M for L-tryptophan, which was matched the previously reported binding affinity reported by

Stojanovic's group (Figure 3.4 C). When TRP-Milan was tested with D-tryptophan, significantly worse affinity was observed with a K_D of 106 μ M, demonstrating a high selectivity towards the nature isomer of tryptophan with a 16-fold increased affinity (Figure 3.4 D). However, when we tested our new aptamer D-TRP 1401, the opposite response was observed. The K_D affinity for L-tryptophan was 85 μ M and 11 μ M for D-tryptophan (Figure 3.4 A,B). These finding suggests that the selection of tryptophan with DNA aptamers does not exhibit a preference for any chiral form of the molecule. This may be due to the larger size of tryptophan and thus has more unique epitopes for binding compared lactate, therefore providing more binding features that allow for selective binding for either isomer.

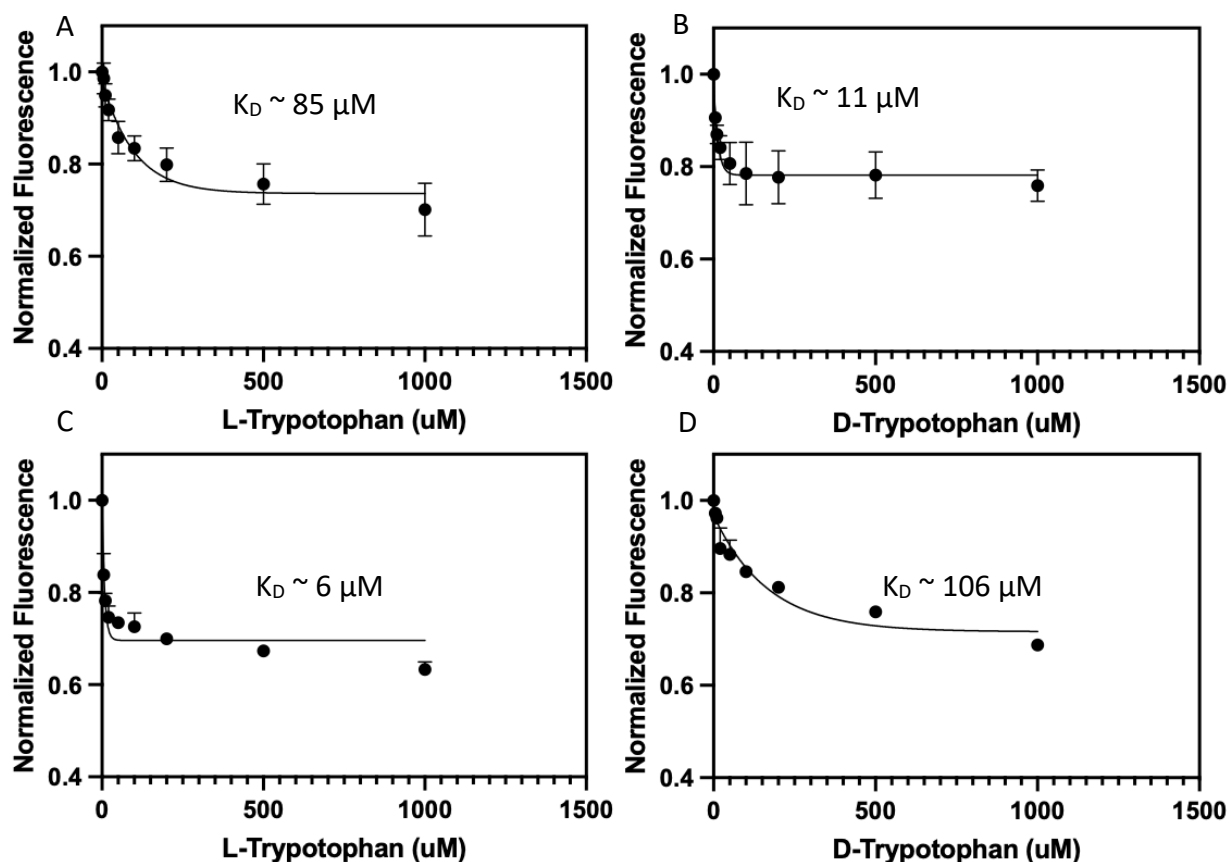


Figure 3.4. Detection of Tryptophan Concentration using ThT Displacement Assay for aptamers obtained the D-Tryptophan Selection. The figure illustrates the fluorescence changes detected for Trp1401 with (A) L-tryptophan and (B) D-tryptophan at various concentrations. The fluorescence changes detected for the aptamer ordered from Milan Stojanovic's selection, TRP-Milan, with (C) L-tryptophan and (D) D-tryptophan at various concentration are shown.

3.3 Summary

From the tryptophan selections, we observed that DNA aptamers can selectively bind to both L-tryptophan and D-tryptophan. Initially, following the inference that was made with the selected lactate aptamer, we originally suspected that DNA aptamers selected with tryptophan will also

lead to aptamers with a preference towards the natural isomer, L-Tryptophan. For our first selection with racemic tryptophan, only enrichment of sequences that either bind better to L-tryptophan or exhibited similar binding affinity between both isomers was discovered. However, when we increased to selective pressure for more high affinity tryptophan aptamer, the selection with just D-tryptophan as the target revealed that it is possible to obtain a selective D-Tryptophan binding aptamer. The K_D of the new aptamer, D-Trp 1401, was calculated to be 11 μM and had a selectivity of approximately 7-folds which is comparable to aptamers selected for L-tryptophan from previous studies. We hypothesize that this is mainly due to the nature of larger size molecule which would allow for more specific interactions between the ligand to the aptamer. Unlike lactate, tryptophan has greater structural complexity that allow for stronger binding that overcome any chiral preferences that might exist within DNA aptamers.

3.4 Experimental Procedure

3.4.1 Chemicals

All the DNA sequences were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA) and are listed in Table 3.1. All chemicals which include L-Tryptophan, D-Tryptophan, sodium hydroxide, hydrochloride acid, magnesium chloride and potassium chloride were purchased from Sigma-Aldrich. The streptavidin agarose bead resin was purchased from Fisher Scientific (Ottawa, ON, Canada). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and its sodium salt form were purchased from Biobasic Inc. (Markham, ON, Canada). Taq DNA polymerase, Taq Reaction buffer and dNTPs solution mix were purchased from New England

Biolabs (Ipswich, MA, USA). SsoFast EvaGreen supermix was purchased from Bio-Rad. Milli-Q water was used to prepare all solutions and buffers.

Table 3.1. DNA sequences used for the tryptophan selection.

DNA Names	Sequences 5' to 3'
FP n36	GGA GGC TCT CGG GAC GAC
RP n36	AGA CCA TTC AAG TGA TCG GGA CG
Biotin-RP n36	5Biosg/ AGACCATTCAAGTGATCGGGACG
Biotin-column	GTCGTCCCGAGAGCCATA/3BioTEG/
N36 Library	GGAGGCTCTCGGGACGAC-n36- GTCGTCCCGATCACTTGAATGGTCT
TRP 1501	GACGACGATCAGTGTAACGAGTACtGAAGGTATTGAGTCGTC
TRP 1503	GACGACGGGTGTTAACAACACTGAATGCAATTCTCAGTCGTC
D-TRP 1401	GACGACGGGGGCTTGTTTCCTGGTAGTCACTTGACGAATGACGTCGTC
TRP-Milan	CGACGGAACCGGTGGTGTAGTTCCGGCGTGGGGAAGGTTCGTCG

3.4.2 SELEX Selection

The selection using 1 mM of racemic Tryptophan and 1 mM of D-tryptophan follows the procedure used for lactate in chapter 2.4.2. For the selection using D-tryptophan, the library was changed from a 30-nt library to a 36-nt library alongside the use of correct corresponding primers. The selection (SELEX) buffer was prepared containing 500 mM NaCl, 10 mM MgCl₂, 10 mM KCl, 50 mM HEPES at a pH 7.5.

3.4.3 Binding with ThT Assay

The measurement was performed using the spectrometer (FluoroMax-4, Horiba Scientific). A 1:1 ratio of aptamer and ThT was prepared in a cuvette with a final concentration of 1 μ M in 500 μ L of SELEX buffer. The wavelength parameters were set at 420 nm excitation and 490 nm

emission. When the cuvette was inserted into the spectrometer, the fluorescence signal was incubated for approximately 30 minutes to allow the background signal to stabilize. Once the signal was stable, increasing concentration of tryptophan was added and the change in fluorescence intensity was recorded. The fluorescence kinetics of each target addition was monitored until a stable signal was achieved between each addition.

Chapter 4 Conclusions and Future Directions

Exploring the interactions that DNA has on chiral molecules can greatly improve the field of therapeutics in areas like biosensing and drug development. Previous studies by other groups have extensively discussed that the structure of RNA is the root cause for the dominance of one enantiomer to be found for many essential biomolecules such as amino acids and sugars.

Building on this hypothesis, my thesis investigates the enantioselectivity of DNA aptamers against the isomer of two targets: lactate and tryptophan. Despite that both targets are essential biomolecules commonly found in the body, the enantioselectivity of the DNA aptamers for these targets are found to not always have a bias towards the target's "natural" isomer.

In chapter 2, we compared the resulting aptamers from two separate selection with different isomer for the lactate target. Separate DNA libraries were used for the selection of 1 M concentration of L-lactate and 100 mM concentration of D-lactate to reduce the outcomes of possible contamination between experiments. Regardless of the use of different libraries, many of the emerging sequences were identical and only demonstrated enantioselectivity towards on the L configuration. The aptamer that was measured with the best binding affinity was selected with D-lactate as a selection target and had a K_D of 0.23 mM with L-lactate. Some of our speculation on why this was the case includes the lower concentration used in the selection process, the selective pressure that comes with a more challenging binding target, or a combination of both factors.

In chapter 3, we obtained the tryptophan-binding aptamers from a selection using 1 mM racemic mixture of tryptophan and another selection using 1 mM solution of pure D-tryptophan. Between these two selections, the aptamers produced by the latter yielded the best aptamers with a K_D of 11 μ M. We were surprised to discover that this aptamer also had a 7-fold selectivity towards D-tryptophan, the “non-natural” isomer. Past studies have also shown that DNA aptamers with high enantioselectivity for L-tryptophan can also be obtained, which lead us to conclude that DNA is not restricted to a particular enantiomer for tryptophan. It is possible that the larger size of the tryptophan structure may provide more binding motifs that allows DNA to overcome the binding barriers of chiral preference.

Regarding the discrepancy observed between the selection of these two biomolecules, further selections need to be explored of molecules of varying structure. Building on our conclusion that the DNA's affinity for lactate was affected by the isomer that was present, the types of interactions that affect the binding between DNA and ligand should be investigated. One aspect to focus on is how size can affects the chiral bias that DNA may have. Future studies should target other essential biomolecules with similar size to lactate. One such biomolecule is alanine, an amino acid that shares the same three carbon structure of lactate. Additionally, larger chiral molecules can also be investigated to gauge how large a molecule must be to provide some influencing factor on DNA's inherent enantioselectivity. Beside investigating varying sizes of molecules, future selections should also focus on how different binding features can influence the enantioselectivity of DNA. Molecules like thiolactic acid, which closely resembles lactic acid but interchanges a hydroxyl group with a thiol group, would be an interesting case for

examining whether the chiral preference of DNA would be influenced in this single change of functional group.

References

- [1] J. Gal, *Helv Chim Acta* **2013**, 96, 1617–1657.
- [2] L. D. Barron, *Strategies of Life Detection* **2008**, 187–201.
- [3] D. G. Morris, Royal Society of Chemistry (Great Britain), **2001**, 170.
- [4] D. G. Blackmond, *Philosophical Transactions of the Royal Society B: Biological Sciences* **2011**, 366, 2878.
- [5] D. P. Glavin, A. S. Burton, J. E. Elsila, J. C. Aponte, J. C. Aponte, J. P. Dworkin, *Chem Rev* **2020**, 120, 4660–4689.
- [6] “Biological Homochirality: One of Life’s Greatest Mysteries | by Sebastian Wellford | Cell Your Soul | Medium,” can be found under <https://medium.com/a-spoonful-of-sugar/biological-homochirality-one-of-lifes-greatest-mysteries-2031f4700c4b>, **n.d.**
- [7] Y. Yi, L. Liu, Y. Wu, G. Zhu, *Analytical Sciences* **2021**, 37, 275–281.
- [8] F. Applications, J.-J. Dai, G.-Y. Chen, L. Xu, H. Zhu, F.-Q. Yang, *Molecules* **2024**, Vol. 29, Page 3376 **2024**, 29, 3376.
- [9] D. Winogradoff, P. Y. Li, H. Joshi, L. Quednau, C. Maffeo, A. Aksimentiev, *Advanced Science* **2021**, 8, DOI 10.1002/ADVS.202003113.
- [10] N. Hermien, T. Wiyatini, *Kesehatan Masyarakat* **2021**, 252.
- [11] P. Cintas, Ed. , **2013**, 333, DOI 10.1007/978-3-642-37626-9.
- [12] S. Nikfar, A. F. Behboudi, *Encyclopedia of Toxicology: Third Edition* **2014**, 78–82.
- [13] “(S)-(+)-Carvone - American Chemical Society,” can be found under <https://www.acs.org/molecule-of-the-week/archive/c/s-carvone.html>, **n.d.**

- [14] D. Damiañ, D. Pergentino, D. E. Sousa, F. Ferreira De Farias No'breaga, N. No'breaga, R. No'breaga, D. E. Almeida, *Chirality* **2007**, *19*, 264–268.
- [15] P. Maho, C. Herrier, T. Livache, G. Rolland, P. Comon, S. Barthelmé, *Biosens Bioelectron* **2020**, *159*, 112183.
- [16] M. Pohanka, *Biomed Res Int* **2020**, *2020*, DOI 10.1155/2020/3419034.
- [17] J. Ceramella, D. Iacopetta, A. Franchini, M. De Luca, C. Saturnino, I. Andreu, M. S. Sinicropi, A. Catalano, *Applied Sciences* **2022**, *Vol. 12*, Page 10909 **2022**, *12*, 10909.
- [18] I. Ali, V. K. Gupta, H. Y. Aboul-Enein, P. Singh, B. Sharma, *Chirality* **2007**, *19*, 453–463.
- [19] R. U. McVicker, N. M. O'Boyle, *J Med Chem* **2024**, *67*, 2305–2320.
- [20] M. E. Franks, G. R. Macpherson, W. D. Figg, *Lancet* **2004**, *363*, 1802–1811.
- [21] “Thalidomide - American Chemical Society,” can be found under <https://www.acs.org/molecule-of-the-week/archive/t/thalidomide.html>, **n.d.**
- [22] N. Vargesson, T. Stephens, *Expert Opin Drug Saf* **2021**, *20*, 1455–1457.
- [23] A. Ballard, S. Narduolo, H. O. Ahmad, D. A. Cosgrove, A. G. Leach, N. J. Buurma, *Expert Opin Drug Discov* **2019**, *14*, 527–539.
- [24] B. S. Sekhon, *J Pestic Sci* **2009**, *34*, 1–12.
- [25] A. W. Garrison, *ACS Symposium Series* **2011**, *1085*, 1–7.
- [26] D. Mangelings, Y. Vander Heyden, *J Sep Sci* **2008**, *31*, 1252–1273.
- [27] G. Gübitz, M. G. Schmid, *J Chromatogr A* **2008**, *1204*, 140–156.
- [28] Q. Cheng, Q. Ma, H. Pei, Z. Mo, *Sep Purif Technol* **2022**, *292*, 121034.
- [29] J. Teixeira, M. E. Tiritan, M. M. M. Pinto, C. Fernandes, *Molecules* **2019**, *24*, DOI 10.3390/MOLECULES24050865.

- [30] C. Viet Bui, T. Rosenau, H. Hettegger, C. V Bui, T. Rosenau, H. Hettegger, *Cellulose* **2022** 30:2 **2022**, 30, 915–932.
- [31] B. Chankvetadze, *TrAC Trends in Analytical Chemistry* **2020**, 122, 115709.
- [32] G. K. E. Scriba, *J Chromatogr A* **2016**, 1467, 56–78.
- [33] A. R. Ribeiro, P. M. L. Castro, M. E. Tiritan, *Environmental Chemistry Letters* **2012** 10:3 **2012**, 10, 239–253.
- [34] T. J. Ward, A. B. Farris, *J Chromatogr A* **2001**, 906, 73–89.
- [35] J. Haginaka, *J Chromatogr A* **2001**, 906, 253–273.
- [36] H. Hofstetter, O. Hofstetter, *TrAC Trends in Analytical Chemistry* **2005**, 24, 869–879.
- [37] V. Thivyanathan, D. G. Gorenstein, *Proteomics Clin Appl* **2012**, 6, 563.
- [38] V. E. Bochenkov, T. I. Shabatina, V. E. Bochenkov, T. I. Shabatina, *Smart Nanosystems for Biomedicine, Optoelectronics and Catalysis* **2020**, DOI 10.5772/INTECHOPEN.93661.
- [39] S. J. Yoo, Q. H. Park, *Nanophotonics* **2019**, 8, 249–261.
- [40] J. Ruta, C. Ravelet, I. Baussanne, J. L. Décout, E. Peyrin, *Anal Chem* **2007**, 79, 4716–4719.
- [41] X. Tao, X. Wang, B. Liu, J. Liu, *Biosens Bioelectron* **2020**, 168, 112537.
- [42] K. S. Park, *Biosens Bioelectron* **2018**, 102, 179.
- [43] C. Tuerk, L. Gold, *Science (1979)* **1990**, 249, 505–510.
- [44] H. Yu, O. Alkhamis, J. Canoura, Y. Liu, Y. Xiao, *Angew Chem Int Ed Engl* **2021**, 60, 16800.
- [45] R. Nezlin, *Immunol Lett* **2014**, 162, 252–255.
- [46] S. Cai, J. Yan, H. Xiong, Y. Liu, D. Peng, Z. Liu, *Analyst* **2018**, 143, 5317–5338.
- [47] M. Michaud, E. Jourdan, C. Ravelet, A. Villet, A. Ravel, C. Grosset, E. Peyrin, *Anal Chem* **2004**, 76, 1015–1020.

- [48] P. J. J. Huang, J. Liu, *Angewandte Chemie International Edition* **2023**, 62, e202212879.
- [49] E. Peyrin, *Chiral Recognition in Separation Methods: Mechanisms and Applications* **2010**, 275–288.
- [50] A. Shoji, M. Kuwahara, H. Ozaki, H. Sawai, *J Am Chem Soc* **2007**, 129, 1456–1464.
- [51] P. J. J. Huang, J. Liu, *Angewandte Chemie International Edition* **2023**, 62, e202212879.
- [52] A. Geiger, P. Burgstaller, H. Von der Eltz, A. Roeder, M. Famulok, *Nucleic Acids Res* **1996**, 24, 1029.
- [53] M. Michaud, E. Jourdan, A. Villet, A. Ravel, C. Grosset, E. Peyrin, *J Am Chem Soc* **2003**, 125, 8672–8679.
- [54] J. Ruta, C. Ravelet, C. Grosset, J. Fize, A. Ravel, A. Villet, E. Peyrin, *Anal Chem* **2006**, 78, 3032–3039.
- [55] E. Węgrzyn, I. Mejdrová, F. M. Müller, M. Nainytė, L. Escobar, T. Carell, *Angewandte Chemie - International Edition* **2024**, DOI 10.1002/ANIE.202319235.
- [56] A. Vater, S. Klusmann, *Drug Discov Today* **2015**, 20, 147–155.
- [57] C. Ravelet, E. Peyrin, *J Sep Sci* **2006**, 29, 1322–1331.
- [58] T. A. Feagin, D. P. V. Olsen, Z. C. Headman, J. M. Heemstra, *J Am Chem Soc* **2015**, 137, 4198–4206.
- [59] Y. Ding, J. Liu, *J Am Chem Soc* **2023**, 145, 7540–7547.
- [60] Y. Liu, J. Liu, *Analysis & Sensing* **2022**, 2, e202200010.
- [61] R. Nutiu, Y. Li, *J Am Chem Soc* **2003**, 125, 4771–4778.
- [62] W. Zhou, J. Ding, J. Liu, *Org Biomol Chem* **2017**, 15, 6959–6966.
- [63] W. Zhou, Z. Yu, G. Ma, T. Jin, Y. Li, L. Fan, X. Li, *Analyst* **2019**, 144, 2284–2290.

- [64] F. Yeasmin Khusbu, X. Zhou, H. Chen, C. Ma, K. Wang, *TrAC Trends in Analytical Chemistry* **2018**, *109*, 1–18.
- [65] Y. Zhao, A. Z. Li, J. Liu, *Environment & Health* **2023**, *1*, 102–109.
- [66] C. K. Chou, Y. T. Lee, S. M. Chen, C. W. Hsieh, T. C. Huang, Y. C. Li, J. A. Lee, *J Pharm Biomed Anal* **2015**, *116*, 65–70.
- [67] M. Pohanka, *Biomed Res Int* **2020**, *2020*, DOI 10.1155/2020/3419034.
- [68] J. D. Rabinowitz, S. Enerbäck, *Nature Metabolism* **2020**, *2*, 566–571.
- [69] C. W. Lee, *Fibers and Polymers* **2007**, *8*, 571–578.
- [70] Y. Zhang, P. V. Vadlani, *Bioprocess Biosyst Eng* **2013**, *36*, 1897–1904.
- [71] D. Choi, J. Kim, S. Ha, K. Kwon, E. H. Kim, H. Y. Lee, K. S. Ryu, C. Park, *FEBS J* **2014**, *281*, 5447–5462.
- [72] H. Hove, P. B. Mortensen, *Dig Dis Sci* **1995**, *40*, 320–330.
- [73] M. Pohanka, *Biomed Res Int* **2020**, *2020*, DOI 10.1155/2020/3419034.
- [74] C. S. Pundir, V. Narwal, B. Batra, *Biosens Bioelectron* **2016**, *86*, 777–790.
- [75] B. S. Ferguson, M. J. Rogatzki, M. L. Goodwin, D. A. Kane, Z. Rightmire, L. B. Gladden, *European Journal of Applied Physiology* **2018**, *118*, 691–728.
- [76] Y. Ding, J. Liu, *Anal Chem* **2023**, *95*, 14651–14658.
- [77] R. D. Jenison, S. C. Gill, A. Pardi, B. Polisky, *Science (1979)* **1994**, *263*, 1425–1429.
- [78] M. Zuker, *Nucleic Acids Res* **2003**, *31*, 3406–3415.
- [79] J. Hu, C. J. Easley, *Analyst* **2011**, *136*, 3461–3468.
- [80] A. Lozoya-Colinas, Y. Yu, J. C. Chaput, *J Am Chem Soc* **2023**, *145*, 25789–25796.

- [81] J. M. Gostner, S. Geisler, M. Stonig, L. Mair, B. Sperner-Unterweger, D. Fuchs, *Neuropsychobiology* **2020**, *79*, 89–99.
- [82] I. Davis, A. Liu, *Expert Rev Neurother* **2015**, *15*, 719.
- [83] A. Tsuji, Y. Ikeda, S. Yoshikawa, K. Taniguchi, H. Sawamura, S. Morikawa, M. Nakashima, T. Asai, S. Matsuda, *Int J Mol Sci* **2023**, *24*, DOI 10.3390/IJMS24065742.
- [84] J. J. A. J. Bastings, H. M. van Eijk, S. W. O. Damink, S. S. Rensen, *Nutrients* **2019**, *11*, DOI 10.3390/NU11092205.
- [85] K. Yang, N. M. Mitchell, S. Banerjee, Z. Cheng, S. Taylor, A. M. Kostic, I. Wong, S. Sajjath, Y. Zhang, J. Stevens, S. Mohan, D. W. Landry, T. S. Worgall, A. M. Andrews, M. N. Stojanovic, *Science (1979)* **2023**, *380*, 942–948.