Selection and characterization of DNA aptamers for therapeutic drugs: voriconazole and carbamazepine

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 author and several scientific collaborations. Contributions from each scientist and the resulting publications are listed in detail below.

Chapter 1, parts of the literature review presented will be published in a review paper as: Zhang, X.; Kamel, A.; Liu, J. "Recent advancements in aptamer selection for monitoring therapeutic drugs." All drug related information was collected by the author.

Chapter 2, most of the thioflavin-T titrations assays were performed by an undergraduate student, Hongwei Sun, under the supervision of the author. All SELEX, data analysis and other work was conducted by the author.

Chapter 3 will be published as: Kamel, A.; Sun, H.; Liu, J. Binding of voriconazole aptamers and modulation of binding by cyclodextrins, *Nanotechnology*, in press. Some of the main thioflavin-T titrations were performed by the author and the rest were performed by Hongwei Sun. All data analysis and other work were completed by the author.

Abstract

Therapeutic drug monitoring (TDM) is currently a time-consuming and repetitive process of monitoring a drug's concentration in the blood to ensure an effective level is achieved and avoid potentially dangerous sub-therapeutic or toxic levels. To date, this process relies on centralized medical laboratories equipped with expensive instrumentation and highly trained personnel. Aptamers, used as target binding for biosensors, are proposed as a solution for fast and cheap point-of-care-testing of the drugs that require TDM. However, only a few aptamers are available in the literature for such drugs, and not all the reported aptamers are well characterized and validated. My thesis aims to fill in this gap by performing aptamer selection for carbamazepine and characterizing the binding of aptamers for voriconazole.

In this thesis, I first used the capture-SELEX method to select new aptamers for the antiepileptic drug carbamazepine (CBZ) as explained in chapter 2. After 17 rounds of selection and deep sequencing of the final library pool, I collected the common motifs from the top 61 sequences and grouped them into a structurally related group. Then, three potential aptamer candidates were selected for further binding characterization using Thioflavin-T fluorescence spectroscopy and isothermal titration calorimetry. Unfortunately, both deep sequencing results analysis and binding assays confirmed that capture-SELEX failed to enrich high-affinity aptamers specific to this target. CBZ seems to be one of the challenging small-molecule targets because of its non-planarity and the steric crowding around its only functional group. The DNA sequences could not form strong non-covalent bonds with CBZ under the used conditions and current capture-SELEX protocol employed in this work.

In chapter 3, we used Thioflavin-T fluorescence spectroscopy to characterize some aptamers previously selected by our group for another challenging small-molecule target: the antifungal drug voriconazole. A new aptamer was identified called VO3 with a K_D value of 26 μM, high selectivity against similarly structured small molecules, and good stability in different buffer conditions and complex matrices such as serum. In addition, we used this aptamer to probe the nanopolymers cyclodextrins and learn more about the different interactions of α- and β-cyclodextrins (CD) with VOZ. As expected α-CD did not interact with VOZ, but β-CD seemed to have a greater affinity for VOZ than its affinity with the aptamer VO3. We could not use another orthogonal binding assay methods such as isothermal titration calorimetry or circular dichroism because of constraints related to VOZ's poor water solubility. Nevertheless, I believe VO3 is a good candidate for further validation and development assays.

Finally, in chapter 4, the conclusion and some suggested future directions were included. Through this thesis, we explore some of the challenges and setbacks for developing aptasensors, specially for the purpose of point-of-care-testing for drug requiring TDM. Since all these drugs fall under the category of small-molecule targets, selecting specific high-affinity aptamers for most of them is still a challenge. Using more advanced SELEX methods, modified DNA sequences or a specially structured library motif can help facilitate selection for aptamers of challenging targets such as CBZ. Even after selecting the aptamers, rigorous characterization assays should be implemented to confirm and validate the binding affinity of the aptamer and its suitability for the final application. For VO3 for example, we recommend confirming its binding with further NMR or FRET assays before further development into electrochemical platforms.

Acknowledgements

I am deeply grateful to my supervisor, Dr. Juewen Liu, whose guidance, patience, and steady encouragement have been invaluable throughout the course of this research. His thoughtful insights, rigorous standards, and consistent support shaped both the development of this thesis and my growth as a researcher. I am sincerely thankful for the opportunity to learn under his supervision and for the generosity with which he shared his expertise.

I would also like to thank my lab partners especially Dr. Jin Wang, Dr. Yibo Liu, Dr. Kaya Wong, Dr. Sihan Wang, Dr. Yuzhe Din, Dr. Xiaohan Zhang, Stefen Stangherlin and Yachen Xie for helping me and answering all my questions; my advisory committee members Dr. Subha Kalyaanamoorthy and Dr. Mahla Poudineh for taking the time to read and evaluate my thesis; my sponsors Fluid AI and NSERC for their financial support; last but not least my family especially my husband, my father and my mother for always encouraging me and believing in me.

Dedication

I dedicate this thesis to my daughter and sons: Khadiga Ahmed, Ismail Ahmed and Sulaiman Ahmed. May you always follow your passions with determination and kindness.

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List of Abbreviations

CBZ	Carbamazepine
CD	Cyclodextrin
DMSO	Dimethyl sulfoxide
EMIT	Enzyme-Multiplied Immunoassay Technique
FOZ	Fluconazole
FPIA	Fluorescence Polarization Immunoassay
FRET	Fluorescence resonance energy transfer
HEPES	hydroxyethylpiperazine-N′-2-ethanesulfonic acid
HMAIA	Homogenous Microparticle Agglutination Immunoassay
HPLC	High Performance Liquid Chromatography
KIMS	Kinetic Interaction of Microparticles in a Solution
MB	Methylene blue
NMR	Nuclear magnetic resonance
POCT	Point of Care Testing
SELEX	Systematic Evolution of Ligands by Exponential enrichment
TDM	Therapeutic Drug Monitoring
ThT	Thioflavin T
VOZ	Voriconazole

Chapter 1 Introduction

1.1 Therapeutic drug monitoring

Therapeutic drug monitoring (TDM) is the process of measuring the drug's concentration in the blood for the purpose of avoiding dangerous sub-optimal or toxic drug levels. [1–6] Not all drugs need TDM, it is required only when the drug has a narrow-therapeutic-index, high inter- or intra- patient variability, serious side effects that should be avoided or when monitoring patient compliance is a necessity. These drugs can be grouped into 2 main groups; the first group includes drugs that treat chronic diseases such as epilepsy, heart diseases, psychotic disorders and chronic obstructive pulmonary disease.[7] The second group contains drugs that have severe permanent or life-threatening side effects even if taken for a short period of time such as certain members of antibiotics, antifungals, antineoplastics, and immunosuppressants.

1.1.1 Chronic-use drugs

A major example of the chronically used drugs are the anti-epileptic drugs. They are among the most monitored drugs, especially the first generation that includes phenytoin, phenobarbital, ethosuximide, primidone, carbamazepine and valproic acid.[8] Mainly because seizures occur in irregular intervals and antiepileptic drugs are prescribed for prophylaxis; by consequence, it is difficult to assess the treatment plan only by clinical symptoms observation. Moreover, both suboptimal and toxic drug concentrations can lead to increased seizure episodes thus in case of worsening symptoms, the physician's decision for dose adjustment would be challenging without knowing the current blood concentration of the antiepileptic drug and comparing it to the patient's effective drug concentration range.

Cardioactive drugs especially antiarrhythmic drugs are another example of heavily monitored group of drugs because both under- and over- dosing can cause the same symptom,

life threatening arrhythmia. [9]Antiarrhythmics are classified into 4 classes, and the need for drug monitoring varies along these classes. Class I drugs that includes procainamide, quinidine, lidocaine, mexiletine, flecainide and propafenone, though not commonly prescribed nowadays, are always monitored to protect the patient from their severe toxic effects. Amiodarone from Class III is also regularly monitored to assess patient compliance and avoid pulmonary toxicity.

From the antipsychotic drugs group, antidepressants including amitriptyline, nortriptyline, clomipramine, desipramine, doxepin, fluoxetine, impramine, and trimipramine are routinely monitored. Monitoring the antidepressant drug concentrations in patients' blood give the physicians objective data to be able to derive their clinical decisions on instead of relying only on subjective assessment since psychotic symptoms cannot be measured by any biological indicators like blood pressure.[10] These clear drug concentration measurements help guide dose tapering at the beginning of the treatment, assess patient compliance and avoid serious adverse effects of antipsychotic drugs.

Theophylline is an example of bronchodilators where monitoring was found to be beneficial for elderly patients.[11] Theophylline intoxication can easily occur in patients over 80 years old with non-specific symptoms like nausea, vomiting and arrythmia. The risk is high in older patients because of their age-related decrease in hepatic blood flow and decreased activity of the enzyme P450 causing lower drug clearance, thus higher accumulation. Elderly patients usually consume several concurrent medications to treat comorbidities, any drug that affects the enzyme P450 activity will affect theophylline's concentration in the blood and consequently its therapeutic or toxic effects. Moreover, female patients with 30% less body weight suffer from higher risks if receiving standard doses. Thus, age, sex and drug-drug interactions are all factors to be considered for theophylline dose adjustment.

1.1.2 Hospital-use drugs

An example where drug monitoring is employed for short-term used drugs is monitoring antibiotics treatment in hospitals for severe bacterial infections. Large doses of antimicrobial agents are given to critically ill patients in ICUs to prevent sepsis and death from septic shock. Since the pharmacokinetics of any drug is relatively different in critically ill patients compared to normal patients, monitoring the drug concentrations can help the healthcare professionals maintain an effective drug level sufficient to clear the infection, but not high to the extent of causing serious side effects.[12] For example, vancomycin and aminoglycosides like amikacin, gentamycin and tobramycin are monitored, typically in laboratories inside of the hospitals, to reach therapeutic doses quickly while avoiding their severe ototoxic and nephrotoxic side effects.[13]

Monitoring antifungal therapy during invasive fungal infections is an additional example where monitoring antimicrobial agents in severely ill patients is beneficial, especially when one of the triazole antifungal drugs itraconazole, posaconazole or voriconazole is used.[14] Patients at risk of opportunistic systematic fungal infections are immunocompromised patients. Immune dysfunction can occur as a result of different factors such as receiving chemotherapy, chronic administration of corticosteroids, uncontrolled diabetes, bone marrow transplant, solid organ transplant and severe illness. The antifungal drugs suffer from unpredictable pharmacokinetics in these patients, by consequence drug monitoring is proven to be advantageous.

It is empirical that anticancer drugs necessitate monitoring since sub-therapeutic doses leave the patients at risk of worsening cancer prognosis and overdoses cause severe cytotoxic effects that can be manifested in cardiotoxicity, myelosuppression, hepatic cirrhosis or gastrointestinal mucositosis.[15] Nonetheless, only a few cytotoxic drugs such as methotrexate, busulfan and 5-fluorouracil are routinely monitored by measuring their concentration in the

patients' blood.[16] Primarily because the lack of availability of useful assays for many of the antineoplastics, the therapeutic concentration ranges were not well established or the relationship between the blood concentration and clinical efficacy was not identified.[17]

Another group of drugs that require monitoring in hospitals are immunosuppressants (e.g. cyclosporin, everolimus, sirolimus, tacrolimus and mycophenolic acid) used after solid organ transplantation.[18] Keeping the immunosuppressive drug concentration in the narrow effective therapeutic range after an organ transplant is crucial because sub-dosing results in organ rejection and over-dosing can cause serious infections, neurotoxicity, nephrotoxicity and cardiotoxicity. Since all these life-threatening conditions can develop in a few hours and may result in death, having a rapid insight of the drug's level for each individual patient can help save lives, reduce hospital stay, avoid the psychological, financial and health consequences of organ rejection or serious medication adverse effects.

1.2 Current analytical methods

TDM is currently an iterative process where a blood sample is drawn by a nurse and sent to a specialized medical laboratory. In the laboratory, trained laboratory technicians prepare the sample for analysis, perform the suitable assay, write a report about the results and send them to the prescribing physician. Upon receiving the results, the physician can use the data along with other clinical symptoms and patient related history to make informed clinical decisions. This process is usually expensive, time-consuming, requires special laboratory equipment and training for professional personnel. By consequence, the benefits of TDM for a certain drug must outweigh these drawbacks for application in real life situations. [19] There is no doubt that TDM for narrow therapeutic index drugs has been repeatedly proven beneficial specially when the relationship between the drug concentration in the blood and the clinical

outcomes is clear and when inter- and intra- patient pharmacokinetic variability of the drug is a problem.

One of the main limitations of TDM is the analytical methods used for drug-level detection.[20] To date, most tests employ methods involving chromatography like LC-MS/MS (as seen in Table 1.1) because they are accurate and precise.[21] However, these tests can only be performed in special laboratories that have the required expensive and bulky instruments and trained medical laboratorians. For point-of-care testing, immunoassays are the better choice because they are less expensive, less time-consuming and easier to perform. Nevertheless, they suffer from cross reactivity, matrix effect and less availability.[22]

Table 1.1 Some important therapeutic drugs with a narrow therapeutic window and current analytical methods.

Drug name	**MW (g/mol)**	**Therapeutic Window (mcg/mL)**	**Current Analytical Method**	**Turnaround (days)**
Anticonvulsants				
Brivaracetam	212.29	0.2-2.0	LC-MS/MS	1 to 2
Carbamazepine	236.27	4.0 - 12.0	HMAIA	1
Clobazam	300.74	30-300 ng/mL	LC-MS/MS	1 to 5
Clonazepam	315.71	29-75 ng/mL	LC-MS/MS	7 to 11
Felbamate	238.24	30-80	LC-MS/MS	1 to 3
Gabapentin	171.24	2.0-20.0	LC-MS/MS	1 to 2
Lamotrigine	256.09	3.0-15.0	LC-MS/MS	1 to 2
Levetiracetam	170.21	10-40	LC-MS/MS	1 to 2
Oxcarbazepine	252.27	10-35	HTLC-MS/MS	1 to 3
Perampanel	349.34	180-980 ng/mL	LC-MS/MS	2 to 5
Phenobarbital	232.23	20.0-40.0	KIMS	1
Phenytoin	252.27	10.0-20.0[a]	KIMS	1
Pregabalin	159.23	2.0 - 5.0	LC-MS/MS	2 to 7
Primidone	218.25	5.0-12.0	Immunoassay	1
Rufinamide	238.19	5.0-30	LC-MS/MS	3 to 5
Topiramate	339.36	5.0-20.0	LC-MS/MS	1 to 4

Trimethoprim	290.32	>2.0	LC-MS/MS	2 to 5
Valproic acid	144.21	50-125[b]	Immunoassay	1 to 2
Antidepressants				
Amitriptyline	277.4	80-200 ng/mL	LC-MS/MS	2 to 5
Nortriptyline	263.37	70-170 ng/mL	LC-MS/MS	3 to 5
Clomipramine	314.85	230-450 ng/mL	LC-MS/MS	3 to 5
Desipramine	266.38	100-300 ng/mL	LC-MS/MS	3 to 5
Doxepin	279.4	50-150 ng/mL[c]	LC-MS/MS	3 to 5
Duloxetine	297.4	30-120 ng/mL	LC-MS/MS	1 to 8
Fluoxetine	309.33	120-500 ng/mL[d]	LC-MS/MS	1 to 8
Impramine	280.41	175-300 ng/mL[e]	LC-MS/MS	3 to 5
Sertraline	306.23	30-200 ng/mL	LC-MS/MS	3 to 7
Trimipramine	294.43	150-300 ng/mL	LC-MS/MS	3 to 5
Antipsychotics				
Lithium	73.89	0.5-1.2 mmol/L	Colorimetric	1
Clozapine	326.82	350-600 ng/mL	LC-Ms/MS	3 to 5
Antiarrhythmics				
Amiodarone	645.3	0.5-2.0	LC-MS/MS	2 to 5
Flecainide	414.34	0.2-1.0	LC-MS/MS	2 to 5
Lidocaine	234.34	1.5-5.0	EMIT	1 to 2
Mexiletine	179.26	0.5-2.0	LC-MS/MS	2 to 5
N-acetylprocainamide	277.36	12.0-18.0	Homogeneous Enzyme Immunoassay	1 to 2
Porpafenone	341.44	0.5-2.0	LC-MS/MS	2 to 5
Procainamide	235.33	4.0-10.0	Homogeneous Enzyme Immunoassay	1 to 2
Cardiac drugs				
Digitoxin	764.9	10-30 ng/mL	Immunoassay	9 to 11
Digoxin	780.9	0.6-1.2 ng/mL	Electrochemilumineschent Immunoassay	1 to 2
Antibiotics[f]				
Amikacin	585.6	<8.0	KIMS	1
Gentamicin	447.6 / 1390.7	<2.0	Turbidimetric Immunoassay	1
Sulfamethoxazole	270.3	>50	LC-MS/MS	2 to 5
Tobramycin	467.5	<2.0	Immunoassay	1 to 2
Vancomycin	1449.25	10.0-20.0	Immunoassay	1 to 2
5-Flucytosine	129.09	>25.0	LC-MS/MS	3 to 8

Antifungals				
Itraconazole	705.6	> 1	LC-MS/MS	1 to 3
Posaconazole	700.8	>700 ng/mL	LC-MS/MS	2 to 5
Voriconazole	349.31	1.0-5.5	LC-MS/MS	1 to 2
Antineoplastic Agents				
Busulfan	246.3	AUC 900-1500 mcmol/L	LC-MS/MS	1 to 3
Methotrexate	454.4	<0.1 mcmol/L	Competitive Homogenous Immunoassay or EIA	1
Mitotane	320	14-20	GC-MS	2 to 7
Bronchodilators				
Caffeine	194.19	8.0-20.0	EMIT	1 to 2
Theophylline	180.16	8.0-20.0	KIMS	1 to 2
Immunosuppressants				
Cyclosporin	1202.63	100-400 ng/mL	LC-MS/MS	1
Everolimus	958.24	3-8 ng/mL	LC-MS/MS	1 to 2
Hydroxychloroquine	335.87	>500 ng/mL	LC-MS/MS	3 to 7
Mycophenolic Acid	320.3	1.0-3.5	LC-MS/MS	1 to 3
Sirolimus	914.2	4-20 ng/mL	LC-MS/MS	1 to 3
Tacrolimus	804.02	5.0-15.0 ng/mL	LC-MS/MS	1 to 3
Antirheumatics				
Leflunomide	270.21	>40	HTLC-MS/MS	3 to 5

Note: The molecular weights for each drug were retrieved from PubChem[15] database and cross checked with Drug Bank[16] database. The therapeutic ranges, analytical methods and turnaround times were obtained from Mayo Clinic laboratories[17] test catalog. KIMS: Kinetic Interaction of Microparticles in a Solution; EMIT: Enzyme-Multiplied Immunoassay Technique; HMAIA: Homogeneous Microparticle Agglutination immunoassay; a. When taking daily 6 mg oral dose; b.Total serum; c. doxepin + nordoxepin; d. fluoxetine + norfluoxetine; e. impramine + desipramine; f. Trough samples except 5-flucytosine peak samples.

1.3 Aptasensors as a possible alternative

Aptamers are short chains of oligonucleotides that undergo conformational changes and bind to a specific target just like an antibody binding to its target.[23–27] Unlike antibodies, aptamers are simple and cheap to produce, easy to modify, stable on the shelves, have less batch-to-batch variability and less immunogenicity problems. These excellent properties along with the wide range of possible targets that includes small molecules and span to whole-cells

or body organs, gave rise to various applications such as therapeutics,[28–33] drug delivery,[34,35] diagnostics[28,30,32,36–39] and biosensors that can be used for environmental monitoring,[33,40,41] wearable health monitoring,[42–44] and point-of-care testing (POCT).[45,46]

Biosensors that employ aptamers as their target recognition elements are known as aptasensors. A wide range of aptasensor mechanisms has been extensively investigated in the literature over the past three decades. Focusing on small-molecule applications, aptasensors were developed for sensing (1) heavy metal ions like lead, cadmium, and mercury; (2) mycotoxins like aflatoxins, ochratoxins, fumonisin, and zearalenone; (3) pesticide residue like organophosphorus pesticides, neonicotinoid insecticides, carbamates, and pyrethroids; (4) antibiotics like macrolides, aminoglycosides, quinolones, tetracyclines, sulfonamides, and lincosamides; and (5) disease related biomarkers like vitamins, neurotransmitters, ATP, and mineral elements. [47]

All aptamers are isolated using systematic evolution of ligands by exponential enrichment (SELEX). [48–51] This iterative process has evolved over time and is generally classified into 2 categories: homogenous SELEX and heterogenous SELEX.[25] Heterogenous SELEX involves the immobilization of either the target of interest or the library of oligonucleotides onto a solid substrate for partitioning, while in homogenous SELEX both the target and the library are free flowing in the matrix. Homogenous SELEX techniques have a higher separation efficiency for protein targets, but they are less applicable to small-molecule targets. The separation is difficult because the mobility of small-molecule target-aptamer complexes is very similar to that of unbound nucleotides. Selecting aptamers that bind to small-molecule targets with high affinity and specificity is particularly challenging because a small molecule has a small surface area with less functional groups to interact with the aptamer or to be linked with the beads for separation. Early work focused on immobilizing the small-molecule target by covalently attach it to a solid support like beads, a few examples of

successful selections using this technique are aptamer selected for ATP, theophylline, ethanol amine and arsenite (Figure 1.1A-D).[38] Nonetheless, target-immobilized SELEX is not ideal for small-molecule targets, as attaching such molecules to solid supports is difficult and can result in aptamers that bind the free target with lower affinity.[52,53] That's why library-immobilized SELEX, also called capture-SELEX, is the separation method of choice for small-molecule targets and it is the technique that was employed in this project (Figure 1.1E). Moreover, capture-SELEX is ideal for biosensor applications because it enriches aptamers that change structure upon target binding, which is exactly what biosensors need to generate a measurable signal.

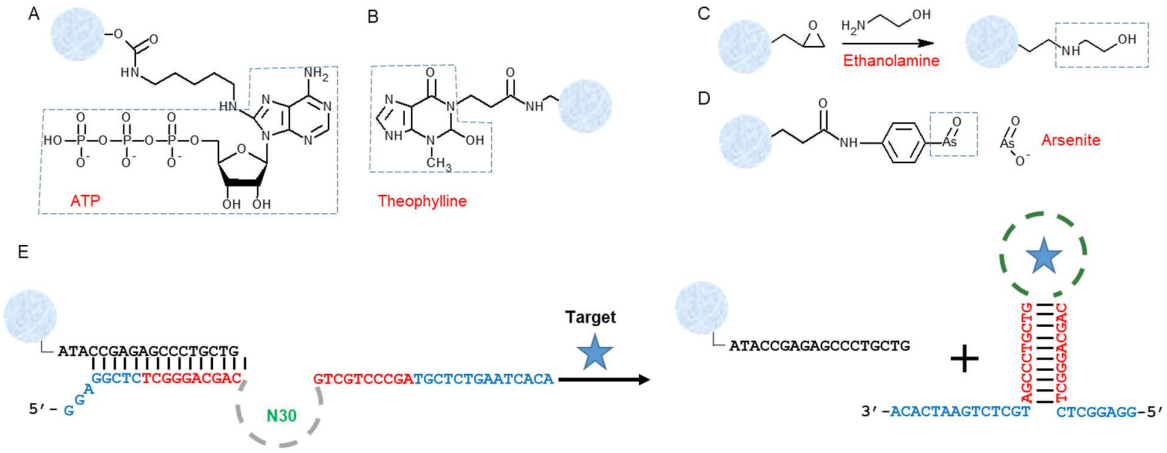


Figure 1.1 Different small-molecule targets vs DNA library attached to solid support.

(A) ATP covalently linked to beads; (B) theophylline attached to a solid support; (C) ethanolamine covalently linked to the solid phase and (D) arsenite attached to a solid support and a comparison to the structures of free target molecules. (E) Capture-SELEX method where DNA library is attached to the solid support and the target is unmodified and free flowing. *Figure reproduced from reference.[38] Open access.*

1.3.1 Electrochemical aptasensors

Similar to glucose meters, which detect blood glucose levels using electrochemical detection, aptamers can be used to fabricate an aptasensor. Electrochemical aptasensors do not require a light source, high-voltage power supplies or other sophisticated equipment.[37]

Therefore, they are suited for POCT applications, especially for wearable or in-vivo biosensors. The sensor is usually constructed by modifying a methylene blue (MB) label on the aptamer attached to a gold electrode through a thiol group. Without the target present, the aptamer chain is flexible making the MB label close to the electrode surface leading to high electron-transfer efficiency. When the target is added, the aptamer folds into its inherent structure, thus inhibiting electron-transfer and decreasing the current. As a result, the current difference is measured and related to the target concentrations.

The first electrochemical aptamer-based (E-AB) biosensor was reported by Xiao et al. for the detection of thrombin.[54] The design is shown in Figure 1.2. The thrombin aptamer was labeled with MB dye, and it was based on a signal-off mechanism. This sensor achieved a limit of detection of 11 nM. Although thrombin is not a therapeutic drug, this study has established the feasibility of this sensing mechanism. Other mechanisms have been suggested throughout the years following that, but this one remains the simplest and easiest platform for POCT applications.

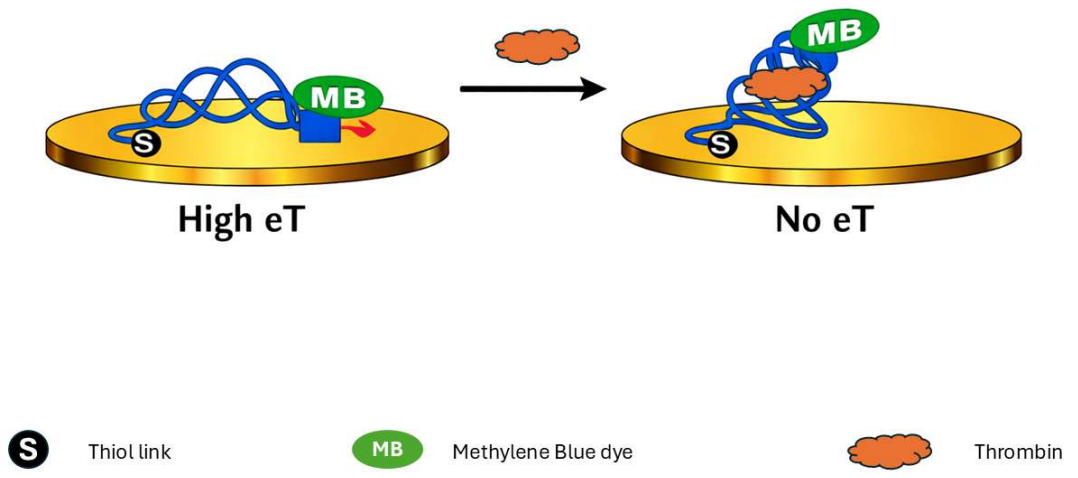


Figure 1.2 Schematic of the first electrochemical aptasensor designed to detect thrombin.

The thrombin aptamer is attached to the gold electrode by a thiol covalent bond (S) and attached to a methylene blue (MB) dye from the other side. At first the aptamer is free flowing and bending next to the

gold electrode allowing electron transfer (eT). When the thrombin protein is present, the aptamer binds to it moving the MB away from the electrode and by consequence decreasing the electron transfer. *This figure is Ai generated using the Copilot application*.

An example of E-ABs designed to detect small-molecule drug substances is the one developed by the Plaxco group for the detection of the antibiotic vancomycin (VAN).[55] The original aptamer they selected for VAN had a K_D value of 0.1 μM but was too rigid for use in biosensor applications, so they removed a few bases and tested it again in whole blood samples. The truncated more flexible aptamer (Figure 1.3a) had a K_D value of 45 μM and was able to detect VAN (Figure 1.3b) in whole blood sample within the desired therapeutic range of 6-35 μM.

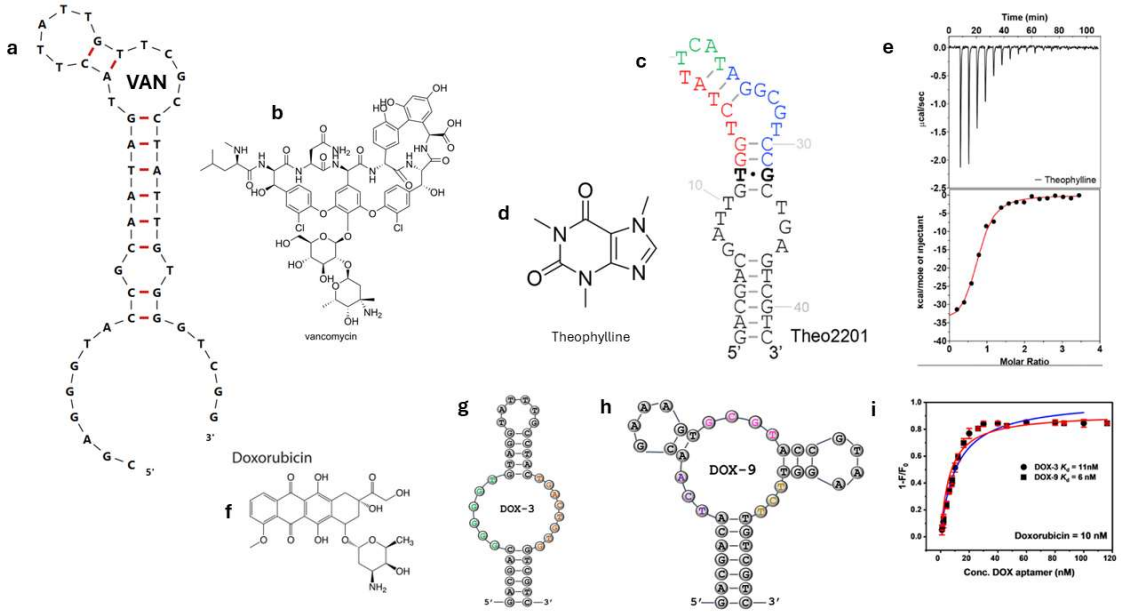


Figure 1.3 Some examples of aptamers selected for drugs that need TDM.

(a) Vancomycin aptamer redrawn with a software developed in our lab;[55] (b) vancomycin chemical structure; (c) theophylline aptamer;[56] (d) theophylline chemical structure; (e) ITC results for theophylline aptamer vs. theophylline; (f) doxorubicin chemical structure; (g) and (h) aptamers for doxorubicin;[57] (i) ThT titration assays for doxorubicin aptamers vs. doxorubicin. *(c) and (e) Reproduced with permission from ACS Chemical Biology, via RightsLink (Order No. 6313791080592). (g), (h) and (i) reproduced with permission from ACS Chemical Biology, via RightsLink (Order No. 6314071286776).*

1.4 Aptasensors for TDM

From the large list of drugs that requires TDM presented in Table 1.1, only a limited number have aptamers selected for use in aptasensor development, most of which fall within the antibiotics category. Notably, these aptasensors were initially proposed for environmental monitoring rather than clinical TDM. Several obstacles contribute to this shortcoming, including the limited availability of high-affinity aptamers for many clinically relevant drugs, challenges associated with aptamer performance in complex biological matrices, and the lack of validated biosensor platforms suitable for routine clinical use.[58]

In addition to the vancomycin example mentioned above, another example of high-affinity aptamer selected for a drug that requires TDM is a theophylline (Figure 1.3d) DNA aptamer (Figure 1.3c) selected by our group with a K_D of 510 nM determined by ITC (Figure 1.3e). This aptamer showed a significant selectivity for theophylline against its structural analogue caffeine, compared to the RNA aptamer previously reported, and is one of the few aptamers that had its 3D structure resolved.[59] Using capture-SELEX, our group selected high-affinity aptamers to the chemotherapy drug doxorubicin (Figure 1.3f) and 2 high-affinity aptamers were identified.[57] They were named DOX-3 (Figure 1.3g) and DOX-9 (Figure 2.3h) and K_D values of 11 and 6 nM respectively (Figure 1.3i). These aptamers can be used for biosensor applications as well as functional strategies such as blocking the cellular uptake of doxorubicin.

1.5 Carbamazepine and voriconazole

In this work, 2 target drugs were chosen from the list of drugs that need TDM: the antiepileptic drug carbamazepine from the chronic-use group and the antifungal drug voriconazole from the hospital-use group.

1.5.1 Carbamazepine

Carbamazepine (CBZ) is an anticonvulsant drug used to treat different types of seizures, pain from trigeminal neuralgia and some symptoms of bipolar 1. [60] It is a narrow-therapeutic-index drug with 4 to 12 mg/L therapeutic window (Table 1.1). A 20-year retrospective study found that only about 71% of CBZ's plasma samples were within the therapeutic range, highlighting frequent subtherapeutic or supratherapeutic levels.[61] The authors concluded that routine TDM is essential across all ages and sexes to optimize efficacy and safety in epilepsy treatment. TDM is currently done in medical laboratories using methods involving chromatography such as HPLC, or immunoassay techniques for more rapid results. Homogenous microparticle agglutination immunoassay is preferred for CBZ's blood level detection (Table 1.1), but it suffers from cross-reactivity with CBZ's active metabolite. Moreover, immunoassays rely on proprietary reagents and analyzers, which can be costly and subject to supply issues. An aptamer has been previously selected for CBZ to be used for POCT, but it was too long (72-mer) with an extensive duplex part as seen in Figure 1.4a.[62] These two characters are not optimal for biosensor applications.[62] A shorter, well characterized aptamer is still not selected to our knowledge. Chapter 2 discusses a capture-SELEX method used for an attempt to select a higher quality aptamer for CBZ.

1.5.2 Voriconazole

Voriconazole (VOZ) is a broad-spectrum antifungal drug used to treat invasive fungal infections that can exacerbate in immunocompromised or critically ill patients.[63] This drug also has a narrow therapeutic window of 1-5.5 mg/L (Table 1.1), and any overdose can cause serious adverse effect such as hepatotoxicity and neurotoxicity.[64] TDM is recommended when using this drug because of the potentially fatal consequences of under-dosing and over-dosing. LC/MS-MS is the gold standard for measuring VOZ's (and most of the drugs that need TDM)

level in blood samples since it is a reliably accurate, selective and sensitive assay. Although antibodies were developed for VOZ,[65] immunoassays are not routinely used for analytical tests in hospitals due to VOZ's stability issues and cross-reactivity risks. An aptamer was selected by the Stojanovic group using a complex method of functional group-guided SELEX, since it was deemed to be a challenging target for aptamer selection.[66] This aptamer was turned into a quenching-FRET sensor (Figure 1.4c) and detected CBZ to concentrations as low as 3 μM. Additionally, our group selected some aptamers for VOZ, one of them was similar to the aptamer previously selected (Figure 1.4b-d) but new families also emerged.[67] However, further characterization of these new aptamers was not performed. Chapter 3 explains the importance of aptamer characterization and how we characterized these aptamers and used them to probe other nano polymers such as cyclodextrins.

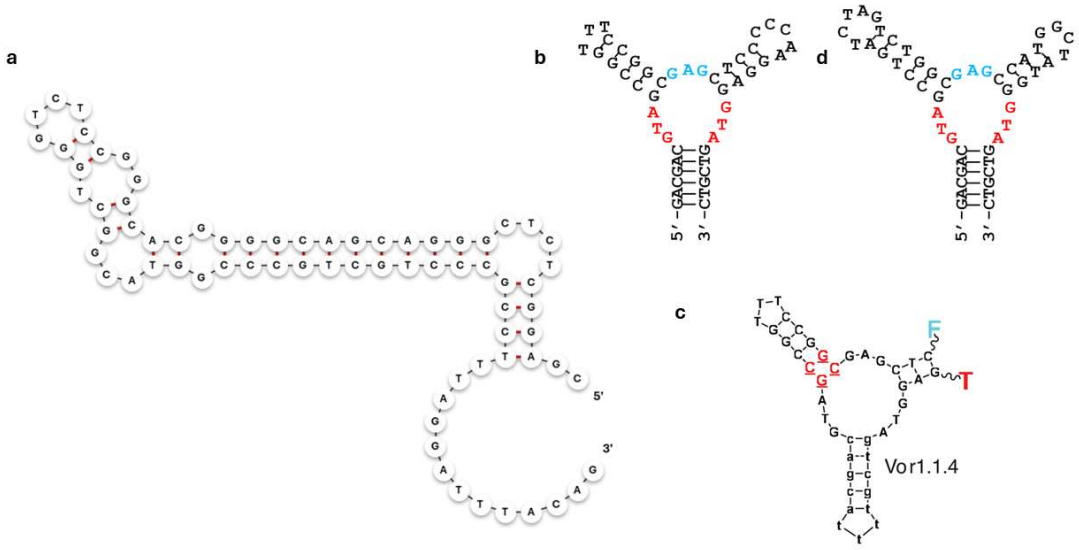


Figure 1.4 Carbamazepine and voriconazole aptamers.

(a) 2D structure prediction of carbamazepine aptamer reported in 2022, redrawn by an application designed in our lab;[62] (b) voriconazole aptamer selected by the Stojanovic group;[66] (c) FRET sensor of the voriconazole aptamer designed by the Stojanovic group;[66] (d) voriconazole aptamer selected by our group.[67] *(b) and (d) Open Access(c) Reproduced with permission from Science, AAAS. via RightsLink (Order no. 6313790133040.)*

Chapter 2 Selection of aptamers for carbamazepine

2.1 Introduction

Approximately 65 million people around the world suffer from epilepsy, including 1 out of 100 Canadians as indicated by the Canadian Chronic Disease Surveillance System in 2023.[68] Carbamazepine (CBZ) is one of the drugs used to control seizures in epilepsy,[61] it is also used to treat trigeminal neuralgia,[69] and to manage some psychotic symptoms like the mania phase in bipolar disorder.[70] As a narrow therapeutic index drug with a therapeutic range of 4-12 mg/L, CBZ's drug level in the blood has to be constantly monitored through TDM.[61] Analytical methods used to detect or quantify CBZ differ depending on the purpose of the analysis. Although a plethora of methods has been proposed, only a few are being used systematically in medical, environmental or academic laboratories. For detection in blood samples or serum, the two most employed methods are methods involving chromatography like high performance liquid chromatography (HPLC) and immunoassays like fluorescence polarization immunoassay (FPIA).[71] Even though instrumentation methods provides rich and accurate information, they have the drawbacks of sample preparation and a long turnaround time (often days). For immediate point-of-care results (e.g. in hospitals or at home) and minimal sample volume, biosensors are the solution. They also have additional advantages such as fast detection, ease-of-use, portability, cost-effectiveness and ease of mass production. An aptamer has been selected for CBZ to serve as a bioreceptor for a biosensor that can be used for POCT. However, the details of the sequencing results and aptamer characterization were not disclosed leaving no clear evidence of the binding ability, selectivity nor stability of the claimed aptamer. Moreover, the aptamer had unfavorable properties for biosensor applications such as being 72 nucleotides long and having an extensive duplex formation. A shorter aptamer is cheaper to synthesize and exhibits more efficient folding and unfolding, enabling a faster response rate. However, a short high-quality aptamer for CBZ has yet to be identified.

2.1.1 Aptamer selection

As shown in Figure 2.3, CBZ is a small molecule with only 1 polar amide functional group. We previously explained in chapter 1 that capture-SELEX is the method of choice for aptamer selection when it comes to small-molecule targets.[52,53] Since this method successfully enriched high-affinity aptamers for various small-molecule targets in our laboratory before, we decided to use it to enrich aptamers for CBZ.

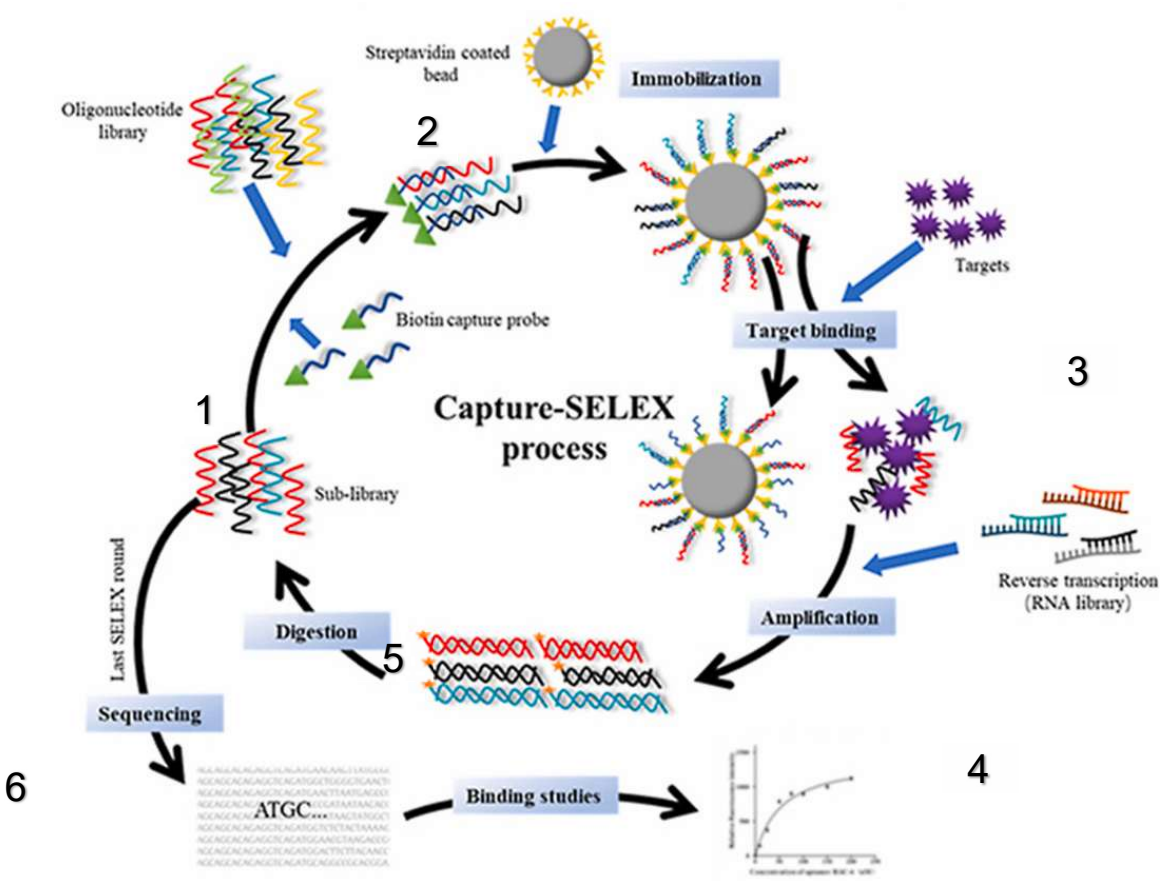


Figure 2.1 Capture-SELEX process general scheme.

(1) Biotin capture probes are incubated with the oligonucleotide library (i.e. 30-mer ssDNA sequences), (2) the biotinylated DNA sequences are added to streptavidin beads for immobilization, (3) the target is added to release potential aptamer sequences, (4) the eluted sequences are amplified using PCR, (5) the amplified sequences are digested into single strands and the reverse sequences are discarded, (6) the SELEX rounds stops when sufficient enrichment is achieved then the sequences obtained are sent for sequencing. *Figure reproduced with permission (order Number: 6312331314588) from Reference*[53] *Talanta, copyright Elsevier.*

2.1.2 Library design

RNA libraries were predominantly used in the very early stages for aptamer development.[72] Nowadays, they are mostly used in genomic SELEX which is a type of SELEX mainly employed for functional studies. For all other types of SELEX, ssDNA libraries are generally preferred because DNA is more chemically and biologically stable, easier to synthesize, and it has a more robust shelf-life. Moreover, DNA-based SELEX is more cost and time effective because it eliminates the need for an extra reverse transcription step essential for RNA-based SELEX.

The ssDNA oligomer size choice is another important parameter for finding the high-quality aptamer desired. It was found that short aptamer libraries have various advantages such as (1) they are easier and more cost effective to synthesize, (2) they reduce PCR by-product formation, and (3) they are simpler to manipulate for later truncation and application. Usually, the original library contains a randomized region flanked by two fixed primer-binding sequences on both ends enabling amplification via PCR. These fixed primers are around 20 nucleotides long and aptamers with 30 to 50 nucleotides in the random region are preferred as demonstrated by a comparative study using a variety of pool lengths.[73] Aptamers with shorter random regions rely much more heavily on primer binding sites for structure and function which can cause PCR bias. Based on these findings, the library chosen was composed of random 30 nucleotides long ssDNA sequences.

2.1.3 Target concentration

According to a recent study by Alkhamis and Xiao (2023), choosing a low target concentration is essential for obtaining a high-quality aptamer.[74] Keeping in mind that "low" can be a relative term, the decision of the amount should be made according to the selection target identity. The physiochemical properties of the target influence the level of affinity that

aptamers can have for that target. For example, larger molecules like proteins have several moieties to interact with the aptamer, thus improve the binding. Additionally, the polarity of the target can affect how well it binds to the negative DNA aptamer sequence. Since we cannot use too high concentration due to solubility constraints, we chose 100 μM for CBZ's starting concentration.

2.1.4 Buffer optimization

The buffers used during the SELEX process can have a great influence on the structural conformation of an individual aptamer sequence, specifically the buffers' pH value, the types of ions they contain and their concentrations.[50] Magnesium, for example, is known to contribute to the hairpin and duplex structures in general which facilitates aptamer-target interactions. Sodium or potassium in the buffer can weaken electrostatic forces which is necessary to get rid of any non-specific electrostatic binding between the ssDNA sequences and the target. However, in high concentration, these 2 ions can weaken even specific electrostatic interactions between the target and the potential aptamer. The pH value of the buffer can influence the conformational structure of the aptamer by protonation of the bases that are involved in the structure formation.[75] As a result, the buffer environment can influence greatly how the aptamer forms and how it binds to the target. The final purpose of the aptamer should be kept in mind when choosing these buffers to avoid non-functional aptamers if the selection conditions were different than the intended-use conditions. Based on the above and knowing that CBZ's aptamer will be used for CBZ's detection in the blood, we used NaCl, $MgCl_2$ and N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) at pH 7.5 for SELEX buffer and NaCl and HEPES at pH 7.5 for separation buffer.

2.2 Materials and Methods

2.2.1 Chemicals

All DNA used in these experiments was purchased from Integrated DNA Technologies (Coralville, IA, USA) and collected in Table 2.1.

Table 2.1 DNA sequences used in this work.

DNA name	Sequence from 5' to 3'
Library	GGAGGCTCTCGGGACGAC$_{(18)}$-N$_{30}$-GTCGTCCCGATGCTGCAATCGTAA$_{(24)}$
Forward Primer	GGAGGCTCTCGGGACGAC
Reverse Primer	(bio)AGACCATTCAAGTGATCGGGACG
Biotin Reverse Primer	/5Biosg/CTGTAAATCCTAAAGGCGGGACG
Biotin Column	GTCGTCCCGAGAGCCATA/3BioTEG/
CB23	GACGACCAAATTACAAGTGGTACTTGCGTATACTGAGTCGTC
CB50	GACGACGTATAGAACCAACAATATGGTATAGGGATTGTCGTC
CB51	GACGACGCTCCTAGGTTGGAGAATTCAGTGTATCCTGTCGTC
G1	GACGACGGGTCTGTAATGGATTGTTCTCAACCAACTGTCGTC
VO4	GACGACGTAGCTGATCTAGTCTGCGAGCCATGGCTATGGGTAGTCGTC

Sodium chloride and magnesium chloride were purchased from Amerseco (Framingham, MA, USA). Carbamazepine was purchased from Millipore-Sigma (Oakville, ON, Canada). Streptavidin agarose resin was purchased from Fisher Scientific, 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 buffers and solutions were prepared with Milli-Q water. SELEX buffer contains 20 mM HEPES, 150 mM NaCl and 2 mM $MgCl_2$ (pH 7.6); while the strand separation buffer contains 20 mM HEPES, and 150 mM NaCl only (pH 7.6).

2.2.2 Capture-SELEX

The library design (Table 2.1) and the capture-SELEX method was previously reported in our lab[76] and by the Stojanovic group.[77] The initial library contained a random region of 30 nucleotides flanked by 2 constant regions for binding to PCR primers. For each round, the ssDNA library was annealed with a 3-fold excess of biotinylated capture strands at 95°C for 5 mins then left to cool to room temperature in SELEX buffer. Concurrently, 200 μL of agarose resin was introduced into a microchromatography column and washed 6 times with 500 μL SELEX buffer. Next, 20 μL of streptavidin-modified agarose resin was added to the column, subsequently the biotinylated ssDNA library was added to be immobilized on the streptavidin beads.[78] After immobilization of the DNA library on the streptavidin resin, the column was washed 12 times to remove any unbound library strands. Then, 750 μL of the target CBZ dissolved in SELEX buffer was added, left to flow through the column and reloaded 3 times to ensure proper interaction with the DNA library. When CBZ was added to the library immobilized on the beads, a very small fraction of the sequences bound to it underwent conformational changes and by consequence were released from the beads. The released sequences were collected, concentrated using a 10 kDa molecular weight cutoff filter and washed with Milli-Q water 3 times. A biotinylated reverse primer was used to synthesize 1 mL of PCR products which was then incubated with 50 μL of streptavidin agarose resin for 15 mins and washed 10 times with strand separation buffer. To separate the double-stranded amplicons, 600 μL of 0.2 M NaOH was added to the column and incubated for 10 minutes. 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). These collected forward strands were used as the library for the next round of selection. For each round, the amount of target eluted was quantified using quantitative real-time PCR to monitor the progress of the

selection. When enough enrichment was observed, the rounds were stopped and the sample was sent for deep sequencing.

2.2.3 Deep sequencing results analysis

After 17 rounds, the enriched sample was sent to the genomic facility at McMaster University for deep sequencing. The sequences sent back from McMaster University were analyzed using Geneious Prime software and aligned using ClustalW program. The 50 most abundant sequences were individually analyzed for their secondary structures using mFold. The similarly structured aptamers were grouped into families and 3 major families emerged. Aptamers CB50, CB51 and CB23 were chosen for further analysis as representative for Family 1, Family 2 and Family 3, respectively.

2.2.4 ThT Fluorescence spectroscopy

Using Thioflavin T (ThT) Fluorescence spectroscopy to assess binding affinity is a cheap and reliable method that we use in our lab for primary affinity tests.[79] ThT is a dye that fluoresce upon intercalating with DNA, therefore measuring the resulting fluorescence of ThT-aptamer interaction can be used as a starting point for measuring the change in fluorescence observed after gradually titrating increasing concentrations of the target. The Horiba Fluoromax-4 fluorometer was used for all ThT titration assays with an excitation wavelength of 420 nm and an emission wavelength of 490 nm. The concentrations used were 500 nM for the aptamer and 1 μM ThT added to the same SELEX buffer used for selecting CBZ and brought to a final volume of 500 μL.

2.2.5 Isothermal Titration Calorimetry

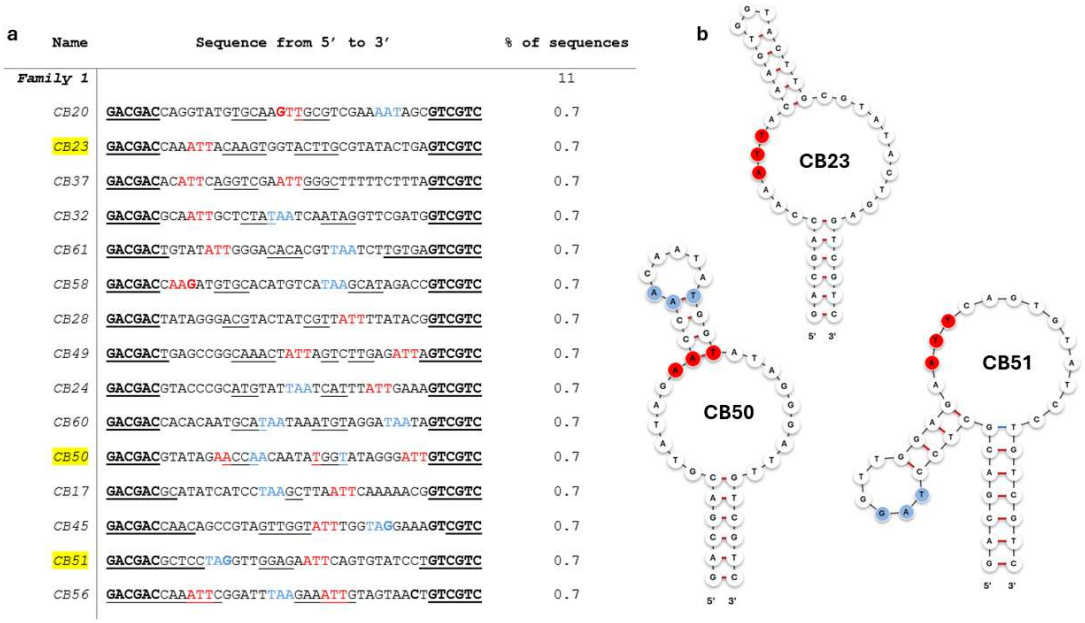
The isothermal titration calorimetry (ITC) experiment was conducted using MicroCal iTC200. Both the aptamer and target were dissolved in test buffers (20 mM HEPES pH 7.5, 150 mM NaCl, 2 mM MgCl) and degassed by sonication for 5 min before the start of the experiment. The cell chamber was loaded with 300 μL aptamer solution (100 μM), while the syringe contained 200 μL of target solution. The titration experiment was conducted at a temperature of 25 ° C. The obtained titration curve was fitted using a one-site binding model by Origin software.

2.3 Results and Discussion

2.3.1 Deep sequencing results

The resulting PCR amplicon from round 17 was deep sequenced and 46,984 sequences were obtained using the Geneious software. Only 264 sequences were assembled into contigs using the De Nove assemble command suggesting a high variability in the sample even after 17 selection rounds. Any sequences that contained numerous degenerate nucleotide bases or were not the length of around 40-mer were considered too mutated for analysis and thus excluded from the evaluation. The top 61 sequences were individually analyzed for their secondary structures and grouped into 1 family based on common motifs as seen in Figure 2.2a. Most of the sequences had an abundance of A & T nucleotides (from 50-75 %) especially in the order ATT and TAA, suggesting that A & T play a crucial role in the interaction with CBZ. The enrichment of each sequence was low, mostly 2 reads for each sequence even after 17 rounds of SELEX. This low enrichment result may be caused by a failed capture-SELEX experiment or an incompatibility of the program Geneious Prime with aptamer enrichment detection since it was not designed for this purpose. From our lab experience, we found that

the De Novo assembly feature can easily detect highly enriched sequences but fails to recognize challenging aptamers with lower enrichment patterns.



a

Name	Sequence from 5′ to 3′	% of sequences
Family 1		11
CB20	GACGACCAGGTATGTGCAAGTTGCGTCGAAAATAGCGTCGTC	0.7
CB23	GACGACCAAATTACAAGTGGTACTTGCGTATACTGAGTCGTC	0.7
CB37	GACGACACATTCAGGTCGAATTGGGCTTTTTCTTTAGTCGTC	0.7
CB32	GACGACGCAATTGCTCTATAATCAATAGGTTCGATGGTCGTC	0.7
CB61	GACGACTGTATATTGGGACACACGTTAATCTTGTGAGTCGTC	0.7
CB58	GACGACCAAGATGTGCACATGTCATAAGCATAGACCGTCGTC	0.7
CB28	GACGACTATAGGGACGTACTATCGTTATTTTATACGGTCGTC	0.7
CB49	GACGACTGAGCCGGCAAACTATTAGTCTTGAGATTAGTCGTC	0.7
CB24	GACGACGTACCCGCATGTATTAATCATTTATTGAAAGTCGTC	0.7
CB60	GACGACCACACAATGCATAATAAATGTAGGATAATAGTCGTC	0.7
CB50	GACGACGTATAGAACCAACAATATGGTATAGGGATTGTCGTC	0.7
CB17	GACGACGCATATCATCCTAAGCTTAATTCAAAAACGGTCGTC	0.7
CB45	GACGACCAACAGCCGTAGTTGGTATTTGGTAGGAAAGTCGTC	0.7
CB51	GACGACGCTCCTAGGTTGGAGAATTCAGTGTATCCTGTCGTC	0.7
CB56	GACGACCAAATTCGGATTTAAGAAATTGTAGTAACTGTCGTC	0.7

b

Figure 2.2 Deep sequencing results and secondary structures.

(a) One family of aptamers grouped from the top 61 sequences obtained from deep sequencing of the 17th round of capture-SELEX; it was chosen according to similar motifs and common bases in non-duplex regions. (b) The secondary structures of 3 representatives CB23, CB50 and CB51 obtained by mFold and drawn using an application designed in our lab.

2.3.2 Secondary structure analysis

In general, the sequences had a simple stem loop motif with a bigger internal loop which most probably represents the pocket for target interaction (Figure 2.2b). A and T nucleotides were most common among the top sequences, suggesting that these 2 nucleotides are important in the interaction with CBZ, especially the combinations of ATT or TAA that were repeated in most of the aptamer's loops where most of the small-molecule targets reside and interact with their aptamers. At first glance when we look at the chemical structure of CBZ, we see that it has a high potential to interact with ssDNA by π-π interactions, or hydrogen bonds through the amine or carbonyl group. However, when we dive deeper and look at its 3D structure, we find

that the overall molecular geometry restricts such interactions.[80] The non-planar conformation of the tricyclic scaffold and the out-of-plane orientation of the amide group introduce steric and electronic constraints that hinder parallel ring alignment. As a result, π–π interactions are weakened and occur less frequently than in fully planar polyaromatic systems. No specific sequences were able to highly amplify using capture-SELEX with the chosen parameters because aptamers usually form a pocket surrounding the small-molecule target using numerous non-covalent interactions such as π-π, electrostatic, van der walls and hydrogen bonds. By consequence, it seems that CBZ is interacting with many sequences equally and weakly.

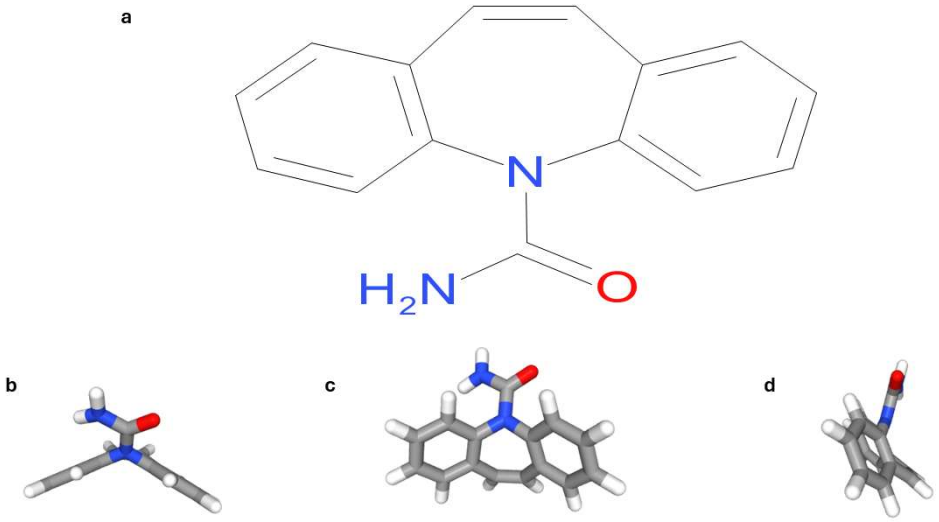


Figure 2.3 Structure of carbamazepine.

(a) 2D chemical structure of a dibenzazepine derivative bearing a formamide substituent, with heteroatoms highlighted (nitrogen in blue, oxygen in red); (b) the formamide fragment is shown in a side-on view;(c) the 3D rendering reveals the non-planarity of the dibenzazepine scaffold, where the fused rings adopt a twisted geometry. The attached formamide substituent projects out of the aromatic plane, illustrating steric crowding between the substituent and the bulky ring system;(d) this orientation shows how the fragment deviates from planarity when interacting with neighboring groups. The rotation emphasizes torsional strain and steric hindrance, demonstrating that the molecule cannot adopt a fully flat geometry due to spatial clashes.

2.3.3 Binding assay results

2.3.3.1 ThT fluorescence spectroscopy

Thioflavin T (ThT) is a dye that fluoresces upon binding to DNA by intercalation. This property is useful to detect apparent K_D values of DNA aptamers that can fold into G-quadruplex or non-G-quadruplex structures. We use ThT titration assays frequently in our lab to determine K_D values of the aptamers we select because it is a reliable and cheap method that gives results similar to ITC results.[79] When used for the 3 potential aptamers of CBZ, the fluorescence drop was not significant, less than 30% for all the aptamers. Additionally, the fluorescence drop occurred at two stages. In the low concentration region (below 100 μM), there was a more rapid drop, which could be assigned to the binding of the aptamers. Further increase in the CBZ concentrations led to a slower drop, which could be due to nonspecific fluorescence quenching. Thus, the fitting of binding was mainly based on the first quenching stage. Even though, the 3 aptamers showed similar K_D values from 12 – 19 μM, these values could not be trusted because of the low fluorescence intensity change. When we used two previously selected aptamers from our lab as negative controls, VO4 (voriconazole aptamer) and G1 (guanine aptamer), similar K_D values were obtained. These results imply that CBZ interacts weakly and equally with all these sequences (CB50, CB51, CB23, VO1 and G1) and that no specific strong binding is observed with any of these sequences. Thus, none of the 3 sequences CB23, CB50 nor CB51 appear to be a true aptamer using this characterization technique.

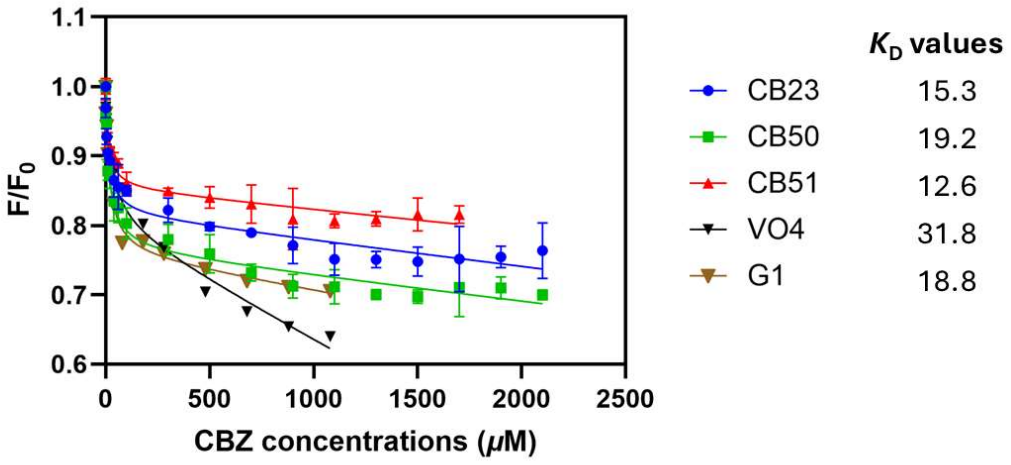


Figure 2.4 Binding affinity of CBZ aptamers vs negative controls.

ThT titration assays were performed for the 3 potential aptamers CB23, CB50 and CB51 using 500 nM aptamer concentration and 1 μM ThT in 500 μL SELEX buffer. The aptamers VO4 (voriconazole aptamer) and G1 (guanine aptamer) were used as negative controls. Experiments were performed in triplicates except for negative control VO4 and G1.

2.3.3.2 Isothermal titration calorimetry

Since ThT titrations only give us apparent K_D values and the results of CBZ aptamers assays using ThT fluorescence spectroscopy was not conclusive, we decided to use isothermal titration calorimetry (ITC) assays. ITC is the gold standard for measuring K_D values of aptamers, but it cannot be used as a primary tool because it only works with low K_D values in the nanomolar or low micromolar range. In our lab, we usually use ThT titration assays for primary determination of apparent K_D values, then move to ITC when the values are promising or when ThT results are questionable like in the case of CBZ aptamers. Another obstacle for using ITC is the solubility of the target; since higher concentrations are usually needed for ITC experiments, DMSO or other organic solvents can be used in very small quantities to assist the solubility of non-soluble target like CBZ, and the same quantities has to be matched in the DNA solution mixture.

We tested CB51 using ITC since it was the most promising aptamer in the ThT titration assays data. As seen in Figure 2.5, ITC shows a very weak and non-specific binding, confirming the deep sequencing and ThT assays results. By consequence, none of these selected aptamers (CB23, CB50 or CB51) appears to bind CBZ strongly and specifically.

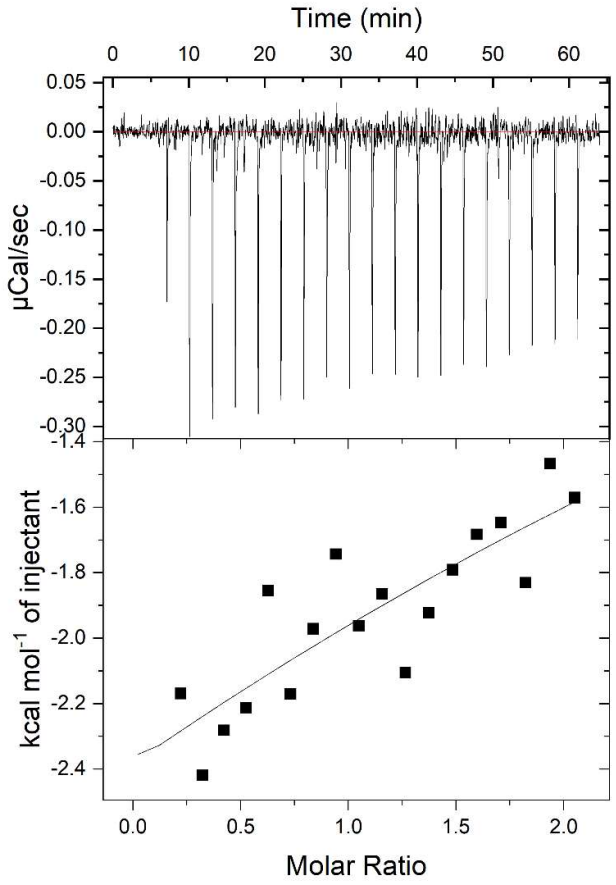


Figure 2.5 ITC results for CB51 vs. CBZ. 500 μM CBZ was titrated against 50 μM CB23 aptamer, both solutions contained 1% DMSO.

2.4 Conclusion

In this chapter, CBZ was used as a target for aptamer selection. After 17 rounds of capture-SELEX, a very low enrichment was observed. We grouped the similar sequences into

1 group and chose 3 representative aptamers CB23, CB50 and CB51. Next, we tested the binding affinity of these potential aptamers using ThT fluorescence spectroscopy and ITC. None of these aptamers showed high affinity or specific binding to CBZ.

Based on the poor sequence enrichment and the binding assays, it is likely that the selection of aptamers for CBZ has failed. Aside from my attempt, two other lab members have also conducted capture-SELEX experiments for CBZ, but neither enriched aptamers. It appears that CBZ is a difficult target for aptamers to bind. Not only because of its small size and the fact that it has only 1 functional group to interact with DNA, but its 3D structure also confirms non-planarity and steric hindrance around its only functional group disallowing the DNA aptamers to form a proper pocket for CBZ's interaction.

Future studies should explore other variations of the SELEX methods, modified DNA sequences, or a starting library with a chosen motif that preference stronger interactions with DNA and CBZ.

Chapter 3 Biochemical characterization of voriconazole aptamers and modulation of cyclodextrins

3.1 Introduction

Voriconazole (VOZ, marketed as Vfend®) is a broad-spectrum triazole antifungal agent used to treat life-threatening fungal infections, including invasive candidiasis, invasive aspergillosis, and other emerging fungal diseases.[63] These infections can progress rapidly, particularly in critically ill or immunocompromised patients.[81] Therapeutic drug monitoring (TDM) is therefore recommended because VOZ exhibits a narrow therapeutic window (1–5 mg/L), substantial inter- and intra-patient pharmacokinetic variability, frequent drug–drug interactions, and potentially severe adverse effects, including photophobia and QT interval prolongation.

High-performance liquid chromatography (HPLC) is the gold standard for measuring VOZ's concentrations in clinical laboratories.[82] While highly accurate, it requires costly instrumentation, laborious sample preparation, and skilled operators, leading to turnaround times of one or more days that are incompatible with point-of-care testing (POCT). Immunoassays have also been developed for VOZ's detection and are more amenable to POCT, but they depend on the expensive and time-consuming production of target-specific antibodies.

Aptamers, short single-stranded DNA or RNA molecules first reported in the early 1990s, have emerged as attractive alternatives to antibodies.[36,38,41,83–85] They offer several advantages, including high chemical and thermal stability, rapid and inexpensive chemical synthesis, facile chemical modification, excellent batch-to-batch reproducibility, and low immunogenicity.[23] An aptamer for VOZ was selected by the Stojanovic group using a sophisticated functional group guided approach. Later this aptamer was converted into a fluorescence resonance energy transfer (FRET) sensor by labelling the ends of the aptamer,

which was able to detect VOZ down to 3 μM.[66] To facilitate the selection of aptamers against small molecules such as VOZ, our group previously investigated the effect of target–library incubation time during capture-SELEX. Two independent selections were performed: one with a 10 min incubation between the target and the DNA library before partitioning, and the other without any pre-incubation.[67] The two selections yielded one aptamer family that was consistent with the earlier results of the Stojanovic group, along with several previously unreported families. However, none of these aptamers underwent rigorous follow-up characterization, leaving their binding affinities, selectivities, and application potential largely unexplored.

Although thousands of aptamers have been reported in the literature, only a few have succeeded in real-life applications.[30] To date, only two aptamer therapeutics—pegaptanib and avacincaptad pegol—have received FDA approval, both for the treatment of sight-threatening retinal diseases.[86,87] This disparity between academic research and practical applications is partly driven by the absence of rigorous and systematic validation for newly discovered aptamers.[88] Consequently, reliably distinguishing high-affinity, functional aptamers from weak or false-positive sequences remains a significant challenge. Additionally, an aptamer can bind its target well in the selection conditions (buffer, metal ions, pH, temperature, etc.) but fails to bind the same target in the designated final application such as in serum or blood samples. Rigorous biochemical characterization is essential to determine whether a reported aptamer is suitable for its intended application. Here, we systematically evaluated previously selected VOZ aptamers by assessing their binding affinity, selectivity, and stability, with the goal of identifying candidates suitable for POCT biosensing.

Cyclodextrins are classical nanoscale host molecules.[89] β-cyclodextrin has been commonly used in VOZ drug formulations.[90,91] However, it is difficult to directly monitor the binding between cyclodextrin and VOZ because neither molecule produces a measurable

optical signal upon complex formation, and the moderate binding affinity combined with VOZ's limited solubility prevents the use of standard biophysical techniques such as isothermal titration calorimetry (ITC) or circular dichroism spectroscopy. In this study, we demonstrate for the first time that aptamers can serve as indirect probes to compare the relative binding affinities of nanoscale host molecules, such as cyclodextrins, toward VOZ. This simple and general approach provides a new strategy for investigating host–guest interactions and expands the utility of aptamers beyond molecular recognition and biosensing.

3.2 Experimental

3.2.1 Materials

The DNA samples used in this study were purchased from Integrated DNA Technologies (Coralville, IA, USA) with the following sequences 5′-GACGAC TGGGCGTAGTTGTGCGACGAATAAGTTCGTAGGCCTGTCGTC for VO1, 5′-GACGAC TTAAAGGGTTTGTTCCTTGTACCAGGAATGGATTTAGTCGTC for VO3, and 5′-GACGACGTAGCTGATCTAGTCTGCGAGCCATGGCTATGGGTAGTCGTC for VO4. Sodium chloride and magnesium chloride were purchased from Amerseco (Framingham, MA, USA). Voriconazole and human serum were purchased from Millipore-Sigma (Oakville, ON, Canada). All buffers and solutions were prepared using Milli-Q water.

3.2.2 Methods

3.2.2.1 Sequences analysis

The sequencing results from the two previous VOZ selection experiments done in our laboratory were analysed using Geneious Prime (version 11.0.0 (84)) and the top 20 sequences in terms of abundance from each selection were aligned using ClustalW and compared. Then, the secondary structures of 3 representative aptamers were analysed with the help of mFold.

3.2.2.2 ThT fluorescence spectroscopy

Horiba Fluoromax 4 fluorometer was used for all the ThT binding assays experiments with excitation at 420 nm and emission at 490 nm. Generally, 500 nM of the aptamer and 1 μM of Thioflavin T (ThT) were added to a buffer or serum solution to reach 500 μL final volume in a quartz cuvette. The SELEX buffer was composed of 20 mM HEPES, pH 7.6, 150 mM NaCl and 2 mM $MgCl_2$. Human serum was diluted using the SELEX buffer to the desired concentrations when needed. At 25ºC, the fluorescence was left to stabilize before starting a gradual titration of the sample. The titration was stopped after a plateau was reached and the fluorescence values at 490 nm were recorded for computation. Binding curves were generated by plotting normalized fluorescence (F/F_0) versus ligand concentration. Data points represent the mean of three independent replicates. Nonlinear regression was performed in GraphPad Prism (version 11.0.0 (84)) using a one-site binding model to estimate the dissociation constant (K_D).

3.2.2.3 Effect of cyclodextrins

To test the effect of α- and β-cyclodextrin titrations, a solution containing 500 nM VO3 aptamer, 1 μM ThT and SELEX buffer were left to equilibrate. Then, 30 μM VOZ (near VO3's K_D value) was added and left to stabilize before starting the corresponding cyclodextrin titration. To test the relative affinities of the cyclodextrins in a different approach, VOZ was titrated as normal in a solution containing 500 nM VO3 aptamer, 1 μM ThT and SELEX buffer after adding a constant concentration of α- or β-cyclodextrin and waiting for the sample to reach equilibrium. The concentrations chosen for the cyclodextrins were 0, 2, 6 and 10 mM.

3.3 Results and discussion

3.3.1 Analysis of sequencing results

Our lab previously performed two capture-SELEX experiments using VOZ as the target, differing only in the inclusion of a 10 min incubation period between the target and the DNA library before partitioning.[67] The deep sequencing results are summarized in Figures 3.1a and 3.1b. Closely related sequences were grouped into families, representing variants of the same underlying aptamer motif (Figure 3.1d). Members of each family share conserved sequence motifs and are therefore expected to adopt similar structures and recognize the target through a common binding mechanism. For example, in the selection incorporating the 10 min incubation (Figure 3.1a), 18 of the top 20 enriched sequences belong to a single dominant family (Family 3.1). Secondary structure prediction indicates that these sequences fold into a three-way junction, with the three connecting loops containing the highly conserved GTA–GAG–GTA motif.

Despite the difference in incubation time during SELEX, the two selections yielded overlapping sequence families. For subsequent characterization, we picked the following three sequences from the 10-min-incubation selection: VO1, VO3, and VO4. VO1 and VO3 are unique sequences that do not belong to any enriched family and therefore each represent a distinct aptamer motif, whereas VO4 is a dominant representative of Family 3.1.

VO3 and VO4 were also identified in the no-incubation selection as nVO1 and nVO4, respectively. The most notable difference was observed for VO3: it accounted for only 2.8% of the enriched pool in the 10-min incubation selection but became the dominant sequence (65%) in the no-incubation selection (Figure 3.1c). This enrichment pattern suggests that the VO3 family has a kinetic advantage during capture-SELEX, likely because it dissociates more rapidly from the capture strand upon target binding. Although many sequences may bind to a

specific target with good affinity, the most rapid one to bind the target amplifies exponentially along the SELEX rounds.[67] In both selections, VO3 appeared as a single enriched sequence, with no closely related variants that could be assigned to the same family, suggesting that this aptamer tolerates little sequence variation. Like VO4, VO3 is predicted to fold into a three-way junction structure, although its conserved sequence motifs are distinct.

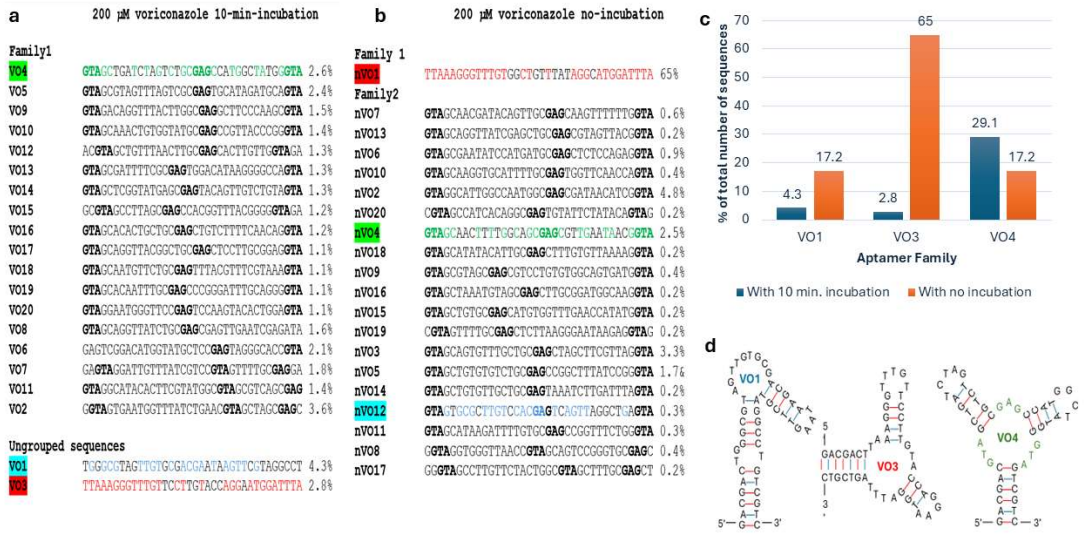


Figure 3.1 Deep sequencing results of the two capture-SELEX experiments previously done by our group for VOZ.

The N30 region of the first 20 sequences obtained by Geneious Prime (version 11.0.0 (84)) and aligned with ClustalW for (a) the 10-min-incubation selection, and (b) the no-incubation selection.[67] Identical nucleotides shared by related sequences from the two SELEX experiments are highlighted in the same color. The common nucleotides in each family were in boldface. (c) The abundance of the three families that each of the 3 aptamers belongs to, from the two selections. The secondary structures of the 3 aptamers obtained by mFold are shown in (d).

3.3.2 Affinities and selectivity of the VOZ aptamers

To measure binding affinity of the aptamers, we used the label-free thioflavin T (ThT) based binding assay.[79] This assay exploits the fluorescence enhancement of ThT upon binding to DNA aptamers and monitors the fluorescence change, typically a decrease, upon target binding. Through extensive use of ThT in our laboratory to characterize both G-quadruplex

and non-G-quadruplex aptamers, we have found that ThT binds non-G-quadruplex aptamers with substantially lower affinity than G-quadruplex-forming aptamers. For many non-G-quadruplex aptamers, the results were similar to ITC results,[92] and the concentrations of ThT did not affect the K_D values. As expected, the 3 sequences (VO1, VO3 and VO4) all showed target-dependent fluorescence drop suggesting binding with K_D values ranging from 20 to 30 μM as seen in Figure 3.2a.

Among these aptamers, only VO1 was predicted to have a non-three-way-junction structure. Instead, it was predicted to have a two-way junction with a big loop. One of the stem regions has a mismatched part as marked by a red box in Figure 2d. Therefore, we wondered if this mismatched region can be mutated to a fully base paired region. If so, then this predicted secondary structure in Figure 3.2e is likely to be correct. However, when we made this stem fully base paired, this VO1s mutant showed markedly weaker binding with more than 5-fold increase in the apparent K_D value (Figure 3.2b), which suggests that the predicted folding of this aptamer may not be correct.

When the three aptamers were titrated with fluconazole (FOZ), a structural analogue of VOZ, all exhibited weaker binding than with VOZ. Notably, VO3 and VO4 showed little concentration-dependent fluorescence change in the presence of FOZ (Figure 3.2c), indicating good selectivity for VOZ. Based on their superior affinity and selectivity, VO3 and VO4 were selected for further characterization.

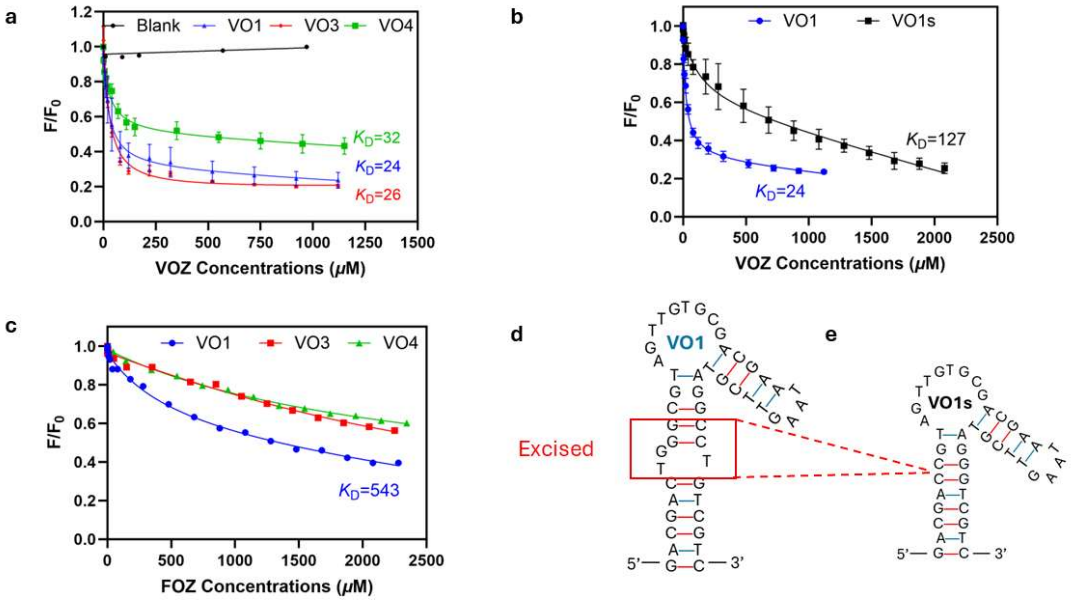


Figure 3.2 ThT fluorescence based binding assays result.

(a) VOZ and (c) FOZ were titrated into a solution containing 500 nM of one of the VOZ aptamers (VO1, VO3 or VO4), double concentration of ThT (1 μM) and SELEX buffer. (b) VOZ was titrated into a truncated VO1 aptamer as shown in (d, e). Experiments were performed in triplicate, except in (c).

3.3.3 Effect of buffer components and serum on VO3 and VO4 aptamers

To evaluate the robustness of the two most selective aptamers, VO3 and VO4, we repeated the ThT fluorescence binding assays under different buffer conditions and in diluted human serum.

3.3.3.1 Effect of magnesium ions

Divalent metal ions often play critical roles in aptamer binding by shielding the negative charges of the DNA phosphate backbone, thereby reducing electrostatic repulsion, stabilizing the folded structure, and facilitating target recognition. In the SELEX buffer, 2 mM Mg^{2+} was used. To examine the effect of Mg^{2+} on the aptamers' affinity, a side-by-side comparison was made by titrating VOZ into the aptamers in the presence or absence of 2 mM Mg^{2+}. Without Mg^{2+}, the K_D value almost tripled for VO3 and doubled for VO4 as seen in Figures 3.3a and

3.3b respectively, which suggest that Mg^{2+} supports the aptamer's structure to some extent, but it is not essential for the final functional structure of the aptamers since they all maintained a micromolar K_D affinity to VOZ even without any Mg^{2+} present.

3.3.3.2 Effect of sodium ions

A NaCl concentration of 150 mM is commonly used in SELEX buffers to approximate physiological ionic strength. To evaluate the effect of ionic strength on aptamer binding, ThT titration experiments were repeated for VO3 and VO4 in SELEX buffer containing 1 M NaCl instead of 150 mM, and the resulting K_D values were compared. VO3 was essentially unaffected by the large increase in NaCl concentration, whereas VO4 exhibited only a modest decrease in affinity, with its K_D increasing by less than twofold. This slight reduction in affinity at higher ionic strength is consistent with previous observations for protein-binding aptamers, where increased salt concentrations partially screen favourable electrostatic interactions involved in target recognition.[93] These results suggest that the ionic strength did not strongly affect the binding property of the VO3 aptamer, although the VO4 aptamer was more affected as shown in Figures 3.3c and 3.3d, respectively.

3.3.3.3 Serum effect

Keeping the final application as a biosensor in mind, we wanted to test the 2 aptamers (VO3 and VO4) in more complex media, like serum. VO3's affinity did not change at all in 5% serum while VO4's affinity decreased almost 2-fold at the same serum concentration as observed in Figures 3.3e and 3.3f, respectively. One limitation to this test is the know interaction between ThT and proteins in serum like albumin.[94] The higher the concentration of serum, the less ThT available to interact with DNA aptamers and thus less fluorescence to begin with. Serum and whole-blood experiments are better performed using the final

application format (such as an electrochemical biosensor),[55] where the readout does not rely on ThT fluorescence.

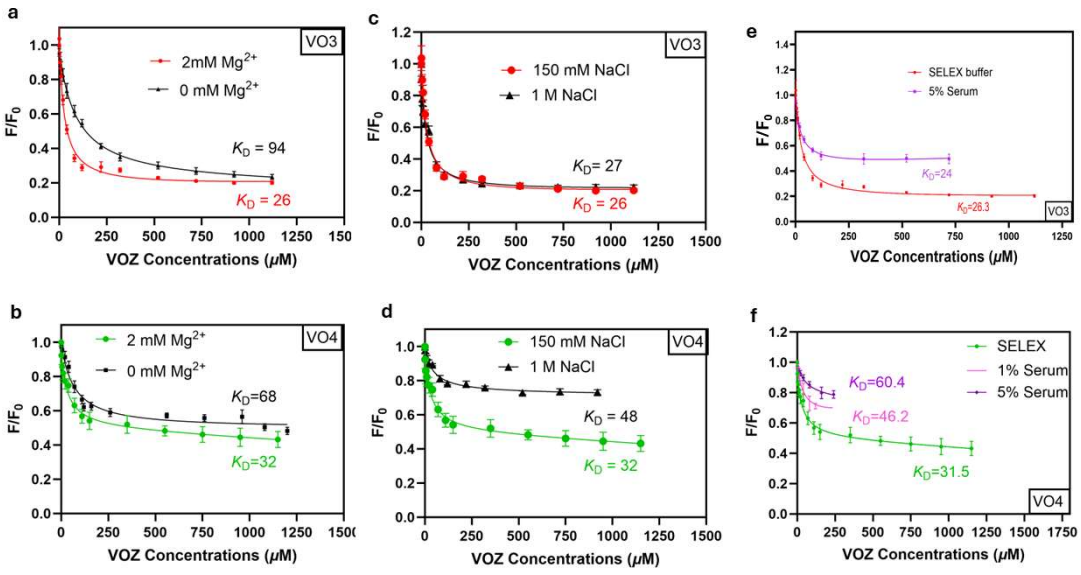


Figure 3.3 Effect of buffer components and serum on the VO3 and VO4 aptamers.

The effect of Mg^{2+} on the binding of the (a) VO3 and (b) VO4 aptamers using the ThT titrations. Effect of sodium chloride concentration on the binding of the (c) VO3 and (d) VO4 aptamers. Effect of serum on the binding of the (e) VO3 and (f) VO4 aptamers. Experiments were performed in triplicate.

3.3.4 Selectivity of the VO3 aptamer

So far, we have identified VO3 as an optimal aptamer considering its binding affinity, specificity and robustness. To further test the selectivity of the aptamer VO3, different small molecules that have some structural similarities to VOZ and could be present in human serum were titrated using the ThT fluorescence technique. The normalized fluorescence at 500 μM concentration was collected for adenine, uracil, thymine, cytidine, thymine and compared to the same concentration for VOZ and a blank (Figure 3.4). While most of the small molecules induced a maximum 20% decrease in fluorescence, VOZ was able to reduce the fluorescence by 80%. Although VOZ is typically prescribed as a monotherapy, its structural analogue FOZ was included to assess potential cross-reactivity. The minimal response observed for FOZ

indicates that the aptamer VO3 exhibits high selectivity toward VOZ. In this work, we primarily used ThT fluorescence to characterize aptamer binding, which is mainly due to the poor solubility of VOZ and the relatively low affinity of the aptamer, disallowing us to use ITC.

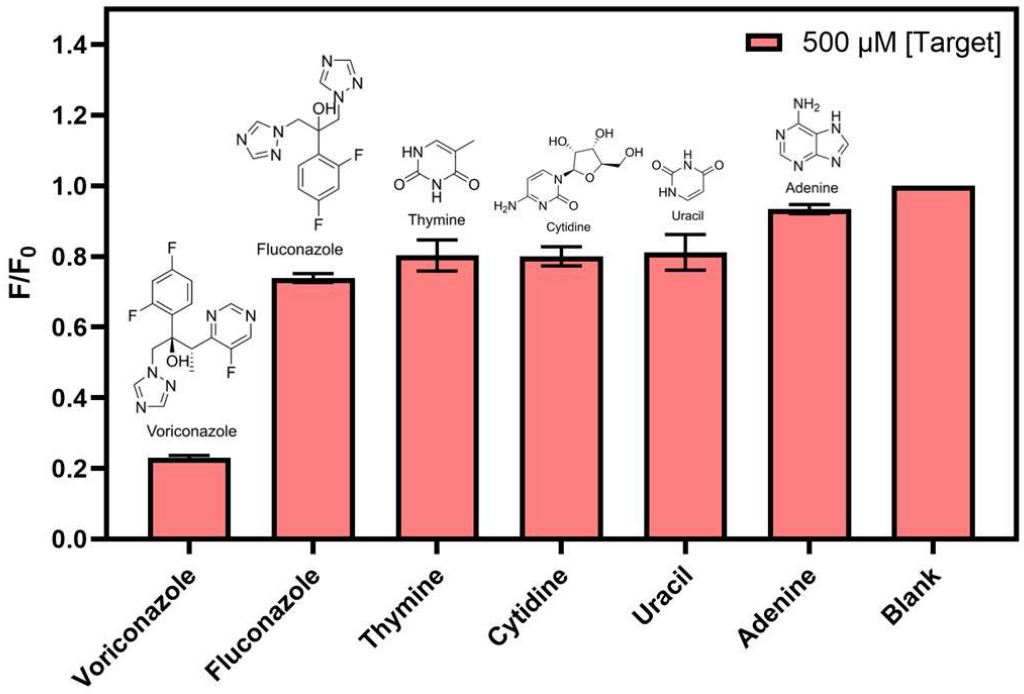


Figure 3.4 Selectivity of the VO3 aptamer in the presence of 500 μM voriconazole, structural analogue fluconazole and various small molecules.

Data represent mean ± SD from n = 3 independent measurements. One-way ANOVA with Tukey's post-hoc test showed that voriconazole generated significantly lower fluorescence than all other analytes (adjusted $p < 0.001$), confirming strong selectivity of VO3.

3.3.5 Using the VO3 aptamer to probe biding of cyclodextrins to VOZ

Thousands of aptamers have been reported in the literature with a variety of potential applications proposed like diagnostics, therapeutics, biosensing, imaging, targeted drug delivery and environmental monitoring.[23] Nonetheless, using aptamers as research tools to study other nanopolymer was not previously suggested. In this work, we used the VO3 aptamer to study the relative affinity of different cyclodextrin (CD) polymers to VOZ. CDs are circular

sugar polymers that are widely used in drug formulations and derivatives of β-CD are known to be used in VOZ formulations like the use of CBE-β-CD in Vfend®.[95] Though the interaction of β-CD with VOZ is established, most research focused on the increased solubility effect of the inclusion of VOZ in β-CD and derivatives. β-CD forms a 1:1 inclusion complex with VOZ by encapsulating its hydrophobic fluorophenyl ring within the cyclodextrin cavity, stabilized by hydrophobic and van der Waals interactions, resulting in increased solubility and reduced crystallinity. In this study, we used the VO3 aptamer to examine the affinity of β-CD and α-CD to VOZ. 30 μM VOZ, a concentration slightly higher than VO3's apparent K_D value, was added to a solution containing 500 nM VO3 aptamer, 1 μM ThT and SELEX buffer to 500 μL. After equilibrium was reached, different CD concentrations were titrated to the solution, and the titration curves were compared in Figure 3.5b. As expected, α-CD did not show any affinity with VOZ as no change in fluorescence was observed when adding higher concentrations of α-CD. Although the β-CD titration curve showed a response in the form of decrease in fluorescence upon adding higher concentrations of β-CD, the K_D value could not be determined. This is mainly because of the water solubility constraint of β-CD with a solubility of 1.8 g/100 mL corresponding to around 16.4 mM. Higher concentrations of β-CD were not soluble in water and thus could not be used for the ThT titration assays to get a meaningful titration curve and calculate the relative K_D value of β-CD. In a different attempt to study the affinities of α- and β-CD to VOZ, VOZ was titrated as usual in a series of solutions containing different concentrations of α- or β- CD: 2 mM, 6 mM and 10 mM and keeping all other component constant. The K_D values of VO3 to VOZ increased gradually with increasing the β-CD concentrations as seen in Figure 3.5d, suggesting that the affinity of β-CD to VOZ is higher than the affinity of VO3 aptamer to VOZ. When the same experiment was repeated using α-CD instead of β-CD, the K_D did not change much (as shown in Figure 3.5c) because VOZ does not interact with α-CD.

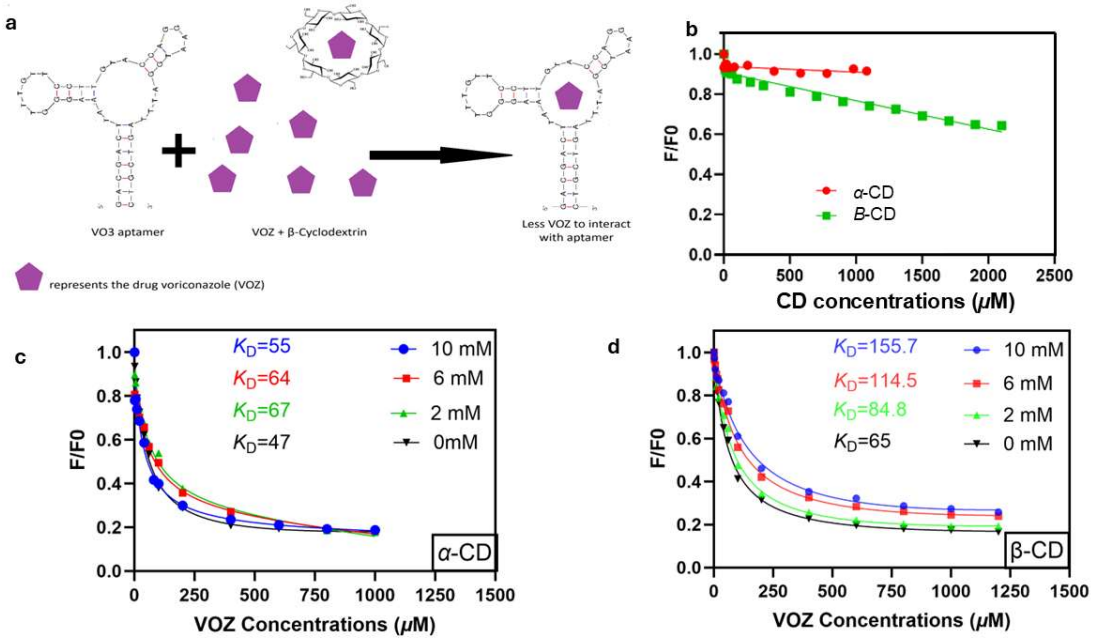


Figure 3.5 Study of the affinity of *α*- and *β*- CD with VOZ using its relative affinity to the VO3 aptamer.

A schematic representation of how *β*-cyclodextrin inhibits the aptamer-target interaction as depicted in (a). *α*- or *β*- CD was titrated in a solution containing 30 *μ*M VOZ, ThT, VO3 aptamer and SELEX buffer (b). In another approach, VOZ was titrated as usual in a series of solutions containing different concentrations of *α*- or *β*-CD (0, 2, 6 and 10 mM) and the decrease in the absolute fluorescence was observed and compared (c) and (d) for *α*- and *β*- CD, respectively.

3.4 Conclusion

In this work, ThT fluorescence-based assays were used to study the binding between VOZ and its aptamers. It's worth noting that these assays can be used as a label-free biosensor for the detection of VOZ. Previously selected VOZ aptamers were grouped into families, and three representative sequences were studied. After binding characterization for affinity, specificity and serum stability, the VO3 aptamer was found to have the best characteristics for potential biosensor applications. Characterization of aptamers already reported in the literature is essential and could help verify the functional ones and push them towards their final desired applications. Additionally, the characterized aptamer was used as a research tool to assess the

affinity of VOZ to the polymers α- and β- CD. Interesting insights regarding α- and β- CD were observed suggesting the possibility to use similar approaches with other aptamers and their targets. These approaches broaden the applications scope of the aptamers reported in the literature and open the door of using the various aptamers as fundamental research tools. Future studies can be focused on the development of advanced signaling methods by involving nanomaterials,[96–99] electrochemistry[100,101] and understanding the effect of biological sample matrix.

Chapter 4 Conclusions and Future Directions

Aptamer-based biosensors (aptasensors) have emerged as promising analytical tools for point-of-care testing (POCT) because of their high specificity, low cost, and compatibility with portable sensing platforms. They are particularly attractive for therapeutic drug monitoring (TDM), where rapid and frequent measurement of drug concentrations can improve treatment efficacy while minimizing toxicity. Despite this promise, progress has been limited by the lack of reliable aptamers for clinically important drugs. Most TDM drugs have no reported aptamers, and many published aptamers have not undergone rigorous validation, raising concerns about their reliability and reproducibility. In this thesis, I address this unmet need by developing and thoroughly characterizing DNA aptamers for two widely used TDM drugs, carbamazepine and voriconazole.

In chapter 2, capture-SELEX was used to select an aptamer for the drug carbamazepine (CBZ). After 17 rounds of selection, binding assays confirmation and the repetition of the selection by 2 different members from our group, no aptamers were enriched for CBZ. It seems that CBZ is a hard target for aptamer enrichment using the capture-SELEX method. Small-molecule targets are challenging for aptamer selection in general because they have fewer epitopes to interact with DNA, but CBZ was even more challenging because its hydrophobic rings are non-planar weakening the π-π interactions, and its only functional group suffers from steric hindrance disallowing hydrogen bonds formation. CBZ was interacting only weakly and non-specifically with the different DNA sequences since it could not form the usual non-covalent interactions necessary to form a binding pocket. Future SELEX experiments should focus on overcoming the

non-planarity challenge of CBZ by using novel selection techniques or modified DNA library to begin with.

In chapter 3, biochemical characterization assays were performed to validate the already selected aptamer for the antifungal drug voriconazole (VOZ). Using ThT fluorescence spectroscopy, we detected an aptamer with good potential for future biosensor application: VO3. The new aptamer has a K_D value of 26 μM, high selectivity even against VOZ/s structural analogue fluconazole, and good stability in more complex environment such as serum. One limitation was the inability to validate the K_D value with another orthogonal method, such as ITC, because of the chemical characteristics of the drug VOZ. Another weakness is the relatively high K_D value of the aptamer because it is higher than the therapeutic range of VOZ (3-16 μM). However, this problem can be easily solved with high amplification of the signal in the final biosensor platform design. Future studies can focus on attaching the new aptamer to an electrochemical electrode and designing an electrochemical biosensor. Furthermore, the affinity, selectivity and stability in serum and whole blood could be confirmed using electrochemical assays using the new platform. This chapter emphasizes the importance of the characterization of the hundreds of non-validated aptamers reported in the literature to help distinguish the high-quality ones from the fake or non-functional ones, and by consequence see more aptamers used in real life applications.

As a novel approach to using aptamers as research tools, I used VOZ's validated aptamer (VO3) to probe the nanopolymers cyclodextrins. Different insights were observed using a very simple technique of competitive binding assays and ThT fluorescence spectroscopy. Using such

techniques with other aptamers and nanopolymers that interact with their targets can help us learn more about important nanopolymers characteristics and dynamics.

Based on the experiments prepared in this thesis, some future research directions are summarized below. 1) The failure of the CBZ selection needs to be revisited. New selections can be conducted under different conditions such as using a longer library, a higher target concentration or a higher magnesium concentration. Selection of molecules related to CBZ can also be informative to provide insights into the feasibility of obtaining aptamers for CBZ. 2) Currently, the voriconazole aptamers were only characterized using the ThT assay. Future work can be done by using other assay methods such as NMR and FRET.

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