Screening the structure and binding affinity of ochratoxin A aptamers

by

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Abstract

Mycotoxins are fungal secondary metabolites that contaminate a wide range of agricultural commodities worldwide. Ochratoxin A (OTA) is of particular interest as it is one of the most abundant food-contaminating mycotoxins. Produced by numerous fungal species from the *Aspergillus* and *Penicillium* genera, research suggests that OTA may be teratogenic as well as immunotoxic to humans and carcinogenic to rodents. In addition to its toxicity, OTA is a stable molecule that can resist most food processing and does not result in any visible damage to crops, therefore careful testing is required of all crop samples prior to processing. Aptamers are single-stranded oligonucleotides, typically DNA or RNA, that are capable of specifically interacting with high affinity to a desired target. Recently, several groups have developed aptamers for OTA. We have aimed to understand the secondary structures of these aptamers using a DNase I assay. It is also useful for future applications of these aptamers, to compare their affinities using the same analytical techniques and conditions. The affinity of each aptamer to OTA has been tested using a DNase I assay, as well as a magnetic bead affinity assay. We found that using K_d affinity methods to compare aptamers was unreliable and instead developed several direct competitive affinity tests. Initial results appear promising, however further testing is required. Once the optimal conditions are found, the optimal aptamer can be chosen for use in existing antibody technology. This can be done to develop useful sensors and detection methods, such as the preparation of a lateral flow assay for cost efficient, onsite detection of OTA. The production of these novel detection platforms will allow rapid detection of this problematic mycotoxin,

reducing the amount of grain waste and the cost of detection as well as ultimately reducing the exposure of mycotoxins to humans.

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List of Abbreviations

6-FAM – 6-carboxyfluorescein phosphoramidate

ATP – adenosine triphosphate

AuNP – gold nanoparticle

BPA – Bisphenol A

CPG – controlled pore glass

CD – circular dichroism

CE – capillary electrophoresis

CV – crystal violet

DNA – deoxyribonucleic acid

DNase I – deoxyribonuclease I

EDC – 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride

EDTA – ethylenediaminetetraacetic acid

ELAA – enzyme-linked aptamer assay

ELISA – enzyme-linked immunosorbent assay

ESI– electrospray ionization

EU – European Union

FLD – fluorescence detection

FDA – US Food and Drug Administration

GAP – good agricultural practice

GC – gas chromatography

GMP – good manufacturing practice

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC – high performance liquid chromatography

HRP – horseradish peroxidase

IDT – Integrated DNA Technologies

IDV – integrated density value

K_d – dissociation constant

LC-MS/MS – liquid chromatography coupled with tandem mass spectrometry

LFA – lateral flow assay

MES – 2-(N-morpholino)ethanesulfonic acid

MS – mass spectrometry

NOEL – no observed effect level

OTA – ochratoxin A

OTB – ochratoxin B

PAGE – poly(acrylamide) gel electrophoresis

PCR – polymerase chain reaction

PEG – polyethylene glycol

RNA – ribonucleic acid

SPCE – screen printed carbon electrode

SPE – solid phase extraction

SPR – surface plasmon resonance

SELEX – systematic evolution of ligands by exponential enrichment

TBE – tris(hydroxymethyl)aminomethane/borate/ethylenediaminetetraacetic acid buffer

TEMED – N,N,N',N'-tetramethylethylenediamine

TLC – thin layer chromatography

tris – tris(hydroxymethyl)aminomethane

UV-Vis – Ultraviolet-visible spectrophotometry

Chapter 1: Introduction

1.1 Mycotoxins

Mycotoxins are fungal secondary metabolites, which can contaminate a wide range of agricultural commodities worldwide¹. Mycotoxins were first discovered in the 1960's, when it was discovered that turkeys ingesting peanut meal were becoming sick from aflatoxin contamination². This discovery led to extensive research occurring in the field, and in the fifteen years following, a wide variety of mycotoxins were discovered³. Today 300 to 400 compounds have been identified as mycotoxins⁴. The most common mycotoxin-producing species of fungi are from the *Fusarium*, *Aspergillus* and *Penicillium* genera⁴. It is known that mycotoxin production is not essential for the growth or survival of fungi; however, their purpose is not fully understood. One possibility suggested in literature is that this fungal virulence helps protect the fungus from other organisms that may feed on it⁵. Some of the major mycotoxins which infect crops are summarized in table 1-1.

Most mycotoxins have a low acute toxicity. For example, aflatoxin B1 has an LD₅₀ in male rats of 5.5mg/kg, while botulinum toxin B has an LD₅₀ in mice of 10⁻⁶mg/kg⁶. The main concerning issue of mycotoxin exposure is their chronic toxicity. Prolonged exposure to mycotoxins can cause major health problems in animals and humans⁶. In rats, aflatoxin has been described as one of the world's most potent carcinogens⁷.

While classified together based on their propensity to cause mycotoxicosis in animals, mycotoxins are actually quite diverse molecules that can have different toxic effects². The diversity of mycotoxin structures make it difficult to develop a single method for simultaneous detection⁸. However, since many fungi are capable of

producing more than one type of mycotoxin, it is likely that crops will be contaminated with multiple compounds. More resources then must be used to monitor crop contamination to ensure public safety, resulting in more funds going toward crop analysis.

Table 1-1. Primary mycotoxins of concern⁹.

Mycotoxin Group	Most common toxin	General Structure	Fungi Genera
Aflatoxin	<i>Aflatoxin B1</i>		<i>Aspergillus</i>
Fumonisin	<i>Fumonisin B1</i>		<i>Fusarium</i>
Ochratoxin	<i>Ochratoxin A</i>		<i>Penicillium</i> and <i>Aspergillus</i>
Trichothecenes	<i>Deoxynivalenol</i>		<i>Fusarium</i>
Zearalenone	<i>Zearalenone (F2 toxin)</i>		<i>Fusarium</i> and <i>Gibberella</i>

1.1.1 Mycotoxin Occurrence

The production of mycotoxins is highly dependent on environmental factors including temperature and availability of water⁹. Often the compounds are formed during the growing season, however, they can also be formed during storage, transportation, and processing under certain conditions¹⁰. Another factor in mycotoxin occurrence is crop damage by insects. This stress on the crop makes it easier for fungi to proliferate and produce mycotoxins¹⁰. The ideal locations for the production of mycotoxins include temperate to subtropical climates. Therefore, mycotoxin contamination in developing countries is of the highest concern due to their climate conditions being optimal for fungal growth¹¹. In these poorer regions a large percentage of agricultural activities is subsistence farming and therefore does not face stringent quality control practices¹². Furthermore, there is a lack of sophisticated storage facilities, as well as little consumer or producer knowledge on the issue of mycotoxins¹⁰.

Given that mycotoxin contamination is very heavily dependent on climate, it is inevitable that climate change will have an effect on the occurrence of these toxins. Currently, this issue is not examined thoroughly in literature, however it has been suggested that the occurrence of mycotoxins in temperate regions, such as parts of Europe and the United States of America will be affected the most by climate change¹³. The temperate climate in these areas is expected to become warmer, generating a more optimal environment for mycotoxin production, in particular aflatoxin production, which is a mycotoxin of high concern¹⁴. At the same time, these temperate regions could become too warm for the production of some *Penicillium* and *Aspergillus* toxins, such as ochratoxin A, and therefore their occurrence may decrease¹⁴. The rising

temperature in regions which are already very warm, such as Australia, may result in decreased mycotoxin production¹⁴. Regions such as Canada and Russia, which are cold climates, may see an increase in toxins such as ochratoxin A and patulin, which prefer temperate climates¹³. Although the effects of climate change cannot be known for certain, it is clear that the change in conditions may potentially impact the global issues of mycotoxin contamination.

1.1.2 Economic implications

There are multiple impacts of mycotoxins on society from an economic standpoint. There are effects felt from the loss of profit due to a lower quality product, and from the loss of product that is rejected and must be destroyed¹². This can be detrimental to farmers whose crop production is their livelihood. Limited crop availability is also problematic for processors and companies who need crops to manufacture their products. Furthermore, a limited supply with the same demand will result in higher priced products. On a larger scale this affects global trade and therefore global economy. With different region-specific regulations, commodities produced for trade in one country may exceed the mycotoxin concentration limit of a trade partner¹². In the USA hundreds of millions of US dollars in trade losses occur every year due to mycotoxin contamination of crops¹⁵. The other main effect is on the health care economy. The consumption of mycotoxins resulting in diseases adds an economic burden to the health care system, with an increasing need for healthcare professionals, and pharmaceuticals¹⁵. Finally livestock are also susceptible to mycotoxicosis and as a result the loss of livestock or contamination of meat products can occur¹⁵.

1.2 Ochratoxin A

Ochratoxin A (OTA) {N-[(3R)-(5-chloro-8-hydroxy-3-methyl-1-oxo-7-iso-chromanyl) carbonyl]-L-phenylalanine} (CAS 303-47-9) is a highly abundant food-contaminating mycotoxin (Figure 1-1)¹⁶. OTA was first isolated in 1965 from the mold species *Aspergillus ochraceus*¹⁷, but is also produced by *Penicillium verrucosum*¹⁸. OTA is a colourless crystalline material with a molecular weight of 403.81g/mol¹⁹. The compound has a blue fluorescence under alkaline conditions²⁰ and a green fluorescence under acidic conditions¹⁵. The chemical structure consists of a dihydroisocoumarin moiety modified with a *para*-chlorophenolic group, linked through an amide bond to L-phenylalanine²¹. Since its discovery, OTA has grown to be one of the most studied mycotoxins in food safety and regulation²². OTA is primarily produced during storage of crops. The fungi *A. ochraceus* prefers to grow in warmer conditions around 40°C, while *P. verrucosum* can grow in cooler conditions (0 to 30°C) and is often responsible for OTA production in cooler temperate zones. Both fungi have optimal growth in high water activity conditions, around 0.9 a_w , while still being able to grow down to levels of 0.8 a_w ²³.

Figure 1-1: Ochratoxin A (OTA) molecular structure.

1.2.1 Toxicology of OTA

Exposure to OTA is predominantly through ingestion of contaminated foodstuffs. OTA ingested by mammals is absorbed through the gastrointestinal tract and then quickly distributed through the blood stream to the kidneys where it can accumulate¹⁵. A study monitoring OTA administered to rats intravenously found that OTA was distributed through a two compartment system, slowly moving from the central to peripheral compartments²⁴. OTA metabolites were eliminated more quickly than the parent compound, but both were mainly excreted through the urine²⁴. In male F344/N rats the no observed effect level (NOEL) was determined to be 21 µg/kg²⁵.

The structure of OTA (Figure 1-1) contains a phenylalanine group which is thought to be responsible for its toxicity. This allows OTA to bind to phenylalanine receptors, inhibiting the production of phenylalanine-tRNA synthetase, resulting in problems with protein synthesis²⁶. It has been demonstrated that OTA acts as an immunosuppressant²⁷, teratogen²⁸, hepatotoxin²⁹, and as a potent nephrotoxin³⁰ in many animal studies. Carcinogenicity of OTA has also been studied and results have concluded that kidney carcinomas do form in mice and rats²⁵. However, OTA has been classified a Group 2B carcinogen, as there has been no sufficient data indicating that OTA causes tumours in humans¹⁵.

1.2.2 Human exposure

OTA affects many different crops around the world with cereals (i.e. maize, wheat, barley etc.) being the most impacted³¹. Other crops affected are, grapes³², coffee beans³³, cocoa beans, meat³³, and spices³⁴.

OTA is a highly stable molecule and is not easily eliminated through processing or cooking conditions. It has been demonstrated that OTA in wheat is not fully destroyed when exposed to temperatures as high as 250°C³⁵. Furthermore, OTA in whole grain wheat that had been cleaned, scoured and then baked into bread was only reduced by approximately 40%³⁶. These studies are indicative of a need to prevent heavily OTA contaminated foodstuffs from reaching the public since it cannot be completely removed.

There are multiple ways to proactively prevent the occurrence of OTA in crops. Fungicides can be used to protect crops from being infected with molds prior to harvest, while storage and transportation conditions can be monitored and controlled closely, limiting the moisture content. Additionally, crop damage can be reduced by using pesticides, bird deterrents and by following crop rotation procedures³⁷. Although these methods can reduce contamination, complete prevention of OTA production in crops is not possible. Therefore, there is a need for highly sensitive and accurate detection techniques.

1.2.3 OTA detection techniques

Currently, many different detection techniques for OTA exist. Usually a sample must undergo pre-treatment which involves extraction and cleanup methods. Liquid-liquid extraction was previously used, however this has been mostly replaced with solid phase extraction (SPE) techniques such as immunoaffinity SPE³⁸. Common separation methods used include thin layer chromatography (TLC), gas chromatography (GC) and capillary electrophoresis (CE)³⁹. The most commonly used method is high performance

liquid chromatography (HPLC). HPLC can be coupled with fluorescence detection (FLD) or tandem mass spectrometry (MS) using electrospray ionization (ESI) which offers higher selectivity than fluorescence detection⁴⁰. It has been reported that OTA exhibits optimal precursor ion signal when using positive ion ESI^{8,41}. When using LC-MS/MS it is beneficial to use an internal standard such as isotope labelled OTA or structurally similar compounds such as ochratoxin B (OTB)^{40,42}, as well as a Certified Reference Material (CRM), such as the wheat blank, BCR-CRM 471³⁹. Using these standards makes it possible to quantitatively analyze samples with higher accuracy.

Rapid screening methods have also been developed for OTA analysis, and are often used alongside chromatography methods. The most common form of these tests are enzyme-linked immunosorbent assay (ELISA) kits. These tests utilize antibodies in a competitive assay format⁴³. The benefits of these kits are that they require little sample and are usually portable, however they are not as sensitive as LC-MS/MS methods⁴³ and can have cross-reactivity problems⁴⁴.

1.2.4 Regulating OTA Contamination

The European Union (EU) has set maximum OTA limits on commodities such as unprocessed cereals and coffee beans at 5µg/kg, processed cereals for baby foods for infants and young children at 0.5µg/kg, grape juice and wine at 2µg/kg. Italy has set limits for food commodities like coffee 8µg/kg, beer 0.2µg/kg, and baby food 0.5µg/kg. Switzerland regulates spices and dried fruit at 20µg/kg and infant formula at 0.5µg/kg²². Canada does not currently have set regulations for OTA, but Health Canada has

proposed regulations similar to the EU, with baby foods around 0.5µg/kg and other commodities such as cereal products at 7µg/kg⁴⁵.

To limit the occurrence of OTA and other mycotoxins, there are a few regulated procedures in place. Good Agricultural practice (GAP) have many different definitions in place depending on the region, but in general serves as a guideline for production of healthy, sustainable crops¹⁵. This is especially important for mycotoxin regulations in terms of drying techniques since moisture provides excellent growth conditions for fungal species. Good manufacturing practice (GMP) governs the practices for handling of crops to ensure high quality products¹⁵. In the case of mycotoxins this refers to sorting through crops to remove anything that is suspected to be contaminated¹⁵.

1.2.5 Difficulties in OTA detection

Through years of research, detection techniques for OTA have been greatly improved resulting in efficient and sensitive techniques. However, there are still many difficulties in the field that need to be addressed.

Mycotoxins are produced by fungal species; therefore there can be physical signs of fungi growth, indicating that mycotoxins may be present. However, OTA contamination often does not cause any physical symptoms to an infected crop⁴⁶. This makes OTA detection much more difficult, since there is no indication of which parts of the crop may be infected, thus increasing the need for very thorough sampling processes⁴⁵.

One of the leading challenges in mycotoxin analysis is the issue of sampling. Sampling is of the utmost importance for ensuring accurate detection of OTA in crops.

Before a product can be shipped for processing, intensive analysis must be completed. OTA is not always evenly distributed throughout a crop. This means taking only one sample from a large batch may not accurately represent the overall contamination. This issue has been termed distributional heterogeneity⁴⁵. To account for this issue, crops are split up into different lots and multiple samples from each lot are required to be tested using a reasonably representative sampling plan¹⁵. Rapid on site tests are very expensive, for example the estimated cost for a 20-tonne grain truck load would be \$100 or more (personal communication Canadian National Millers Association). Furthermore, lots that test positive, require additional testing through sensitive laboratory detection techniques. These costs accumulate quickly and make sampling a highly complex and important factor in mycotoxin analysis.

Rapid on-site detection methods using ELISA have been developed in an effort to reduce the amount of samples that need to be sent for more sensitive testing. ELISA also requires less intensive clean-up methods and smaller samples⁴³. However these tests also have difficulties. First of all, ELISA test kits are for a single use only, and therefore still contribute significantly to analysis costs. Another disadvantage of ELISA based tests are that they often produce false negative, and false positive results⁴⁷. Furthermore, onsite personnel may not be as well trained in how to perform ELISA testing which can contribute to incorrect results. Samples that have false negative results can lead to OTA contamination reaching the consumer affecting the public's health. Also antibodies can sometimes react with molecules that are structurally similar to their targets which can contribute to false positives. For example, the structural difference between OTA and ochratoxin B (OTB) is one chlorine group, so if OTB is

present it can artificially enhance the OTA signal⁴⁸. Furthermore, antibodies are unstable and have limited shelf lives⁴⁹.

If a sample does exhibit positive OTA results, it must be sent for further offsite testing by highly sensitive separation techniques which were described in section 1.2.3. To prepare a sample for these analysis methods it must first undergo sample clean up and extraction procedures³⁹. It is important to be particularly diligent at this step as it will affect the purity of the sample⁵⁰. However, sample preparation is often the most time consuming step in analysis, as it can take up to 2/3 of the analysis time⁵⁰. These analysis procedures require highly trained personnel, and expensive equipment which drastically increases the cost of OTA detection.

As this section has outlined, the detection of OTA is important for human and animal health, however preventing and detecting this mycotoxin is very challenging. It is an extremely lengthy and expensive process to monitor crops for mycotoxin analysis. These challenges indicate the need for a new, efficient and cost effective method of OTA detection.

1.3 Aptamers

Aptamers are defined as short, single stranded DNA or RNA oligonucleotides that are able to form intricate tertiary structures, and exhibit binding affinity to specific targets⁵¹. These complex structures can be exploited for use in molecular recognition and sensor applications. They are especially useful in this regard due to their ability to bind to a target with strong affinity and high selectivity⁵². Aptamers are developed from a random nucleic acid library in a process called Systematic Evolution of Ligands by

Exponential Enrichment (SELEX)⁵³. It is possible to use this process to select aptamers for many different types of targets. Over the past 2 decades aptamers have successfully been developed for viruses⁵⁴, proteins⁵⁵, cells⁵⁶, and small molecules⁵⁷.

1.3.1 Aptamers as “chemical antibodies”

Antibodies have been widely used as the molecular recognition platform of choice for the past 3 decades⁵⁸. Since their development, aptamers have continuously been compared to antibodies due to their similar ability to bind to specific targets. However, aptamers offer several advantages over antibodies as molecular recognition molecules. Firstly, while antibodies must be developed *in vivo*, aptamers are developed *in vitro* offering much more control over binding conditions⁵⁷. Chemical synthesis also allows for more reproducibility with very little batch-to-batch variability. Furthermore, while antibodies are limited to non-toxic substances and molecules that illicit an immune response, aptamers can be developed for virtually any target⁵⁹. The production of aptamers is much less expensive than that of antibodies, and does not require the use of animals. Furthermore, the sequence of an aptamer can be shortened to include only the sites important for binding to its target, producing a “minimer” which further reduces the cost of aptamers⁶⁰. Another advantage of aptamers is that they are much more stable under harsh conditions and have very long shelf lives compared to antibodies⁵⁹. Aptamers can reversibly change conformations making it possible to develop aptamers in conditions with varying pH, temperatures and ionic strengths which would cause antibodies to be irreversibly denatured⁵¹. Modifications to aptamers can be applied during synthesis to increase stability and increase nuclease resistance, or allow for

different functionalities⁶¹. Additionally, aptamers can be tagged with different molecules to allow for fluorescent detection or immobilization onto a specific platform⁶¹.

These benefits have brought a lot of interest into the field of aptamers, and many different targets have been explored. This research has allowed for many different aptamers-based applications to be developed.

1.3.2 Measuring binding efficiency

Once an aptamer is developed it is necessary to test the binding efficiency it has for its target. To accomplish this, the dissociation constant (K_d) of the aptamer-target complex is measured. A dissociation constant in terms of aptamers is described as the propensity of the aptamer-target complex (C) to dissociate into individual components, aptamer (A) and target(T)⁶². This can be expressed as the equilibrium Equation 1-1.

Considering this equation the dissociation constant can be described as:

Equation 1-2⁶³:
$$K_d = \frac{[A][T]}{[C]}$$

Thus, a low K_d is desirable for an effective aptamer and in general K_d values range from the picomolar (1×10^{-12}) to nanomolar range ($1 \times 10^{-9} \text{M}$)⁶⁴.

In general, the K_d is determined by constructing a curve (binding isotherm). This is done by titrating either the aptamer or target with various increasing concentrations of the other⁶³. For example, fraction of bound target (f_t) can be determined if the initial

concentration of the target is kept constant and is then titrated with increasing concentrations of the aptamer. This can be demonstrated using Equation 1-3.

Equation 1-3⁶³:

$$f_t = \frac{[A]}{K_d + [A]}$$

The K_d can then be estimated by using a non-linear regression analysis of the curve produced from Equation 1-3 which forms a rectangular hyperbola with a saturation of 1 when graphed (Figure 1-3)⁶³.

Figure 1-2: Example of a binding isotherm constructed using Equation 1-3

Another way to demonstrate the dissociation constant for the equation 1-1 is by considering the ratio of the forward (k_{on}) and backward (k_{off}) reaction rates of the aptamer. Although two aptamers may have a similar K_d value, one aptamer may have a higher k_{on} or k_{off} rate than the other. This relationship is described by Equation 1-4.

Equation 1-4:

$$K_d = \frac{k_{off}}{k_{on}} = \frac{[A][T]}{[C]}$$

There are numerous methods that have been developed to determine the K_d of an aptamer, and more are still being developed. The method used is dependent on the target and conditions that the aptamer would be used in.

Separation-based methods are the most used for K_d Determination. These techniques include using high performance liquid chromatography (HPLC), equilibrium dialysis, and affinity chromatography⁵⁷ for separating the aptamer-target complex from free components. The K_d can then be calculated using methods such as fluorescence detection. However, these techniques are based on the change in size that happens when the aptamer-target complex forms, making this technique ineffective for small molecules⁵⁷. Other techniques that are commonly used include fluorescence polarization, surface plasmon resonance (SPR), and circular dichroism (CD)⁶³.

When studying interactions between an aptamer and its target it is important to limit any possibility of non-specific binding. Non-specific binding can occur due to electrostatic or hydrophobic interactions between an aptamer and materials used in the study⁶⁵. These interactions can result in an artificially improved K_d value.

1.3.3 Aptamer applications

The properties described in section 1.2.2 make aptamers ideal for use in many different applications. Current research is moving towards applications that can apply previously selected aptamers. To-date, aptamers have been used in drug delivery systems⁶⁶, drug therapy⁶⁷, bio-imaging⁶⁸, stationary phases for affinity

chromatography⁶⁹, and cancer therapy⁷⁰, however, aptamers are most commonly employed in biosensors⁶¹.

A biosensor is comprised of two main components: the bio recognition element, (in this case, the aptamer) and a transducer that can transform a signal from the analyte into a detectable signal⁷¹. When aptamers are employed, they are sometimes called aptasensors⁷¹.

Optical biosensors are the most common aptasensor. These biosensors often involve fluorescence detection⁶¹. This type of detection can be achieved by modifying the aptamer with a fluorophore-quencher pair. For example, a sensor can be designed such that when the aptamer is unbound, the quencher molecule is too far from the fluorophore to have any effect. However, when the target is present, the aptamer will fold and bring the quencher close to the fluorophore, and a loss of fluorescence can be observed⁵¹. This type of sensor has been used to detect L-argininamide with maintained aptamer affinity⁷². This type of biosensor can also be constructed using quantum dots⁷³. Colourimetric biosensors utilizing gold nanoparticles (AuNPs) are also possible. Aptamers can be conjugated with AuNPs so that when target is present and binding occurs there is a visible colour change in the solution. This type of sensor has been used to detect the presence of platelet-derived growth factors⁷⁴. Another prevalent type of optical sensor uses dyes that will non-covalently bind with the aptamer such as crystal violet dye (CV). This interaction increases the originally weak fluorescence of CV. When the target is present it causes the aptamer to fold and reduces the fluorescence of CV. This type of sensor has been demonstrated with thrombin binding aptamer⁷⁵.

Lateral flow assay devices have also been constructed with aptamers. The aptamer can be substituted for the antibodies normally used in these devices. This method was used to detect thrombin using two different aptamers for the target by Xu *et al* in 2009⁷⁶. The device was designed so that when the target migrated up the device it could bind a primary aptamer conjugated with gold nanoparticles. This complex then continued to migrate and would be stopped at the testing site when it reached the immobilized secondary aptamer. The accumulation of the complex would cause the conjugated gold nanoparticles to aggregate and form a visible red line. Any excess gold nanoparticle conjugated aptamer that continued to migrate would be stopped once it reached the control zone on the test and again would form a red line. Therefore, if two lines appear there was target present in the sample, and if only one line appeared there was no target present⁷⁶. Another type of LFA is designed to only produce a visual test line if the target is not present. In this type of test, target can be immobilized at the test site. If target is present in a sample, then it will bind with the aptamer as it migrates up the strip before it reaches the test site preventing it from stopping to form a test line. Therefore only the control line will appear if target is present. This type of test has been used to design a device for detecting the Hepatitis C virus⁷⁷.

Aptamers can also be used in an ELISA analogous biosensor called an enzyme-linked aptamer assay (ELAA). In a direct competitive ELAA the aptamer is immobilized to the wall of a microtiter well and then incubated with a mixture of the sample containing target and enzyme conjugated target. The enzyme-conjugated target will produce a colour change and this colour change can be measured. The higher this signal the lower the amount of target was present in the sample. An indirect competitive

ELAA can also be designed with immobilized target that has to compete with free target to bind with enzyme-conjugated aptamers. These methods were used by Baldrich *et al* in 2004 to develop biosensors for thrombin⁷⁸, and also by Barthelmebs *et al* in 2011 for OTA in wine⁷⁹.

1.4 Aptamers for alternative OTA detection

Aptamer-based biosensors offer a possible solution to some of the current issues in OTA detection offering improved quality control in food safety. Currently five aptamers have been developed for OTA using SELEX and many researchers are investigating implementing them in possible biosensors. The current aptamers and their reported K_d values can be seen in table 1-2.

Table 1-2: OTA aptamers, their sequences and reported K_d values.

DNA Aptamer	Sequence	Aptamer Mass g/mol	Reported K_d value for OTA (nM)
1.12.2	5'-GATCGGGTGTGGGTGGCGTAAAGGGAGC ATCGGACA-3'	11311.3	50 ⁸⁰
H12	5'-GGGAGGACGAAGCGGAACCGGGTGTGGG TGCCTTGATCCAGGGAGTCTCAGAAGACACGC CCGACA-3'	20569.3	96 ⁷⁹
H8	5'-GGGAGGACGAAGCGGAAGTGGGTGTGGGG TGATCAAGGGAGTAGACTACAGAAGACACGCC CGACA-3'	20690.4	130 ⁷⁹
A08	5'-AGCCTCGTCTGTTCTCCCGGCAGTGTGGGC GAATCTATGCGTACCGTTTCGATATCGTGGGGA AGACAAGCAGACGT-3'	23507.2	N/A*
B08	5'- AGCCTCGTCTGTTCTCCCGGCGCATGATC ATTCGGTGGGTAAGGTGGTGGTAACGTTG GGGAAGACAAGCAGACGT-3'	23627.3	N/A*

* Dissociation constants have not yet been published for these aptamers

1.4.1 OTA aptamer 1.12.2

The first aptamer developed for OTA was selected using SELEX by Cruz-Aguado and Penner, in 2008⁸⁰. This aptamer was optimized to the resulting minimizer aptamer 1.12.2, which contains 36 bases. This aptamer exhibited a K_d value of 200nM to OTA when using an equilibrium dialysis technique. Further optimization studies revealed the importance of both pH and the presence of divalent cations. The introduction of calcium in the aptamer buffer reduced the K_d value to 50nM⁸⁰. It is theorized that binding of the aptamer to OTA is enhanced in presence of calcium due to the formation of a coordination complex between the calcium ion and the carboxyl and 8-hydroxyl groups

of OTA. Furthermore, it was shown that at an acidic pH the binding efficiency of the aptamer was compromised, likely due the protonation of the 8-hydroxyl group on the OTA molecule. The selectivity of the aptamer was shown by a lack of affinity to both n-acetyl-L-phenylalanine, and Warfarin which both have similar structures to OTA. Moreover, the aptamer showed a 100 time decrease in affinity for ochratoxin B (OTB) which structurally differs from OTA by only one chlorine group⁸⁰.

The 1.12.2 aptamer has been incorporated into numerous biosensor platforms since its development. Many optical sensors have been developed, including colorimetric sensors utilizing gold nanoparticles¹⁶ and horse radish peroxidase (HRP)⁸¹. Numerous fluorescence based sensors have also been created using 6-carboxyfluorescein (6-FAM) measuring a quenching effect^{82–84} or a conformational change that effects fluorescence signal⁸⁵. An ultrasensitive fluorescence sensor was also developed using real-time quantitative polymerase chain reaction (PCR) which had a limit of detection of 1fg/mL⁸⁶. Recently an optical sensor has been developed using DNA-scaffolded silver-nanoclusters as the signal transducer meaning no labelling was required⁸⁷.

Electrochemical biosensor applications have also been explored. Sensors utilizing a “signal off”^{88,89} or “signal on” approach have been developed⁹⁰. Loop-mediated isothermal amplification has been implemented with 1.12.2 in an electrochemical biosensor for ultrasensitive detection of OTA⁹¹. It has also been shown that electrochemical biosensors can be developed using biotin-streptavidin interactions⁴⁷. A flow-based aptasensor using a screen printed carbon electrode (SPCE) functionalized with magnetic beads has been demonstrated⁹². Finally sensors have

been designed using aptamers immobilized to an SPCE by a polyethylene glycol (PEG) spacer⁹³ and hexamethyldiamine spacer⁹⁴.

The examples highlighted here outline the current ability to use aptamers for efficient cost effective mycotoxin analysis. These sensors offer a possible improvement on current antibody based rapid detection techniques. However, currently only one commercial kit is available from the company NeoVentures, “OTA-Sense Detection”⁹⁵. Further research in this field is necessary to broaden the availability of commercial grade detection systems for OTA.

1.4.2 Other OTA aptamers

Additional aptamers were developed for OTA by Barthelmebs *et al* in 2011. The SELEX yielded a pool of 25 aptamers with H12 and H8 exhibiting the best binding affinity and least cross reactivity with phenylalanine and OTB. Both of these sequences contained a consensus sequence, AGGGAGT, which is thought to be important for binding to OTA⁷⁹. The K_d values determined for H12 and H8 were 96nM and 130nM respectively. The aptamers were tested in both a direct and indirect competitive Enzyme-Linked Aptamer Assay (ELAA). These were both colorimetric based tests, which relied on the ability of HRP to oxidize 3,3',5,5'-tetramethylbenzidine (TMB). In the direct competitive ELAA, the OTA analyte had to compete with free OTA bound with HRP for the immobilized aptamer. In the indirect competitive ELAA, OTA was immobilized and free HRP labeled aptamer would bind with it or the free OTA analyte. The results of these assay testing indicated that the affinity of the aptamer for OTA was higher when it was immobilized in the direct competitive ELAA⁷⁹.

Further SELEX performed by Bardóczy and Mészáros generated the aptamers A08 and B08⁹⁶. These aptamers were both shown to be guanine rich sequences, and had the consensus sequence of GTGGG.

1.5 Thesis Objectives

This work described in this thesis aims to further investigate the aptamers presently available for OTA. First, the structure of these aptamers will be investigated using both a DNase enzyme test, as well as melting point studies. Since OTA occurs in very low quantities, it is important to determine which aptamer has the best binding affinity to ensure high sensitivity in future aptamer applications. This is investigated by determining each aptamer's K_d values using a magnetic bead assay as well as the DNase enzyme test. Additionally, several novel competitive assays are designed to directly compare aptamers. The aptamer with the highest affinity for OTA can be implemented into a biosensor for OTA, introducing a new robust, sensitive and cost effective method for OTA detection.

Chapter 2: Initial structural characterization of Ochratoxin A aptamers

2.0 Statement of Contribution

The DNase I enzyme assays were performed by McKenzie Smith and analyzed by Kayla Hill.

2.1 Introduction

2.1.1 Aptamer structures

Aptamers have been shown to be very useful receptors or molecular recognition elements for sensor purposes and have already been used in many applications. Prior to using an aptamer for a new application, it is useful to understand details about its secondary and tertiary structure. Aptamers form three dimensional structures that are typically important in target binding⁶¹. For example, aptamers can form loops, bulges, and double stranded helices⁹⁷. These structures are stabilized by hydrogen-bonding, as well as electrostatic and hydrophobic interactions^{98,99}. However, the presence of the target is often required for these intricate structures to form⁹⁸.

It is possible to characterize nucleic acids using their spectroscopic properties. For example, DNA absorbs ultraviolet light with a maximum absorption around 260 nm¹⁰⁰. Therefore, using the Beer-Lambert law, aptamers can be quantified. Monitoring the denaturation of nucleic acids while increasing temperature yields a thermal denaturation curve, also called a melting curve, and can provide information about the physical properties of nucleic acids. At temperatures below their melting point, an aptamer exists in the structured form and exhibits lower absorbance due to hypochromism. Hypochromism is observed due to the parallel stacking of the dipoles from each nucleotide. This will lower the overall transition dipole, and lower the

absorption¹⁰¹. Therefore this is observed when an aptamers structure changes from single stranded to double stranded. The opposite effect, hyperchromism, is observed when the dipoles are arranged linearly which increases the overall transition dipole and increases the absorption¹⁰¹. This is observed as an aptamer is denatured and exists in a single stranded form.

Another common structural motif found in aptamers with guanine rich sequences is a complex, four stranded structure, known as a G-quadruplex¹⁰² (Figure 2-1). Guanine bases can associate through Hoogsteen hydrogen bonding instead the typical Watson-Crick base-pairing¹⁰². This allows the formation of a square planar tetrad. Multiple g-tetrads on top of each other results in the formation of a G-quadruplex. The 15 base aptamer selected for the protease thrombin is the most well-known example of a G-quadruplex aptamer^{103,104}.

It is possible to determine the presence of a G-quadruplex structure using an ultraviolet absorption denaturation experiment by monitoring the absorbance of a known concentration of the aptamer sequence as the temperature is increased¹⁰⁵. Instead of monitoring the absorption of DNA at 260nm, as done for denaturation studies¹⁰⁰, it is necessary to monitor the absorbance at a wavelength of 295nm¹⁰⁵. This is the optimal wavelength to observe the hypochromism caused by the “melting” of the G-quadruplex structure¹⁰⁶. This method is a very simple and effective way to determine if an aptamer with a guanine rich sequence forms a G-quadruplex. Limited initial studies on the first OTA aptamer, 1.12.2 have shown that it likely forms a G-quadruplex as well¹⁶.

Figure 2-1: Structure of a G-quadruplex

2.1.2 DNase I

In order to make aptamer synthesis and production cost efficient, its length is often minimized from the originally selected aptamer⁶⁴. When doing this, the sequence that is essential in target binding must be determined. To date, most aptamers are minimized by synthesizing and testing variable length regions of the full aptamer. This method is not high throughput and often expensive.

Enzymatic studies, such as footprinting, may be used to monitor region-specific interactions between an aptamer and its target⁵⁷ and thus facilitate the aptamer minimization process. In this technique, a fluorescently labeled aptamer is digested by the enzyme. The resulting digested fragments can be separated and imaged using gel

electrophoresis. The pattern is dictated by the aptamer structure. Both, regions more accessible to the enzyme and those that are double stranded will be more favourably digested. If the aptamer is incubated with its target and the same digestion is performed, the target may prevent the binding regions of the aptamer from being cleaved by the enzyme¹⁰⁷. Alternatively, changes in secondary and tertiary structure triggered by target binding could lead to changes in the fragment pattern. Comparing the fragment patterns in the absence of target with increasing concentrations of the target makes it possible to identify the regions which are involved in binding. In particular a change in band intensity, or even the complete loss of bands may occur¹⁰⁸.

A commonly used enzyme for footprinting studies is the nuclease deoxyribonuclease I (DNase I). DNase I is a large (30.4 kDa) endonuclease which, in the presence of magnesium and calcium ions, interacts with the minor grooves of DNA and cleaves the phosphodiester backbone¹⁰⁹. Although this means DNase I will non-specifically cleave any sequence of DNA, its cleavage pattern depends on the varying DNA structures¹¹⁰. Furthermore, DNase will cleave both single stranded and double stranded DNA, however it cleaves single stranded DNA at a much slower rate¹⁰⁷.

Figure 2-2: DNase I in the presence of magnesium and calcium ions will digest an aptamer producing different fragments. Fragments containing fluorescence can be imaged using gel electrophoresis.

The binding affinity of an aptamer can also be determined using DNase I to digest an aptamer-target complex. This can be done by measuring the fluorescent intensity of each digested band and plotting the change in each band against the target concentration.

2.1.3 Chapter Objectives

Currently, five aptamers exist for OTA (Table 1-2). However, there have been no studies of the newly selected OTA aptamers A08 and B08. This chapter reports the initial structural studies of these two aptamers. The predicted structures for A08 and B08 were determined using Mfold which predicts the lowest free energy structure . These are found in figure 2-2; however, nucleic acid structural prediction software is limited in that it cannot predict certain secondary structures such as G-quadruplexes. Given, that the first OTA aptamer (1.12.2) was reported to possibly form a G-quadruplex structure upon OTA binding⁹² we first sought to determine if these other OTA aptamers also formed G-quadruplex structures using an ultraviolet absorption thermal melt experiment. More detailed structural studies of the OTA aptamers were performed using a DNase I assay with separation by poly acrylamide gel electrophoresis (PAGE) as described in section 2.1.2. The DNase studies were aimed at determining the important sequence and structural motifs for binding to OTA. The DNase studies also permitted the measurement of the affinity of each aptamer in solution by analysing the change in band intensity with varying OTA concentration.

2.2 Methods

2.2.1 Buffer Compositions

Table 2-1: Buffer compositions

Buffer	Components	pH
TBE	45mM Tris-HCl 45mM boric acid 1mM EDTA	8.3
A08/B08	10mM Na ₂ HPO ₄ 2.0mM KH ₂ PO ₄ 2.7mM KCl 137mM NaCl	7.4
H12/H8	137mM NaCl 2.7mM KCl 10mM Na ₂ HPO ₄ 2.0mM KH ₂ PO ₄ 1.0mM MgCl ₂	7.5
1.12.2	10mM HEPES 120mM NaCl 5mM KCl 10mM CaCl ₂	7.0
MES	0.1mM 2-(N-morpholino)ethanesulfonic acid	4.5
DNase I reaction	10mM Tris-HCL 2.5mM MgCl ₂ 0.5 mM CaCl ₂	7.0

2.2.2 Preparation of five OTA aptamers

Table 2-2: Aptamers modified with fluorescein (F)

DNA Aptamer	Sequence	Aptamer Mass g/mol
1.12.2F	5'-FGATCGGGGTGTGGGTGGCGTAAAGGGAGCATCGGACA-3'	11848.8
H12F	5'-FGGGAGGACGAAGCGGAACCGGGTGTGGGTGCCTTG ATCCAGGGAGTCTCAGAAGACACGCCCCGACA-3'	21106.8
H8F	5'-FGGGAGGACGAAGCGGAAGTGGGTGTGGGGTGATCA AGGGAGTAGACTACAGAAGACACGCCCCGACA-3'	21227.9
A08F	5'-FAGCCTCGTCTGTTCTCCCGGCAGTGTGGGCGAATCTA TCGTACCGTTTCGATATCGTGGGGAAGACAAGCAGACGT-3'	24044.6
B08F	5'-FAGCCTCGTCTGTTCTCCCGGCGCATGATCATTGGGTG GGAAGGTGGTGGTAACGTTGGGGAAGACAAGCAGACGT-3'	24164.7

The five OTA aptamers from table 1-2 were purchased from IDT. The fluorescently-labelled aptamers seen in table 2-2, were prepared on a BioAutomation Mermade 6 DNA synthesizer following standard phosphoramidite chemistry¹¹¹ using reagents purchased from Glen Research. Due to the length of the desired aptamers, 1000Å controlled pore glass (CPG) columns (BioAutomation) were used. The aptamers were modified by adding fluorescein (6-FAM) to the 5' end of the aptamers during synthesis.

The DNA was cleaved from the beads after synthesis by mixing 1 mL of ammonium hydroxide with the beads and incubating at 55°C for 24 hours. The DNA was then isolated using centrifugation to form a pellet of the beads and pipetting out the supernatant. To ensure the maximum amount of DNA was collected, the process was repeated using deionized water to wash the beads. The DNA solution was then dried down overnight using a Savant AE2010 SpeedVac on cryopump setting.

The synthesized DNA was purified using denaturing polyacrylamide gel electrophoresis (PAGE). The 12% PAGE gel was prepared by mixing 31.5g of urea (0.5245mol), 23.5mL of acrylamide solution (acrylamide/bis-acrylamide, 40% solution 19:1), 15mL of 5x TBE buffer and 14mL of deionized water. The solution was then heated to 37°C and filtered by gravity using a Whatman No. 1 filter paper. The filtrate was allowed to cool to room temperature, at which point 450µL of freshly prepared 10% ammonium persulfate and 35µL of N,N,N',N'-tetramethylethylenediamine (TEMED) was added to the mixture. The gel was poured between two glass plates with a 1 well comb and allowed to polymerize for 30 minutes. The DNA samples were prepared for analysis by adding 300µL of deionized water, 300µL of formamide and heating to 90°C for 5 minutes. The gel was pre-equilibrated in 1xTBE buffer using a SE 600 Chroma Standard Dual cooled gel electrophoresis unit at 300V for 15 minutes. The DNA samples were loaded onto the gel. To monitor the movement of the samples, a fluorescently-labelled 18 nucleotide DNA marker was also loaded on the gel. A constant ampere setting was used to run the gel at 300V until the fluorescently labeled marker had travelled $\frac{3}{4}$ of the way down the gel. The gel was then imaged on a 20x20 cm silica, fluorescein doped, TLC plate in an Alpha Imager Multi Image Light Cabinet (Alpha

Innotech) using the epi UV setting ($\lambda_{\text{excitation}}=254\text{nm}$). The fluorescent bands in the gel were also imaged using an excitation wavelength of 302nm. The bands containing the desired aptamer lengths were excised and crushed. The DNA was then extracted from the gel with 25mL of deionized water and incubated at 37°C in a New Brunswick Scientific Innova 40 Incubator shaker overnight. The mixture was filtered through a 0.22 μm cellulose acetate syringe filter to remove the gel pieces and was then freeze-dried using a Labconco Freezone lyophilizer. The DNA was desalted using Amicon-Ultra 0.5 mL 3kDa centrifuge units. To quantify the DNA, it was dissolved in 100 μL of deionized water and then diluted by approximately 100 times. The absorbance at 260nm was then measured using a Varian Cary 300 Bio UV-Vis spectrometer.

2.2.3 Thermal denaturation studies

The DNA was diluted into 3mL of A08/B08 buffer for an approximate concentration of $2 \times 10^{-6}\text{M}$, and put into a 10mm open top quartz cuvette. A Varian Cary 300 Bio UV-Vis spectrometer with a 6x6 Peltier-Thermostatted Multicell Holder, was set to heat the DNA to 20°C and then heated it up to 80°C while measuring the absorbance at 260nm at a ramp rate of 5°C/min. The same process was repeated using the same sample and setting the absorbance to be read at a wavelength of 295°C. These processes were repeated using different temperature ramp rates of either 5°C/min or 0.20°C/min. The experiment was also performed using DNA that had been mixed with OTA in a 1:5 (DNA:target) ratio prior to the melting point study. The absorbance values measured were plotted against temperature using GraphPad Prism 5, using a nonlinear regression curve.

2.2.4 OTA preparation

OTA from *Petromyces albertensis* $\geq 98\%$ (Sigma Aldrich) was dissolved in 1 mL of MES buffer. The OTA solution was quantified by measuring the absorbance at 333nm on a Varian Cary 300 Bio UV-Vis spectrometer.

2.2.5 DNase I enzyme assay

OTA dilutions of 10^{-5}M , 10^{-6}M , 10^{-7}M , 10^{-8}M and 10^{-9}M were prepared in deionized water. Fluorescently labeled DNA (table 2-2) was prepared to a dilution of $1 \times 10^{-5}\text{M}$ using the appropriate aptamer buffer from table 2-1. The DNA was allowed to bind with OTA by mixing 10 μL of DNA with 10 μL of each OTA dilution and shaking on a vortex for 30 minutes. After mixing, 80 μL of 1x DNase I reaction buffer was added to each mixture. Two controls containing just 10 μL of DNA were also prepared. The first, the negative control, had 82 μL of 1xDNase I reaction buffer added. The second, the target negative control, had 81 μL of 1xDNase I reaction buffer added. One unit of DNase I (1 μL) was then added to the target negative control sample, as well as to each of the aptamer-target mixtures. This was vortexed briefly and incubated at 37°C for precisely 3 minutes. The DNase I was inactivated by adding 1 μL of 90°C 0.5M EDTA solution, vortexing and incubating at 90°C for 10 minutes. The samples were mixed with 102 μL of formamide and heated to 55°C for 5 minutes. The DNA fragments after digestion were separated on a 19% denaturing PAGE gel by loading 80 μL of each sample into separate wells.

To analyze the data from the gel, AlphaView software was used. The integrated density value (IDV) of each band observed was divided by the IDV of the whole lane to

obtain a ratio (band:lane). The ratio of each band in a row was then plotted against OTA concentrations with GraphPad Prism 5 using a nonlinear regression with Equation 2-1.

Equation 2-1

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{((LogEC50 - x) * HillSlope)}}$$

2.3 Results and discussion

2.3.1 Predicted Structures by Mfold.

Figure 2-3: Predicted structures for A) A08 aptamer, B) B08 aptamer, C) H8 aptamer, and D) H12 aptamer, using Mfold Web Server¹¹².

2.3.2 Melting point studies on A08 and B08

The aptamers A08 and B08 both have guanine rich sequences hence we wanted to determine if these aptamers formed G-quadruplex structures. We examined the absorbance of each aptamer using ultraviolet spectroscopy, as the temperature was increased.

Figure 2-4: Melting point curve for A08 aptamer demonstrating hyperchromicity at a wavelength of 260nm and using a ramp rate of 5°C/min.

Figure 2-5: Melting point curve for B08 aptamer demonstrating hyperchromicity at a wavelength of 260nm and using a ramp rate of 5°C/min.

Figure 2-6: Melting point curve for A08 aptamer at a wavelength of 295nm and using a ramp rate of 5°C/min. The curve does not demonstrate hypochromicity which would be represented by a decrease in absorbance. This indicates that a G-quadruplex was not present.

Figure 2-7: Melting point curve for B08 aptamer at a wavelength of 295nm and using a ramp rate of 5°C/min. The curve does not demonstrate hypochromicity which would be represented by a decrease in absorbance. This indicates that a G-quadruplex was not present.

Figure 2-8: Melting point curve for A08 aptamer at a wavelength of 295nm with increasing temperature using a slower ramp rate of 0.20°C/min. The curve does not demonstrate hypochromicity which would be represented by a decrease in absorbance. This indicates that a G-quadruplex was not present.

Figure 2-9: Melting point curve for A08 aptamer at a wavelength of 295nm with increasing temperature using a slower ramp rate of 0.20°C/min. The curve does not demonstrate hypochromicity which would be represented by a decrease in absorbance. This indicates that a G-quadruplex was not present.

The change in absorbance of A08 and B08 with an increasing temperature rate of 5°C/min was monitored at a wavelength of 260nm. As expected, we observe hyperchromicity in figures 2-4 and 2-5, caused by the melting of duplex structures in the DNA. The melting points for A08 and B08 from this experiment were determined to be 52.28±0.12°C and 53.65±0.23°C. The obtained values compare well to the melting points predicted using Mfold version 3.5¹¹², as can be seen in Table 2-3.

Table 2-3: Melting points predicted and observed for A08 and B08 aptamers¹¹².

Aptamer	Melting point from predicted structure in Mfold T_{pm} (°C)	Observed melting point T_{om} (°C)
A08	56.5	52.3±0.12
B08	62.4	53.7±0.23

The same samples were analyzed at a wavelength of 295nm using the same temperature increase rate. The resulting melting point curves can be seen in figures 2-6 and 2-7. It is expected if an aptamer forms a G-quadruplex, hypochromism will be observed, caused by the denaturation of the structure into a single stranded structure¹⁰⁵. This effect was not observed with either aptamer and instead an increase in absorbance with increasing temperature is observed. These results suggest that no G-quadruplex structure is formed. The experiment was then repeated with a slower temperature ramp rate, as increasing the temperature too fast may not allow sufficient time for the aptamer solution to reach the desired temperature. Therefore, the temperature recorded in the experiment may not reflect the actual experimental

temperature... Again the melting point curves obtained for A08 (figure 2-8) and B08 (figure2-9) had positive slopes indicating that no G-quadruplex was present.

Figure 2-10: Melting point curve for A08 aptamer in the presence of OTA at a wavelength of 295nm and using a ramp rate of 5°C/min. The curve does not demonstrate hypochromicity which would be represented by a decreased in absorbance. This indicates that the aptamer did not form a G-quadruplex even when target was present.

Figure 2-11: Melting point curve for A08 aptamer in the presence of OTA at a wavelength of 295nm and using a ramp rate of 5°C/min. The curve does not demonstrate hypochromicity which would be represented by a decreased in absorbance. This indicates that the aptamer did not form a G-quadruplex even when target was present.

As discussed in section 2.1.1, many studies have found that aptamers may only form a G-quadruplex structure in the presence of their target. Therefore, although our results indicate that both A08 and B08 do not form into a G-quadruplex, the presence of OTA may be required to induce folding. To test this, each aptamer was combined with OTA, in a 1:5 (DNA:Target) ratio. The melting point curve of each aptamer was then obtained using a wavelength of 295nm, and increasing the temperature by 5°C/min. The results for both A08 and B08 again were not consistent with the melting of a G-quadruplex, as can be seen in figure 2-10 and 2-11.

From the results one can conclude that despite their high guanine content, neither the A08 or B08 aptamer forms a G-quadruplex structure in either the presence or absence of their target.

2.3.3 DNase I assay

As mentioned previously, to minimize cost and improve the efficiency of producing aptamers it is beneficial to minimize the length of their sequence, by removing regions that are not required for binding. As described in section 2.1.2 the DNase I assay may help identify regions required for target binding, by monitoring the change in digestion fragments of the aptamer when it is bound or unbound to OTA.

Figure 2-12: Gel electrophoresis image of B08 Trial 1 digestion with DNase. DNA was run on a 19% PAGE gel for 3 hours and imaged using an Alphamager with AlphaView software. The predominant fragments produced by digestion with DNase I enzyme can be seen in rows A to F.

Figure 2-12 shows the results of B08F aptamer being tested using the DNase I enzyme assay. The first lane, the negative control, shows the fluorescent aptamer that was not digested or bound with target. Since this sample contains the undigested aptamer sequence, it has a high molecular weight and moves slowly through the gel. There was very little streaking, indicating that the DNA used was pure, containing only the full sequence. Lane 2 shows the negative target control which contains DNase I-digested aptamer. The absence of a band at the top of the gel in this lane indicates that there is no longer any complete aptamer after digestion by the enzyme, demonstrating that the enzyme is highly efficient in digesting the aptamer. It is important to run this control to establish the fragment pattern that occurs in the absence of target and is dictated only by the initial secondary and tertiary structure of the aptamer. Figure 2-12 shows that even when there is no target present, different sized spacing between fragments is observed. The variations in band spacing are dependent on the free aptamer structure. Although DNase I is known to non-specifically cleave double and single stranded DNA at all bases, different structural formations can affect the cleavage efficiency of the enzyme, therefore it is expected that there will be variable intensities for each row. In lanes 3 to 7 the aptamer was allowed to bind with increasing OTA concentrations prior to digestion. It is possible that where OTA interacts with the aptamer, enzymatic cleavage efficiency is reduced due to protection of the region by the target. This should result in a decrease in certain fragments. Binding of OTA to the aptamer may also result in a conformational structure change, resulting in formation or breaking of double stranded regions that were previously cleaved by DNase I. Therefore, a target-induced structural change may result in the decrease or increase of

aptamer fragments. Overall, with increasing OTA concentration, we expect to see a greater effect in the change of fragment occurrence. This effect is observed by comparing the intensity of bands in each of the samples containing various OTA concentrations. The fragments that migrate at similar rates appear in a row, this can be seen in figure 2-12 where there are 6 rows, labeled A-F. Note that some rows, for example row C may have contained multiple digests. For this experiment if rows were difficult to resolve, they were combined as one row. It is then possible to measure the visual intensity, in the form of an integrated density value (IDV), of each lane and band. We then take a ratio of the band to lane IDV (band:lane), and plot this ratio vs the OTA concentration used for each lane, giving us a binding isotherm. The resulting binding isotherms obtained from the gel analysis in figure 2-12 are found in figure 2-13.

Figure 2-13: Binding isotherms for rows A-F from B08 enzyme assay Trial 3 seen in Figure 2-12. Each curve was analysed using GraphPad Prism 5, using a nonlinear regression fit with equation 2-1.

As can be seen in Figure 2-13, not every row from the gel resulted in a binding isotherm that results in a predicted K_d . This is because, as described in section 2.2, only certain residues are typically involved in direct binding with target. An appropriate binding isotherm that describes a specific binding interaction of the aptamer and target will resemble a sigmoidal function when plotted using with a logarithmic x-axis scale. A row that results in a linear trend with a change in concentration, or that cannot be fit to the binding model (equation 2-1), indicates that there is no relevant change in the bands

observed. This may represent a fragment where the DNase I cleavage was not involved in binding to the target. In figure 2-13 we see that the nonlinear regression for row F was not possible, as the data did not fit the parameters of equation 2-1. Although the rest of the rows were analyzed and a nonlinear regression was calculated, it is clear that some of the rows do not show binding. For example both row A and B, show a linear trend with the exception of one point, while row C exhibits a completely linear trend. Although the fit does predict a K_d value for these curves seen in figure 2-13, by looking at the gel, and the curve, it is evident that there is very little change in band intensity and these bands do not indicate binding.

Rows D and E of the gel image in figure 2-12 both display a change in intensity with increasing OTA concentration. This is further seen in the expected sigmoidal curve shaped K_d curves observed in figure 2-13. As there is an increase in row intensity with increased concentrations of OTA, it is likely that this region has undergone a significant conformational change allowing more efficient DNase I cleavage at this site. Therefore, it is possible that the change in conformation is due to target binding. Therefore, this data can be used to calculate a K_d value for the curve. For row D the K_d was calculated to be 862nM, while the K_d for row E was 1081nM. These values are relatively consistent, within the error of the assay, which further suggests that these fragments are potentially involved in the OTA-bound conformation of the aptamer. Two more trials analyzing B08F produced similar results with rows D and E always producing binding curves in all trials. The data from these trials can be seen in appendix A figure A-1. The average K_d obtained from these experiments can be seen in Table 2-4. The consistency of the bands D and E continuously producing K_d plots, with changing band intensity,

provides strong evidence that the regions containing these fragments are important in aptamer folding. Since the fragments that are visible on the gel must contain the fluorescein tag and therefore have the 5' end of the sequence, it would be useful to start designing and testing minimizer aptamers by removing part of the sequence at the 3' end. This can increase the efficiency of minimizer testing by providing some initial insight.

Table 2-4: Results obtained from DNase I assay for OTA aptamers, including bands indicative of a binding event and the corresponding K_d values.

DNA Aptamer	Studied bands	Dissociation Constant K_d (nM)	Error (nM)
B08F	D, E	989	792
A08F	D	481	503
H12F	N.B	N.B*	N.B
H8F	N/A [†]	865	213
1.12.2F	N.B	N.B*	N.B

*N.B = no change in banding pattern observed

[†]N/A = There was only 1 gel with useable results

A08F resulted a similar trend to B08F, with row D continuously resulting in a changing intensity and a K_d curve (data seen in figures A-3 and A-4 of appendix A). The average K_d value obtained for A08F was 481 ± 503 nM, which is comparable to the affinity that B08F demonstrated for OTA. These results were more interesting however with regards to aptamer structure. As with B08F, we can conclude that row D contains an aptamer fragment that is likely important in aptamer binding and this would be taken

into consideration when trying to design a minimizer for this aptamer. Furthermore it is interesting that these aptamers, which were selected in the same SELEX process, both seem to involve regions close to the 5' end of the aptamer in target binding. This finding underscores the importance of aptamer structure in target binding and validates our method for structure and binding determination.

Having validated this method for K_d determination using the A08 and B08 aptamers, we applied this method to the other 3 previously characterized OTA aptamers. Some limited structural information on these aptamers is available; however this method has not been applied to these aptamers. In particular, the aptamers H8 and H12 have not been minimized and this method may provide insight into the structural features important for these sequences.

The gel images obtained for H12F can be seen in appendix A figure A-5. These images show that the digestion of the aptamer in the target negative control did not result in an accumulation of any distinct fragments, as there are no separated bands visible and instead, there is just a long smear. This indicates that H12F may not be a highly structured aptamer and therefore the DNase I digested all of the aptamer fairly consistently, resulting in a smear instead of distinct bands that we can measure. Furthermore, a lack of distinct bands is also observed in the digested aptamer mixed with target. This could be due to a few different factors. First it is possible that the conditions, such as buffer composition and pH, used in this test were not ideal for the aptamer to form a stable structure. Since structure is important in aptamer folding this could prevent the aptamer from properly folding and furthermore prevent OTA binding. Additionally, low structured aptamers may be difficult to probe with this assay, as

sufficient intricate structures are required to observe fragmentation by DNase I. On the other hand, H8 (Appendix A, figures A-6 and A-7), did have some distinguishable bands seen in its gel images, however only the first trial showed any binding results, with two rows that had a slight observable change in intensity. From these two binding isotherms the K_d was calculated to be $865 \pm 216 \text{ nM}$. However, it appears that, similarly to H12F, the lack of structural complexity of the aptamer in this test, results in insufficient information to predict a possible structure for testing a minimizer sequence.

The aptamer 1.12.2 in this test did not show any change in band pattern. In the absence of OTA, the digestion did result in some separated bands in the images (Appendix A figure A-8), however with increasing concentration of target, the intensity of each band in a row did not drastically change, resulting in linear curves, or data that could not be fit with equation 2-1. These results indicate that 1.12.2 does fold into a highly structured conformation, even without the presence of target, however does not undergo any significant conformational changes when bound to the target. This has been shown with many other aptamers¹¹³. Compared to the other aptamers that have been discussed so far, 1.12.2 has a much shorter sequence as it was already optimized by Cruz-Aguado and Penner. It is possible that since the aptamer has already been minimized, the entire sequence is important for binding. It is possible that the optimization of this aptamer has resulted in it continuously existing in a highly structured form, and further folding is not required or possible in binding to the aptamer. Furthermore, there is a very intense band in row A of every sample, which can be observed in appendix A figure A-8, trials 2 and 3, indicating that there is still a lot of undigested aptamer present in the sample. This further signifies that the 1.12.2 aptamer

is highly structured, and this affects the ability of the DNase I to digest the aptamer efficiently.

In addition to the challenges already discussed, another important consideration is the timing of enzyme cleavage. As explained in section 1.3.2, the formation of the aptamer-target complex is in equilibrium with the free aptamer and target, thus if the enzyme digests the aptamer too quickly, the digestion pattern may not be affected by the binding of the target. DNase I has been shown in literature to be much slower at digesting single stranded DNA than double stranded DNA¹⁰⁷. Taking this into consideration, it is possible that OTA has a relatively slow on and fast off rate for binding to 1.12.2 and therefore the binding event may not be captured by the cleaving enzyme. It would be useful then, to study the kinetic relationship between an aptamer and its target prior to choosing an enzyme for this experiment.

Through this work we have concluded that using an enzyme such as DNase I to analyse an aptamer's structure is possible and we have suggested regions that may be important in binding for both A08 and B08 aptamers. This can allow for higher efficiency in determining minimiser structures for aptamers, lowering the cost and time of this process. This method, being based on previous DNase I footprinting techniques, is useful for small molecules, but also for many other types of targets such as proteins and viruses. Challenges of the technique have also been identified. In particular, DNase I may not be an appropriate enzyme to probe all aptamer structures. Lack of cleavage by DNase I suggests that a nucleic acid is of low structural complexity. However, it is unlikely that an aptamer with low structural complexity can bind to its target in solution. Since this test failed on aptamers that have been previously characterized to bind to

OTA, this suggests that DNase I may not be able to resolve the structures formed by these aptamers, under the binding conditions used in the assay. Further work will be done to determine if the lack in H12 fragmentation was a result of the buffer conditions or the enzyme activity.

2.3.4 Future work

Although it was concluded that it is unlikely that A08 and B08 form G-quadruplex structures, further analysis would be needed to confirm these findings and to accurately determine the structure of these aptamers.

It would be useful to compare the results of more highly structured and simple aptamers to confirm whether DNase I is more useful with highly structured aptamers. The test may be optimized with different conditions to allow for the detection of smaller conformational changes by changing the enzyme or target concentrations. Furthermore, since DNase I is slower at cleaving single stranded DNA, it may be beneficial to optimize the method using different enzymes. Having multiple enzymes with different functions would allow the test to be tailored depending on the type of aptamer being used. To further investigate the accuracy level in which this test is able to provide useful information in predicting important binding regions of an aptamer, the full length aptamer 1.12, developed by Cruz-Agado and Penner, could be analyzed. It would then be possible to check if the binding regions predicted from the test are in fact present in the actual minimizer sequence, 1.12.2.

**Chapter 3: Using a magnetic bead assay for the determination of the binding
affinity of aptamers for ochratoxin A**

3.0. Statement of contribution

McKenzie Smith assisted by performing some of the magnetic bead assays.

3.1 Introduction

3.1.1 Multiple Aptamers for one target

Aptamers are selected, through a process called SELEX⁵³, under specific conditions to bind with a high affinity and selectivity to a molecular target. This process has been shown to be highly successful for a variety of targets, resulting in aptamers that can bind with dissociation constants in the nanomolar range such as thrombin¹⁰³, bisphenol A (BPA)¹¹⁴, and tetracycline¹¹⁵.

The selection method can be tailored by changing certain conditions, such as temperature, selection buffer, or pH. With different conditions the selection can produce different aptamer sequences for the same target⁵¹. For example, multiple aptamers have been reported for the well-studied target, thrombin. The DNA aptamer for thrombin was first selected in 1992¹⁰³. This aptamer binds with a high affinity ($K_d \sim 200\text{nM}$)¹⁰³, and folds into a G-quadruplex¹¹⁶. Although this K_d was in the nanomolar range, further aptamers were later selected for thrombin by Macaya *et al*, in 1995¹¹⁷. These aptamers also form G-quadruplex structures, however, they also formed a bridged duplex, connecting the 5' and 3' ends of the structure. These aptamers demonstrated improved affinity to thrombin, with K_d values between 10-25 nM¹¹⁷. A third selection done two years later resulted in the selection of a high affinity aptamer for thrombin with a K_d of 0.5nM¹¹⁸. Another example includes the RNA aptamer for thrombin that forms a stem loop structure¹¹⁹, and binds to thrombin with a K_d value of 37nM⁶⁰. This aptamer was

found to bind to the exosite I, or heparin recognition site of thrombin, while the original DNA aptamer is known to bind to the exosite II, fibrinogen binding site of thrombin¹¹⁹.

There are many other examples of thrombin-binding aptamers that display a variety of affinity and selectivity characteristics for the target. This provides precedent that it is beneficial to perform multiple selections for the same target, as aptamers with an improved binding affinity or different functionality may be obtained. Obtaining aptamers that bind to different sites of a target can also allow versatility for designing aptamer-based applications. For example it would be beneficial to use the DNA thrombin aptamer if the aim of the application was to inhibit thrombin from converting fibrinogen to fibrin¹¹⁸. However, if the aptamer was meant to protect thrombin from heparin-binding serpins, then the RNA aptamer would be more suited¹²⁰. For small molecule applications, aptamers binding to different functional groups may facilitate the use of unique detection applications, such as sandwich based assays^{65,121}.

3.1.2 Magnetic bead assay

Magnetic beads are useful tools that have been employed for many applications including binding assays for determining aptamer affinity. The uniform, superparamagnetic beads, have a 2.7µm diameter, and are composed of iron oxide. Each bead is coated with highly cross-linked polystyrene, and a hydrophilic layer of glycidyl ethers. This layer reduces non-specific interactions with the bead surface. These beads can be covalently modified with many different substrates, which can be used to immobilize an aptamer target. It is then possible to introduce aptamers to the –

target-conjugated beads, and use a magnet to easily separate unbound from bound aptamer.

In this study, the magnetic beads were obtained coated with primary amine groups, through short hydrophilic linkers. These amine groups can be reacted with the carboxylic acid moiety present on the OTA molecule. Through a reaction with N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) a highly stable amide bond is formed between the bead and the OTA molecule.

To perform a binding assay, fluorescently- labelled aptamers are incubated with the OTA surface modified beads. Using a supermagnet, the beads are pulled to the side and any unbound aptamer can be removed (Figure 3-1). The bound aptamer can then be eluted from the beads. Measuring the fluorescence, allows for the construction of a binding isotherm from which one can determine the K_d .

There are numerous benefits to using magnetic beads in determining the affinity of an aptamer for its target. First, many aptamers that are selected using a solid support matrix, such as sepharose, may exhibit affinity for the components of the matrix¹²². It is difficult to completely eliminate non-specific binding during the selection process, therefore, it may be useful to use a different solid support during binding assays. Using magnetic beads to immobilize the target lowers the occurrence of non-specific interactions and therefore more accurately reflect specific binding to the target. Moreover, the beads do not interact with each other, and do not retain any residual magnetism once the magnet has been removed¹²³. Therefore, it is easy to remove unbound aptamers and then perform several washes to remove any non-specifically

bound aptamers from the beads. Magnetic beads have already been used successfully in measuring the affinity of aptamers for small molecular targets such as fumonisin B1 ($K_d=100\text{nM}$)⁶⁵ and homocysteine ($K_d=600\text{nM}$)¹²².

Figure 3-1: Measuring an aptamers binding affinity to OTA using magnetic beads.

3.1.3 Chapter objectives

Many groups are focusing on incorporating the OTA aptamer 1.12.2, into sensors for OTA detection, some of which are described in section 1.4.1. However, given that there are four other aptamers that have been selected for OTA, it would be useful to determine which aptamer has the highest affinity for OTA. To effectively compare the performance of these aptamers and their applicability in a potential sensor, they must be characterized using the appropriate selection buffers, under the same conditions,

within the same assay¹²⁴. Magnetic bead based assays have been employed many times to effectively characterize aptamer affinity for a variety of targets. Thus it is likely a useful platform for characterizing the panel of OTA aptamers. However, it is first important to also ensure that the magnetic beads are a reliable method for determining the binding of these aptamers. Therefore, control experiments were used to evaluate the non-specific interactions that may occur between the aptamer and the beads.

Using this assay, the K_d values for each aptamer were determined. Since OTA occurs in low quantities, it is imperative that a sensor designed for the detection of OTA would have very high sensitivity. It follows then that the aptamer with the highest affinity is likely to be the best candidate to employ in aptasensors for OTA. It is expected that employing the highest affinity aptamer will increase the possibility of developing a successful OTA sensor.

3.2 methods

3.2.1 Additional Buffers used in chapter 3

Table 3-1: Buffer compositions

Buffer	Components	pH
MES	0.1mM 2-(N-morpholino)ethanesulfonic acid	4.5
NaHCO ₃	0.1mM Sodium bicarbonate	8.5
Tris	1.0 Tris(hydroxymethyl)aminomethane	7.4
Geng	10mM Tris-HCl 120mM NaCl, 20mM CaCl ₂	7.0

3.2.2 Attaching OTA to amine-functionalized magnetic Dynabeads®

Hydrophilic magnetic Dynabeads® beads functionalized with amine groups were separated into 1mL aliquots (approximately 2×10^9 beads) for a total of 5mL of beads, and re-suspended in MES buffer. A DynaMag-2 magnet was used to pellet the beads to the side allowing the supernatant to be pipetted out. This process was repeated twice more for a total of three washes. A 1:2 mixture of 1M EDC and 6×10^{-5} M OTA with a total volume of 7.5mL was reacted with the beads on a rocker for 2 hours at room temperature. The magnet was used again to separate the beads from the supernatant which was removed and filtered using spin-x cellulose acetate microcentrifuge filter tubes. The unbound OTA was then quantified by measuring its fluorescence on a Fluorolog Fluorescence Spectrophotometer (Horiba Jobin Yvon, USA) with a SpectrAcq controller and compared to the original concentration of OTA to determine the binding efficiency.

The OTA-conjugated beads were then washed twice using 5mL of carbonate buffer (0.1M NaHCO_3 , pH 8.5). Unreacted amine groups on the beads were protected by reacting with 100mg of sulfo-NHS-acetate dissolved in NaHCO_3 buffer on a shaker at room temperature for 2 hours. 5mL of Tris buffer was used to wash the beads three times and finally the beads were washed with 5mL of the appropriate aptamer binding buffer (table 2-1).

3.2.3 Measuring aptamer dissociation constants using a Dynabeads® based affinity chromatography assay

The beads were prepared for testing by washing 90uL aliquots three times using 90uL of the buffer specified for the aptamer being tested (table 2-1). The modified aptamer was prepared at various concentrations ranging from 8×10^{-9} M to 5×10^{-7} M, heated to 90°C and cooled to room temperature. The prepared DNA was incubated with the OTA conjugated beads by shaking for 1 hour at room temperature. The unbound aptamer was removed from the beads and collected as “flow through” samples. Non-specifically bound DNA was removed by washing the beads with 90uL of buffer at RT 4 times. The bound DNA was eluted from the beads using buffer heated to 90°C and incubating at 90°C for 10 minutes. The recovered DNA was then removed and filtered through spin-x cellulose acetate microcentrifuge filter tubes yielding the “eluate” sample. The elution process was repeated for a second time to ensure maximum recovery of the bound DNA. The combined “eluate” samples for each dilution were analyzed using a Fluorolog Fluorescence Spectrophotometer (Horiba Jobin Yvon, USA) with a SpectrAcq controller. The dissociation constant (K_d) was determined by plotting the fluorescence intensity vs. Concentration and fitting the data using GraphPad Prism 3, using a one site saturation binding curve using equation 3-1.

Equation 3-1:
$$Y = \frac{B_{max} * X}{K_d + X}$$

In the case of the H12 aptamer, a subsequent DNA elution was attempted with 90°C 6.76M urea. This recovered DNA was also analyzed using a Fluorolog Fluorescence Spectrophotometer (Horiba Jobin Yvon, USA) with a SpectrAcq controller.

The concentration of “flow through” and “washing” collections were calculated using a calibration curve prepared with varying H12 aptamer concentrations. This analyzed using a Fluorolog Fluorescence Spectrophotometer (Horiba Jobin Yvon, USA) with a SpectrAcq controller.

3.2.4 Control tests

K_d studies for 1.12.2 aptamer were completed using 1.12.2 buffer and a second buffer, Geng buffer¹²⁵. The procedure from section 3.2.3 was repeated for each buffer separately.

Fluorescently labelled dopamine aptamer was provided by Erin McConnell at Carleton University. DNA dopamine aptamer (5'-FGTCTCTGTGTGCGCCAGAGACAGTGGGGCAGATATGGGCCAGCACAGAATGAGGCCC-3') was reacted with OTA-conjugated beads following the procedure outlined in section 3.2.3.

The non-specific binding control test was conducted utilized Dynabeads without OTA. The beads were first reacted with sulfo-NHS-acetate and then mixed with Aptamers 1.12.2, A08, and B08 following the procedure outlined in section 3.2.3.

3.3 Results and discussion

Currently five aptamers have been selected for OTA. As discussed previously, detection of OTA contamination at low concentrations is necessary and it is likely that aptamers with the best binding affinity to OTA would be best suited for the development of sensitive OTA detection assays. Therefore, it is necessary to determine which of the panel of OTA aptamers has the highest affinity for the target. It is possible to do this by comparing the dissociation constant that each aptamer yields for OTA using a magnetic bead assay. To ensure that the results are meaningful, it was important to also evaluate if the method produced reliable binding results or if the measured interactions were a result of non-specific interactions. This was done by running control tests for this method to measure non-specific interactions between aptamers and the beads.

3.3.1 Magnetic beads assay control tests

To determine the level of non-specific binding between aptamer sequences with the OTA-conjugated magnetic beads, an aptamer that has been demonstrated to be selective for dopamine was tested using the magnetic bead assay. The dopamine aptamer should not be able to specifically bind to OTA and thus any binding detected to the OTA beads in the assay is the result of non-specific binding. Various concentrations of dopamine aptamer modified with fluorescein were incubated with a constant number of OTA-covered beads (2×10^5). The bound aptamer was eluted with 90°C buffer and the fluorescence was measured.

Figure 3-2: DNA dopamine aptamer control binding isotherm obtained using the magnetic bead assay. Fluorescently labelled-DNA dopamine aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The data was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3.

Figure 3-2 shows that the dopamine aptamer produce a binding isotherm that is not representative of target binding, and a K_d value cannot be determined as the amount of aptamer that is eluted from the OTA-conjugated magnetic beads remains relatively constant despite increasing the concentration of aptamer incubated with the beads. This indicates that the dopamine aptamer does not bind to the OTA-conjugated beads with high affinity. The fact that there is a fluorescence signal in the eluate samples indicates that some dopamine aptamer did bind to the beads. Since the intensity is very low it is most likely due to very weak non-specific interactions between the DNA and the beads, the bead linker arm or the OTA. As a result, it can be concluded that any high affinity aptamer binding measured using the magnetic bead assay is due to a specific affinity for the target, OTA.

To further investigate the effect of non-specific interactions between aptamers and the magnetic beads, the beads assay in section 3.2.3 was done by incubating A08F aptamer with unmodified beads. At the neutral pH (7.4) used in this assay, the primary amine functional groups on the beads will be positively charged. This positive charge is known to non-specifically interact with the negatively charged phosphate-deoxyribose backbone of aptamers¹²⁶. To prevent this electrostatic interaction, the beads were reacted with sulfo-NHS-acetate, which caps the amine groups with an acyl group.

Figure 3-3: Negative control binding isotherm for A08F obtained using a magnetic bead assay to observe any non-specific binding. Fluorescently-labelled A08 aptamer concentration was varied using a constant number of beads (2×10^5) reacted with sulfo-NHS-acetate, without immobilizing target. The data was fit using a non-linear regression equation 3-1 in GraphPad Prism 3.

The results in Figure 3-3, demonstrate that a small amount of aptamer was eluted from the beads and therefore bound to the beads even in the absence of OTA. However at the aptamer concentration below 5×10^{-7} M, the amount of eluted aptamer

was very low and is thus negligible. At higher concentrations, the non-specific interactions seem to increase, which is a typical trend with non-specific binding.

While the surface of the magnetic beads is supposed to reduce non-specific interactions, previous studies have shown that some aptamers still non-specifically interact with either the linker arm or the surface of the beads⁶⁵. The observed non-specific binding may also be a result of incomplete protection of the amine groups with sulfo-NHS-acetate. With the OTA-specific binding tests, this non-specific interaction may be reduced as both OTA and sulfo-NHS-acetate will be conjugated to the beads, and hopefully completely saturate the free amines.

To ensure that the beads assay reflects specific interaction with the target and not weak non-specific interactions with the beads, concentrations of aptamer at and below $5 \times 10^{-7} \text{M}$ that result in negligible non-specific interaction will be used. Through the control experiments, it has been demonstrated that the DNA dopamine aptamer does not exhibit binding to OTA, and that non-specific binding of aptamers is minimal at concentrations below $5 \times 10^{-7} \text{M}$. Therefore, we can conclude that using the magnetic bead assay with concentrations of aptamer below this level is reliable for measuring aptamer binding interactions.

3.3.2 Magnetic bead assay results for OTA aptamers

Figure 3-4: A08F binding isotherm obtained using a magnetic bead assay. A08F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3. The K_d was determined to be $27 \pm 8 \text{ nM}$.

The binding isotherm depicted in Figure 3-4 was obtained for A08F aptamer by following the procedure outlined in section 3.2.3. The K_d calculated for this curve was $27 \pm 8 \text{ nM}$. The curve demonstrated the expected trend for an aptamer binding to its target; the amount of DNA eluted from the beads increases until all the possible OTA molecules are saturated with aptamer, as can be seen by the plateau in the curve. The average K_d values for all five aptamers can be seen in Table 3-2.

Table 3-2: Dissociation constant values determined for OTA aptamers using a magnetic bead assay. Individual trial binding isotherms can be seen in Appendix B.

Aptamer	Dissociation constant K_d (nM)	Number of trials
A08F	34±15	6
B08F	73±41	4
H12F	114±118	3
H8F	NB	N/A
1.12.2F	243±184	5

The aptamers A08F and B08F, which were selected during the same SELEX process, exhibited the highest binding affinity for OTA, as can be seen by the low K_d values in Table 3-2. This is the first study of the affinity of these aptamers for OTA, therefore it is not possible to compare the results to previously reported K_d values. However, these results are very encouraging as the K_d values reported for aptamers with small molecular targets tend to be much higher⁵⁷.

The aptamer with the next highest affinity for OTA was H12 with an average K_d value of 114±118nM. This aptamer was selected along with the aptamer H8, by Barthelmebs *et al.* in 2011. At the time of selection this aptamer was tested by incubating with biotinylated OTA, and then separating the aptamer-OTA complex with streptavidin modified magnetic beads⁷⁹. The fluorescently-labelled aptamer was then eluted and the K_d was determined to be 96nM, which is within the same magnitude as the calculated K_d in this study. The K_d for H8 was not determined in this study as none of the trials showed a plateau in binding; therefore it was difficult to distinguish specific from non-specific binding.

Finally, the first selected aptamer for OTA, 1.12.2F, was tested using the magnetic bead assay. The results obtained produced an average K_d value of 243 ± 184 nM. Interestingly, out of the four aptamers that demonstrated binding in this study this aptamer had the highest calculated K_d value but was reported to bind to OTA with the best K_d in literature. In the study by Cruz-Aguado and Penner, the aptamer affinity was measured using equilibrium dialysis. This method utilizes the intrinsic fluorescence of OTA, and monitors the amount of OTA in two separate chambers at equilibrium⁸⁰. The aptamer-OTA complex will be too large to diffuse between chambers and the difference in each chamber can be used to calculate the K_d ⁵⁷. This method resulted in 1.12.2 having a K_d value of 50 nM, which is much lower than the value obtained in this study. One of the main differences between the method presented in this study compared to that used by Cruz-Aguado and Penner, is that here, the target is immobilized to a solid support. It is possible that the carboxylic acid that is reacted to form the stable amide bond with the bead is important in binding to the aptamer. It is also possible that the OTA immobilization hinders the ability of the aptamer to fully interact with OTA. Furthermore, in this study it was necessary to label the aptamer with fluorescein, and this may also affect the ability of the aptamer to bind to its target.

Although it was important for this study to measure the binding affinity for each aptamer under the same conditions, it is necessary to use the appropriate buffer designed for each aptamer (Table 2-1). The presence of divalent cations can affect the ability of the aptamer to bind with its target. This was investigated by Geng *et al* by investigating the effects of different buffer compositions on the binding of OTA with

1.12.2¹²⁵. This study found that a variation of the original buffer used to select OTA (Geng buffer, Table 3-1), produced a K_d value of $53 \pm 9 \text{ nM}$ ¹²⁵. The effect of this buffer was investigated with the magnetic bead assay used in this study.

Figure 3-5: 1.12.2F binding isotherm obtained using a magnetic bead assay. 1.12.2F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C Geng buffer (Table 3-1), and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3.

Figure 3-5, demonstrates that using the Geng buffer (Table 3-1), did not result in a measurable K_d value for 1.12.2F. The data obtained indicates that the amount aptamer eluted from OTA remained constant despite increasing the amount of aptamer incubated with the beads. This is similar to the results obtained in the DNA dopamine aptamer control test (Figure 3-2), indicating that using the adjusted buffer composition prevented the 1.12.2 aptamer from binding with OTA. It has previously been reported the aptamers binding affinity for OTA is highly dependent on the presence of calcium.

Therefore adjusting the amount of calcium in the buffer used for the magnetic bead assay may hinder the aptamer from binding with OTA in our.

The concentration of aptamer that binds to OTA is eluted using the appropriate 90°C buffer. Although the eluate samples do demonstrate fluorescence, indicating that the aptamer has in fact been eluted from the target, it is difficult to ensure that all of the bound aptamer is eluted. To investigate this challenge, beads used for the Trial 1 H12F magnetic bead assay (Figure B-10) were further eluted with 90°C 7M urea. The resulting samples demonstrated that although some aptamer was still bound to OTA after elution with 90°C buffer, the amount of aptamer further eluted with urea was consistent with the original trend. Therefore, it was concluded that further elution with urea was not necessary. The flow through and washing samples from this test were also analyzed, in an attempt to calculate the concentration of aptamer that did not bind to OTA. However, the amount of aptamer present in these samples were too low to measure reliable results using a Fluorolog Fluorescence Spectrophotometer (Horiba Jobin Yvon, USA) with a SpectrAcq controller.

Using the magnetic bead assay to determine an aptamer's affinity for a target does appear to be a useful method. However, there are significant challenges to consider. First, the experimentation process is very time consuming and not high-throughput. Furthermore, the assay requires that the aptamer being studied is modified with a fluorescent label, and that the target is immobilized. These modifications may affect the binding ability of the aptamer.

Furthermore, as demonstrated by the large standard deviations in the measured K_d values there is a lot of variability associated with this assay. This is likely because this assay requires many steps which introduces a high possibility for error. It is difficult to ensure that the concentration of target immobilized is consistent for each batch of beads, as the method requires an indirect measurement of unbound target to determine the reaction efficiency. Furthermore, targets that are not-fluorescent or have other spectroscopic properties cannot be easily quantified without the use of expensive equipment such as LC-MS. The beads themselves introduce a great deal of error, as it is very easy to lose beads through washing and pipetting steps. Although the beads are used to minimize non-specific binding, as we demonstrated, they do not eliminate the interactions completely, and this reduces the range of concentrations that can be investigated with this method. Therefore, it would not be possible to determine the binding affinity for an aptamer that binds with a K_d in the micromolar range. This work also demonstrates the K_d value of an aptamer is influenced by the method used to calculate it. This is clearly exhibited by the difference between the K_d values obtained for OTA aptamers using the magnetic bead assay (Table 3-2) and the values obtained using the DNase I assay (Table 2-4). The K_d values for A08 and B08 are a magnitude higher using the DNase I assay compared to the magnetic bead assay. Furthermore there are no results to compare for H12, H8, and 1.12.2, as the DNase I assay was unable to determine the K_d for H12 or 1.12.2, and the magnetic bead assay was unable to determine the K_d for H8. This is a result of aptamers behaving differently under varying conditions. It may be that certain aptamers have a higher binding affinity for a free target, while others may exhibit better binding to a bound target. This suggests that

it is imperative that the future application of the aptamer be considered when testing the binding affinity of an aptamer. For example if the aptamer being studied is going to be used in a sensor to detect OTA in a wheat sample, the magnetic bead assay may not be useful as it does not reflect aptamer binding to OTA in solution

3.3.3 Future Directions

This study aimed to determine the aptamer with the best binding affinity to OTA through the use of a magnetic bead assay. Although the assay was able to determine the aptamer K_d values for OTA, it is not sufficient to choose the best aptamer for OTA solely based on this one method. We and others have shown that the method and conditions used to determine the binding affinity of an aptamer may affect the resulting K_d values. Thus, it is clear that comparing aptamers based on their K_d alone is insufficient. Perhaps to reliably characterize and compare aptamers for a given target, multiple affinity methods should be employed. To-date, no comprehensive studies have been performed that evaluate the effect of aptamer affinity method on the resulting K_d . Extending our in-solution and immobilization-based aptamer assays to a larger set of aptamers might be the initial steps required to provide insight into the effects these different strategies have on aptamers affinity characterization, This work might also help guide aptamer researchers to choose the best aptamer candidates for their application of interest.

Chapter 4: Developing a novel competitive testing method for studying multiple aptamers for one target

4.1 Introduction

4.1.1 Enzyme-linked immunosorbent assay (ELISA)

As discussed in section 1.3.1, aptamers function similarly to antibodies, in that they bind to a desired target with high affinity and selectivity. Considering these similarities, many aptamer applications are designed to imitate the already well-established antibody based applications. ELISA (enzyme-linked immunosorbent assay) is a commonly used technique designed to determine the presence of an antigen in a sample. There are three main types of ELISA, direct/indirect, sandwich and competitive.

In direct and indirect ELISA (Figure 4-1A) the sample containing the antigen of interest is added to ELISA microtiter plates which will cause all proteins in the sample to adhere to the walls of the plate. The primary monoclonal antibody, which is specific for the antigen of interest, is added to the plate and will bind only to the antigen. The primary antibody can be linked to an enzyme, which can react with a substrate to form a colour change (direct ELISA), or it can be used as an antigen for a secondary antibody, which would be linked to an enzyme which would react with the substrate to cause the colour change (indirect ELISA)¹²⁷.

A sandwich ELISA (Figure 4-1B) is similar to the process of an indirect ELISA, however instead of immobilizing the antigen of interest to the walls of the ELISA microtiter plate, a known amount of antibody for the desired antigen is immobilized to the walls of the plate¹²⁷. Therefore when the sample is added to the wells, the antigen will bind to the antibody. To detect the antigen an enzyme linked antibody is added to the wells which also binds to the antigen, and will produce a colour change when the

substrate for the enzyme is added. For this method it is necessary that the target has multiple epitopes so that more than one antibody can bind to it at the same time¹²⁸.

The third type of ELISA is a competitive based assay (Figure 4AC). In this method, an antibody is immobilized on the wells of an ELISA microtiter plate. The sample is mixed with a solution containing an enzyme conjugate version of the desired antigen (enzyme conjugate). This mixture is added to the wells, and the enzyme conjugate and antigen compete for binding to the antibodies¹²⁷. The enzyme on the enzyme conjugate will produce a colour when mixed with a substrate. In this case, the higher intensity of colour indicates a lower concentration of antigen present in the sample, while a lower intensity of colour indicates a higher concentration of antigen in the sample. This type of assay is particularly useful if an antigen has only one epitope, and a sandwich ELISA cannot be used¹²⁸. This concept of two molecules competing for binding with a molecular recognition probe would be an interesting concept to incorporate for studying aptamers.

A) Direct/Indirect ELISA

C) Sandwich ELISA

B) Competitive ELISA

Figure 4-1: Antibody based ELISA systems

4.1.2 Single microbead SELEX

The majority of SELEX methods involve exposing a pool of random oligonucleotides to a packed column with immobilized target present⁶⁵. This process is time consuming because many sequences that are able to bind to the high concentration of target molecules present (as well as the column material). In an effort to increase the efficiency of SELEX, Tok *et al* developed a method that utilized a single target-conjugated microbead¹²⁹. In this study two *Clostridium botulinum* neurotoxin-related targets were used for selection, one of which being *Clostridium botulinum* toxoid. By using one microbead for this selection, there was a limited amount of target molecules for the random pool of oligonucleotides to interact with. Therefore, it was expected that aptamers that weakly bound to the target would be displaced by sequences which bound with higher affinity. This method drastically reduced the number of selection rounds and therefore time needed to carry out the selection process, successfully producing an aptamer for *Clostridium botulinum* toxoid with a K_d in the nanomolar range¹²⁹. This is another example of a method of using molecular recognition probes in a competitive context. This direct competition of aptamers for the same target could be useful in choosing aptamers to use in specific applications.

4.1.3 Chapter objectives

Currently aptamers are selected for an application based on affinity by comparing reported K_d values. Although measuring the binding affinity of an aptamer by considering its K_d value is useful, we found from the results in chapter 3 that direct

comparison of aptamers through measured K_d values is not always reliable. We hypothesize that using a method that investigates the ability of a set of aptamers to compete for target binding, may provide insight into which aptamer is more useful for aptamer-based applications. As there are currently no existing methods for this in literature, this study investigates several competition based methods that utilize the magnetic bead assay from chapter 3.

4.2 Methods

4.2.1 Aptamers purchased from IDT

Table 4-1: Aptamers modified with TYE705 (TYE)

DNA Aptamer	Sequence	Aptamer Mass g/mol
1.12.2TYE	5'-TYEGATCGGGTGTGGGTGGCGTAAAGGGAGCATCGG ACA-3'	11928.1
A08TYE	5'-TYEAGCCTCGTCTGTTCTCCC GGCGCATGATCATTTCGGTGGGTAAAGGTGGTGGTAACGTT GGGGAAGACAAGCAGACGT-3'	24123.9

4.2.2 Gel electrophoresis competitive test with 1.12.2, H12, and A08

Modified aptamers, 1.12.2, H12 and A08, were each prepared to a concentration of $6 \times 10^{-7} \text{M}$ in separate microcentrifuge tubes, heated to 90°C and then cooled to room temperature. 320uL of each of the three aptamers were mixed together to bring each to a concentration of $2 \times 10^{-7} \text{M}$ in 960uL of 1.12.2 buffer (Table 2-1). The mixed dilutions

were incubated with the OTA conjugated beads as described in section 3.2.3. The same procedure was used for washing and eluting the DNA from beads. The eluate samples were combined and then dried down using a Savant AE2010 SpeedVac overnight. The flow through samples, and 200uL of the original aptamer mixture were also dried down overnight. The ten samples were each re-suspended in 35μL of deionized water and 35μL of formamide. All samples were run on a 19% PAGE gel for approximately 3 hours. The fluorescent bands in the gel were imaged using an Alpha Imager Multi Image Light Cabinet (Alpha Innotech) with an excitation wavelength of 302nm. Band intensity was analyzed using AlphaView software.

4.2.3 Competitive displacement assay

DNA dilutions were prepared using 1.12.2F aptamer (table 2-2) in 1.12.2 buffer (Table 2-1) and A08 aptamer (Table 1-2) in A08/B08 buffer (Table 2-1). All dilutions were heated to 90°C and then cooled to room temperature. 90uL of 1.12.2F aptamer was added to 8 tubes of beads (herein named 1.12.2F beads), and 90uL of A08 was added to a second set of 8 tubes of beads (herein named A08 beads). These were left to react on a shaker at room temperature for 1 hour. The flow through was removed and the beads were washed with 90uL of the appropriate buffer four times. A08 aptamer was then added to 1.12.2F beads, while 1.12.2F aptamer was added to the A08 beads. This competitive reaction was left on a shaker for 1 hour at room temperature. The flow through was again removed and the beads were washed 4 times with 90uL of the buffer corresponding to the second added aptamer (Table 2-1). The bound DNA was eluted twice using 90°C buffer and incubating for 10 minutes. These eluate samples were

filtered using spin-x cellulose acetate microcentrifuge filter tubes and the amount of fluorescently-labelled aptamer present was analyzed on a Fluorolog Fluorescence Spectrophotometer (Horiba Jobin Yvon, USA) with a SpectrAcq controller.

This procedure was repeated using a competition of 1.12.2F with B08, A08F with 1.12.2, and B08F with 1.12.2.

4.2.4 Double dye competitive test using fluorescein and TYE705

A08F (Table 2-2) was mixed with 1.12.2TYE (Table 4-1) in 1.12.2 buffer (Table 2-1). The fluorescence spectrum of the fluorescein in the mixture was measured with an excitation wavelength of 490nm and an emission wavelength of 520nm, while the TYE705 fluorescence spectrum was measured with an excitation of 686nm and emission wavelength of 704nm. Both measurements were taken using a Fluorolog Fluorescence Spectrophotometer (Horiba Jobin Yvon, USA) with a SpectrAcq controller. The DNA dilutions were mixed with OTA-conjugated beads and the eluate samples were analyzed as per the procedure outlined in section 3.2.3. The intensity of the fluorescence emitted by the fluorescein and TYE705 present in the eluate samples were compared to the intensity observed in the aptamer mixture using Equation 4-1.

Equation 4-1: % of original aptamer eluted from OTA =

$$\left(\frac{\text{Fluorescence from eluted aptamer mixture}}{\text{Fluorescence from aptamer in original mixture}} \right) \times 100\%$$

This procedure was repeated using 1.12.2F and A08TYE.

4.3.5 Competitive assay vs magnetic bead assay

Dilutions of 1.12.2F (Table 2-2) and A08 (Table 1-2) aptamer were prepared as described in section 3.2.3. In this case, the dilutions were first mixed together and then the mixture was incubated with OTA-conjugated beads on a shaker for 1 hour at room temperature. The bound DNA was eluted and analyzed on the Fluorolog Fluorescence Spectrophotometer (Horiba Jobin Yvon, USA) with a SpectrAcq controller. A simple (non-competitive) magnetic bead assay, utilizing 1.12.2 modified aptamer, was performed simultaneously following the procedure described in section 3.2.3 and the results of the two experiments were compared using Equation 4-1. This was subsequently repeated using A08F with 1.12.2 for the competition portion of the experiment and A08F aptamer in the magnetic bead assay portion of the experiment.

Equation 4-2: *Loss of fluorescence in competitive assay =*

$$\text{Fluorescence of magnetic bead assay} - \text{Fluorescence of competitive assay}$$

This competitive test was repeated using 1.12.2 and H8 aptamers. Furthermore, a control test was completed following this procedure using the DNA dopamine aptamer that was obtained from Erin McConnell for section 3.2.5 and 1.12.2 aptamer.

4.3 Results and Discussion

As demonstrated in chapter 3, separate screening of aptamers for a target does not provide reliable comparison of their binding affinity. Considering this issue, several direct competitive assays were designed and preliminary testing was completed to compare the 5 aptamers available for OTA.

4.3.1 Gel electrophoresis competitive test with 1.12.2, H12, and A08

We investigated a method where a larger set of OTA aptamers could be compared for affinity binding using a shotgun approach with the magnetic bead assay from section 3.1.2. Aptamers that have different sequence lengths will migrate through a denaturing acrylamide gel at different rates based on size and charge, and can therefore be separated, identified and quantified.

The labelled aptamers were combined in equimolar amounts and a fraction was incubated with OTA-conjugated beads. The eluted samples were combined into one tube, and this sample, as well as a fraction of the original mixture of aptamers (ie. total aptamer), were then separated by gel electrophoresis. The percentage of aptamers that preferentially bind to OTA in the eluted samples can be quantified by measuring the corresponding band intensity and comparing to the intensity of the bands from the original sample mixture.

Of the five aptamers selected for OTA, aptamers A08 and B08 are the same length (ie.76 bases), and H12 and H8 aptamers are the same length (ie. 65) and 1.12.2 is 36 nts long. Therefore, the aptamers A08F, H12F, and 1.12.2F (Table 1-2) could be compared at once as they could be sufficiently separated and identified by gel electrophoresis.

It is also possible to separate the flow through fractions by gel electrophoresis to determine the fraction of aptamer that did not bind to the target. These results should correspond with the eluate sample to ensure reliability of the test.

Figure 4-2: Aptamers from gel electrophoresis competitive assay separated on a 19% denaturing acrylamide gel. A fraction of the original aptamer mixture as a control, was run in the first lane, while the combined eluate samples were run in the second lane. However, there was not a sufficient amount of aptamer eluted to visualize on the gel. The last 8 lanes display the flow through samples from all trials.

Figure 4-2 displays the separated bands from the three aptamers, A08F, H12F, and 1.12.2F. The aptamers in this study were modified with fluorescein as unlabelled DNA is very difficult to detect. We hoped that imaging the labelled DNA would provide sufficient detection sensitivity. Unfortunately, it was not possible to observe the aptamers that were eluted from the beads, as no bands can be seen in the eluate sample lane. This does not necessarily mean that the aptamers did not bind to OTA, but that the small fraction of eluted aptamers could not be visualized with a gel imager. The visualization of such a small concentration of aptamer may be improved by labelling the aptamers with a radioactive isotope. The aptamer concentrations present in the flow

through samples from all eight trials were present in sufficient levels and can be observed in Figure 4-2. The percentage of non-binding aptamer was determined by calculating the IDV for each aptamer band divided by the total aptamer (Lane : Original mixture of aptamers). The average percent of each aptamer present in the flow through samples can be seen in figure 4-3.

Figure 4-3: Comparing the binding affinity of A08F, H12F, and 1.12.2F using a gel electrophoresis competitive assay. The flow through samples containing all three aptamers were separated on a 19% PAGE gel, and the percent of the original aptamer sample that did not bind to the beads was calculated. The 8 trials were averaged and the standard deviation is represented by the error bars on each bar.

The results in Figure 4-3 indicate that H12F had the lowest percent of original aptamer present in the flow through. By subtraction, this suggests that the H12F aptamer out-competed the other aptamers for binding to OTA-conjugated beads.

Following this reasoning, the aptamer with the next highest fraction of binding to OTA beads was 1.12.2F and finally A08F displayed the least amount of binding. These results are completely inconsistent with the results in the DNase I assay (Table 2-4) where A08 was the only aptamer to demonstrate binding out of these three aptamers. They are also contrary to the K_d values determined using the magnetic bead assay (Table 3-2), where A08 exhibited higher binding affinity than the other two aptamers. However, with the error, the results are likely not statistically significant.

There are many challenges present with this method that need to be considered. Although the flow through samples were clearly visible on the gel, using these samples as an indirect method to determine the amount of each aptamer that bound to the target, introduces a lot of error. This is due to fact that after the flow through samples are removed, the beads are washed several times to remove non-specifically bound aptamers. Therefore, it is possible that some percentage of aptamer that is not present in the flow through samples was not actually bound, and was removed in the washing step. It may be useful then, to run the washing samples on the gel as well, however, it is likely that like the eluate samples, they would not produce visible bands on the gel.

Furthermore, the three aptamers were each diluted to $6 \times 10^{-7} \text{M}$, so that when mixed they will each have a concentration of $2 \times 10^{-7} \text{M}$. This is done to produce samples concentrations that are slightly above the expected K_d value for each aptamer based on the previous K_d studies performed in chapters 2 and 3. However, each aptamer has the potential to non-specifically bind to the beads, and when the concentration of the aptamers is considered together, their combined concentration of $6 \times 10^{-7} \text{M}$. This is above the concentration that introduces unreliable results determined in section 3.3.1.

These challenges suggest that the results from this test cannot be considered reliable for comparing which aptamer demonstrates the highest affinity for OTA. Given the resolution of this assay, it is likely that to efficiently compare aptamer binding in this way that the affinity of each aptamer must be drastically different (orders of magnitude). We have demonstrated that the affinity of these OTA aptamers, within error, are relatively similar and thus this one-point method could probably not sufficiently compare these small affinity differences.

4.3.2 Competitive displacement assay

A new competitive test was designed to compare the affinity of two aptamers at a time. This test also utilized the magnetic bead assay from section 3.1.2. First a fluorescently-labelled aptamer was incubated with the OTA-conjugated beads, and any aptamers that did not bind, or that non-specifically bound with the beads were removed. A second aptamer (unlabelled) was then incubated with the same beads (Figure 4-4A). This would allow the second aptamer to competitively displace the first aptamer from OTA. Again the beads were washed to remove any non-specifically bound aptamers and then the two bound aptamers could be eluted (Figure 4-4A).

Figure 4-4: Competitive assay design for comparing two aptamers affinity to OTA. A) The fluorescein (yellow star) labelled aptamer of interest is incubated with the beads first, followed by the non-labelled aptamer which will competitively displace some of the first aptamer to bind to the target. B) The aptamer that was not labelled in part A of the test is now labelled with fluorescein and incubated with the beads first.

Only the fluorescently labelled aptamer can be detected by measuring the fluorescence. Therefore the non-labelled aptamer present in the eluate sample would lower the fluorescence. It is not possible to measure the amount of non-labelled aptamer present. Therefore, to determine which aptamer binds to more of the OTA molecules, it is necessary to repeat the test using the oppositely labelled aptamer and

non-labelled aptamer (Figure 4-4B). Since the second test results display the fluorescence intensity from the second aptamer, it is possible to compare the amount of fluorescence from both tests. The fluorescently-labelled aptamer that demonstrates the higher fluorescence intensity then has a high enough affinity to prevent the second aptamer from competitively displacing it.

Figure 4-5: Competitive test with 1.12.2 and A08 aptamers by comparing the intensity of the fluorescently-labelled aptamer after being competitively displaced by the non-labelled aptamer. In this test, A08F demonstrated a higher fluorescent intensity at all concentrations, and therefore was not competitively displaced as much as 1.12.2F

Based on the results obtained in chapter 3, the aptamer A08 was chosen for initial testing. Since 1.12.2 has been previously used in many applications in literature it was chosen as the second aptamer for this test. The results demonstrated by a bar graph in Figure 4-5, show that the fluorescence intensity measured for A08F is higher than that measured for 1.12.2F, for every concentration tested. This means that 1.12.2

was not able to competitively displace as much A08F from the OTA-conjugated beads compared to the amount of 1.12.2F that was displaced by A08. Therefore, A08 appears to have a higher affinity for OTA than 1.12.2. To further confirm these findings the procedure was repeated but with the non-labelled aptamer being incubated with the beads first (Figure 4-6). The data expressed in the bar graph seen in figure 4-7 again shows that A08F was able to competitively displace more 1.12.2 than 1.12.2F was able to displace A08.

Figure 4-6: Competitive assay design for comparing two aptamers affinity to OTA. A) The non-labelled aptamer of interest is incubated with the beads first, followed by the fluorescein (yellow star) labelled aptamer which will competitively displace some of the first aptamer to bind to the target. B) The aptamer that was labelled in part A of the test is then used unlabelled and incubated with the beads first.

Figure 4-7: Competitive test with 1.12.2 and A08 aptamers by comparing the fluorescence intensity of the fluorescently labelled aptamer after competitively displacing the non-labelled aptamer. In this test A08F demonstrated a higher fluorescent intensity at all concentrations, and therefore was able to competitively displace 1.12.2 more successfully than 1.12.2F was able to displace A08.

The same method was used to compare B08 and 1.12.2 aptamers. The results from this test can be seen in Figure 4-8 and Figure 4-9. In the first part of the test the fluorescently labelled aptamers were incubated with the OTA-conjugated beads first, and then were competitively displaced by the non-labelled aptamers. Figure 4-8 clearly demonstrates that in this case not as much B08F aptamer was competitively displaced as 1.12.2F was, indicating that B08 has a higher affinity for OTA than 1.12.2.

Figure 4-8: Competitive test with 1.12.2 and B08 aptamers by comparing the fluorescence intensity of the fluorescently labelled aptamer after being competitively displaced by the non-labelled aptamer. In this test B08F demonstrated a higher fluorescent intensity at all concentrations, and therefore was not competitively displaced as much as 1.12.2F

In part two of the test, the non-labelled aptamers were incubated with the beads first and then competitively displaced by the fluorescently labelled aptamers. The results seen in figure 4-9, demonstrate that at lower concentrations below 8×10^{-8} M, B08F was able to competitively displace more of 1.12.2, than 1.12.2F was able to displace B08. However, at concentrations starting at 8×10^{-8} M and higher, it becomes less obvious which aptamer competitively displaced more unlabelled aptamer, as the fluorescence intensities are very similar.

Figure 4-9: Competitive test with 1.12.2 and B08 aptamers by comparing the fluorescence intensity of fluorescently labelled aptamer after competitively displacing a non-labelled aptamer. In this test it was difficult to determine if the fluorescent aptamer was able to displace the non-labelled aptamer.

When the second aptamer is incubated with beads that have already bound with the first aptamer, the second aptamer can competitively displace the first aptamer to bind with OTA. However since aptamer-target complexes are constantly associating and dissociating, this displacement is in equilibrium. This may affect the results, as there will be a much higher concentration of the second aptamer than the first aptamer competing for binding. Comparing the reporter affinities and some of our affinity results for all of these OTA aptamers, all five of these aptamers display affinities relatively within the same range, thus in all cases, the presence of significantly large concentrations of a 2nd OTA aptamer will likely be able to displace the first aptamer that

is bound to the OTA-conjugated beads. Therefore, the equilibrium will favour the second aptamer, and this could artificially cause more of the second aptamer to bind to the target, regardless of discrete difference in aptamer affinity. The effect of the equilibrium favouring the second aptamer, can be observed if you consider the different intensities of fluorescent aptamer when it is added first to the beads compared to when it is added second. For example, the fluorescence intensity of the 1.12.2F aptamer, when it is added second to the beads after A08 (figure 4-7), shows a much higher fluorescence than when it is added before A08 (Figure 4-5).

The previously measured K_d values between 1.12.2 and A08 aptamers did not demonstrate a large difference in affinity. However, it is possible that the difference in binding affinities between these aptamers is sufficiently able to overcome the effect caused by the equilibrium in both cases (Figures 4-5 and 4-7). However to obtain reliable results, the challenge of the aptamer equilibrium must be addressed. It would be beneficial to mix the aptamers together prior to incubating with the beads. Therefore this method was concluded to be unreliable to compare aptamers of similar affinities and new methods were explored.

4.3.3 Double dye competitive test using fluorescein and TYE705

In both of the previously designed competitive tests, the only fluorescent label used was fluorescein, therefore only one aptamer can be distinguished and measured in solution at a time. In section 4.3.1, the aptamers were separated based on their lengths, however this limited the test to only be able to compare aptamers which had

sequences that were distinctly different lengths. In the second competitive test, described in section 4.3.2, only the aptamer modified with fluorescein was able to be measured, therefore it was necessary to perform multiple tests for each aptamer which was very time consuming. We therefore explored the possibility of including multiple dyes for aptamer labelling. A second dye, TYE705 was chosen as it has an excitation wavelength of 686nm, and emission wavelength of 706nm, which is sufficiently red-shifted from fluorescein ($\lambda_{ex}=490\text{nm}$, $\lambda_{em}=520$), to prevent spectral overlap. Therefore two aptamers could be tested at a time, one with a fluorescein label, the second with a TYE705 label.

First, a mixture of both aptamers at various concentrations was prepared, and the fluorescence of both dyes was measured for each concentration. The mixed aptamer samples were then incubated with the OTA-conjugated beads following the procedure outlined in section 3.1.2, which would allow the two aptamers to compete to bind to the target. The obtained eluate sample was analyzed for both the fluorescently-labelled aptamer and the TYE705 aptamer. The resulting fluorescence intensity obtained for each aptamer was then compared to the starting sample fluorescence intensity to determine the percent of original aptamer eluted from the target (equation 4-1). It can then be inferred that the aptamer which demonstrates the higher percent of original aptamer eluted from OTA was able to bind to the most OTA molecules. These results were plotted using a bar graph to visualize the data.

Figure 4-10: Comparison of 1.12.2TYE and A08F binding affinities for OTA Trial 1. The percent of both aptamers in an original mixture that were eluted from OTA after being competitively incubated with OTA-conjugated beads in 1.12.2 Buffer (Table2-1) are shown. These results indicate that 1.12.2TYE was able to bind to more OTA than A08F.

The first attempt at comparing aptamers using this method investigated TYE705-labelled 1.12.2 aptamer (1.12.2TYE) and fluorescein-labelled A08 (A08F). The results from this test can be seen in Figure 4-10. These results suggest that more 1.12.2TYE aptamer was able to bind to OTA than A08F. An important factor of the test was that 1.12.2 buffer (Table 2-1) was used throughout the test. Since these buffer conditions were optimized for 1.12.2 binding and not A08 binding, this could have affected to results. To test whether this was a factor, the procedure was repeated using A08/B08 buffer (Table 2-1).

Figure 4-11: Comparison of 1.12.2TYE and A08F binding affinities for OTA Trial 2. The percent of both aptamers in an original mixture that were eluted from OTA after being competitively incubated with OTA-conjugated beads in A08/B08 Buffer (Table2-1) are shown. These results indicate that 1.12.2TYE was able to bind to more OTA than A08F.

The results displayed in Figure 4-11, demonstrate that by using the buffer optimized for A08 aptamer, more A08F aptamer was eluted from the beads, which indicates it was able to bind more successfully to OTA under these conditions. However, this test also that support the findings that 1.12.2TYE was able to bind to more OTA than A08F. As in section 3 the possibility that all of the aptamer was not being eluted with 90°C buffer was addressed by further eluting with 90°C 7M urea, however, the results still indicated that a higher percent of 1.12.2TYE had eluted from the beads than A08F. The method was repeated for a third time continuing to use

A08/B08 buffer (Table 2-1) and again the results indicated that 1.12.2TYE was outcompeting A08F for OTA binding (Figure 4-12).

Figure 4-12: Comparison of 1.12.2TYE and A08F binding affinities for OTA Trial 3. The percent of both aptamers in an original mixture that were eluted from OTA after being competitively incubated with OTA-conjugated beads in A08/B08 Buffer (Table2-1) are shown. These results indicate that 1.12.2TYE was able to bind to more OTA than A08F.

The method was further investigated by exchanging the dye labels used on each aptamer. A08 was modified with TYE705 (A08TYE) and 1.12.2 was modified with fluorescein (1.12.2F). The same method was followed, again using A08/B08 buffer (Table 2-1).

Figure 4-13: Comparison of A08TYE and 1.12.2F binding affinities for OTA Trial 1. The percent of both aptamers in an original mixture that were eluted from OTA after being competitively incubated with OTA-conjugated beads in A08/B08 Buffer (Table2-1) are shown. These results indicate that over all A08TYE was able to bind to more OTA than A08F.

The results obtained when labelling A08 with TYE705 instead of 1.12.2 displayed in Figure 4-13, interestingly conflicted with the previous results. These results suggest that A08TYE was able to bind to more OTA molecules than 1.12.2F was. The test was repeated to confirm the results, and again there appeared to be more A08TYE binding to OTA than 1.12.2F (Figure 4-14).

Figure 4-14: Comparison of A08TYE and 1.12.2F binding affinities for OTA Trial 2. The percent of both aptamers in an original mixture that were eluted from OTA after being competitively incubated with OTA-conjugated beads in A08/B08 Buffer (Table2-1) is shown. These results indicate that over all A08TYE was able to bind to more OTA than A08F.

The conflicting results implied that the aptamer that was labelled with TYE705 was likely to always demonstrate more binding to OTA than the aptamer labelled with fluorescein. It was observed that the fluorescence intensity of the TYE705 label at any given concentration was significantly lower (always at least one magnitude lower) than the same concentration of fluorescein label. When the concentration of TYE705 labelled aptamer in the original mixture was increased the change in TYE705 fluorescence intensity only increased slightly. It may be then, that since the change in fluorescence is

so low, any small change in intensity exaggerates the calculation of the percentage of original aptamer eluted from the beads. These results indicate that this method of comparing aptamers is not reliable.

4.3.4 Competitive assay vs magnetic bead assay

The fourth method designed for comparing the OTA aptamers was based on the method in 4.3.2, with some variations. For this method, a competitive assay was performed by first mixing two aptamers, one fluorescently labelled and the other unmodified, at equal volumes prior to incubating with the OTA-conjugated beads. At this point the competitive assay was completed in the same manner as the magnetic bead assay procedure described in section 3.1.2. Simultaneous to this test a magnetic bead assay was also completed using only the fluorescently labelled aptamer. The eluted samples from both of these tests were then analyzed using only the fluorescently-labelled aptamer monitoring only the fluorescently labelled aptamer. In the competitive assay, the aptamers had to compete to bind to the OTA molecules. Therefore, due to the presence of non-labelled aptamer, the fluorescence intensity was lower for the competitive assay eluate samples than for magnetic bead assay (Figure 4-15). Similar to the method used in section 4.3.2, the same test was completed a second time using the oppositely labelled aptamer and non-labelled aptamer. Therefore, the difference in fluorescence intensity of the competitive assay and magnetic bead assay were calculated for both tests. It is assumed that the fluorescently-labelled aptamer used in

the test that demonstrated the lower difference in intensity between the competitive assay and the magnetic bead assay demonstrated a higher affinity for the target.

Figure 4-15: Magnetic beads (conjugated with OTA) incubated with a single fluorescently-labelled aptamer, or a mixture of a fluorescently labelled aptamer and a non-labelled aptamer. The addition of two aptamers with affinity to OTA will cause the aptamers to compete for binding with the target. In a separate test the modified aptamer is incubated with the beads. It is then possible to compare the fluorescence of the eluate samples to monitor the difference in fluorescence between each test. The competitive assay will be lower due to the competitive binding of the non-fluorescent aptamer.

The first two trials using this method compared the aptamers 1.12.2 and A08. The difference in fluorescence intensity between the competitive assay and magnetic bead assay eluate samples were calculated using Equation 4-1, for a test using A08F

and a separate test using 1.12.2F. These results demonstrated that tests using A08F had a lower difference in fluorescence intensity than the 1.12.2F tests (Figure 4-16 and 4-17). These results suggest that A08 demonstrated a higher binding affinity towards OTA under the conditions of the test.

Figure 4-16: Comparison of A08 and 1.12.2 binding affinities for OTA by comparing a competitive assay and a magnetic bead assay. Trial 1. Loss of fluorescence was observed in the competitive assay compared the fluorescence observed in the magnetic bead assay. These results indicate that overall the test using fluorescently labelled A08 resulted in a lower loss of fluorescence indicating that it was able to bind to more OTA than 1.12.2.

Figure 4-17: Comparison of A08 and 1.12.2 binding affinities for OTA by comparing a competitive assay and a magnetic bead assay. Trial 2. Loss of fluorescence was observed in the competitive assay compared the fluorescence observed in the magnetic bead assay. These results indicate that overall the test using fluorescently labelled A08 resulted in a lower loss of fluorescence indicating that it was able to bind to more OTA than 1.12.2.

The method was then tested using 1.12.2 and H8 aptamers. The results from this test seen in Figure 4-18 were not what were expected. Instead of observing a loss of intensity in the eluate samples from the competitive assay compared to the magnetic bead assay, an increase was observed. This can be seen for several of the samples from the 1.12.2F competitive assay and the lowest concentration from the H12 competitive, by the negative values obtained when subtracting the competitive assay fluorescence readings from the magnetic bead assay readings. These results suggest

that there was more fluorescently labelled aptamer bound to the OTA-conjugated beads when competing with the non-labelled aptamer, than when it was incubated with the beads alone. The same amount of beads were used for each concentration of aptamer tested therefore there was equal amount of OTA available for binding both assays. Furthermore the same concentration of fluorescently-labelled aptamer is present in both the competitive and magnetic bead assay, hence there was not more aptamer present for binding in one test over the other. However, when repeated several times, the results consistently demonstrated that the competitive assay yielded a higher amount of fluorescently bound aptamer than the magnetic bead assay.

Figure 4-18: Comparison of H8 and 1.12.2 binding affinities for OTA by comparing a competitive assay and a magnetic bead assay. Loss of fluorescence was observed in the competitive assay compared the fluorescence observed in the magnetic bead assay. These results suggest that at certain concentrations, more of the fluorescently labelled aptamer was able to bind to OTA in the competitive assay than in the magnetic bead assay.

It is possible that there is increased non-specific binding occurring in the competitive assay due to the fact that the combined concentration of OTA aptamers is exceeds the determined threshold for non-specific binding suggested in section 3.3.1. The fact that the increase in fluorescence is predominantly observed with the higher concentrations supports this.

Similar to non-specific binding, it is possible that interactions between any complementary regions of the two aptamers being tested could result in a higher fluorescence intensity in the competitive assay elute samples. This could occur if when the non-labelled aptamer binds to the target it also interacts with the fluorescently labelled aptamer, meaning extra fluorescently labelled aptamer is present in the samples eluted from the beads. Furthermore, multiple complementary regions could cause more than one fluorescently labelled aptamer to interact with the non-labelled aptamer bound to OTA. This would result in higher fluorescence intensity from the competitive assay eluate samples than the magnetic bead assay samples.

Finally, it is possible that the different aptamers may bind to different regions of the OTA molecule, and therefore the test would be analogous to a sandwich ELISA method and would not actually require the aptamers to compete for binding.

To further investigate these results, the test was repeated with the original competition between A08 and 1.12.2. The initial results seemed promising (Figures 4-16 and 4-17). However when repeated for a third time the results again indicated that more

fluorescently labelled aptamer was binding during competition to OTA, than when not forced to compete (Figure 4-19).

Figure 4-19: Comparison of A08 and 1.12.2 binding affinities for OTA by comparing a competitive assay and a magnetic bead assay Trial 3. Loss of fluorescence was observed in the competitive assay compared the fluorescence observed in the magnetic bead assay. These results suggest that at certain concentrations, more of the fluorescently labelled aptamer was able to bind to OTA in the competitive assay than in the magnetic bead assay.

Further work with this method is required as the results were inconclusive despite having promising initial data. Possibly, the comparison of the competitive assay to the magnetic bead assay may not be a reliable method for this type of analysis as the addition of a second aptamer may change the conditions too drastically compared to incubation of the aptamer with target alone.

4.3.5: Future Directions

We tested several novel methods for comparing aptamers without the need to rely on K_d values. The results provided valuable insight for further experimentation with these types of aptamer testing methods.

The most reliable results were obtained with the competitive assay for two aptamers in section 4.3.2. Although this assay suggested that A08 may be the best suited aptamer for OTA detection under the conditions used in the testing method, there is concern that the results could be affected by the addition of the two competing aptamers at different steps in the assay. To overcome this it would be useful to repeat the test using a solution of the two aptamers that is mixed prior to incubating with the beads. This would potentially overcome the possibility of the second aptamer displacing the initially added aptamer due to the equilibrium being in its favour. This would allow the results to be interpreted with more certainty.

It would be useful to further validate this method using well-characterized aptamers from other small molecule targets displaying a range of binding affinities. Control experiments that used a non-specific DNA sequence with a selected aptamer for OTA would also be useful in validating the reliability of the method and help us identify and normalize for non-specific interactions.

Since antibody-based screening methods are currently used for OTA detection, it would be useful to alter this test to be able to directly compare a particular aptamer to antibodies available for OTA. This type of testing would help demonstrate that aptamers are an excellent alternative to using antibodies as molecular recognition probes.

Further applications of this method could include comparing the affinity of a designed minimizer to its original sequence, or comparing aptamers selected in the same SELEX, instead of using separate screening techniques. Also, the test could be designed to compare the selectivity of aptamers by immobilizing molecules similar to their target, for example OTB could be used for the OTB aptamers. This would be particularly useful if an application was more focused on a highly selective aptamer, and less concerned about the binding affinity.

Chapter 5: Contributions to knowledge and future studies

The detection of OTA is of the utmost importance in regards to public health, and also global economy. With regulations for OTA maximum limits being considered in Canada, it is imperative that new, robust, cost efficient methods of detection be explored. Aptamers offer a possible alternative to current detection methods, and currently five aptamers for OTA have been developed. In order to successfully utilize one of these aptamers in an application for OTA detection, their properties need to be investigated. This study aimed to do that by examining the aptamers structural properties and their affinity to OTA.

Investigation into the structures of A08 and B08 aptamers demonstrated that it is likely that they do not form a complex G-quadruplex structure. However, preliminary results obtained from the DNase I assay could be used to begin testing the aptamers for a minimizer sequence. This work established that the design of the DNase I assay is useful in studying the conformational changes of an aptamer. It would be useful to optimize the test by studying aptamers whose structures have already been elucidated in literature. Furthermore, it would be beneficial to explore the use of other nucleases.

The magnetic bead assay was successful in measuring the dissociation constants of the OTA aptamers, and it was determined that A08 exhibited the highest binding affinity under the conditions of the test. The test was not able to reproduce the reported K_d value that was reported for the first OTA aptamer, 1.12.2. the K_d was found to be a magnitude higher than the value originally reported by Cruz-Aguado and Penner⁸⁰. This demonstrates the strong influence that the method conditions have on the affinity of an aptamer for its target. This revelation indicates that less of an

importance should be put on the K_d value of an aptamer. Instead it is necessary to develop a new way to investigate aptamers and compare their ability to bind to a target.

In order to compare aptamers for a target in a way that does not depend on calculating a K_d value, several competitive assays were designed and preliminary testing was done. The most promising design involved monitoring the amount of a fluorescently-labelled aptamer that was able to bind to OTA, while competing with a non-labelled aptamer. By also reversing the labelled and non-labelled aptamer and repeating the test, it is possible to compare which aptamer resulted in higher fluorescence intensity, suggesting it exhibits a higher binding affinity for the target under the test conditions. The preliminary results of this method were promising, however more work needs to be done in order to optimize the method and determine the aptamer which demonstrated the highest affinity for OTA in the desired conditions. Prior to further studies with the OTA aptamers it would be beneficial to investigate these methods using aptamers that have previously been demonstrated to exhibit significantly different binding affinities for the same target in order to optimize the method. Successfully designing and utilizing a competitive test to study aptamers would revolutionize the study of aptamers. Therefore, it is imperative that this research continues forward and new methods are investigated.

The aim of comparing the binding affinity of the OTA aptamers is to determine which aptamer would be most appropriate for use in a lateral flow assay-based detection device. This device would be useful for detecting OTA on site and would decrease the current cost of this type of detection, which is mostly attributed to the use of expensive antibodies. As mycotoxins cause harmful effects in animals, and are also a major

source of global economic cost, improving the detection of these toxins is of high priority, and the work presented in this study has contributed to this goal.

Appendices

Appendix A: DNase I assay results (chapter 2)

B08

Figure A-1: DNase I assay results for B08 Trial 1 (A) and Trial 2 (B). DNA was run on a 19% PAGE gel for 3 hours and imaged using an Alphamager with AlphaView software. The predominant fragments produced by digestion with DNase I enzyme can be seen in rows A to F for both trials.

Figure A-2: Binding Isotherms for DNase I assay with B08 from (A) Trial 1 using rows D and E, and B) Trial 2, rows C, D and E. Each curve was analyzed using GraphPad Prism 5, using a nonlinear regression, log(agonist) vs. response -- Variable slope fit.

A08:

Figure A-3: DNase I assay results for A08 Trial 1 (A), Trial 2 (B) and Trial 3 (C). DNA was run on a 19% PAGE gel for 3 hours and imaged using an Alphamager with AlphaView software. The predominant fragments produced by digestion with DNase I enzyme can be seen in rows A to D for trial 1, A-L for trial 2 and A-F for trial 3.

Figure A-4: Binding Isotherms for DNase I assay with A08 from (A) Trial 1 using rows C and D and B) Trial 2, rows D and F, and C) Trial 3 using rows D and G. Each curve was analyzed using GraphPad Prism 5, using a nonlinear regression, $\log(\text{agonist})$ vs. response -- Variable slope fit.

H12:

Figure A-5: DNase I assay results for H12 Trial 1 (A) and Trial 2 (B). DNA was run on a 19% PAGE gel for 3 hours and imaged using an Alphamager with AlphaView software. Each sample produced a long streak on the gel. There are some rows (A, B and C) that may be an accumulation of fragments produced by digestion with DNase I enzyme, however none of these produced a binding isotherm.

H8:

Figure A-6: DNase I assay results for H8 Trial 1 (A), Trial 2 (B) and trial 3 (C). DNA was run on a 19% PAGE gel for 3 hours and imaged using an Alphamager with AlphaView software. The predominant fragments produced by digestion with DNase I enzyme can be seen in rows A to E for Trial 1 and rows A and B of trials 2 and 3.

Figure A-7: Binding Isotherms for DNase I assay with H8 from Trial 1 using rows C and E. The curve was analyzed using GraphPad Prism 5, using a nonlinear regression, $\log(\text{agonist})$ vs. response -- Variable slope fit.

1.12.2:

Figure A-8: DNase I assay results for 1.12.2 Trial 1 (A), Trial 2 (B) and Trial 3 (C). DNA was run on a 19% PAGE gel for 3 hours and imaged using an Alphamager with AlphaView software. The predominant fragments produced by digestion with DNase I enzyme can be seen in rows A to D of Trial 1 and Trial 2 and rows A to E of Trial 3.

Appendix B: Binding isotherms from magnetic bead assay tests (chapter 3)

Figure B-1: Trial 2 A08F binding isotherm obtained using a magnetic bead assay. A08F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3. The K_d was determined to be 21 ± 12 nM.

Figure B-2: Trial 3 A08F binding isotherm obtained using a magnetic bead assay. A08F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3. The K_d was determined to be 18 ± 9 nM.

Figure B-3: Trial 4 A08F binding isotherm obtained using a magnetic bead assay. A08F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3. The K_d was determined to be 47 ± 17 nM.

Figure B-4: Trial 5 A08F binding isotherm obtained using a magnetic bead assay. A08F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3. The K_d was determined to be 56 ± 36 nM.

Figure B-5: Trial 6 A08F binding isotherm obtained using a magnetic bead assay. A08F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3. The K_d was determined to be 36 ± 19 nM.

Figure B-6: Trial 1 B08F binding isotherm obtained using a magnetic bead assay. A08F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3. The K_d was determined to be 56 ± 39 nM.

Figure B-7: Trial 2 B08F binding isotherm obtained using a magnetic bead assay. A08F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3. The K_d was determined to be $200 \pm 93 \text{ nM}$.

Figure B-8: Trial 3 B08F binding isotherm obtained using a magnetic bead assay. A08F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3. The K_d was determined to be $27 \pm 9 \text{ nM}$.

Figure B-9: Trial 4 A08F binding isotherm obtained using a magnetic bead assay. A08F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3. The K_d was determined to be 12 ± 4 nM.

Figure B-10: Trial 1 H12F binding isotherm obtained using a magnetic bead assay. A08F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3. The K_d was determined to be 275 ± 219 nM.

Figure B-11: Trial 2 H12F binding isotherm obtained using a magnetic bead assay. A08F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3. The K_d was determined to be 14 ± 11 nM.

Figure B-12: Trial 3 H12F binding isotherm obtained using a magnetic bead assay. A08F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3. The K_d was determined to be 53 ± 17 nM.

Figure B-13: Trial 2 1.12.2F binding isotherm obtained using a magnetic bead assay. A08F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3. The K_d was determined to be 333 ± 470 nM.

Figure B-14: Trial 2 A08F binding isotherm obtained using a magnetic bead assay. A08F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3. The K_d was determined to be 117 ± 48 nM.

Figure B-15: Trial 3 1.12.2F binding isotherm obtained using a magnetic bead assay. A08F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3. The K_d was determined to be 247 ± 209 nM.

Figure B-16: Trial 4 A08F binding isotherm obtained using a magnetic bead assay. A08F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3. The K_d was determined to be 495 ± 226 nM.

Figure B-17: Trial 5 1.12.2F binding isotherm obtained using a magnetic bead assay. A08F aptamer concentration was varied using a constant number of OTA immobilized beads (2×10^5). Bound aptamer was eluted with 90°C aptamer buffer, and measured using fluorescence. The curve was fit using a non-linear regression Equation 3-1 in GraphPad Prism 3. The K_d was determined to be 25 ± 12 nM.

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