Fluorescence aptasensor development for aflatoxin B₁

by

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Abstract

Using a previously selected and characterized aptamer (known as 3MR5) for aflatoxin B₁ (AFB₁), a Förster Resonance Energy Transfer (FRET) based optical biosensor for AFB₁ was constructed. Experiments were conducted utilising the organic chromophores Fluorescein (FAM) and Black Hole Quencher 1 (BHQ₁) that had been incorporated into a complementary oligonucleotide sequence or capture probe (RCP), and the 3MR5 sequence, respectively. Fluorescence experiments were conducted with the hope that the FRET based quenching caused by close proximity of the chromophores would be disrupted upon introduction of AFB₁, and that this change in fluorescence occurred after addition of AFB₁ and so an alternative FRET pair was tested. This new FRET pair was that of 525 nm emitting Quantum Dots (QDs) and spherical gold nanoparticle (AuNPs). Hybridization of RCP conjugated QDs and 3MR5 conjugated AuNPs resulted in the quenching of the QD fluorescence, however addition of AFB₁ did not result in any recovery of fluorescence. Potential causes for this lack of fluorescence recovery are discussed.

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List of Abbreviations

AFB₁: Aflatoxin B₁

AFB₂: Aflatoxin B₂

AFG₁: Aflatoxin G₁

AFG₂: Aflatoxin G₂

AuNP: Gold nanoparticle

BHQ: Black hole quencher

CD: Circular dichroism

CPG: Controlled pore glass

Cy3: Cyanine 3

Cy5: Cyanine 5

DNA: Deoxyribonucleic acid

DTT: Dithiothreitol

ELISA: Enzyme-linked immuno sorbent assay

FAM: Carboxy fluorescein

FRET: Förster resonance energy transfer

GO: Graphene oxide

HPLC: High-performance liquid chromatography

IARC: International Agency for Research on Cancer

LSPR: Localised Surface plasmon resonance

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

QD: Quantum dots

SELEX: Systematic Evolution of Ligands by Exponential enrichment

Sulfo SMCC: Sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate

TAMRA: Carboxytetramethylrhodamine

TCEP: Tris(2-carboxyethyl)phosphine

TEAA: Triethylammonium acetate

TFA: Trifluoroacetic acid

1. Introduction

1.1 Mycotoxins

Mycotoxins are a group of small molecular weight compounds $(<700 \text{ Da})^1$ produced by genera of fungi such as Aspergillus, Penicillium, Fusarium, Alternaria, and Claviceps.² These compounds are able to cause adverse health effects to livestock and humans in low doses. Mycotoxins commonly contaminate commercial food products such as maize, wheat, barley and spices³. The fungi can grow on numerous crops and contamination can occur during pre-harvest, harvest or post-harvest. The nature of the fungi dictates how contamination occurs but in general plants become susceptible if they have been compromised by other animals or climate.⁴ There are numerous identified mycotoxins, but the major common ones of importance to human health are aflatoxins, ochratoxin A, patulin, fumonisins, zearalenone and nivalenol/deoxynivalenol (see Table 1). Each of these toxins can cause both acute poisoning as well as long term damage leading to cancer among other things. Exposure can occur by direct ingestion of contaminated products or by accumulation of mycotoxins or mycotoxin metabolites within livestock products such as meat, milk and eggs that are later ingested by humans. The acute and chronic effects of mycotoxin exposure are referred to as mycotoxicosis. The effects of mycotoxicosis varies based on the toxin in question, the dosage as well as the health and age of the individual.⁵ Diseases caused by mycotoxins are abiotic in nature and are thus unable to be treated with drugs or antibiotics.⁶ Most mycotoxins were discovered after acute poisoning events of animals, such as the discovery of aflaxtoxin B₁ (AFB₁) after the "Turkey X" incident.

Mycotoxin Class	Principle Fungal Producers	Contaminated Foodstuffs
Aflatoxins	A. flavus	Groundnuts, maize,
	A. parasiticus	cottonseed
Ochratoxins	A. ochraceus	Beans, chickpeas, pecans,
	P. verrucosum	pistachios, green coffee
		beans, cereals
Fumonisins	F. verticillioides	Maize
	F. proliferatum	
Trichothecenes	F. graminareum	Wheat, barley, triticale
(DON, NIV)	F. culmorum	
Zearalenone	F. graminareum	Wheat, barley, triticale
	F. colmorum	

Table 1: Important mycotoxin classes, their fungal producers, and commonly contaminatedfoodstuffs⁵

There are three main factors to consider when evaluating mycotoxins: the identity of the fungi producing the toxins, what substrates the fungi colonize, and the toxicity of the mycotoxins produced.⁷ While hundreds of mycotoxins have been identified, most are not of concern because the fungi that produce them do not colonize animal feed or human food. On a similar note, some fungi produce so little mycotoxins that the risk is non-existent. Lastly, some mycotoxins require large doses to be harmful, and are therefore benign when exposure occurs naturally. As an example, the mycotoxin Sterigmatocystin is by *Aspergillus versicolor*. This mycotoxin shares structural similarities with AFB₁, as well as similar bioactivity. Due to its poor solubility in water and acidic media it is however 100-fold less toxic then AFB₁.^{5,8}

1.1.1 Aflatoxins

Aflatoxins are a class of mycotoxins produced as secondary metabolites by two Aspergillus species. These two species, *Aspergillus flavus* and *Aspergillus parasiticus*, are molds that can contaminate various food products. These fungi generally affect cereal crops such as corn, rice, wheat, oilseeds such as peanuts, certain spices and tree nuts. They were first identified in the 1960's following investigations into the Turkey X disease epidemic.⁹ This was a devastating event in southern England which resulted in the death of over 100,000 poults. It later spread to farmed duck and pheasant, leading to the cause of death being discovered to be infected feed. Imported groundnut meal from Brazil had been contaminated with *Aspergillus flavus* resulting in the aflatoxin contamination.¹⁰ Since this incident aflatoxins have become one of the most studied mycotoxins.²

These molds favour hot and humid conditions seen in tropical and sub-tropical regions.⁴ Growth of these molds tends to follow the growth cycle of the crops being infected. Growth can also occur during storage under ideal conditions. Thus, improper storage or poor screening can lead to contamination of cereal crops distributed to consumers. This can result in ingestion of these dangerous compounds by the population.

Aflatoxins are considered to be the most dangerous mycotoxins, and generally result in damage to the liver. The four major aflatoxins are Aflatoxin B₁, B₂, G₁, and G₂, named for their blue/green fluorescence (structures shown in Figure 1). *Aspergillus flavus* produces primarily Aflatoxin B₁ and B₂, while *Aspergillus parasiticus* produces Aflatoxins B₁, B₂, G₁, and G₂. Other aflatoxins include Aflatoxin M₁ and M₂ which are metabolized forms of B₁ and B₂, and Aflatoxicol. Aflatoxins are difuranceoumarin compounds synthesized through the polyketide pathway. This is a highly regulated process with 17 genes controlling 12 enzymatic conversions in the synthesis pathway of aflatoxin/sterigmatocystin.¹¹



Figure 1: Structures of aflatoxin B₁ (2,3,6aR,9aS-tetrahydro-4-methoxy-1H,11Hcyclopenta[c]furo[3',2':4,5]furo[2,3-h][1]benzopyran-1,11-dione) MW=312.28 g/mol, aflatoxin B₂ (2,3,6aR,8,9,9aS-hexahydro-4-methoxy-cyclopenta[c]furo[3',2':4,5]furo[2,3-h][1]benzopyran-1,11-dione) MW=314.29 g/mol, aflatoxin G₁ (7aR,10aS)-3,4,7a,10a-tetrahydro-5-methoxy-1H,12H-furo[3',2':4,5]furo[2,3-h]pyrano[3,4-c][1]benzopyran-1,12-dione) MW=328.28 g/mol, and aflatoxin G₂ (7aR,10aS)-3,4,7a,9,10,10a-hexahydro-5-methoxy-1H,12H-furo[3',2':4,5]furo[2,3-h]pyrano[3,4-c][1]benzopyran-1,12-dione) MW=330.29 g/mol.

Aflatoxins are known carcinogens, with Aflatoxin B₁ being classified as a Class 1 Carcinogen.⁵ Aflatoxin metabolites can intercalate into DNA and cause damage. AFB₁ toxicity first occurs by activation into an electrophilic species that will react with DNA. This oxidation is accomplished via cytochrome P450 mixed function oxidases, and results in the formation of both exo- and endo-8, 9-epoxides. It has been shown that the endo isomer is relatively non-toxic and that genotoxicity is mostly from the exo isomer.¹² This toxicity of only the exo epoxide is due to the orientation of the activated species during intercalation with DNA. The exo epoxide is oriented in a way that allows for a reaction between N⁷ of guanine bases and C8 of the epoxide.¹² These adducts leads to mutagenic effects such as G to T transversions, lesions, and tumour formation. Hepatocellular carcinomas in humans have been linked to G to T transversion at codon 249 of the p53 tumor suppressor gene.^{5,13} Chronic exposure to AFB₁ has also been shown to stunt the growth of youth.¹⁴ Aflatoxins are excreted from the body by a reactive glutathione *S*transferase system found in the cytosol and microsomes that catalyzes the conjugation of activated aflatoxins with reduced glutathione.⁸ Due to the danger of such compounds it is necessary to have accurate methods of detection.

1.1.2 Impact of Mycotoxins

Mycotoxins have two major economic impacts globally. The first is related to the health risk they pose to consumers due to their toxicity. The second major impact is the financial losses that occur from contaminated food products in the market.

The health risk that aflatoxin and mycotoxin contamination pose in general is usually only felt by low-to-middle income countries without the tools or resources to uphold higher food safety standards. In regions that may have high mycotoxin levels food quality is unregulated and untested. Other times food production is strained resulting in contaminated food being ingested locally instead or sent to poorer markets.^{5,15} These regions typically suffer from food scarcity and thus are unable to prioritize food safety standards.

In more developed countries, effective screening and other practices result in a much lower health impact. It is in these countries that the more prominent impact is the financial loss on markets from contamination. This manifests as the loss in value of products and decreases in supply. In just the US, it was estimated that losses of \$163 million for maize farmers and \$500 million for maize and other crop growers occur annually from aflatoxin contamination alone.¹⁶ The cost due to contamination from other mycotoxins only adds to the financial losses experienced.

1.1.3 Methods of Detection

The serious health risk that mycotoxins present means that accurate methods of detection are of vital importance. Since mycotoxin toxicity exists even at small doses, these detection methods need to be very sensitive and specific.¹⁷

Mycotoxin detection generally relies on techniques such as thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) or enzyme-linked immuno sorbent assay (ELISA) as screening methods. ¹⁸ ELISA is used as a fast screening method with a high throughput for many mycotoxins. ELISA has been used to detect numerous mycotoxins at relatively low cost, such aflatoxins, fumonisins, ochratoxin A, zearalenone, and trichothecenes.^{19,20,21} One issue with ELISA, however, is that matrix dependence can alter recognition events, leading to false negative results. Cross reactivity can also occur resulting in false positives. This has led to using other methods as confirmation tools for positive results from ELISA tests.²²

In general, sample preparation is intensive for methods such as TLC or HPLC. Training and cost are also intensive for these methods. Sample extraction followed by purification is required.¹⁸ Purification typically is done using immunoaffinity chromatography. Due to the varied nature of mycotoxins, specific extraction and purification steps are often required for different mycotoxin samples. Detection of mycotoxins usually either targets individual mycotoxins or specific mycotoxin families. In the case of aflatoxins, the most common method used is still liquid chromatography with fluorescence detection.²² An ELISA based system for AFB₁ detection was reported by Ammida et al. The system relied on screen printed carbon electrodes and anti-aflatoxin B₁ monoclonal antibodies. Their system was able to reach a limit of detection of 90 pg/mL.²³

1.2 Optical sensing

Optical sensing is one tool used often in detection methods for molecules. These sensors are able to convert light into an electric signal that can be used for detection. Examples of optical based sensors include those that exploit fluorescence/phosphorescence, IR, UV, and colorimetric (visible) spectral changes. All these rely on a change in light energy or intensity and convert them into a detectable signal.

1.2.1 Forster Resonance Energy Transfer (FRET)

FRET is a photon-less transfer of energy between a donor and acceptor when in close proximity (See Figure 2).



Figure 2: A donor (D) is excited by a photon in 1. This is followed by relaxation (2) to the lowest excited state. The donor could return to the ground state (3), and if an acceptor (A) is close enough it may be excited by the energy from the donor group. This is a non-radiative process and is called resonance. The acceptor then relaxes to the lowest excited state (4), and can either emit a photon when returning to the ground state (5) or return to the ground state via non-radiative processes (6). Modified with permission from https://chem.libretexts.org/Bookshelves/Physical_and_Theoretical_Chemistry_Textbook_Maps/Supplemental_Modules (Physical_and_Theoretical_Chemistry)/Fundamentals/Fluorescence Resonance Energy Transfer #:~:text=Fluorescence%20Resonance%20Energy%20Transfer%20(FRET,is%20smaller%20than%2010%2_Onanometers.

FRET results from the transfer of energy from an excited state of the donor to a resonant

state in the acceptor by coupling of two transition dipole moments. FRET requires an overlap

between the absorption of the acceptor and the emission of the donor, with stronger overlap

resulting in higher FRET efficiency.

FRET efficiency (E) can be defined mathematically as the quantum yield of the energy transfer transition. It is defined as

$$E = \frac{k_{ET}}{k_f + k_{ET} + \sum k_i}$$

where

- k_{ET} is the rate of FRET
- k_f is the rate of radiative relaxation (i.e., fluorescence)
- k_i are the non-radiative relaxation rates (e.g., internal conversion, intersystem crossing, external conversion etc).

FRET efficiency can also be related to donor/acceptor pair distance using a point dipole-

dipole approximation. This defines the FRET efficiency as

$$E = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6}$$

where

- r is the distance between donor and acceptor chromophores and
- R_0 is the characteristic distance (the Förster distance or Förster radius) with a 50% transfer efficiency

Note that FRET efficiency is inversely proportional to the 6th power of the distance between the acceptor and donor, making it a short range interaction. Efficient FRET, in general, can occur when the FRET pair is usually within 1-10 nm of each other. FRET results in a decrease in donor fluorescence intensity based on efficiency, and results in an increase in the acceptor fluorescence intensity. Some FRET pairs commonly used include Carboxy fluorescein (FAM) /

Carboxytetramethylrhodamine (TAMRA) and Cyanine 3 (Cy3)/ Cyanine 5 (Cy5). Ryohei et al utilised the Cy3/Cy5 FRET pair when conducting experiments attempting to elucidate the conformation during rotation of F₁-ATPase. In their experiment, Cy3 was attached to one of the β subunits, while Cy5 was attached to the γ unit. By measuring the FRET that occurred (or did not) they were able to determine the conformation during rotations.²⁴ In the context of AFB1 sensing, FRET pairs have been employed in optical sensors for the detection of AFB1. Chen *et al* used the FRET pair of FAM and TAMRA when creating their aptamer based sensor for AFB1.²⁵ The creation of a duplex brings the two dyes in close proximity allowing the quenching of FAM by TAMRA. This duplex would be disrupted in the presence of AFB1 resulting in recovery of FAM fluorescence. Other FRET pairs of note are FAM being quenched by Graphene Oxide(GO)²⁶ and Quantum dots (QD) being quenched by GO.²⁷

FRET pairs like that of FAM and TAMRA result in an increase in the accepter fluorescence, in that case an increase in TAMRA fluorescence. A relatively new class of acceptors have been discovered called dark quenchers. Before, most FRET based systems would involve two dyes, which would result in higher background levels due to the emission of the acceptor dye. On the other hand, these dark quenchers are accepters that relax nonradiatively after excitation, dissipating the energy as heat, resulting in lower background fluorescence. Many quenchers have been synthesized covering different wavelengths in the visible and NIR spectrum, with the Black Hole Quencher (BHQ) having a structure capable of absorbing across the entire visible spectrum. Sreenadh *et al* have used a QD/BHQ₁ FRET system for biosensing applications, previously. In that system, QD fluorescence was quenched by proximity of BHQ₁.

The FRET pair was mediated by peptides that could be cleaved by matrix metallopeptidases produced by cancer cells, creating a biosensor.²⁸

Other candidates for FRET that have become of increasing used are nanoparticle reporters. QD have risen as promising candidates along with carbon dots as FRET donors thanks to their highly tunable properties.^{29,30} Wang et al reported a system using carbon dots and MnO₂ sheets as a FRET donor accepter pair. They were able to create a sensor for the detection of glutathione in whole human blood samples with high sensitivity using this system. ³¹ For FRET acceptors, AuNPs have also been explored as an alternative to organic dyes thanks to their stability and optical properties.²⁹

1.2.2 Nanoparticles

Nanoparticles generally refer to small particles whose diameter is between 1-100nm. These particles exhibit properties different from that of bulk material due to their small size. Nanoparticles have been used as far back as 4th century Rome in the Lycurgus Cup, but were not understood until modern times. There unique properties have made them popular for many different applications. Some notable examples include metal nanoparticles such as gold or silver, semiconductor crystals like quantum dots and carbon nanotubes.

1.2.2.1 Gold Nanoparticles

Gold nanoparticles (AuNP) are often used in sensing applications due to the unique optical properties they possess. AuNP can support a phenomenon known as localized surface plasmon resonance (LSPR) (see Figure 3). This is a size and shape-dependent phenomenon that results in strong extinction of light of a specific wavelength. The extinction is a result of a

resonance between incoming electromagnetic radiation and the oscillation of electrons of the metal particle. For spherical AuNP with a radius of 13 nm in water, the resonance condition is met by 520 nm light. Deviations in size can cause a broadening of the absorption band. Larger particles will cause the resonance wavelength to shift to longer wavelengths. The refractive index of the media in which the particles reside also plays a large role in the resonance condition, such that changing the aqueous media or adsorption onto the surface of the particles can cause shifts in resonance wavelength. AuNPs are useful in biosensing thanks to their low toxicity, and easy of conjugation by reacting thiols directly to the Au surface. The sensitivity of the LSPR they can support has also made them popular for sensing applications. This LSPR can be used in SPR or in FRET based experiments as the accepter of a FRET pair.



Figure 3: General Depiction of Surface Plasmon Resonance of Metal Nanoparticles

1.2.2.2 Quantum Dots

Quantum Dots (QD) are small semi-conductor nanoparticles that exhibit unique properties. One important property is the discrete almost atom-like energy levels present, resulting in unique electrical and optical properties. Charge carriers within these semiconductor nanocrystals experience quantum confinement due to the small size of the crystals. This causes discrete energy levels as opposed to a continuous energy band. These discrete energy levels result in the ability to emit light within a narrow wavelength range. The emission wavelength is highly tunable based on the semiconductor metal used, as well as the size of the nanoparticle. Increasing the size results in a redshift in the emission wavelength allowing a wide range of wavelengths to be chosen based on size of QD alone.

1.3 Aptamers

The discovery of DNA and RNA as more than simply being a storage unit for biological systems is a recent event. In 1990, Tuerk & Gold described a generalizable in vitro technique which was used to selectively amplify an RNA pool to favour the binding of T4 DNA polymerase. Their method was described as an in vitro selection or evolution process referred to as SELEX (Systematic Evolution of Ligands by Exponential enrichment). Almost simultaneously, the term aptamer was coined by Ellington & Szostak to describe the nucleic acids that were produced using this method.^{32,33} The term comes from the Latin word aptus meaning "to fit" and the Greek word meros meaning "part". In Ellington and Szostak's research they described small RNA sequences that would bind to organic dyes.

Aptamers are synthetic, single stranded oligonucleotides. They can be comprised of either RNA or DNA and are able to bind to specific targets with high selectivity and affinity. The

SELEX process can be modified to produce aptamers for many different targets. Aptamers have been reported capable of binding to targets such as cells, ions, proteins and small molecules. These small molecules include toxins, antibiotics, heavy metals, drugs and biomarkers.^{34,35}

Aptamers are analogous to antibodies, but offer several advantages over them. Aptamers are selected through an in vitro process, which offers greater freedom when applying selection conditions than their antibody counterparts. They also are able to be synthesized with high accuracy and little batch to batch variation. The in vitro selection conditions allow aptamers to be functional at non-physiological conditions, whereas antibodies are produced in vivo and thus are limited to physiological conditions.³⁶ One disadvantage of aptamers is their rapid degradation by nucleases, requiring modification for use in vivo or else digestion will occur in blood. There is also only 4 nucleobases as opposed to the 20 amino acids making them less chemically diverse than proteins.

1.3.1 SELEX

Aptamers are selected through a process known as SELEX, where only candidates with strong affinity can be separated from other random nonbinding sequences in a pool of oligonucleotides. SELEX allows screening of large libraries of chemically synthesized oligonucleotide sequences. The diversity in these sequences allows evolutionary pressures to be applied using experimental parameters. The selection process relies on the large diversity of sequences and their structural complexity to yield only a few sequences with the ability to bind effectively to the desired target. SELEX is a 3 step process, selection, partition, and enzymatic amplification.^{34,37} The initial library is usually selected with a central random region anywhere from 30 to 80 bases long, flanked by fixed primer binding regions for PCR amplification. The

library is first incubated with the target of interest. Washing is used to remove nonbinding sequences from the pool. Next, partitioning is done to separate binding sequences and nonbinding sequences. The method used for partitioning is typically dependant on the target molecule. Larger target molecules such as proteins can be partitioned using methods such as centrifugation and capillary electrophoresis.³⁸ Small molecule targets generally require immobilization of the target to a solid support. Examples of solid supports used are acrylic, magnetic and sepharose beads. Once partitioning is finished, the remaining sequences are amplified using PCR to give an enriched aptamer pool. This enriched pool can undergo further selection rounds using more stringent parameters to give even higher-affinity aptamers. In order to remove aptamers that bind to similar molecules to the target of interest, or to the solid support, counter selections are also employed. When finished, the remaining oligonucleotides are sequenced and characterized.^{37,39}

1.3.1.1 Capture SELEX

Capture SELEX (see Figure 4) is generally employed in the selection process for small molecule aptamers. Small molecule aptamer target complexes generally do not have a large enough mass difference to be partitioned using mass or size partitioning methods. An alternative is using wash based methods, but these require one of either the target or aptamer to be immobilized onto a solid substrate, and washing is used to partition binding sequences. Some issues arise when immobilization is performed on the target. Aptamers may have a strong affinity for the modified target rather than the unmodified target. This also leads to issues if the aptamer is meant to bind to target free in solution. Immobilization of the aptamer sequence can bypass these issues. Notably, by immobilizing the aptamer sequence, this design of aptamer selection is useful for selecting "structure switching aptamers". This method of selecting structure switching aptamers had first been reported by Nutiu and Li. The intent behind immobilizing the pool instead of the target was to allow selection of unmodified aptamers that could be incorporated into signalling probes.⁴⁰ This is achieved via a small capture region in the central region that is complementary to a biotinylated probe sequence attached to magnetic beads, while random regions flank on either side. Sequences are immobilized to the bead via hybridization to the capture probe. Sequences that would detach in favor of binding to a target would be amplified after washing. Nonspecific binding can be limited by using negative selection rounds against sequences that would bind to other targets and would not be amplified. Aptamers selected in this way have a greater likelihood to require a conformational or structural change upon target binding due to the fact that the sequence must detach from the bead in order to be retained and amplified. Structure-switching aptamers are useful in biosensor designs that will be described in section 1.3.3 Aptasensing.

This approach was recently used to find aptamers that had strong affinity for aflatoxins.⁴¹ The aptamer template contained a fixed central region and randomized flanking regions, followed by PCR primer binding region. The DNA library was immobilized onto a solid support through a 12-base complementary region incorporated into the fixed central domain. An antisense, sequence 24-nt in length, containing a 5' biotinylated poly T-tail (12-nt), referred to as the capture probe (CP), and allowed the DNA library to be immobilized onto streptavidin functionalized agarose beads. One family of aptamers that was found using this method was referred to as 3MR (see Table 2), and was 100-nt in length, containing a 19-nt central region and

25-nt random regions. All 3MR candidates contained the same central region highlighted in Table 2. 3MR5, in particular, is further described in section 1.3.3.1.

Sequence (5' to 3')	Length (nt)	G content (%)
GAGGTCAGATGCAATCCAAACTTTGTTACTCGTC	. 100	22.0
AGAAA <u>TTCGTTCGAGGC</u> TTCCACGGCGCTTTAAA		
ATAGTTGTTATACCTATGCGTGCTACCGTGAA		
GAGGTCAGATGGGTCTGTTGAACACGACCAGAA	A A 100	27.0
AACTGG <u>TTCGTTCGAGGC</u> TTCCACGGCGCTTTAA		
AATAGTTGTTATACCTATGCGTGCTACCGTGAA		
CAGCACAGAGGTCAGATGTGGCGCCTCACACGT		
GCAATCGAATGAC <u>TTCGTTCGAGGC</u> TCATAATCGT	100	25.0
CCTCGGTCTATGATGTATCCCTATGCGTGCTA		
	Sequence (5' to 3') GAGGTCAGATGCAATCCAAACTTTGTTACTCGTC AGAAA <u>TTCGTTCGAGGC</u> TTCCACGGCGCTTTAAA ATAGTTGTTATACCTATGCGTGCTACCGTGAA GAGGTCAGATGGGTCTGTTGAACACGACCAGAA AACTGG <u>TTCGTTCGAGGC</u> TTCCACGGCGCTTTAA AATAGTTGTTATACCTATGCGTGCTACCGTGAA CAGCACAGAGGTCAGATGTGGCGCCTCACACGT GCAATCGAATGAC <u>TTCGTTCGAGGC</u> TCATAATCGT CCTCGGTCTATGATGTATCCCTATGCGTGCTA	Sequence (5' to 3')Length (nt)GAGGTCAGATGCAATCCAAACTTTGTTACTCGTC AGAAATTCGTTCGAGGCTTCCACGGCGCTTAAA ATAGTTGTTATACCTATGCGTGCTACCGTGAA100GAGGTCAGATGGGTCTGTTGAACACGACCAGAA AACTGGTTCGTTGAGGCTTCCACGGCGCTTAAA100AATAGTTGTTATACCTATGCGTGCGCCTCACAGAA CAGCACAGAGGTCAGATGGGCGCCTCACACGT GCAATCGAATGACTTCGTTCGAAGGCTCAGAGGTCAGATGGCGCCTCACACGT CCTCGGTCTATGATGTATCCCTATGCGTGCGCCTCACACGT100



Figure 4: Capture SELEX process⁴² A) Capture sequence is immobilized onto solid support matrix (agarose bead) via biotin-streptavidin interaction B) Sequences contain a known central region (red) flanked by two random randomized regions C) Sequences are immobilized via hybridization between the known region and immobilized capture sequence D) Elution with target is used to evoke structural changes and the release of binding sequences E) Partitioning of binding sequences (potentially structure-switching aptamers) and amplification.

1.3.2 Determining Aptamer Affinity

An aptamer's ability to bind strongly to a target ligand is generally described using its dissociation constant, K_d. The dissociation constant describes a binding equilibrium between an aptamer, ligand and aptamer-ligand complex system with a 1:1 ratio. Such a system would be expressed as:

$$A + L \rightleftharpoons C$$
$$[A] \cdot [L] \cdot k_{on} = [C] \cdot k_{off}$$

Where A represents the aptamer, L is the target ligand and C is the aptamer-ligand complex. In such a system, K_d represents the dissociation constant. The K_d can be described both using thermodynamics and kinetics as such:⁴³

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

As such, the lower the K_d of an aptamer ligand complex, the stronger the binding affinity of the aptamer.

There are numerous methods possible for determining the dissociation constant of aptamers.⁴⁴ Some common methods used include equilibrium dialysis, gel electrophoresis, fluorescence anisotropy, and surface plasmon resonance. All these techniques however, require that the aptamer-target complex undergo a significant conformational change or change in mass. This makes these methods of K_d determination suffer from the same limitations that occur for partitioning during SELEX when the target is a small molecule. Other techniques can be utilized, such as spectroscopic techniques that rely on intrinsic fluorescence of the target, or labelled DNA, in order to characterize the binding of these small molecule targets.

1.3.3 Aptasensing

In order to turn aptamers into useful biosensors, the binding event must be coupled to a detectable signal.⁴⁵ Due to their similarity to antibodies, many antibody-based systems can use aptamers instead as the recognition element. Thanks to the low cost and ease of modification and synthesis of aptamers, many aptamers-based sensors have been developed, leading to their current position as useful molecular recognition elements. The ability to specifically modify certain sites and to develop aptamers that have a conformational change, along with their ease of synthesis, makes them appealing for biosensing. Many different methods have been employed previously, such as mass based, electrochemical, and optical.^{46,47}

Mass-based methods rely on the sensitivity of phenomena such as SPR to provide a signal for the binding event. Slight shifts in signals can be attributed to target binding. Common optical based methods include colorimetric or fluorometric methods. Examples of colorimetric methods involve the aggregation of gold nanoparticles to cause a colour change.

One common method of signal transduction using aptamers is exploiting the ability of aptamers to change conformation upon target binding. Many such structure switching aptamers with different designs have been reported.⁴⁸ Ewa Heyduk and Tomasz Heyduk reported such a structure switching aptamer system for the detection of thrombin. Two aptamers binding different regions of thrombin protein were labelled with fluorophores. Coassociation would bring the aptamers and fluorophores together, resulting in a change in FRET occurring.⁴⁹ Li and Nutiu also reported a structure switching aptamer utilising strand displacement for the detection of ATP. Their system was a tripartite system, using two-stem duplex assembly. The main DNA sequence would contain two stem forming regions. One stem would bind to a complementary sequence containing a modified fluorophore. The second stem would bind to a complementary sequence containing a quenching moiety. This second stem would also contain the aptamer binding. The resulting in displacement of the quenching strand upon target binding. The resulting change in fluorescence could then be measured.⁵⁰

Numerous methods for optical signal transduction in aptamers have been reported. For example, Sabet et al reported a FRET based aptamer biosensor for AFB₁. The system involved QD conjugated to the aptamer sequence. DNA was incubated with AuNP and adsorption onto the AuNP surface brought the FRET pair in close proximity. The introduction of AFB₁ causes desorption of the aptamer sequence resulting in a recovery of QD fluorescence. This system had a limit of detection of 3.4 nM.⁵¹ Other methods take advantage of the conformational change that occurs when an aptamer-target binding event occurs. These sensors are often called "Structure switching aptasensors".⁵⁰ Seok et al reported on one such system. Split halves of a hemin-binding DNAzyme⁵² (a DNA-based enzyme) was combined with an aptamer for AFB₁. These DNAzyme split halves have peroxidise mimicking activity when combined. When no AFB₁ is

present, the split halves of the DNAzyme can interact with the aptamer to form a complex and the peroxidise activity results in a color change. When AFB₁ is present, the aptamer binds preferentially to AFB₁ resulting in deformation of the complex and reduced peroxidise activity, with a limit of detection of 0.1 ng/mL.⁵³

Some techniques require immobilization of either recognition elements or the target to function. This can be labor and cost intensive, so systems that do not require such steps are preferred. Homogenous fluorescent assays solve this issue by removing the many preparation steps while also removing non-specific interactions and even reducing cost.^{54,}

DNA does not exhibit fluorescence naturally; fluorescence in the system must come from other sources. Covalently binding fluorophores to specific sites in DNA allows the development of fluorescence-based aptasensors. The ability to attach these fluorophores means that the ligand of interest also does not need to be inherently fluorescent. With the large variety of fluorophores available many different sensor systems are possible.

1.3.3.1 Structure Switching Aptamer 3MR

As described in section 1.3.1, several aptamers (3MR family) for AFB₁ were recently selected using the capture SELEX design.

Circular dichroism (CD) was previously used to examine the structure of these aptamers and compare them to an oligonucleotide sequence that is known to form a G-quadruplex structure, sequence PS2.M (Figure 5). G-quadruplexes are one of many secondary structures that can form in oligonucleotides. They are formed by repeating guanine rich regions in a sequence. The G-quartet motif is formed by Hoogsteen hydrogen bonding between four guanines.⁵⁵ The G- quadruplexes are formed by two or more stacked planes that are then stabilized by various π - π interactions.



Figure 5: CD spectrum of 3MR aptamer candidate sequences in 20 mM Tris buffer (100 mM NaCl, 5 mM KCl, 1mM CaCl₂, 1 mM MgCl₂), as monitored by UV-CD using an Olis Cary 17 CD spectropolarimeter. Used with permission from reference 41

None of the 3MR5 aptamers were found to be G-quadruplexes, only stem containing structures. There are many different G-quadruplex structures, but the main two are parallel and anti-parallel. The parallel and anti-parallel G-quadruplexes each have distinct characteristics that can be used to determine this kind of secondary structure in the sequence from the CD of the sample. In the case of parallel G-quadruplexes, there is a tendency to see a maximum around 260 nm, followed by a smaller negative band at 240 nm.⁵⁶ Anti-parallel G-quadruplexes will,
however, show a positive band at 295 nm, a negative band at 260 nm and a positive band at 245 nm in their respective CD spectra.⁵⁶

Further, these sequences were studied for their affinity and selectivity for AFB₁. 3MR5 was the only 3MR aptamer shown to bind AFB₁ with high affinity. The K_d value was determined to be $1.4 \pm 0.7 \mu$ M. Figure 6 shows the raw and normalized binding curves for 3MR5 and AFB₁. 3MR5, 3MR3, and 3MR2 were also tested against AFB₂, AFG₁, and AFG₂ but they were all determined to be nonbinding for the additional aflatoxins tested.



Figure 6: A) Binding curve for 3MR5 and AFB₁ B) Binding curve for 3MR5 and AFB₁ normalized to fraction bound. Used with permission from reference 41

Previously, a structure switching assay was designed for 3MR5. This structure switching assay took advantage of the conformational change that would occur when the aptamer bound with its target. The small capture probe previously used for capture SELEX had been truncated to remove the 5' biotinylated poly T-tail (12-nt), and then was modified with BHQ₁. This capture probe was termed RCP. The 3MR5 aptamer had also been internally modified to include FAM near the capture region. The hope was that FRET between BHQ₁ and FAM would result in quenching of FAM fluorescence after hybridization. Then a recovery of fluorescence would be seen after the conformational change upon target binding. A general depiction of the proposed system is shown in Figure 7.



Figure 7: Schematic representation of the structure switching system to detect AFB₁. Hybridization of the capture probe and aptamer results in quenching (Left). Upon addition of AFB₁, release of the complementary capture probe would provide recovery of fluorescence (Right). Used with permission from reference 41

This was not observed however, as instead of a recovery of fluorescence, what was seen was further quenching of the 3MR5_6FAM aptamer. Varying ratios of aptamer to capture probe were tested against 1µM AFB₁ as shown in Figure 8, and all showed either insignificant recovery or further quenching. Finally, the aptamer sequence itself was tested against AFB₁ solely. The results (Figure 8F) showed that even without the BHQ₁ tagged capture probe, that quenching still occurred upon addition of AFB₁. AFB₁ is named for its blue fluorescence, and as such its absorption should not have resulted in the quenching of fluorescein as there is not any spectral overlap.



Figure 8: Previous fluorescent experiments conducted using the fluorophore on the 3MR5 sequence. Fluorescence signal decrease with the addition of 1µM AFB1 to the quenched 3MR5_FAM signalling aptamer at varying Apt/CP ratios A) 1:1, B) 1:2, C) 1:3, D) 1:4, E) 1:5, F) 3MR5_FAM and AFB1 only. Used with permission from reference 41

1.4 Thesis Objectives

The goal for this thesis is to develop an aptamer-based assay for AFB₁ using the 3MR5 aptamer described above. This aptamer candidate, previously selected through capture SELEX for detection of aflatoxins, was studied for its use in a fluorescence-based assay. Previously, the aptamer 3MR5 had been selected via SELEX as a binding partner for the AFB₁. Fluorometric experiments had been performed using 3MR5 and RCP tagged with a FRET pair, fluorescein and black hole quencher. In this experiment, 3MR5 was tagged with fluorescein and RCP was tagged with BHQ₁. These were first incubated together, leading to a loss of signal due to quenching. It was thought that upon addition of AFB₁, that fluorescence would be recovered as target binding would lead to the release of the capture sequence, but that was not observed. We hypothesized that this could have been due to internal quenching from the secondary structure adopted by the aptamer/target complex. This led to two questions that were the focus of this thesis: 1) Will swapping the location of the fluorophore and quencher create a functional aptasensor? and 2) Will replacing blackhole quencher and fluorescein with AuNP and QD, respectively, yield a working sensor and improve the efficiency?

Characterization of the aptamer after modification with fluorophores was performed. The interactions of the aptamer and its viability as a component for sensing aflatoxin B_1 were studied using fluorometry. The aptamer was used as part of a FRET-based hybridized signalling system looking at the effect of the fluorophore quencher pair.

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2. Methods

2.1 DNA preparations

2.1.1 Materials

Oligonucleotide sequence 3MR5-IDT was purchased HPLC purified from IDT. Phosphoramidites, sequence modifiers, activator, deblock, capping agents, and oxidizing agents were purchased from Glen Research. All buffers and solutions were prepared with EMD Millipore, Milli-Q deionized water (18.2 M Ω ·cm @ 25°C).

2.1.2 Aptamer Synthesis

Oligonucleotide 3MR5-BHQ₁, RCP-3FAM and RCP-Thiol were synthesized using standard phosphoramidite chemistry. 0.067 M solutions of guanosine, thymidine, adenosine, and cytidine phosphoramidites were prepared by dissolving 100 µmol samples in 1.5 mL of HPLC grade acetonitrile (Glen Research). 0.067 M Thiol-Modifier C6 S-S (Glen Research) modifier was prepared with anhydrous acetonitrile. 0.067 M Black Hole Quencher 1 (BHQ-1-dT) modifier was prepared with a 1:3 (v/v), anhydrous acetonitrile/dichloromethane diluent. Phosphoramidite solutions and 1000Å CPG support columns were loaded onto a BioAutomation Mermade 6 synthesizer. Sequences were synthesized following manufacturer suggested conditions. Coupling time was increased to 10 minutes for all modifiers.

Following synthesis, support columns were removed from the synthesizer. Each column's CPG support beads were carefully transferred to separate microcentrifuge tubes, and allowed to incubate in 1 mL of 28% NH₄OH for 2 hours at 65°C. Following the cleavage and deprotection steps, the tubes were centrifuged, and the supernatant was transferred to a series of clean

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microcentrifuge tubes. The supernatant was then purified on a Glen Pak using manufacturer's instructions for the specified modifier. After purification the samples were collected and dried overnight on a Savant AES2010 SpeedVac cryopump.

Table 3: Sequences synthesized and their modifiers

	Sequence
3MR5-BHQ1	CAGCACAGAGGTCAGATGTGGCGCCTCACACGTGCAATCGAA NGACTTCGTTCGAGGCTCATAATCGTCCTCGGTCTATGATGTAT CCCTATGCGTGCTA
RCP 3FAM	GCCTCGAACGAAF
3MR5-IDT	CAGCACAGAGGTCAGATGTGGCGCCTCACACGTGCAATCGAA NGACTTCGTTCGAGGCTCATAATCGTCCTCGGTCTATGATGTAT CCCTATGCGTGCTA
RCP-Thiol	NGCCTCGAACGAA

2.1.3 Glen Pak Purification/Disulfide Cleavage

RCP-Thiol and RCP-3FAM sequences were purified by Glen-Pak. Six Glen-Pak purification cartridges were placed in the female luer ports of a 12 port SPE type vacuum manifold. The vacuum was adjusted to ~ 10 mm Hg using the vacuum control valve (targeted flow rate of 1 drop per second). Each cartridge was conditioned with 0.5 mL of acetonitrile followed by 1 mL of 2 M tetraethylammonium acetate (TEAA).

Sequences that were purified using the Glen-Pak method were synthesized with DMT-ON. Deprotection of sequences was carried out by incubating the CPG beads in 1 mL of 28% NH₄OH for 2 hours at 65° C. Once deprotected, an additional 1 mL of 100 mg/mL sodium chloride solution was added to each microcentrifuge tube.

To purify the RCP-3FAM oligonucleotide sequences the oligo/salt mixtures were added to the cartridges in 1 mL aliquots. 2 x 1 mL aliquots of 100 mg/mL NaCl salt wash solution were used to wash the failed sequences from the cartridges. Cartridges were then rinsed with 2 x 1 mL of 2% trifluoroacetic acid (TFA), and 2 x 1 mL of deionized water. Clean 15 mL microcentrifuge tubes were placed in the manifold and the purified DNA sequences were eluted from the cartridge using 2 x 1 mL of 50% acetonitrile in deionized water containing 0.5% ammonium hydroxide. Eluted samples were collected and dried overnight on cryopump.

To purify the RCP-Thiol oligonucleotide sequences, the oligo/salt mixtures were added to the cartridges in 1 mL aliquots. 2 x 1 mL aliquots of 100 mg/mL NaCl salt wash solution were used to wash the failed sequences from the cartridges. 2 x 1 mL aliquots of deionized water were used to wash away excess salts. New clean 15 mL microcentrifuge tubes were placed in the manifold and the purified DNA sequences were eluted from the cartridge using 1 x 1mL of 50% acetonitrile in deionized water. Cleavage of the disulfide bond was performed directly afterwards by adding 1mL of 0.2M DTT in 0.1M phosphate buffer and letting sit for 30 minutes.

A second set of cartridges were prepared and conditioned the same as before. 5 mL of 0.1M TEAA, pH 7, was added to each microcentrifuge tube and the solutions were applied to each cartridge in 1mL aliquots. Each cartridge was then washed with 2 x 1mL of 0.1M TEAA, pH 7, and 2 x 1mL of deionized water. The final oligonucleotide was eluted from the cartridge using 10% acetonitrile in deionized water.

2.2 Aptamer Characterization

Structure prediction for the aptamer structures were examined on RNAstructure (rna.urmc.rochester.edu). RCP-Thiol, RCP-3FAM, 3MR5-BHQ₁ and 3MR5-IDT was characterized using UV-Vis spectroscopy. RCP-Thiol, RCP-3FAM, 3MR5-BHQ₁ were characterized by Mass Spectroscopy. Purified RCP-Thiol and RCP-3FAM was diluted before running a UV-Vis spectrum. Extinction coefficient was calculated using the nearest neighbour approximation for 260nm and then the sample was quantified. LC/MS was performed to confirm the identity of the sequences.

2.3 Nanoparticle Synthesis and Characterization

2.3.1 Materials

515 ITK Quantum Dots were purchased from Thermo Fisher. HAuCl₄ and sodium citrate was purchased from Sigma-Aldrich. All solutions and dilutions were prepared using EMD Millipore, Milli-Q deionized water (18.2 MΩ·cm @ 25°C).

2.3.2 AuNP Synthesis

In a 250 mL Erlenmeyer flask, 2 mL of HAuCl₄ solution and 98 mL of deionized water were added. A stir bar was added and flask placed on a hot plate. Flask was covered with aluminum foil, and was heated with stirring until vigorously boiling. Once boiling 10 mL of sodium citrate solution was added. Solution turned dark red and was allowed to cool for 20 minutes then stored in 4° C fridge until quantification.

2.3.3 Nanoparticle Characterization/Quantification

AuNP and QDs were characterized by UV-Vis and TEM using a Cary Spectrophotometer and TEM (JeoL). Quantification of AuNPs was done by applying the Beer-Lambert Law for the absorption peak at 520 nm. The extinction coefficient applied was that for spherical AuNPs 13 nm in diameter. QD quantification was done by using the peak at 350 nm and the corresponding extinction coefficient for that wavelength. Size distribution and EDS were carried out using TEM on both QD and AuNPs.

2.3.3.1 AuNP Characterization/Quantification

AuNPs were characterized and quantified by UV-Vis, TEM and EDS. For UV-Vis quantification, sample was diluted in deionized water to give a 1 in 10 dilution factor. Using the extinction coefficient of 270000000 cm⁻¹M⁻¹ for 13 nm spherical AuNPs at 520 nm wavelength gave an effective concentration of 13.97 nmol/L. TEM analysis was conducted by dropcasting AuNP onto TEM grids and allowing to dry overnight. From TEM software the size of random particles was measured and EDS was performed to confirm the identity of the particles.

2.3.3.1 Quantum Dot Characterization/Quantification

QD samples were characterized using UV-Vis, TEM and EDS. QDs were previously purchased from Fisher, and the UV-Vis spectra taken were compared to that provided by the supplier. Quantification was carried out without needing any dilution. The concentration was determined by using the known extinction coefficient of at 405nm. TEM samples were prepared by drop casting QD solution onto TEM grids and allowing too dry for 24 hours.

2.4 Nanoparticle Conjugation

2.4.1 Materials

Crosslinker Sulfo-SMCC was purchased from Thermo-Scientific.

2.4.2 Quantum Dot Conjugation

Quantum Dots purchased from Fisher were buffer exchanged into a 0.1M PBS buffer (pH 7.2) using an Amicon 3K filter and then quantified. DNA sequence RCP-Thiol was reduced using 10mM TCEP for 30 minutes. Concurrently, crosslinker Sulfo-SMCC was diluted to 2 mL after dissolving 2 mg in 200 µL.

 $50 \ \mu\text{L}$ of QD in 0.1M PBS buffer were incubated with 4 μL of diluted Sulfo-SMCC for 30 minutes on a shaker. Solution was then diluted to $500 \ \mu\text{L}$ with deionized water and desalted using an Amicon 3k filter. Desalting was done 3 times to ensure all unreacted Sulfo-SMCC was removed from QD solution. Afterwards, 13 μ L of 3.8 μ M RCP-Thiol DNA was incubated with the QD solution for 30 minutes on a shaker. The solution was desalted again, using a 30K Amicon filter to remove any DNA that did not react. Final desalt used 0.1M PBS buffer instead of deionized water. The filtrate was kept to quantify the amount of DNA that did not react. Both filtrate and desalted sample were then quantified.

2.4.3 Gold Nanoparticle Conjugation

1.5mL of previously synthesized AuNP was transferred to a 2 mL microcentrifuge tube. To this 6.372 µL of RCP-Thiol DNA was added to give an AuNP/DNA ratio of 1.20. Mixture was allowed to mix for 30 minutes at room temperature on a shaker. Afterwards 50µL of 500 mM sodium citrate buffer, pH 3, was added to the solution and put on a shaker for 30 minutes. Solution was then centrifuged at 12k RPM for 20 minutes resulting in a red pellet forming. Supernatant was removed and collected in a separate clean microcentrifuge tube, while pellet was redispersed using 1.5mL of deionized water. Sample was centrifuged again at 12k RPM for 20 minutes, and the supernatant was discarded. Sample was redispersed one final time in 750µL 0.1M PBS buffer and 750µL deionized water. Sample was stored in a 4°C fridge until needed.

2.4.4 Characterization of Conjugated QD/AuNP

AuNP conjugates were characterized by UV-Vis and colorimetric methods and QD conjugates were characterized by UV-Vis and TEM/EDS.

2.4.4.1 AuNP Conjugates

AuNP conjugation was confirmed by using two methods. First, a simple colorimetric test involving salt based aggregation was performed. To see whether the DNA had successfully conjugated to the surface of the AuNP, 1 mL of bare AuNP was prepared in a 1.5 mL microcentrifuge tube. To this, the same volume of sodium citrate buffer that was used for the conjugation experiment was added, and an immediate color change occurred. After shaking, aggregation of the AuNP was observed. The only difference between the bare AuNP and the conjugated sample was that thiol-containing DNA was also present in the conjugate. To determine how efficient the conjugation was, UV-Vis spectroscopy was performed. As it is difficult to obtain an accurate quantification of the DNA due to the overlap in the AuNP spectrum, the filtrate collected during conjugation was quantified instead. The extinction coefficient provided by the manufacturer for 260 nm was used to determine the concentration and moles of DNA in the filtrate.

2.4.4.2 QD Conjugates

QD conjugation was confirmed using TEM and EDS. Conjugate samples were dropcast onto TEM grids and left to dry for 24 hours before being imaged. EDS was used to determine the presence of DNA conjugates, by looking at the Phosphorus levels.

2.5 Fluorescence Experiments

2.5.1 Materials

All experiments were performed using a 60 μ L quarts cuvette on a Horiba Jobin Yvon Fluorolog spectrofluorometer.

2.5.2 3MR5-BHQ and RCP-3FAM

All samples were allowed to adjust to room temperature for 30 minutes before measurements were taken. 1:1 ratio of 3MR5-BHQ₁ and RCP-3FAM were incubated 30 minutes before measurements were taken. 15, 30 and 60 μ L of 10 μ M AFB₁ were added to samples containing 30 μ L of hybridized DNA and then diluted with buffer to 300 μ L in order to give 100 nM of 3MR5-BHQ₁ and RCP-3FAM with varying concentrations of AFB₁. Excitation wavelength was set to 495 nm and emission measured from 500-600 nm in 0.5 nm increments and an integration time of 0.5 s.

2.5.3 3MR5 AuNPs and RCP QDs

All samples were allowed to adjust to room temperature for 30 minutes before measurements were taken. A control sample to account for QD signal was performed by adding 5 µL of QD to 995 µL of buffer. A 1:1 mixture of AuNP conjugated DNA and QD conjugated DNA was created by taking 860 µL of 1.79 nM conjugated AuNP, 135 µL of 1:1 buffer/DI water and 5 µL of QD solution. This was mixed and separated into two 500 µL samples. One was to measure quenching from hybridization of conjugates. The second sample had AFB₁ 3.86 µL added to create an approximately 1:1:0.5 ratios of AuNP, QD and AFB₁. This sample was allowed to sit for 30 minutes before measuring fluorescence. Time of measurements was taken to account for loss of fluorescence over time. Excitation was set at 405 nm, and emission was measured from 500-600 nm in 0.5 nm increments and an integration time of 0.5 s. All measurements were performed in triplicates.

3. Results

3.1 DNA Structure Prediction

The potential secondary structure of 3MR5 was examined using RNA/DNA structure prediction software RNAStructure from the Mathews lab. This software looks at base pairing and tries to minimize the overall energy of the structure when predicting structures.⁵⁷ The presence of stem loops is predicted as seen in Figure 9 and Figure 10. Three of the lowest energy systems with 3MR5 hybridized to RCP were predicted as shown in Figure 11. All three systems show the retention of stem loops in the secondary structure of 3MR5 even after hybridization occurred.



Figure 9: Lowest free energy predicted structure of 3MR5, using RNAStructure software.



Figure 10: Predicted structure of 3MR5 using an alternative method of structure prediction that generates structures with the most highly probably base pairs.



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Figure 11: Lowest energy structures that were predicted for the duplexes of 3MR5 and RCP. The presence of stem loops is still predicted even when duplexed with RCP.

3.2 DNA Characterization

DNA was characterized by UV-Vis spectroscopy in order to quantify how much DNA was synthesized. The sequences synthesized are listed in Table 3. Sequences 3MR5-BHQ₁, RCP-3FAM, RCP-Thiol and 3MR5-IDT were all quantified by looking at the absorption at 260 nm. From this it was determined that 174 nanomoles of 3MR5-BHQ₁ (Figure 12), 1594 nanomoles of RCP-3FAM (Figure 14), and 386 nanomoles of RCP-Thiol (Figure 16) were synthesized. 3MR5-IDT was ordered and the sample was confirmed to contain 11 nanomoles of the sequence (Figure 18). Using the absorption of BHQ1 at 534 nm 204 nanomoles was calculated instead for the 3MR5-BHQ₁ sequence. Liquid chromatography and mass spectrum revealed that the purity of 3MR5-BHQ1 was estimated to be only 20% purity (Figure 13). This low purity is due to the presence of failed sequences during synthesis. Molecular weight of 3MR5-BHQ1 is 31432.63 g/mol. Peaks before this would be failures during synthesis. Purity was estimated by examining the intensity of MS peaks for failed sequences compared to full length. LC/MS for RCP-3FAM can also be seen in Figure 15. They show very few impurities present in the sample, however there is two different fluorescein peaks present in the UV-Vis spectra from 480-490 nm. Liquid chromatography and mass spectroscopy was also performed for RCP-Thiol, but no purity was estimated (Figure 17).



Figure 12: UV-Vis spectrum of 3MR5-BHQ₁. Sample was quantified using a 1 in 500 dilution and the absorbance at 260 nm. Inset is rescaled to see the absorbance peak of BHQ₁.

.C/MS Chromatogram of R30973_3MRS_BHQ1:



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Figure 13: A) Liquid chromatogram of 3MR5-BHQ₁. B) ESI Mass Spectrum of 3MR5-BHQ₁. C) Close up ESI Mass Spectrum of 3MR5-BHQ₁ near estimated mass. Molecular weight of 3MR5-BHQ₁ is 31432.63 g/mol. Failure sequences that still contain the modifier BHQ₁ will have a molecular weight greater then 18734.2 g/mol



Figure 14: UV-Vis Spectrum of RCP-3FAM. Sample was quantified using a 1 in 1000 dilution and the absorbance at 260 nm. Inset is a close up of the peak for fluorescein. The presence of two peaks hints at free fluorescein in solution.

LC/MS Chromatogram of R30973_RCP-FAM:



Zoom Deconvoluted Mass Spectrum of R30973_RCP-FAM, RT = 0.132 min:



Figure 15:A) LC of RCP-3FAM sequence. B) Mass Spectrum of RCP-3FAM sequence. Molecular weight of RCP-3FAM sequence is 4208.86 g/mol.



RCP-Thiol Quantification Data

Figure 16: UV-Vis Spectrum of RCP-Thiol sequence. Absorbance at 260 nm was used to quantify the amount of DNA present.



Figure 17: Top; liquid chromatogram of RCP-Thiol; Middle and Bottom, mass spectrum of RCP-Thiol. Molecular Weight of RCP-Thiol is 3835g/mol.



Figure 18: UV-Vis Spectrum of sequence 3MR5-IDT. Quantified using a 1 in 200 dilution and the absorbance at 260 nm

3.2 Nanoparticle Characterization

3.2.1 AuNP

Quantification of AuNPs was carried out using the absorption peak at 520 nm (Figure 19). From this absorption the concentration of the stock AuNP solution was found to be 13.97 nmol/L. All further experiments involving AuNP were conducted using dilutions of this stock solution.



AuNP Quantification Data

Figure 19: UV-Vis Spectra of a 1 in 10 Dilution of AuNP solution. Quantification was done using the peak at 520 nm and the extinction coefficient of 270000000 cm⁻¹M⁻¹.

TEM software was used to measure the size of random particles in order to find an average size. Particles ranged in size from 11 nm to 17 nm in diameter (Figure 20). EDS was also performed to confirm that the particles observed were AuNPs, and Au peaks were prominent in the EDS providing confirmation that the particles identities were Au (Figure 21).



Figure 20: TEM image of AuNP drop casted onto TEM grid. Diameter of random particles labelled to show relative size.



Figure 21: EDS of synthesized AuNPs, confirming identity of particles as gold.

3.2.2 QD

QD were quantified using the UV-Vis emission peak at 350 nm and the extinction coefficient 710000 cm⁻¹M⁻¹ provided by supplier (Figure 22). No dilution was used for the quantification. For comparison, the supplier provided UV-Vis spectra is present in Figure 23. Note that the QD originally were suspended in a borate buffer and had to be buffer exchanged into a 0.1M PBS buffer.



Figure 22: UV-Vis spectra of QDs in 0.1M PBS buffer. No dilution was required. Inset displays the higher energy portion of the spectrum.



Figure 23: Supplier absorption and emission spectrum of ITK 525 QD.

TEM and EDS were used to confirm the identity of the particles. The particles were well dispersed and were found to be between 4 and 6 nm in diameter (Figure 24). EDS was performed to further confirm the particles identities. The Zn/S outer core is readily seen in the EDS spectra and the Cd present in the inner core is also detected (Figure 25).



Figure 24: TEM image of bare QDs showing relative size distribution.



Figure 25: TEM (Left) and EDS (Right) of bare QDs dropcast onto a TEM grid. EDS shows presence of both the ZnS outer shell and Cd from inner core.

3.3 Conjugation Experiments

3.3.1 AuNP Conjugation

At first UV-Vis was used to attempt to quantify the DNA present on the AuNPs (Figure

26). Overlap from AuNP absorption resulted in difficulty trying to quantify DNA at 260 nm via

UV-Vis.

UV-Vis spectra of conjugated 3MR5-AuNPs and fitrate



Figure 26: UV-Vis comparison of Conjugated and filtrate. Both were quantified using absorbance at 260 nm without requiring any dilution. The concentration of DNA calculated did not match the amount of DNA used during conjugation.

Thus AuNP conjugation was instead confirmed using a colorimetric salt test. After incubation with 3MR5-IDT, a volume of sodium citrate buffer was added to the sample. This was to aid in conjugation with the thiol containing DNA. A second sample of only AuNP was also prepared and had the same amount of buffer added, and both were compared. A slight darkening occurred in the sample containing DNA, and aggregation occurred in the sample without DNA (Figure 27). Concentration of AuNP after the conjugation experiment was found to be 1.79 nM.


Figure 27: Images of Colorimetric salt test performed after AuNP conjugation. Left image is bare AuNPs in solution. Middle is after adding buffer containing Na⁺ until concentration was 60 mM Na⁺. Right is a comparison between Conjugated AuNPs (Purple-red) and bare AuNPs(Purple-black) after adding buffer to both until Na⁺ concentration was 60 mM Na⁺. The fact that the conjugated AuNPs are protected from salt-induced aggregation suggests that the conjugation was successful.

3.3.2 QD Conjugation

QD conjugation was difficult to confirm due to the overlap in absorbance of DNA and QD at 260 nm. As such, other methods were used determine conjugation. TEM paired with EDS showed that even in one conjugated sample there were two distinct regions. One region appeared clouded on TEM and was seen to contain much higher levels of phosphorus then the second region (Figure 28). Quantification of the QD peak after conjugation showed the QD concentration to be 0.308μ M.



Figure 28: TEM and EDS of two sections of a QD conjugate sample. The top EDS refer to the region labeled eds1 while the bottom refers to eds2. Both show presence of Cd and Se, as well as Zn and S. Differences in P are used to confirm DNA.

3.4 Quenching Experiments

3.4.1 3MR5-BHQ and RCP-3FAM

Quenching experiments were performed using 3MR5-BHQ₁ and RCP-3FAM in a 1 to 1 ratio. Fluorescence intensity decreased upon hybridization with the 3MR5-BHQ sequence as seen in Figure 29. Addition of AFB₁ to create 0.5, 1 and 2 μ M solutions of AFB₁ did not result in any recovery of fluorescence. There was no significant difference between the various AFB₁ concentrations and the hybridized control.



Figure 29: Fluorescent Quenching experiment between 3MR5-BHQ₁ and RCP-3FAM. Control refers to the 1:1 mixture of both RCP-3FAM and 3MR5-BHQ₁ sequences, while Fluorescein refers to a sample only containing RCP-3FAM. Different Concentrations of AFB₁ were tested against the control sample. Inset is all results except for Fluorescein, showing the small difference in fluorescence between the control and samples with AFB₁ added.

3.4.2 3MR5 AuNP and RCP QD

Fluorescence of QD was seen to decay over time, so to correct this, a calibration was performed alongside the quenching experiment. This involved taking additional readings of the QD sample after the Au + QD and Au + QD + AFB₁ samples as seen in Figure 30. These were then used to determine how much the QD signal decreased at the specific wavelength over time. A simple line of best fit for the emission wavelength of the QD would provide a slope to correct for the emission wavelength, but would also increase the perceived emission for all other measured wavelengths. Using statistical functions in a spreadsheet, a calibration for each individual wavelength was calculated and applied for all wavelengths scanned to provide a better image of the fluorescent decay that had occurred. This was applied to the fluorescence measurements resulting in Figure 31. Even accounting for the loss of fluorescence there was still no significant difference between that of the hybridized sample and that containing AFB₁. The small recovery of fluorescence seen was within the error present in the experiment.



Figure 30: Decrease in Quantum Dot Fluorescence over time. Used to generate a correction for QD fluorescence after a prolonged period of time.



Quenching and Recovery Experiment

Figure 31: Quenching Experiment using a 1 to 1 ratio of conjugated QD and conjugated AuNP. Values were corrected using calibration curve for QD fluorescence decay over time. Concentration of AFB₁ was half that of the QD/AuNP.

4. Discussion

As described in the introduction, the aptamer 3MR5 had been explored as part of a FRET-based assay for AFB₁. The assay had two main steps which are shown in Figure 32. First, 3MR5 tagged with fluorescein and RCP tagged with BHQ was incubated together. The hybridization of the aptamer with the capture probe led to the quencher pair being in close proximity, which led to a loss of the fluorescence signal. Upon addition of AFB₁, the capture sequence would have been displaced by the target and the fluorescence should have been recovered as the quencher would no longer have been in close proximity to the fluorophore, disrupting FRET.



Figure 32: Schematic representation of the structure switching system to detect AFB₁. Arrangement pursued in this experiment (Top) and previous experimental arrangement (Bottom). Both arrangements begin with quenched fluorescence when 3MR5 and RCP are hybridized (Left). Upon AFB₁ addition, release of RCP would result in recovery of fluorescence (Right). The arrangement pursed in this experiment had the quenching entity tagged to the aptamer sequence, and the fluorophore tagged to the capture probe. Previous experiments had reversed the position of both. In both cases, presence of target induces a structural change in the aptamer that causes the release of the capture probe. Used with permission from reference 41.

Unfortunately, this was not observed and it was thought that maybe internal quenching

was leading to this effect. By this, it was thought that perhaps the secondary structure formed

from binding to target somehow altered the fluorescence of the attached fluorophore resulting in the decreased fluorescent recovery seen upon addition of AFB₁.

Thus, this project began by exploring whether swapping the location of the quencher and fluorophore would lead to a working assay. It was thought this could lead to better results since the capture probe would be free in solution after 3MR5 had bound AFB₁. This would eliminate the possibility of secondary structure effects causing interference with the fluorophore.

To ascertain whether a functional aptasensor could be developed, quenching experiments where the two probes were swapped were first performed. At first, the aptamer was tagged with the organic quencher BHQ₁ and RCP was labelled with fluorescein. The purification of the BHQ1 modified 3MR5 was difficult due to the hydrophobic nature of the modifier. As such, the 3MR5 sample contained many contaminants from failures at each base after the modifier. This is illustrated in the Mass Spectrum of 3MR5, where impurities were present throughout the spectrum. The inability to successfully remove these failures effectively is what resulted in low purity of the 3MR5 sequence that would later plague all experiments involving 3MR5-BHQ₁. Further evidence of this can be confirmed by using the absorption of BHQ₁. Using the absorption at 534 nm and the extinction coefficient of 34000 cm⁻¹M⁻¹ for BHQ₁, a different concentration is obtained for total DNA. Only one BHQ₁ is modified into each 3MR5 aptamer, so the concentrations should be relatively similar. If one uses BHQ₁ for quantification you would obtain 204 nmol instead of DNA instead of 174 nmol. The purification of RCP-3FAM was conducted with less issue however, resulting in a more pure oligonucleotide sample. The presence of two peaks in the RCP-3FAM spectra at the absorption of fluorescein means it is likely that free fluorescein was also present in the sample. Regardless, experiments were conducted even with the low purity sample of 3MR5 in the interest of time.

These fluorometric experiments showed that upon addition of the aptamer, the

fluorescence of RCP-3FAM was quenched (See Figure 29) as expected. The next step, however, was the key step. As 3MR5 has an affinity for AFB₁, it was postulated that upon addition of the target, the sequence hybridization would be disturbed and recovery of fluorescence would occur. Unfortunately, experiments revealed that no significant recovery of fluorescence occurred upon addition of AFB₁ (Figure 29).

This could have been due to the poor purity of the 3MR5 sequence used as mentioned earlier. During synthesis the hydrophobic BHQ1 modifier resulted in failures in the occurring after the modifier addition. Purification of these failures from the complete sequence was attempted using numerous methods, but purity never rose above 20%. Lack of the sequence past the modifier accounts for 42 bases, of which these bases could have played a key role in the binding affinity for AFB₁. The binding affinity between 3MR5 and AFB₁ was not particularly strong even with the full sequence, so these missing bases may have prevented the successful disruption of RCP from the aptamer. Predictive software showed that multiple stem loops in the random regions of 3MR5 form (Figure 9 and Figure 10). These are likely instrumental in the binding mechanism to AFB₁. The lack of these stem loops in the low purity sequence could result in no binding occurring between AFB₁ and 3MR5.

Another possible answer to why recovery was not seen could be that 3MR5 was able to bind to both AFB₁ and the capture probe simultaneously. As the capture binding region was not randomized, it is unlikely that this region contributes to the binding of target. As mentioned before, predictive software showed the presence of multiple stem loops in the structure of 3MR5. Since these stem loops are likely what results in binding to target, and predictive software also shows that many of these stem loops are still present even when hybridized with the RCP

sequence (see Figure 11) it is possible that both RCP and AFB₁ can bind to 3MR5 simultaneously. In this scenario, addition of AFB₁ would not result in the desired fluorescence recovery. To further investigate the capabilities of the sequence, nanoparticles were then employed to replace the unwieldy organic chromophores. This would also remove the possibility of secondary structure formation interfering with the fluorescence of the fluorophore.

Thus, a system that would remove the organic chromophores and replace them with nanoparticles was devised. This system would ideally not suffer the same issues as the previous one. A depiction of the system can be seen in Figure 33.



Figure 33: Schematic depicting the new FRET based system using nanoparticles. When hybridized and in the absence of AFB₁, the fluorescence of the RCP conjugated QD is quenched by the 3MR5 conjugated AuNP (Left). When AFB₁ is added to the system, disruption of hybridization due to 3MR5 binding to AFB₁ will cause release of RCP conjugated QD resulting in a recovery of fluorescence (Right).

Challenges arose when attempting to perform conjugation experiments, resulting in numerous experiments needing to be conducted. Results from characterization led to positive proof that conjugation was able to occur. AuNP conjugation was confirmed using the colorimetric salt test on both samples containing DNA and not containing DNA. High concentrations of salt are known to cause aggregation of AuNPs, by disrupting electrostatic forces keeping particles apart. Charged ligands attached to AuNP can shield from the effects of additional salt, which was seen when comparing two samples having equal amounts of salt added. While some aggregation occurred in the DNA containing sample, the solution was able to retain its red colour characteristic of AuNP. The bare AuNP solution however was found to aggregate upon addition of the same amount of salt. Quantification of the DNA in the filtrate was attempted, but the overlap at 260 nm of bare Au (Figure 19) with that of DNA resulted in inconsistent concentrations of DNA being calculated.

Confirmation of QD conjugation was more complicated. UV-Vis was attempted to quantify the DNA on the QD samples, but QD absorbance overlaps with that of DNA at 260 nm. Thus, different measures were taken to confirm QD conjugation. TEM and EDS were used in tandem to examine a sample of conjugated QDs. These samples had been desalted to remove as much background as possible. When observing these samples, there were two distinct regions found in the conjugated samples. One region looked similar to bare QD's, while the second appeared clouded on TEM. This clouded region also contained more phosphorus content when examined with EDS. This high phosphorus level was assumed to be the phosphate backbone of DNA, and aided in confirming conjugation.

After successfully performing the conjugation, experiments with 3MR5-tagged AuNP, and RCP-tagged QD were performed to see if a working assay could be developed. During these

experiments, it was seen that the fluorescence of the conjugated QDs decreased over time. QDs are more resistant to photobleaching then organic chromophores, so photobleaching was not thought to be the cause of the fluorescence decay. However, QD stability in solution decreases over prolonged periods of time leading to degradation of the nanoparticles.⁵⁸ These QD were purchased 1 year prior, and as such they may have started to degrade. To counter this, a calibration experiment was done alongside the normal fluorometric experiment in order to account for this loss in fluorescence (see Figure 30). Using statistical functions, a calibration was performed and the results of the fluorometric experiment were corrected. This correction was calculated by taking measurements of the fluorescence of the QD at intervals over the whole spectrum. Time was recorded when measurements began for all samples and thus could be used to determine how much fluorescence had been lost. Since the calibration allowed us to know how fluorescence decreased over time for any wavelength, all one needed to know was how much time had elapsed from taking the first QD measurement and the current sample measurement in order to calculate the corrected fluorescence. This calculation was performed for all samples over all wavelengths measured.

Once again, as expected, decrease in fluorescence was observed after mixing both the QD and AuNP samples (see Figure 31). After mixing AFB₁ with the hybridized sample, recovery of fluorescence was expected, but no significant recovery was observed. While there was some recovery seen, it falls within the error present in the experiment. Fluorescence recovery for this concentration of AFB₁ was expected to be much larger as the concentration of AFB₁ was only half that of the aptamer/capture probe. Maximum recovery would have been expected at 1:1 ratios. RCP-Thiol was examined using mass spec and found to be indeed synthesized, and

3MR5-IDT had been purchased from the supplier directly and confirmed by MS. As such, purity was not considered the primary cause like thought previously.

This lack of fluorescent recovery may be attributed to the binding affinity of the aptamer to the target molecule. The binding affinity of 3MR5 as mentioned earlier is not particularly strong being only $1.4 \pm 0.7 \mu$ M, but other capture assays have been shown to work with worse binding partners.

Another possible scenario is that hybridization is not effectively disrupted upon binding with the AFB₁ target. In this situation, hybridization still occurs even after binding to target. The aptasensor system relies on the hybridization to be disrupted in order for fluorescent recovery to occur. Thus if the capture probe is not released effectively no recovery will be observed even if the aptamer binds to its target. This is further supported by the predicted structures described before. As stem loops still exist even in the hybridized predicted structures, it is possible that like with the organic chromophores AFB₁ is not able to displace RCP even when bound. Failure to do so would result in the same lack of fluorescent recovery.

Another possible reason for the lack of fluorescence recovery could be due to the conjugation itself. It is difficult to simply conjugate a single aptamer only to one nanoparticle. Multiple copies of the aptamer were likely conjugated to each AuNP, as well as multiple copies of the capture probe to each QD. A general depiction of this is seen in Figure 34.



Figure 34: Depiction of cluster formation. Due to multiple RCP being conjugated to a single QD, it is possible for two different 3MR5 conjugated AuNP to hybridize to a single QD (Left). Upon addition of AFB₁ only one of the hybridized 3MR5 AuNP is able to dissociate from the QD, resulting in the continued quenching (Right, Circled). Thus the presence of small concentrations of AFB₁ may not result in recovery of fluorescence.

This could result in nanoparticles being linked by more than one aptamer-

complementary probe unit, meaning that it would require multiple units of AFB₁ to release one complementary probe from the cluster. The experiment was carried out with a 1:1:0.5 ratios of AuNP, QD, and AFB₁, so it is possible that the creation of these clusters is what prevented the recovery of fluorescence from occurring, and that excess amounts of AFB₁ would have been required to see any recovery of fluorescence. The presence of these clusters allows for cooperativity to occur, resulting in an apparent increase in k_{off} for the aptamer/capture probe system. Increases in avidity due to receptor clustering have been explored in the past as a means to increase binding affinity for different applications.⁵⁹ In this scenario however, the increase in avidity would directly hinder the ability of the system to function. The aptamer/capture probe system employed relies on the dissociation of the capture probe from the aptamer when AFB₁ is present in order to measure the fluorescent recovery. An increase in fluorescent recovery, as AFB₁ would not be able to effectively displace the capture probe due to clustering. As conjugating only a single sequence to the large nanoparticles is difficult, the only course of action would be to

increase the concentration of AFB₁ present. Depending on how much higher the concentration of AFB₁ needs to be though, it may render such a sensing system useless for practical applications. Future experiments using the nanoparticle system will need to take the possibility of receptor clustering into account, and use higher concentrations of AFB₁ to compensate for the stronger avidity. Conversely, introducing further mismatches into the capture probe sequence could lower the binding strength, but may prevent effective hybridization from occurring before addition of AFB₁.

Future work

As noted, many issues plagued experiments conducted with 3MR5. The aptamer 3MR5 was incorporated into a FRET based sensor in the hope of exploiting a structure switching that would occur due to binding with target. If further work in pursuit of this goal were to occur, improvements to the experiments conducted would aid in realizing that goal. Utilization of purer sequences would eliminate much of confusion about the lack of fluorescent recovery seen in the organic chromophore based experiments. Experiments could be conducted at higher temperatures in the hope of destabilizing the duplex or denaturants could be added to the hybridized system. These could weaken the hybridization between 3MR5 and RCP enough that fluorescent recovery could occur. The introduction of base pair mismatches could also be used as another way to weaken the duplex formed between 3MR5 and RCP. It is entirely possible that these modifications would show a recovery of fluorescence and the ability of 3MR5 to be used as a novel biosensor for the detection of AFB₁. In the case of the nanoparticle based system, experiments with excess AFB₁ should be conducted to ensure that the aforementioned clustering does not occur. Controls involving non conjugated samples should also be performed to eliminate static quenching as interference. If all such improvements result in still no recovery of fluorescence, the aptamer could be used without exploiting its supposed structure switching functions. Numerous other methods of detection utilizing aptamers exist without requiring structure switching to occur. Simply having an aptamer selective to AFB₁ is enough for these methods of detection to work. Such methods would include the use of colorimetric assays utilizing AuNPs or electrochemical methods that would detect differences in the bound or unbound state. SPR could also be used due to its sensitivity to changes in local environment from a binding event.

Conclusions

This thesis set out to exploit the structure switching functions of the previously characterized 3MR5 aptamer to develop a FRET based optical biosensor for AFB₁. Experiments were conducted utilising organic chromophores FAM and BHQ₁ that had been incorporated into a capture probe (RCP) and 3MR5, respectively, in the hope of making a sensitive and selective biosensor for AFB1 detection. Fluorescence experiments were conducted with the hope that the FRET based quenching caused by close proximity of the chromophores would be disrupted upon introduction of AFB₁, and that this change in fluorescence could be transformed into a novel biosensor. It was discovered that no recovery of fluorescence occurred after addition of AFB1 and so an alternative FRET pair was tested. This new FRET pair was that of 525 nm emitting QDs and spherical AuNPs. The hope was that this new system would be able to avoid any issues that occurred while conducting experiments with organic chromophores, while also improving the possible sensitivity due to the optical properties of the nanoparticles used. While some of the problems faced earlier were avoided by switching to nanoparticles, new issues arose. Fluorescence experiments were conducted to see if this new system could allow the aptamer to be used in a FRET based biosensor. Hybridization of RCP conjugated QDs and 3MR5 conjugated AuNPs resulted in the quenching of the QD fluorescence. Addition of AFB1 did not result in any recovery of fluorescence.

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