DETECTION OF AFLATOXIN M1 USING APTAMER BASED SENSING SYSTEM

THESIS SUBMITTED TO THE

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL

(DEEMED UNIVERSITY)

IN PARTIAL FULFILLMENT OF THE REQUIREMENT

FOR THE AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY
IN
DAIRY CHEMISTRY

BY

GOSEWADE CHETAN CHHAGAN
B. Tech. (Dairy Technology)

DAIRY CHEMISTRY DIVISION
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
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Karnal – 132 001 (Haryana), India

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Approved by

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This is to certify that the thesis entitled "**DETECTION OF AFLATOXIN M1 USING APTAMER BASED SENSING SYSTEM**" submitted by **Mr. Gosewade Chetan Chhagan** in partial fulfillment of the requirements for the award of the degree of **MASTER OF TECHNOLOGY IN DAIRY CHEMISTRY** of the **ICAR-NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY)**, Karnal (Haryana), India, is a bonafide research work carried out by him, under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

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Place:

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ABSTRACT

Aflatoxin-M1 (AFM1) is 4-hydroxy heterocyclic compound and appears in milk as a result of ingestion of feed contaminated with aflatoxin-B1 by cattle. AFM1 is a very potent toxin due to its carcinogenic effect and is classified as group1 carcinogenic agent. FSSAI, Codex and US regulations prescribes the limit of 500 ng/L in milk. Assessment of AFM1 is usually done by complicated and laborious procedures like HPLC, TLC, GC-MS; hence there is need of robust technique for detection of AFM1. Aptamers are single stranded DNA or RNA oligonucleotides which have affinity and specificity towards a particular target. Earlier, we have generated aptamers which show specificity towards AFM1 (AFAS3, AFAS3-tr8 and APM6-tr). In the present work, efforts have been made to develop aptamer based sensing system to quantify AFM1 in buffer and milk. Attempt was made to developed fluorescent based sensing system. DNAzyme approach did visualize the binding of aptamer to target but results can be obtained only at very high concentration of target. Interaction of fuorophore (FAM) modified aptamer (AFAS3-tr9) with quencher (TAMRA) modified counter strand in absence and presence of target provided significant results. Linearity in Tris buffer was observed over concentration range of 0 – 0.5 µM. Experiments with AFB1 indicated no cross-reactivity. An electrochemical based aptamer sensing system was developed using screen printed gold electrode (SPGE). Biotin and TEG modified AFAS3/APM6-tr was immobilized on the surface of SPGE using steps like layering of dithiodipropionic acid, streptavidin and ethanolamine. Decrease in current was observed with increasing AFM1 concentration. Linearity in Tris buffer (using AFAS 3) was observed over concentration range of 0 – 10^5 ppt. Aptamer was found to be specific to AFM1. Cross reactivity with AFB1 was observed at very high concentration. Similar results were observed using APM6-Tr. Results indicated that fluorescence based methods did not work with milk samples, perhaps due to unknown interference from milk matrix. Electrochemical based assay was used for estimation of AFM1 in milk. In this approach, AFM1 was extracted from spiked milk using chloroform and finally AFM1 was brought in buffer. Electrochemical response of SPGE immobilized with AFAS3 was monitored using square wave voltammetry at various concentration of AFM1. The results indicate that the peak current is inversely related to the logarithmic concentration of AFM1 present in milk. The method is capable of measuring AFM1 in milk in the range of 0 to 10^5 PPT. The results were validated using Rosa Reader assay.

सारांश

अफ्लाटोक्सिन एम1 का ऐप्टामर आधारित संवेदन प्रनाली से पता लगाना

अफ्लाटॉक्सिन –एम 1 (एएफएम 1) दूध में पशु के द्वारा अफ्लाटॉक्सिन बी 1 के साथ दूषित फीड के खाने के परिणामस्वरूप प्रकट होता है| एएफएम 1 कैंसरजनक प्रभाव के कारण एक बहुत शक्तिशाली विष है और समूह 1 कैंसरजनक एजेंट के रूप में वर्गीकृत है| एफएसएसएआई, कोडेक्स और यूएस के नियमों ने दूध में 500 एनजी /ली की सीमा निर्धारित की है। एएफएम 1 का मूल्यांकन आमतौर पर एचपीएलसी, टीएलसी, जीसी-एमएस जैसे जटिल और श्रमसाध्य प्रक्रियाओं द्वारा किया जाता है। इसलिए एएफएम 1 की पहचान के लिए मजबूत तकनीक की आवश्यकता है। ऐप्टामर डीएनए या आरएनए ओलिगोनुक्लियोटाइड होते है जो एक विशेष लक्ष्य के प्रति समानता और विशिष्टता रखते है| इससे पहले, हमने ऐसे ऐप्टामर उत्पन्न किए हैं जो एएफएम 1 (एएफएएस 3, एएफएएस 3-टीआर 8 और एपीएम 6-टी) के लिए विशिष्टता दिखाते हैं। वर्तमान कार्य में, बफर और दूध में एएफएम 1 मात्रा निर्धारित करने के लिए एप्टामर आधारित सेंसिंग सिस्टम विकसित करने के प्रयास किए गए हैं। विकसित फ्लोरोसेंट आधारित संवेदन प्रणाली के लिए प्रयास किया गया था। डीएनए एंजाइम से एप्टामर की टारगेट के साथ बंधन देखा गया लेकिन परिणाम केवल टारगेट के बहुत अधिक एकाग्रता पर प्राप्त किए जा सकते हैं| फ्यूरोफोर (एफएएम) के संशोधित एप्टामर (एएफएएस 3-टीआर 9) शमनकर्ता (टीएएमआरए) संशोधित काउंटर स्टैन्ड के साथ मिलके टारगेट की उपस्थिति और अनुपस्थिति ने महत्वपूर्ण परिणाम प्रदान किए| ट्रिस् बफर में रैखिकता 0 - 0.5 माइक्रोन की एकाग्रता सीमा पर देखी गई। स्क्रीन मुद्रित सोना इलेक्ट्रोड (एसपीजेई) का उपयोग करके एक इलेक्ट्रोकेमिकल आधारित एप्तामर सेंसिंग सिस्टम विकसित किया गया था। बायोटिन और टीईजी संशोधित एएफएएस 3 / एपीएम 6-टी को थियोडिप्रोपोनिक एसिड, इथेनॉलअमिन, स्ट्रेप्टाविडिन के लेयरिंग करके एसआईपीजी की सतह पर स्थिर कर दिया गया था | ट्रिस् बफर (एएएएएस 3 का उपयोग करते हुए) में रैखिकता 0 - 10^5 पीपीटी की एकाग्रता सीमा पर पायी गयी |एप्तामर एएफएम 1 के लिए विशिष्ट पाया गया था| एएफबी 1 के साथ क्रॉस जेटी बहुत उच्च एकाग्रता में पायी गयी| परिणामों ने संकेत दिया कि प्रतिदीप्ति आधारित पद्धतियां , संभवतः दूध मैट्रिक्स से अज्ञात हस्तक्षेप की वजह से दूध के नमूनों के साथ काम नहीं करती हैं| इलेक्ट्रोकेमिकल आधारित परख दूध में एएफएम 1 के आकलन के लिए इस्तेमाल किया गया था। इस दृष्टिकोण में, एएफएम 1 को क्लोरोफॉर्म का उपयोग करके दूध से निकाला गया था और अंत में एएफएम 1 को बफर में लाया गया था। एएफएस 3 के साथ स्थिर नहीं हुए एसपीजीई की विद्युत रासायनिक प्रतिक्रिया एफएएम 1 के विभिन्न एकाग्रता पर वर्ग तरंग वाल्टमैट्री का उपयोग करके निगरानी की गई। परिणाम बताते हैं कि पीक करंट दूध में मौजूद एफएएम 1 के लॉगरिदमिक एकाग्रता से व्युत्क्रम संबंधित है| ये विधि 0 से 10^5 पीपीटी की श्रेणी में दूध में एएफएम 1 को मापने में सक्षम है| सारे परिणाम रोजा रीडर के माध्यम से सत्यापित किया गए|

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ABBREVIATIONS

AFB1	Aflatoxin B1
AFM1	Aflatoxin M1
BHQ1	Black Hole Quencher 1
CAC	Codex Aliment Arius Commission
CAC	Codex Aliment Arius Commission
CV	Cyclic Voltammetry
EDC	Ethyl dimethylaminopropyl carbodiimide
ELASA	Enzyme Linked Aptamer Sorbent Assay
ELISA	Enzyme Linked Immuno Sorbent Assay
FAM	Caboxyflurescin
FAO	Food and Agriculture Organization
FSSAI	Food Safety and Standards Authority of India
HPLC	High Performance Liquid chromatography
IARC	International Agency for Research on Cancer
LC-MS	Liquid Chromatography with Mass Spectrometric Detection
MRL	maximum residue level
NHS	N-hydroxysuccinimide
OTA	Ochratoxin A
PEG	polyethylene glycol
ppb	parts per billion
ppm	parts per million
ppt	parts per trillion
PTK7	protein tyrosine kinase 7
ROSA	Rapid One Step Assay
SAM	Self-assembled monolayer

SPGE	Sreen Printed Gold Electrode
SPR	Surface Plasmon Resonance
SWV	Square Wave Voltammetry
TAMRA	Tetramethylrhodamine
TEG	Tetra ethylene glycol
TLC	Thin Layer Chromatography
TMB	Tetramethyl benzedene
V	Volts

CONVERSIONS

1 ppt AFM1 = 1 ng/ kg of AFM1

1 ppt AFM1 = 0.000304 µM of AFM1

CHAPTER – 1

INTRODUCTION

INTRODUCTION

Various foods of plant and animal origins are prone to contamination with microorganisms. Food contaminated with fungi is risky for human consumption because some species of fungi such as *Aspergillus*, *Fusarium*, *Trichoderma* produces toxic substances known as mycotoxin. Mycotoxins are natural chemical secondary metabolite products produced by saprophytic fungi. A number of mycotoxins including aflatoxin, vomitoxin, patulin, ochrotoxin have been identified and characterized. The contamination of food and feed with mycotoxin is a cause of global concern due to associated toxic effects on the human health. Mycotoxin have hazardous effect on functioning of kidney, liver, lungs and cells of immune and endocrine system. Thousand million tons of food lost every year due to contamination of mycotoxin and this loss may equal to one billion US dollars (FAO 2013). Aflatoxins are a type of mycotoxin produced by Aspergillus species of fungi, such as *A. flavus* and *A. parasiticus*. The aflatoxin covers four different types of mycotoxins produced, which are B_1, B_2, G_1, G_2 whereas M_1 and M_2 are metabolic product of B_1 and B_2 respectively. When ruminants eat foodstuffs containing aflatoxins B1 and B2, these toxins are metabolized and excreted as aflatoxin M1 and M2 in milk. Aflatoxin B_1 (AFB1), the most toxic, is a potent carcinogen and has been directly correlated to adverse health effects, such as liver cancer, in animals as well as humans. Aflatoxin M1 (AFM1) is a major metabolite of AFB1 and is secreted in milk, which is formed when dairy animals ingest feed contaminated with AFB1. The toxic potency of AFM1 is relatively low than that of AFB1 however its heat stability is relatively very high. Initially, International Agency for Research on Cancer (IARC) classified AFM1 as a possible carcinogen for humans (group 2b) since toxicological data was limited (IARC, 1993). However, genotoxicity and cancerogenity of AFM1 have been observed *in vivo*, although lower than those of AFB1, and its cytotoxicity has been definitively demonstrated (Caloni *et al*., 2006). As a result of these and other investigations, the IARC moved AFM1 from Group 2B (possible carcinogenic to humans) to Group 1 (carcinogenic to humans as sufficient evidences are there) human carcinogen (IARC, 2002). In order to provide safety for food and human health, regulatory agencies have fixed the maximum residue

level (MRL) of AFM1 in milk and milk products, considering milk as the main nutrient to young infants. Food Safety and Standards Authority of India (FSSAI) and Codex Aliment Arius Commission (CAC) have prescribed maximum residual limit for AFM1 as 500 ng/kg whereas, the European Commission Regulation has fixed the stringent MRL for AFM1 which is 50 ng/kg in milk and milk based products. As per EC Regulation, the maximum permissible limit is even lower for infant milk and baby food which is 25 ng/kg (Worldwide regulations for mycotoxins for food and feed, 2003). Thus, there is need of sensitive, robust and reliable technique for detection of AFM1. Currently, chromatographic methods such as, Thin Layer Chromatography, Gas Chromatography, and High Performance Liquid Chromatography are used for detection of AFM1 in milk and milk products which provides confirmatory results but these techniques are very expensive and requires tedious and extraction and cleanup of the samples. Other analytical methods involves ligand specific to target molecule. Antibody is one of the examples of ligand and is used for developing ELISA and immune sensors. Another ligand 'Aptamer' is now extensively researched for developing and estimation of analytes and have shown various advantages over traditional ligands.

Aptamer word derived from the Latin word '*Aptus'* which means fit and Greek word '*meros'* which means part, are single stranded DNA or RNA molecules that have affinity towards their target molecule (Jayasena *et al*., 1999; Gopinath *et al*., 2013). Aptamers are selected from random library usually from 10^{13} to 10^{15} random sequences. The selection is very tedious and usually achieved through the process called "Systematic Evolution of Ligand by Exponential Enrichment" or SELEX. SELEX involves repetitive cycles wherein in each cycle, oligonucleotides interacting with target are enriched. After 8 to 12 rounds of cycle, one can obtain few aptamers which can be subsequently used for development of tools or methods for detection and estimation of analytes. The recent upsurge in the number of aptamers generation is due to their astounding features. Aptamers are a powerful tool for therapeutic and diagnostic applications. In diagnostics, aptamers are utilized as the sensor agents to capture their cognate targets. The aptamers can be easily derivatized and therefore, these can be employed on plastic

surface or electrode or gold nanoparticles and thus have enormous potential to develop diversified tools/methods.

Aptamers are now increasingly evaluated for development of biosensor which is defined as device capable of giving digital signal on interaction of ligand with target molecule. Aptamers have a great promise to be used as a ligand and are exploited for development of different categories of sensors including electrochemical sensor, quartz crystal microbalance (QCM), surface Plasmon resonance (SPR). These can be conjugated to biotin and thus these have a great potential to exploit avidin-biotin interaction for immobilization of aptamer on plastic or electrode surface. Aptamers can even be produced against toxins and further these do not require animals for their production.

Earlier, in our laboratory, high affinity aptamers were identified for AFM1 (Malhotra et al., 2014). In the present work, some of these identified AFM1 specific aptamers would be utilized for the development of sensing system for the detection of AFM1.

The objectives of my study are as follows:

- To develop aptamer based fluorescent assay for detection of AFM1.
- To develop electrochemical based assay for detection of AFM1.
- To establish the proof of binding of aptamer to AFM1in milk matrix.

CHAPTER – 2

REVIEW OF LITERATURE

The work reported in this dissertation focuses on aptamer and their uses to develop novel assays for the detection of AFM1 in milk. For this purpose, aptamers were modified with fluorophore like FAM, quenchers like BHQ1 and TAMRA etc to develop fluorescent based assay. Further aptamers were modified with biotin to enable them to immobilize on a gold surface through reaction with streptavidin to develop electrochemical based assay. Thus the subject has been revived under following headings such as (i) aflatoxin-M1 in milk and their detection methods, (ii) aptamers, their generation and applications, (iii) aptamer immobilization, (iv) aptamers against aflatoxin M, (v) aptamer based fluorescent assay and (vi) aptamer based electrochemical assay.

2.1 Aflatoxin-M1 in milk and their detection methods

2.1.1 Aflatoxins

Mycotoxin word has been derived from Greek word mukos meaning fungus and toxikon means poison. These are secondary metabolite products of the fungus kingdom. Out of several species of fungi present in nature, three main fungi namely Aspergillus, Penecillium and Fusarium are known to produce mycotoxins. Aflatoxins are mainly produced by aspergillus species like *A. parasiticus, A. flavus.* Fumonosin is produced by Fusarium species like *F. verticillioides* while ochratoxin is produced by Penecillium species such as P. verrucosum. These fungi can grow over food sources such as nuts, corn, cotton seed, wheat, cocoa, dried and animal feeds.

Toxin of our concern *i.e.* aflatoxins are heterocyclic compounds closely related to each other. The term was coined in 1960 in London due to an outbreak of aflatoxicosis (famous as turkey “X” disease) due to contaminated ground nut feed causing death of large number of livestock population (Blount, 1961). Since then the aflatoxins are found in various foods such as in maize (Chakrabarty, 1981) and cottonseed (Sharma et al., 1981). The various forms of aflatoxins i.e. B1, B2, G1, G2, M1 and M2 are closely related to each other and differs slightly in structure (Figure 2.1). Aflatoxin M1 (AFM1) and aflatoxin M2 (AMF2) are hydroxylated forms of B1 and B2 and are produced by lactating animals when they consume feed contaminated with aflatoxin B1 (AFB1) and aflatoxin B2 (AFB2), respectively. These compounds are mainly produced by *Aspergillus species namely A. flauvs and A. parasiticus.*

These fungi contaminate a wide range of food and agriculture commodities under variety of environmental condition. Therefore, most of the food commodities are prone to contamination with aflatoxicogenic fungi at various stages such as production, processing, transportation and storage.

2.1.2 Aflatoxin M1 in milk

Aflatoxin B1 which is a fungal toxin, biotransforms by the hepatic microsomal mixed function oxidase system to initiate a carcinogenic response (Marsi *et al*., 1974; Lin et al,. 1977). AFB1 is converted into AFM1 when a lactating animal ingests the feed contaminated with AFB1. The conversion takes place in the liver through the enzyme cytochrome P450 (Kanugo *et al*., 2011). The amount of AFB1 consumed by animal and which is converted into AFM1 is 1-2% and varies with animal to animal. The AFM1 in milk is detectable after 24 h of ingestion of AFB1 by the animal. When the intake of AFB1 is stopped, the amount of AFM1 detected in milk decreases and reaches undetectable level after 72 h (Egmond et al., 1989). Aflatoxins are one of the major factor which causes hepato-cellular carcinoma. According to IARC, AFM1 is as a class 1 carcinogen. However the carcinogenic effects have shown variation among different species. Toxicological response differs within species and sometimes different response have observed between the male and female of the same species. Since milk is main source of nutrients for infants and children, they are more prone to the toxicological effects of AFM1 and they are more exposed to contamination. Exposure of infants to aflatoxins is associated with growth faltering (Gong et al., 2004). Considering the risk, nearly all developed countries have adopted maximum permissible levels of AFM1 in milk and milk products. European Union has prescribed 50 ng AFM1/kg in liquid, dried or processed milk as maximum permissible level, while Codex Alimentarius Commission and FSSAI permit up to 500 ng AFM1/kg milk.

AFM1 is soluble in moderately polar solvents such as acetonitrile, methanol, chloroform and dimethylsulfoxide. The solubility in water is quite low and ranges from 10-30 mg/L, whereas AFM1 is insoluble in non-polar solvents. Several protocols of extraction of AFM1 from milk describes removal of fat through centrifugation and then passing of middle layer through syringe filters as well as immunoaffinity columns. (Shundo *et al.,* 2006)

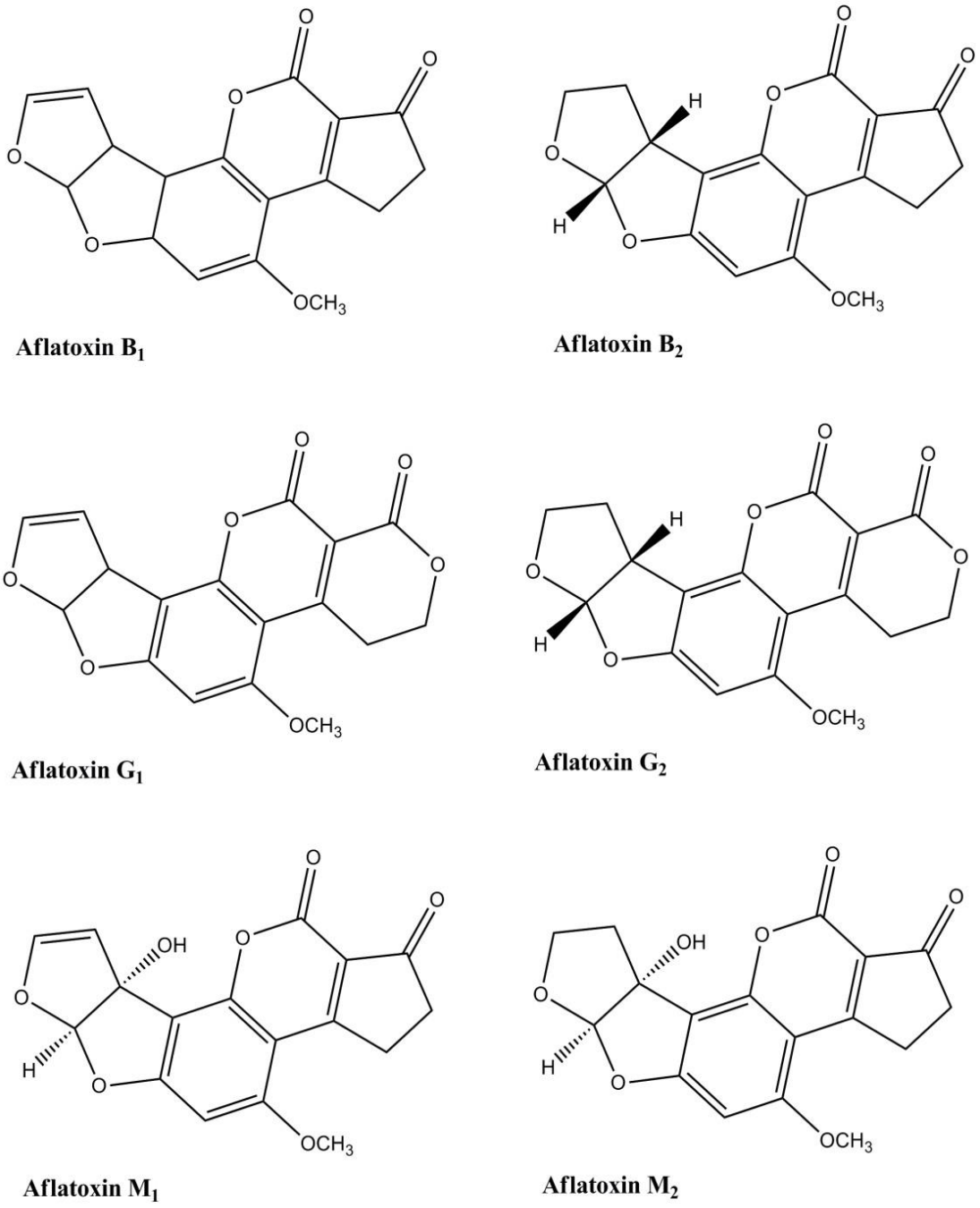

Figure 2.1: Structure of Aflatoxins

2.1.3 Existing detection techniques for AFM1

The presence of AFM_1 in milk and milk product represents a worldwide concern due to high susceptibility of infants and children for aflatoxin (Horn *et al*., 2000). Several different techniques for detection of AFM1 have been developed. Most of them require laborious extraction of AFM_1 from its source, purification and clean up by removing other interfering substances, and finally quantification. Of the various methods for detection of AFM1, some which are found to be more reliable than others and used more commonly includes,

- Thin Layer Chromatography
- High Performance Liquid chromatography
- Liquid Chromatography with Mass Spectrometric Detection (LC-MS)
- ELISA
- Lateral Flow Assay
- Charm ROSA
- Surface Plasmon Resonance

Thin-Layer Chromatography (TLC)

TLC is a widely used chromatographic technique similar to paper chromatography which is used for the separation, purity evaluation and identification of non-volatile mixtures. TLC can be used for detection of aflatoxin (Kamkar, 2005, 2006). TLC can identify and quantify aflatoxin at levels as low as 1 ng/g. It consists of two phases, stationary phase which is of a thin layer of adsorbent like alumina, sillica gel or cellulose immobilized on a glass or plastic plate and solvent acting as a mobile phase. Sample which should be in liquid form is applied as a spot on the stationary phase. After that chromatographic plate is placed vertically in a reservoir in which solvent is place, the solvent moves up on the plate by capillary action. The separated spots are visualized simply with ultraviolet light or by spraying with a suitable reagent. The rate of movement of different components in a mixture is different due to differences in their partitioning behavior. Retardation factor (Rf) for each spot is calculated. It is the ratio of the distance travelled by the sample spot to the distance travelled by the solvent in centimeters.

For identification of analyte, Rf values of standards are compared to those of unknown samples. This method is easy, rapid, sensitive and selective in nature for separation of aflatoxins. For improving the precision, high performance thin-layer chromatography methods are used (Lin *et al*., 1998) in which sample application is automatic and interpretation steps are decreased, which are lacking in the conventional TLC (Stroka and Anklam *et al*., 2002).

High Performance Liquid Chromatography (HPLC)

HPLC is a highly selective, sensitive, precise and automated technique used to separate, identify and quantify each component in a mixture (Kim *et al.,* 2000). HPLC is mainly used for final separation and detection of the aflatoxin (Reiter *et al*., 2009). Prior to detection with HPLC, it is necessary to apply extraction and clean up steps. In HPLC technique, pressurized liquid solvent is used to move the sample through the column in which an immobilized liquid stationary phase is packed. The analyte is then partitioned between the two phases as it passes through the column and thus leading to the separation of compounds due to different partitioning coefficients. Normal phase chromatography and reversed phase chromatography are two types of chromatography commonly used. Various types of detectors are used in HPLC, such as, UV detector, diode array detector (DAD) (Vosough *et al*., 2010) or a fluorescence detector (FLD) (Alcaide-Molina *et al*., 2009). Fluorescence detection utilizes the emission of light (435 nm) from molecules that have been excited to higher energy levels by absorption of electromagnetic radiation (365 nm) for aflatoxin. Elizalde-Gonzalez *et al* (1998) analyzed aflatoxins using HPLC and amperometric detector, and reported that it is possible to detect aflatoxin up to 5 ng.

Liquid Chromatography with Mass Spectrometric Detection (LC-MS)

Liquid chromatography with mass spectrometric detection (LC-MS) is a recent development in aflatoxins detection. It is time consuming method and requires high skill. Extraction and clean-up techniques have to be applied before detection while performing by mass spectrometric detection. In LC-MS, the HPLC effluent enters to an ionization chamber.

Several techniques for ionization like electrospray, thermo spray, chemical, and fast atom bombardment are used. In a collision chamber, fragmentation takes place. The fragments then enter the high vacuum region of the MS where detection takes place. LC-MS/MS methods have been developed for aflatoxins, in most cases, electrospray (ESI) has

been used as the ionization source (Cavaliere *et al*., 2005, Spanjer *et al*., 2008). Aflatoxin determination in corn, milk and milk products have been done using LC-MS method (Sorensen and Elbaek, 2005). Very low level detection (pico-gram levels) can be done using selection-ion-monitoring (SIM) mode.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA is a simple, sensitive and adaptable technique hence most widely used method to detect aflatoxin. ELISA is not only suitable tool for quick and sensitive analysis with high sample throughput, but also cost-effective and require only a small sample volume for analysis (Pei *et al*., 2009; Parker and Tothill, 2009. Detection of AFM_1 in milk, UHT milk, yoghurt, infant formula has been done using this method (Kim *et al*., 2000; Thirumala-Devi *et al.,* 2002; Rastogi *et al*., 2004). Although the antibodies have the advantage of high specificity and sensitivity, matrix effect due to binding of other similar chemical which interact with antibodies leads to cross reactivity which in turn causes alteration of results (Trucksess and Koeltzow, 1995). Additionally, uses of ELISA are limited to matrices for which they are validated (Gilbert and Anklam, 2002). Two types of ELISA are generally used, which are direct competitive ELISA and indirect competitive ELISA. The quantitative determination of aflatoxin present in the sample is done by comparison with the standard curve.

Lateral flow assay

Lateral flow assay or lateral flow immunochromatographic assay is a simple device which does not require any costly equipment for detecting the target analye in a sample matrix. Main components of which are sample pad, conjugate pad, nitrocellulose membrane and absorbent pad. In this method, nitrocellulose membrane is coated with two main regions; test line (AFM_1-protein conjugate) and control line (goat anti-rabbit IgG antibodies). Colloidal gold nanoparticles coated with polyclonal antibodies are usually used as detector reagent. Analyte present in the sample competes with the AFM_1 immobilized on the test line of membrane. Using combination of the one-dot LFA and the smartphone-based reading system, it is possible to conduct a more fast and accurate point-of-care diagnosis. Anfossi *et al*., (2013) optimized the semi-quantitative lateral flow immunoassay for the ultrasensitive detection of AFM_1 in milk. The LOD of proposed method is in the range which is required by the EU legislation in milk but it is not up to the mark for the limit which is set for baby food *i.e.*25 ppt.

Surface Plasmon Resonance

The method for detecting AFM1 in milk by using Surface Plasmon Resonance (SPR) biosensor has also been reported (Karczmarczyk *et al*., 2016). Rapid and sensitive detection of AFM1 can be done using this method. AFM1 is analyzed using an indirect competitive immune assay that is amplified by secondary antibodies which are conjugated with Au nano particles. To prevent the fouling on sensor surface by milk constituents while analysis, an interface of poly(2-hydroxyethylmethacrylate) p(HEMA) brush was employed. The study presents a comparison of performance characteristics of p(HEMA)-based sensor with a regularly used polyethylene glycol based architecture on mixed thiol self-assembled monolayer. Both sensors are characterized in terms of surface mass density of immobilized AFM1 conjugate as well as affinity bound primary and secondary antibodies. The biosensor allowed high sensitivity detection of AFM1 in milk with a limit of detection as low as 18 pg/ml with 55 min of analysis time.

Charm ROSA

Charm Sciences-USA, developed a lateral flow based assay for detection of AFM-1 in bovine milk named Rapid One Step Assay (ROSA) (Salter *et al*., 2006). A ROSA reader quantitatively interprets test strips for measuring AFM1 concentration in parts per trillion (ppt) range. The limit of detection was found to be 400 ppt as concentration below this level are interpreted as negative. The reproducibility of results in different laboratories is quite low. HPLC analysis of the study sample by 5 laboratories showed 38% false negatives with the 500 ppt and 550 ppt AFM1 samples. The reliability of this method is low however results are generated rapidly

Table 2.1: Methods of AFM1 detection and their sensitivity:

Method	Sensitivity	References
Thin Layer Chromatography	300 ng/L	Shotwell *et al*., 1981
High Performance Liquid Chromatography	0.59 ng/L	Pathirana *et al*., 2010
ELISA	5 ng/L	Rastogi *et al*., 2004
Charm ROSA	400 ng/L	Salter *et al*., 2006

The existing methods for detection of AFM1 are expensive and have certain drawbacks.

Table 2.2: Detection techniques of AFM1 and their limitations

Method	Limitations
Thin Layer Chromatography	Extraction is laborious
High performance Liquid Chromatography	Expensive
GCMS-MS	Expensive
Charm ROSA	Lower Reproducibility and repeatability
ELISA	Cross reactivity

2.2 Aptamers their generation and application

2.2.1 Aptamers

Aptamer word is derived from greek word '*aptus*' which means to fit and latin word '*meros*' which means 'part'. Aptamers are basically single stranded DNA or RNA molecule which shows a very strong and specific affinity towards a particular target molecule. Aptamers usually have very complex three dimensional structures which are produced by combined effect of Watson-Crick and non-canonical intramolecular interactions (Gopinath et al., 2013). Aptamers usually contain not more than 80 nucleotides and their molecular weight varies in the range of 6-40 kDa. They are widely used as diagnostic agent due to their high specificity and binding capacity. Apart from their diagnostic use, aptamers are also being used to capture their cognate target (Citartan *et al*., 2016). Aptamer-based capture (AptaCapture) assays makes possible to capture of the target by the aptamers followed by target purification. Due to the very high specificity and high binding capacity of aptamers towards a target, various workers have developed aptamers against different molecules using Systematic Evolution of Ligands using Exponential Enrichment (SELEX) process. SELEX comprises of repetitive cycles wherein in each cycle, oligonucleotides interacting with targets are enriched. Nowadays, in

developing analytical methods, aptamers are preferred ligands over antibodies since they can be derivatized with ease and are commercially available in homogenous form. Aptamers can be used to analyze the natural processes like recognition of nucleic acid–protein, to generate enzyme inhibitors, hormones and toxins with potential pharmacological uses, to detect the presence of target molecules in complex mixtures such as tetracycline (Kim *et al.,* 2010), ochratoxin A (Bonel *et al.,* 2011), tobramycin (Fernandez *et al.,* 2011, β-casomorphine-7 (Parashar *et al.,* 2015). Aptamers can even be generated against toxins (Huang *et al.*, 2015). Aptamers have been also employed for development of biosensor which is defined as an analytical device, used for the detection of an analyte, that combines a biological component with a physicochemical detector digital signal on interaction of ligand with target molecule. Aptamers can also be immobilized on different surfaces to develop electrochemical sensor (Balamurugan *et al.,* 2008; Kim *et al.,* 2010), quartz crystal microbalance (Le *et al.,* 2013) and surface Plasmon resonance (Vance and Sandros, 2014).

2.2.2 Generation of aptamers

Systematic Evolution of Ligands by Exponential Enrichment (SELEX) is an *in vitro* selection process used for the generation of aptamers. It is a process which is conducted by defined protocols and quite robust enzymatic amplification. It involves three main steps: incubation of randomized oligonucleotide library with target, separation of the oligonucleotides which are bound to the target from the unbound and the final step is amplification of the selective oligonucleotides (Gopinath *et al*., 2011). In this method a pool of large number of oligonucleotides either RNA or ssDNA having 10^{13}-10^{15} different sequence comprises of random sequence at the center tagged by defined primer binding site at 5' and 3' terminus. The random single stranded DNA library can be produced with the mixture of phospotidamites in ratio of 1.5:1.25:1.15:1.0 (A: C: G: T) (Ho et al., 1996). The diversity of the library is depend upon the length of the random region, however about 10^{15} is the practical limitation, which corresponds to a length of 25 nucleotides. Only a short part of full aptamer sequence is sufficient for binding to a target (Bock *et al.,* 1992). This suggest that a short and cost effective library can be used for screening of aptamer as well. However for providing long complex structure long random sequences are necessary. Therefore longer library increases the chances of successful aptamer selection. RNA aptamers have extra hydroxyl group in their structure hence they have better capability of folding in 3D structures, However DNA

aptamers are widely used for *in vitro* application since they are more cheaper than the RNA aptamers and no modifications has to be done to increase their stability as in case of *in vivo* applications. The essential steps during a SELEX procedure are binding, selection, amplification and partitioning. In the first step the random DNA library is incubated with the target. Then the bound complex is subsequently separated from the unbound or loosely bound nucleotides. This is one of the crucial step in SELEX to separate high affinity oligonucleotides from the rest of the library. During incubation, target molecule can interact with the oligonucleotide either in the free form or immobilized to a surface. Immobilization of target to a particular surface facilitates easy separation of bound oligonucleotides from the unbound. Several time consuming rounds of SELEX are now converted
into one simple step (Fan *et al*., 2008). There are various SELEX modification protocols are developed which includes genomic SELEX, spiegelmer, *in vivo* SELEX, photo-SELEX, monoLEX, whole cell SELEX and silico SELEX. Using non equilibrium capillary electrophoresis of equilibrium mixture a non-SELEX process has also been developed (Berezovski *et al*., 2006)

2.2.3 Applications of aptamer

Aptamer as a biosensor

Due to its specific and fascinating characteristics along with diagnostic agent, aptamers are nowadays used as a biosensing agent and various biosensing assays are being developed using aptamers for various molecules. Biosensor have various advantages over traditional methods of being simple, rapid, cost effective and portable device that are sensitive and specific for target analytes. These devices can also be designed to be disposable or reusable for several analysis or for continuous monitoring. Using aptamers, biosensors are being developed and are called as aptasensors. Optical aptasensors, electrochemical aptasensors and mass sensitive aptasensors are various type of aptasensors being developed.

Aptamer as a replacement for Antibodies

The enzyme linked immune sorbent assay (ELISA) technique was developed in 1971. ELISA consists of an antigen capturing antibody, an antigen (target) and a detection antibody that produces a signal when the antigen is present. The simplicity and robustness associated with ELISA resulted in its widespread application as a measurement tool in microbiology

(Engvall, 1977), parasitology (Ljungstrom *et al*., 1974), oncology (Lim *et al*., 2010) and microbiology (Engvall, 1977). Among researchers and commercial manufacturers such as Boehringer Mannheim (Germany), Abbott (United States), and Organon Teknika (The Netherlands) (Lequin, 2005) ELISA became a sensation. ELISA is highly specific, can detect antigens at ultra low concentrations (Simeonov,2013), and is safer than radioimmunoassay for a wide variety of tests (Baker *et al*., 2007). Many enzyme detection methods involved in ELISA use standard spectrophotometric detection, hence the need for other sophisticated and expensive equipment is eliminated (Pierce BiotechnologyInc., 2005). However, it is tedious and challenging to generate specific monoclonal antibodies, especially against non-immunogenic molecules.

Table2.3 Comparison between antibody and aptamer as a capturing agent

Parameter	Antibody	Aptamer
Cost of production	Reasonable	Less expensive
Size	Larger	Smaller
Types of target	Only those with high immunogenicity	Almost all kinds
Transportation requirement	Dry ice	Ambient temperature
Chance of causing immunogenic response to host	Higher	Lower
Ability to conjugate to surface	Less variety of surface	Broader
Sensitivity	Similar	Similar
Temperature requirement	4˚C	Tolerate 60˚C
Shelf life	Short	Longer
Cost	Higher	Lower
Reusability	Single use	Repeated use

2.3 Aptamer immobilization for use in AptaCapture assays

Immobilization of the aptamer on the surface platform is the most important criterion of an AptaCapture assay and it is also important that while doing so, affinity for its cognate target should not be compromised. Several conjugation methods have been developed that are based on the biomolecular interaction between the terminal functional groups linked to the aptamer and surface platform conjugated functional groups. The technique which is most widely employed for aptamer immobilization is non-covalent bonding of biotin-streptavidin (Gopinath *et al*., 2014). Semi-covalent interactions formed by gold thiol and non-covalent amine-carboxyl pairs, are also used to immobilize aptamers. In most cases, modification of aptamer is done at the 5'- or 3'-end by these functional groups (biotin, thiol, or amino). These functional groups frame an interaction with their interacting pairs on the platform surface. Immobilization can also be achieved by the interaction of an additional sequence appended to the aptamer with its complementary sequence through Watson–Crick base-pairing. This complementary sequence is conjugated with the functional groups at the 5'- or 3'-end. Importantly, the chosen conjugation strategy should avoid any stearic interference or possible decrease in the binding affinity during the incorporation of the functional groups to the aptamer. Wang *et al*. (2015) recently reported that the accessibility of the aptamer to target living cells is affected by the immobilization orientations, immobilization methods, and spacers. Different surface proteins have different epitope presentations or accessibility to aptamers. Hence, only the most optimum aptamer orientation or immobilization can maximize its recognition of the target.

2.3.1 Streptavidin-biotin system

The streptavidin and biotin is one of the strongest non-covalent interactions in nature. It has translational/rotational entropy (binding energy) of 4.5–6.0 kcal/mol (Lizaridis *et al*., 2002). The binding is very rapid and it is stable over a wide range of temperatures and pH (Ross *et al*., 2014). The isoelectric point of streptavidin is 6.8–7.5, which minimizes non-specific adsorption within this pH range. Kim *et al*. (2016) carried out a study using aptamer-mediated cellular protein co-precipitation (co-range of aptoprecipitation), biotinylated DNA aptamers against human insulin receptor (INSR) and human epidermal growth factor receptor (EGFR) are immobilized on the modified surface streptavidin-coated magnetic beads. These

aptamers specifically captured INSR and EGFR from a complex biological mixture. In addition to high affinity

and specificity, as streptavidin is homotetrameric in nature, binding affinity with biotin is equal of each subunit. In the case of biotinylated aptamers, avidity associated with the homo-tetramericity enhances the target capturing by the aptamers. As an example, Zhao *et al*. (2012) generated a unique 3D network of aptamers that extends into the solution over micrometers of length and was able to efficiently capture the cognate target. Similar to the long tentacles of jellyfish that catch and sting their prey effectively, this 3D DNA network was created by rolling circle amplification from the circular template that contains the DNA aptamer generated against protein tyrosine kinase 7 (PTK7).

2.3.2 Amine-carboxyl and amine-amine system

Covalent amine carboxyl interaction is another robust bio molecular interaction for aptamer immobilization (Cai *et al*., 2013). An aptamer with primary amines such as 5' Amino Modifier C6, Internal amino- C6-dT, or 3' Amino Modifier appended to it can react with succinic anhydride in the presence of EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) and NHS (N-hydroxysuccinimide) to generate activated carboxyl groups (Romhildt *et al*., 2013). Subsequently, the activated carboxyl group can interact with amine-terminated groups such as 3- aminopropyltriethoxysilane (APTES). APTES is usually immobilized on the surface of silanol-terminated silicon through the process known as silanization (Acres *et al*., 2012). It is very essential to remove the unreacted aptamers from the immobilization surface, as carry-over of these unreacted aptamers into the reaction may result in non-conjugated aptamer-target formation. If this happens, the amount of the target captured by the conjugated aptamer in real sample is reduced. During conjugation of aptamer, protein contaminants should be avoided, as these biomolecules can interferes towards the amine or carboxyl-terminated surface.

2.3.3 Thiol-gold system

Thiol gold system is another commonly used system for aptamer conjugation. Aptamer conjugation on a gold surface is actualized via semicovalent interaction between the thiol and gold atom, which has a binding energy of 45 kcal/mol (Ravalli *et al*., 2015). Aptamers needs to get functionalized with thiols prior to their immobilization on the surface of the gold.

Functionalization can be achieved through: (1) a thiol (−SH) or disulphide (−SSR) terminus, (2) a spacer sequence such as poly A20, and (3) the aptamer sequence. A spacer can be replaced with polyethylene glycol (PEG), as in the case of thrombin aptamer immobilized on a gold nanorod surface, which was capable of capturing up to 1 ng of thrombin from a plasma sample (Yasun *et al*., 2012). A self-assembled monolayer (SAM) which is thermodynamically stable and of high order can be formed by large-scale immobilization of thiols on a gold surface (Love *et al*., 2005). SAM formation enables large-scale immobilization of aptamers on the gold surface via the following simple steps. First, the gold surface is rinsed with piranha solution (H_2O_2/H_2SO_4) followed by thorough washing with water. Then, the gold surface is immersed into the solution of aptamer–thiol, and then the vacant sites are blocked by incubation with the lower molecular weight co-thiol. The non-specific interaction of the substrates with the gold surface were reduced by the blocking step. The possibility of the non-specific interaction emphasizes the importance of a 'protein contaminant-free' surface.

2.4 Aptamers against Aflatoxin M1

Aptamers against aflatoxin have also been developed successfully. 5' thiolated sequence was used for development of impedimeteric aptasensor for AFM1 (Dinckaya *et al*., 2011). Same sequence was generated from SELEX and used for developing label free electrochemical aptasensor by institute of Biotechnology, Vietnam (Nguyen *et al*., 2013). In our Institute also, 36 specific sequences of aptamers were developed against AFM1 using SELEX (Malhotra *et al*., 2014).

The dissociation constant of these aptamers were found to be 35-1515 nM. 14 aptamers of which possess dissociation constant of less than 100 nM. These aptamers were divided into 8 groups depending upon the structural analysis using Mfold software wherein classification was done on the basis of possession of same secondary structures. The group 1, group 2, group 3, group 4, group 4, group 5 and group 6 were found to possess the same secondary structure. While group 7 was found to possess G-quadraplex structure and 8^{th} group comprised of aptamers that does not belong to any of the above groups. Recently a researcher from china (Guo *et al*., 2016) have proved the binding of one of this aptamrer to aflatoxin M1 using PCR based technique.

2.5 Aptamers based detection on structure switching concept

Recently structure switching aptamer assay for determination of AFM1 employing the quenching- dequenching mechanism (Sharma et al., 2016). Hybridization of fluorescein labelled anti-AFM1 aptamer (F-aptamer) with TAMRA labelled complementary sequences (Q-aptamer) brings the fluorophore and the quencher into close proximity, which results in maximum fluorescence quenching. On addition of AFM1, the target induced conformational formation of antiparallel G-quadruplex aptamer-AFM1 complex results in fluorescence recovery. Under optimized experimental conditions, the developed method showed good linearity with limit of detection (LOD) at 5.0 ng/kg for AFM1. The specificity of the sensing platform was carefully investigated against AFB1 and ochratoxin A (OTA). The developed assay platform showed the high specificity towards AFM1. The practical application of the developed aptamer assay was verified for detection of AFM1 in spiked milk samples. Good recoveries were obtained from AFM1 spiked milk sample.

Jafari et al. (2017) have developed a DNAzyme based assay for detection of AFM1. In this assay, the AFM1 specific aptamer is bound to oligonucleotide sequence which shows enzymatic activity (Willner *et al.,* 2008). The detection method is based on the conformational changes in the structure of aptamer in the presence of target. The detection limit of this method is quite high hence this method can be used only for the assessment of binding of aptamer to its cognate target.

2.6 Aptamer based electrochemical aptasensors

Nucleic acids aptamers upon binding to their target molecules fold their flexible, single stranded chains into well-defined three- dimensional structures. Aptamers can be immobilized over on a conducting support through series of chemical reactions. Several electrochemical aptasensors have been developed using this strategy. Aptamers labeled with a redox methylene blue (MB) can be immobilized on an electrode (Xiao et al., 2005). The flexible confirmation of the aptamer enables the electrical communication of MB with the electrode. After binding with the target, aptamer self-assembles into a G-quadruplex structure and shields MB from electron transfer communication with the electrode. Another approach used an aptamer tagged with a terminal electroactive with a ferrocene group as the

redox label and a thiol group at the terminus (Mir and Katakis, 2007). The long flexible modified aptamer chain prevent electrical contact of the ferrocene label with the electrode. The formation of a complex of target and the aptamers make the G-quadruplex aptamer configuration rigid and results in the orientation of the ferrocene units towards the electrode. This leads to electron transfer between the electro-active ferrocene units and the electrode, and produced a positive signal in the presence of target.Various workers have developed electrochemical aptasensors against AFM1 and other related molecules which are discussed in detail below.

A DNA probe and GNP based impedimeteric biosensors have been developed by Dinckaya et al., (2011) for detection of AFM1. Self-assembled monolayer of gold nano particle and cysteamine, layer by layer was prepared and thiol modified ssDNA was immobilized, which shows specific affinity for AFM1. Electrochemical assay was performed by electrochemical impedance spectroscopy and $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ solution was used as a redox probe. The biosensor provided linear response against concentration of AFM1 over the range 1 to 14 ng/ml with a standard deviation of 0.36 ng/ml.

An electrochemical aptasensors for detection of tetracycline using ssDNA aptamer which shows specificity towards tetracycline as recognition element have been developed by Kim et al. (2010). Immobilization of ssDNA aptamer was done on streptavidin modified screen printed gold electrode. Then the binding of tetracycline to aptamer was analyzed by cyclic voltammetry and square wave voltammetry. Lower detection limit of this aptasensors was found to be 10 nanomolar to micromolar range. The aptasensors showed high selectivity for tetracycline and can differentiate between tetracycline and other structurally related tetracycline derivatives in a mixture.

Ngugen et al. (2013) have developed an electrochemical aptasensors using Fe_3O_4/ polyaniline polymerized on interdigitated array electrode for AFM1. Aptamer was then immobilized on the surface and detection of AFM1 was performed by electrochemical change acquired by cyclic and square wave voltammetry. The electrochemical aptasensors shows a good sensitivity to AFM 1 in the range of 6-60 ng/ L, with the detection limit of 1.98 ng/L.
AFM1 being small molecule like other mycotoxins viz. ochratoxin A, various electrochemical strategies has been employed to develop aptasensors for ochratoxin A. Bonel et al. (2011) developed an electrochemical competitive biosensor for ochratoxin A based on a DNA biothinylated aptamer, using paramagnetic microparticles beads. The beads functionalized

with OTA binding aptamer were allowed to compete for OTA-HRP conjugated and free OTA. Magnetic beads after separation were placed over disposable screen printed carbon electrode (SPCEs) under a magnetic field. HRP reaction with the substrate was detected with differential-pulse voltammetry. The magnetic aptasensors showed a linear response to OTA in a range 0.78-8.74 ng/mL and a limit of detection of 0.07±0.01 ng/mL.

Su et al. (2012) have developed an impedance aptasensors in which the amine-terminated lipopolysaccharide specific aptamer (K_d=11.9 nM) was immobilized using mercaptopropionic acid (MPA) as a linker. Each step of the modification process was characterized by cyclic voltammetry and electrochemical impedance spectroscopy. A good linear relationship of the changes in the charge-transfer resistance (ΔR_{et}) and the logarithmic value of LPS concentration was demonstrated in a broad dynamnic detection range of 0.001-1 ng/ml. The aptasensors showed high selectivity to LPS and could be regenerated in low pH condition.

Hayat et al. (2013) developed a highly sensitive ochratoxin-A impedimetric aptasensors based on immobilization of azido-aptamer electrografted film via click chemistry. The aptamer was immobilized onto modified screen printed carbon electrode, by electrografting of a protected 4-((Trimethylsily) ethynyl) benzene layer followed by a second one of p-nitrobenzene be means of electrochemical reduction. The aptamer having an azide group from 1, 2, 3-triazole linkage with trimethylsilyl-Ethynyl. Cyclic voltammetry and electrochemical impedance spectroscopy in the presence of ferri/ferricyanide redox probe $[Fe(CN)_6]^{-4/-3}$ were used to characterize each step in the aptasensors development. The increase in electron-transfer resistance (R_{et}) value due to the specific aptamer- OTA interaction was proportional to the concentration of OTA in a range between 1.25 ng/L and 500 ng/L, with a detection limit of 0.25 ng/L.

The aptamers are found to be very significant as a bio-recognition molecules as they can successfully replace antibodies in many antibody based detection assays. As discussed above various approaches have been used to visualize the binding of aptamer to its cognate target and some approaches have also succeed in meeting the lower detection limits of targets prescribed by regulatory bodies. In this research work, efforts will be made to develop aptamer based sensing system to quantify AFM1 in milk. For this, already identified aptamer specific for AFM1 will be employed.

CHAPTER – 3

MATERIALS AND METHODS

MATERIALS AND METHODS

This chapter deals with various materials and instruments employed during the investigation related to "Detection of aflatoxin M1 using aptamer based sensing system".

3.1 Objective 1

To develop Aptamer based fluorescent assay for detection of aflatoxin M1

3.1.1 Materials

Flat black multiwell plate with cover was obtained from Genetix Asia Pvt. Ltd. Aflatoxin M1 (Cat. No. RM-6433-100 µG) was purchased from HiMedia Pvt. Ltd. Aflatoxin B1 (Cat. No. A-6636-1 MG), nuclease free water, hydrogen peroxide (30%), 3,3',5,5'-Tetramethylbenzidine (TMB), dimethyl sulfoxide (DMSO) and hemin were procured from Sigma Aldrich, USA. Tris-HCl, sodium chloride, potassium chloride, calcium chloride, magnesium chloride and sodium hydroxide were of molecular biology grade and were procured from Sigma Aldrich Pvt. Ltd., USA. Ultra-pure water having electrical conductivity of 0.058 µS/ cm was obtained from Cascada Bio water system. Sulfuric acid was obtained from Merck, USA. Aptamer (including their purification) were obtained from (AFAS3-Tr Bs, AFAS3-Tr BsR and AFM1-DNAzyme) Sigma Aldrich, USA and are listed in Table 3.1 and 3.2.

Table 3.1: Sequences of aptamers used in fluorescent based assay

Sr.No.	Aptamer	Sequence (5'-3')	Base Pairs
1	AFAS3-Tr Bs	**FAM** –CCTGCTCT	8
2	AFAS3-Tr BsR	AGAGCAGG **–TAMRA**	8

6FAM = 6-Carboxyfluorescein ; TAMRA = Tetramethylrhodamine

All the plasticware used were of polypropylene. The tips used were obtained from Axygen Inc., USA. Eppendorf tubes were obtained from Hamburg, Germany. Centrifuge tubes of 15 ml and 50 ml volumes were procured from Genetix Asia Pvt. Ltd, India. All glassware used were from Borosil, India and PYREX, Germany. Magnetic stirrer used was from Tarsons and pH meter (Model A420+) used was of Thermo Orion. Multiwell plate reader (Infinite M200 Pro) used was of Tecan.

Two approaches were used for the development of fluorescence based methods for detection of AFM1

3.1.2 Approach 1. Method using 5′ FAM modified Aptamer and 3′ TAMRA modified counter oligonucleotide:

3.1.2.1. Reagents

Binding Buffer (20 mM tris-HCl, 100 mM NaCl, 2 mM $MgCl_2$, 5 mM KCl, 1mM $CaCl_2$, pH 7.6): Binding buffer was prepared by dissolving 0.315 g of Tris-HCl, 0.5844 g of NaCl, 0.0372 g of KCl, 0.190 g of $MgCl_2$, 0.0110 g of $CaCl_2$ in 80 ml of ultra-pure water and then pH was adjusted to 7.6 with dilute NaOH. The volume was made to 100 ml with water. The buffer was autoclaved prior to use.

100 µM Aptamer: 129.2 nmoles of modified aptamer (AFAS3- Tr Bs) was dissolved in 1292 µL (as mentioned in technical datasheet) of nuclease free water and aliquots were prepared and stored at -20 ^{o}C till further use.

100 µM counter oligonucleotide: 80.7 nmoles of modified counter oligonucleotide (AFAS3-Tr BsR) was dissolved in 807µL (as mentioned in technical datasheet) of nuclease free water and aliquots were prepared and stored at -20 ^{o}C till further use

0.5 µM Aptamer: 10 µL of 100 µM Aptamer was diluted to 2 ml with binding buffer of pH 7.6 before use.

0.5 µM Counter oligonucleotide: 10 µL of 100 µM oligonucleotide was diluted to 2 ml with binding buffer of pH 7.6 before use.

Aflatoxin M1 solution: Stock solution of 304.6 µM was prepared by diluting 100 µg (supplied as such) in 1 ml methanol and water solution (9:1). Then 0.5 µL AFM1 solution was prepared by mixing 5 µL stock of AFM1 solution and 3035 µL of binding buffer. Then 0.4 µM AFM1 solution was prepared by mixing 1600 µL of 0.5 µM solution and 400 µL binding buffer. In the same manner solutions of 0.2, 0.1, 0.05 µM were prepared and stored at 4 ^{o}C until use.

3.1.2.2 Method

1. Prior to the use, working aptamer solution of 0.5 µM was given thermal cycle treatment in three steps. The aptamer solution was firstly heated in Eppendorf tube at 95^{o}C for 5 min in a block heater (Techne DRI-block DB3). Then Eppendorf tube were placed in ice

bath for 30 min. Finally aptamers containing tubes were allowed to come at room temperature.

2. Fifty microliter of FAM modified aptamer prepared in binding buffer and undergone thermocycle was added in each well of micro titer plate
3. Fifty microliter of 0.05, 0.1, 0.2, 0.4, 0.5 µM AFM1 dissolved in binding buffer was added in wells of micro titer plates the contents were mixed.
4. The micro titer plate was then incubated at 37 ^{o}C for 45 min in dark.
5. After 45 of min of incubation, 150 µL of TAMRA modified counter oligonucleotide was added into the wells of micro titer plate, the contents were mixed
6. The micro titer plate was incubated for 1 min in dark.
7. Reading was taken in multiwell plate reader using excitation wavelength of 485 nm and emission wavelength of 538 nm. (Sharma *et al*.,2016)

3.1.3 Approach 2: DNAzyme based method for detection of aflatoxin M1

3.1.3.1 Reagents:

100 µM stock of DNAzyme: 105 nmoles of modified aptamer (AFAS3- Tr Bs) was dissolved in 1050 µL (As mentioned in technical datasheet) of nuclease free water and aliquots were prepared and stored at -20 ^{o}C till further use.

AFM 1 solution: Stock solution of 304.6 µM was prepared by diluting 100 µg (supplied as such) in 1 ml methanol and water solution (9:1). Then 10 µM and 20 µM working solution was prepared by subsequent dilution of stock solution

Binding Buffer: (20 mM tris-HCl, 100 mM NaCl, 2 mM $MgCl_2$, 5 mM KCl, 1mM $CaCl_2$, pH 7.6): Binding buffer was prepared by dissolving 0.315 g of Tris-HCl, 0.5844 g of NaCl, 0.0372 gm of KCl, 0.190 g of $MgCl_2$, 0.0110 g of $CaCl_2$ in 80 ml of ultra-pure water and then pH was adjusted to 7.6 with dilute NaOH. The volume was made to 100 ml with water. The buffer was autoclaved prior to use.

Hemin solution- 3.26 mg Hemin was dissolved in 5 ml, 20 mM NaOH. Then 50 µM working Hemin solution was prepared by diluting 1 mM Hemin with Phosphate-Citrate buffer.

3,3',5,5', tertamethylene benzidine (TMB) : 8 mM TMB solution was prepared by dissolving 1.9228 mg TMB in 1 ml DMSO followed by further dilution in DMSO to prepare 6 mM solution.

15 mM H_2O_2: 30 % H_2O_2 (2.538 M) was diluted with Phosphate-Citrate buffer to obtain 15 mM H_2O_2 solution. Molarity of stock H_2O_2 was calculated from molar extinction coefficient (43.6 $M^{-1}cm^{-1}$ at wavelength 240 nm).

1 M H_2SO_4: Concentrated H_2SO_4 (18.385 M) was diluted with milliQ water to obtain 1 M H_2SO_4 solution.

Table 3.2: Oligonucleotide sequence used for DNAzyme aasay

Sr.No.	Aptamer	Sequence (5'-3')	Base Pairs
1	AFM1-DNAzyme	TGG GTA GGG CGG GTT GGG AAT TCT AG	**26**

3.1.3.2 Treatment of Aptamers

Prior to the use, working aptamer solution of 0.5 µM was given thermal cycle treatment in three steps. The aptamer solution was firstly heated in Eppendorf tube at 95°C for 5 min in a block heater (Techne DRI-block DB3). Then Eppendorf tube were placed in ice bath for 30 min. Finally aptamers containing tubes were allowed to come at room temperature.

3.1.3.3 Method:

1) 20 µl of temperature treated 10 µM DNAzyme and 20 µl target (AFM1) were added into 96-well microplate and incubated for 30 min at RT.

2) Then 20 µl Hemin (50 µM) was added and incubated at room temperature for 30 min to form DNA-hemin complex. (Jafari *et al*.,2016)

3)Subsequently, 100 µl Phosphate citrate buffer, 20 µl of 6 mM TMB substrate and 20 µl, 15 mM H_2O_2 (prepared immediately before use) were added.

4) Change in color was observed within 8-10 min.

5) Immediately, 100 µl of 1 M sulfuric acid (stopping solution) was added to stop the reaction.

6) Absorbance values were read at 450 nm in BioTek Eon Microplate UV/VIS Reader.

3.1.4 Cross reactivity of AFM1 aptamer (AFAS3-Tr Bs) with Aflatoxin B1

Cross reactivity AFM1 specific aptamer was checked by fluorescent assay by incubating different concentration of AFB1 (0 to 0.5 µM) with FAM tagged AFAS3 aptamer then TAMRA tagged oligonucleotide is added to wells of micro titer plate and then plate is read in Elisa reader as mentioned in section **3.1.2**

3.2 Objective 2.

To develop electrochemical based assay detection of aflatoxin M1

3.2.1 Materials

Screen printed gold electrodes (Cat. No. DRP-220AT) were procured from DropSense, Spain. 3, 3'-Dithiopropionic acid (Cat. No. 109010-50G), N-(3-dimethylaminopropyl)-N-thylcarbodimide (EDC) (Cat No. 39391-10 ml), morpholineethanesulphonic acid hydrate (MES) (Cat No. M3671-50G), streptavidin from streptomyces (Cat No. S4762-1MG), Tris-HCL, sodium chloride, potassium chloride, calcium chloride, magnesium chloride and sodium hydroxide were of molecular biology grade and were procured from Sigma Aldrich Pvt. Ltd., USA. N-Hydroxysuccinimide (NHS) (Cat No. 56480-50G) was procured from Fluka, USA. Ethanolamine of biological grade was obtained from Sisco Research laboratories Pvt Ltd, India. Aflatoxin M1 (Cat. No. RM-6433-100 µG) was purchased from HiMedia pvt. Ltd. Aflatoxin B1 (Cat. No. A-6636-1 MG), nuclease free water, sodium phosphate monobasic, sodium phosphate diabasic, potassium phosphate monobasic, potassium phosphate dibasic and sodium bicarbonate were procured from Sigma Aldrich, USA. Potassium ferricyanide ($K_3[Fe(CN)]_6$) was procured from HiMedia Laboratories Pvt. Ltd., India. HPLC purified aptamers used for immobilization on screen printed gold electrodes were procured from Avantor India Pvt. Ltd. and are listed in Table 3.2

Table 3.3: Sequences of aptamers used in electrochemical based assay

Sr.No.	Aptamer	Sequence (5'-3')	Base Pairs
1.	AFAS3*	ATCCGTCACACCTGCTCTGACGCTGGGGTCGACCCGGAGAA ATGCATTCCCCTGTGGTGTTGGCTCCCGTAT-**TEG Biotin**	**72**
2.	APM 6 Tr*	**Biotin-TEG-AAAAA**TAATTCTAGGTTA	**18**

*TEG- Tetraethyleneglycol

3.2.2 Reagents

Binding Buffer (20 mM tris-HCl, 100 mM NaCl, 2 mM $MgCl_2$, 5 mM KCl, 1mM $CaCl_2$, pH 7.6): Binding buffer was prepared by dissolving 0.315 g of Tris-HCl, 0.5844 g of NaCl, 0.0372 gm of KCl, 0.190 g of $MgCl_2$, 0.0110 g of $CaCl_2$ in 80 ml of ultra-pure water and then pH was adjusted to 7.6 with dilute NaOH. The volume was made to 100 ml with water. The buffer was autoclaved prior to use.

100 µM Aptamer: 28 nmoles of modified aptamer (AFAS3) in lyophilized form was first centrifuged and then was dissolved in 280 µL (as mentioned in technical datasheet) of nuclease free water and aliquots were prepared and stored at -20°C till further use.

0.1 µM Aptamer: 10 µL of 100 µM aptamer was diluted to 10 ml with nuclease free water and then this dilution was used to make working aptamer solution.

10 nM aptamer: 100 µL of 0.1 µM aptamer was further diluted to 900 µL of water to 1 ml to make 10 nM working aptamer solutions. Working aptamer solutions are given thermocycle treatment as described in 3.1.2.2

10 mM of H_2SO_4: 540 µL of 98% H_2SO_4 was diluted to 10 ml with water.

3, 3'-Dithiopropionic acid (2 mM): 0.42 g of 3, 3'-dithiopropionic acid was dissolved in 10 ml of ethanol and then it was subsequently diluted to 2 mM in ultra-pure water.

N-(3-dimethylaminopropyl)-N-ethylcarbodimide (EDC) (300 mM): 64 µL of EDC was dissolved in 1 ml of ultra-pure water. This solution was freshly prepared prior to use.

N-hydroxysuccinimide (NHS) (3mM): 3.45 mg NHS was dissolved in 10 ml of ultra-pure water.

2-(N- morpholino)ethanesulphonic acid (MES buffer) (100 mM): 1.95 g of MES and 2.92 g of NaCl were dissolved in 80 ml water. The pH was then adjusted to 6.0 and then volume was made to 100 ml with water.

Working EDC/NHS solution: 50 µL each of EDC, NHS and MES buffer were mixed together to get working solution of EDC/NHS.

100 mM ethanolamine: 306.5 µL of ethanolamine was dissolved in 50 ml water.

Phosphate buffered saline (PBS) (pH 7.5): 1.44 g of Na_2HPO_4 and 0.24 g of NaH_2PO_4, 8 g NaCl and 0.2 g KCl were mixed in 900 ml ultra-pure water. The pH was then adjusted to 7.5. The volume was made to 1000 ml with water.

Streptavidin Solution: 1 mg of streptavidin was dissolved in 1 ml of PBS buffer, pH 7.5.

Potassium ferricyanide (5mM) solution: 164.62 mg of potassium ferricyanide ($K_3[Fe(CN)]_6$) and 745.5 mg KCl were dissolved in water to make 100 ml solution.

AFM1 solution: Stock solution of 10^8 ppt (304.6 µM) AFM1 was prepared by dissolving 100 µg AFM1 in 1 ml solution of methanol: water in ratio 9:1. Then 10^6 ppt was prepared by mixing 2 µL 10^8 ppt stock solution of AFM1in 198 µL of TBB. Then 10^5 ppt was prepared by mixing 20 µL of 10^6 ppt and 180 µLTBB. Then 10^4 ppt solution was prepared by mixing 20 µL of 10^5 ppt and 180 µL binding buffer. Similarly 10^3, 10^2 and 10 ppt solutions were prepared and stored in refrigeration in dark until use.

3.2.3 Immobilization of Biotin-TEG modified aptamer on gold working electrode:

1. All three electrodes on screen printed gold electrode (SPGE) were washed with 10 mM H_2SO_4.
2. The electrodes were washed with copious amount of ultra-pure water. Then 100 µL $K_3[Fe(CN)]_6$ solution was gently placed on electrode chip covering all three electrodes. Electrochemical response was measured using cyclic voltammetry under the condition as mentioned in Table 3.3.
3. The electrodes on SPGE were washed with ultra-pure water and then SPGE was placed in chamber in which water was added at the bottom to maintain humid condition. Ten microliter of 2 mM 3, 3'-dithiopropionic acid dissolved in water was placed over gold working electrode to cover entire surface of working electrode. After 30 min, electrodes were washed with 10 ml of ultra-pure water.
4. The carboxylic groups of 3, 3'-dithiopropionic acid were activated by placing 10 µL MES buffer (pH 6.0) containing 100 mM EDC and 1 mM NHS on working electrode. Activation process continued for 1 h at room temperature under humid environment. The electrodes were washed with 10 ml of ultra-pure water.
5. Then, 10 µL of streptavidin (1 mg/ml) prepared in PBS buffer (pH 7.5) was placed over working electrode and incubated overnight at 4 ^oC under humid environment.

6. The electrodes were washed with 10 µL ultra-pure water.
7. The free carboxyl groups over working electrode were blocked by incubation with 10 µL of 100 mM ethanolamine for 20 min at room temperature under humid environment.
8. Then, 10 µL of 10 nM TEG-biotinylated aptamer (AFAS3) was placed on working electrode and incubated for 40 min at room temperature. After 40 min, electrodes were washed with 10 ml ultra-pure water.
9. Electrochemical response of electrode at different stages of electrode modification i.e. after dithiopropionic acid treatment, streptavidin coating and aptamer immobilization was determined.

Table 3.4: Conditions employed for cyclic voltammetry

Parameters	Value
Init E (V)	-0.3
High E (V)	+0.8
Low E (V)	-0.3
Final E (V)	+0.8
Initial scan polarity	Positive
Scan rate (V/s)	0.1
Sweep segments	2
Sample interval	0.001
Quite time (sec)	2
Sensitivity A/V	1.e-004

3.2.4 Measurement of Electrochemical response at various concentrations of AFM1

Aptamer immobilized electrode was treated with known concentration of AFM1 (0, 10, 10^2, 10^3, 10^4, and 10^5 ppt) prepared in binding buffer for 30 min at room temperature. After each treatment, the electrodes were washed with 3 ml of binding buffer. Then, 100 µL of 5 mM $K_3[Fe(CN)]_6$ solution was placed over all three electrodes and square wave voltammetric response was measured under the conditions specified in table 3.4. The electrode was sequentially incubated with AFM1 in its increasing order of concentration. The dose response on aptamer immobilized electrode was then measured.

Table 3.5: Conditions employed for square wave voltammetry

Parameters	Value
Init E (V)	+0.8
High E (V)	-0.3
Incr E (V)	0.004
Amplitude (V)	0.025
Frequency (Hz)	15
Quite time (sec)	2
Sensitivity A/V	1.e-004

3.2.5 Cross reactivity of AFM1 aptamer (AFAS 3) with AFB1

Cross reactivity of AFM1 specific aptamer was checked by taking electrochemical response of aptamer immobilized SPGE after incubating same concentrations of AFB1 as taken for AFM1. Two concentrations of AFB1 were incubated i.e. 10^2 and 10^4 ppt and peak current of SPGE was measured by SWV. Then the current drop occurred by AFB1 was compared with the current drop produced by AFM1

3.3 Objective 3.

Establishment of proof of binding of aptamer in milk matrix

3.3.1 Material

Raw cow milk samples were procured from Institute Livestock Research Center and a local vendor in Karnal (cows fed on green fodder only). A commercial sample of UHT milk was procured from a local shop. Authentic negative milk sample for aflatoxin M1 was obtained from ROSA kit. Chloroform used for extraction was HPLC grade and obtained from Merck, Germany. Other consumables were same as Objective 1 and 2

3.3.2 Preparation of milk samples

The procured milk samples were centrifuged at 6000 g for 10 min at 4 ^{o}C to remove the milk fat. The centrifugation was done thrice to ensure the complete removal of fat from the milk. The skimmed milk thus obtained was then distributed in 15 ml centrifuge tubes and autoclaved at 121 ^{o}C for 30 min. The autoclaved sample was then stored under refrigeration condition until further use.

3.3.3 Establishment of proof

3.3.3.1 Method using spiked milk samples directly in fluorescent based assay

3.3.3.1.1 Reagents

Binding Buffer (20 mM tris-HCl, 100 mM NaCl, 2 mM $MgCl_2$, 5 mM KCl, 1mM $CaCl_2$, pH 7.6): Binding buffer was prepared by dissolving 0.315 g of Tris-HCl, 0.5844 g of NaCl, 0.0372 gm of KCl, 0.190 g of $MgCl_2$, 0.0110 g of $CaCl_2$ in 80 ml of ultra-pure water and then pH was adjusted to 7.6 with dilute NaOH. The volume was made to 100 ml with water. The buffer was autoclaved prior to use.

100 µM Aptamer: 129.2 nmoles of modified aptamer (AFAS3- Tr Bs) was dissolved in 1292 µL (as mentioned in technical datasheet) of nuclease free water and aliquots were prepared and stored at -20 ^{o}C till further use.

100 µM counter oligonucleotide: 80.7 nmoles of modified counter oligonucleotide (AFAS3-Tr BsR) was dissolved in 807µL (As mentioned in technical datasheet) of nuclease free water and aliquots were prepared and stored at -20 ^{o}C till further use.

0.5 µM Aptamer: 10 µL of 100 µM aptamer was diluted to 2 ml with binding buffer of pH 7.6 before use.

0.5 µM Counter oligonucleotide: 10 µL of 100 µM aptamer was diluted to 2 ml with binding buffer of pH 7.6 before use.

Aflatoxin-M1 spiked milk samples: 2 µL of 304 µM of AFM1 was dissolved in 1198 µL of milk in small glass test tube then the tube is ultrasonicated for 5 min to ensure proper mixing. Then 160 µL of spiked milk was mixed with 40 µL of pure milk to obtain 0.4 µM of AFM1 concentration. 0.2 µM AFM1 milk was prepared by taking 80 µL of spiked milk and diluting it to 200 µL with pure milk. Similarly 0.1 and 0.05 µM dilutions were made using 0.5 µM spiked milk sample. All the spiked milk samples were ultrasonicated for 5 min to ensure proper distribution of AFM1 in milk.

3.3.3.1.2 Treatment of Aptamers

Prior to the use, working aptamer solution of 0.5 µM was given thermal cycle treatment in three steps. The aptamer solution was firstly heated in Eppendorf tube at 95^{o}C for 5 min in a block heater (Techne DRI-Block DB3). Then Eppendorf tube were placed in ice bath for 30 min. Finally aptamer containing tubes were allowed to come at room temperature.

3.3.3.1.3 Method

1. 50 µL of FAM modified aptamer prepared in binding buffer and undergone thermocycle was added in each well of micro titer plate.
2. 50 µL of 0.05, 0.1, 0.2, 0.4, 0.5 µM AFM1 spiked skim milk was added in wells of micro titer plates and the contents were mixed.
3. The micro titer plate was then incubated at 37^{o}C for 45 min in dark.
4. After 45 of min of incubation, 150 µL of TAMRA modified counter oligonucleotide was added into the wells of micro titer plate and the contents were mixed
5. The micro titer plate was incubated for 1 min in dark.

6. Reading was taken in ELISA plate reader (NanoQuant) using excitation wavelength of 485 nm and emission wavelength of 538 nm.

3.3.3.2 Method using AFM1 Extraction from spiked milk samples in fluorescent based assay

3.3.3.2.1 Extraction of AFM1 from milk sample

1. Spiked milk samples were prepared as mentioned above and then 1 ml skim milk was mixed with 1 ml chloroform in a glass tube. The contents were mixed and then ultrasonicated for 10 min.
2. The content was left undisturbed to allow the separation of chloroform and aqueous layer.
3. Chloroform layer was removed carefully using glass auto-pipette and transferred to another glass tube.
4. The contents were again extracted twice using 1 ml chloroform each time. The chloroform layer was transferred and pooled together in glass tube.
5. The test tube with the extracted chloroform was then placed over water bath maintained at 45 ^{o}C until the solvent evaporates completely. In place of chloroform acetonitrile or methanol can also be used but, biotin from milk will also get dissolved in acetonitrile and interfere in electrochemical assay hence chloroform is used.
6. After complete removal of solvent, 1 ml binding buffer was added

3.3.3.2.2 Method

1. 50 µL of FAM modified aptamer prepared in binding buffer and undergone thermocycle was added in each well of micro titer plate
2. 50 µL of 0.05, 0.1, 0.2, 0.4, 0.5 µM AFM1 spiked in skim milk was added in wells of micro titer plates the contents were mixed.
3. The micro titer plate was then incubated at 37^{o}C for 45 min in dark.
4. After 45 of min of incubation, 150 µL of TAMRA modified counter oligonucleotide was added into the wells of micro titer plate, the contents were mixed
5. The micro titer plate was incubated for 1 min in dark.

6. Reading was taken in ELISA plate reader (NanoQuant) using excitation wavelength of 485 nm and emission wavelength of 538 nm.

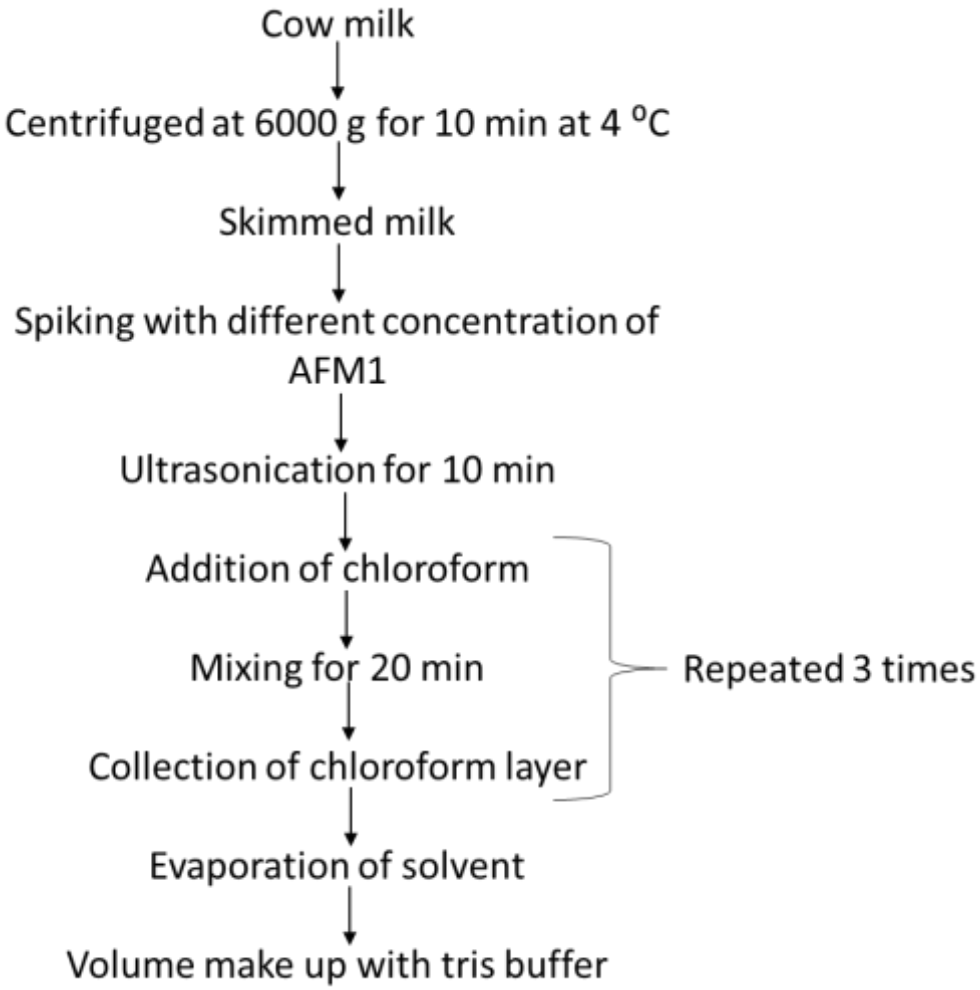

Figure 3.1. Protocol for extraction of aflatoxin M1 (AFM1) spiked milk for its estimation by electrochemical assay

3.3.3.4 Measurement of electrochemical response in milk samples spiked with AFM1

3.3.3.4.1 Reagents: As mentioned in Section 3.2

3.3.3.4.2 Preparation of AFM1 spiked milk samples: AFM1 spiked skim samples (10 – 10^6 ppt) were prepared.

1. 2 µL of 10^8 ppt stock solution of AFM1 (prepared in methanol:water; 9:1) was added in 198 µL of skim milk followed by ultrasonication to obtain 10^6 ppt AFM1 concentration.

2. 120 µL of 10^6 ppt spiked milk was dilute to 1200 µL with skim milk to obtain AFM1 concentration of 10^5 ppt.
3. 120 µL of 10^5 ppt spiked milk was dilute to 1200 µL with skim milk to obtain AFM1 concentration of 10^5 ppt.
4. Similarly other AFM1 spiked skim samples (10^3, 10^2 and 10 ppt) were prepared. Before each subsequent spiking the milk solution is mixed well and ultrasonicated for 5 min to ensure proper mixing of AFM1 in milk.

3.3.3.4.3 Method

1. One milliliter spiked skim milk was mixed with 1 ml chloroform in a glass tube. The contents were mixed and then ultrasonicated for 10 min.
2. The contents were left undisturbed to allow the separation of chloroform and aqueous layer.
3. Chloroform layer was removed carefully using glass auto-pipette and transferred to another glass tube.
4. The contents were again extracted twice using 1 ml chloroform each time. The chloroform layer was transferred and pooled together in glass tube.
5. The test tube with the extracted chloroform was then placed over water bath maintained at 45^{o}C until the solvent evaporates completely.
6. After complete removal of solvent, 1 ml binding buffer was added
7. Then electrochemical responses of different concentrations were taken on SPGE as discussed above.

3.3.3.5 Validation of the Electrochemical based method with Rosa Assay kit for detection of AFM1

AFM1 spiked milk samples were tested using ROSA as per the manufacturer's protocol. The method in brief was as follows:

3.3.3.5.1 Method

1. 300 µl of milk sample was diluted with buffer (1:1) provided with ROSA kit and incubated in ice bath for 10 min.

2. An AFM1 detection strip was removed from the pack and was placed in an incubator maintained at 37 ^{o}C 300 µl of diluted sample was dispensed on sample compartment of the AFM1 detection strip.
3. The sample compartment was sealed and the strip was incubated for 8 min but not more than 10 min.
4. After incubation, strip was removed from the incubator and results were visualized with eye followed by taking reading in the Rosa Reader.
5. Result interpretation: Test results from ROSA strips may be read and officially recorded using the Charm EZ system, or the ROSA Pearl-X Reader.

CHAPTER – 4

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

The work reported in this dissertation is divided into three parts. Part 1 deals with the development of aptamer based fluorescent assay for detection of aflatoxin M1 (AFM1). The recognition of binding of aptamer to AFM1 was evaluated by generation of fluorescence by FAM tagged aptamer in presence of TAMRA tagged counter nucleotide. Part 2 deals with the development of aptamer based electrochemical assay for detection of AFM1. In this part, the aptamer was immobilized over a surface of screen printed gold electrode and the recognition of binding of aptamer with AFM1 was done by evaluating the drop in current of an electrode by taking electrochemical response in an electrochemical station. The specificity of selected aptamers towards mycotoxins was also checked by both the developed methods by evaluating the cross reactivity with another closely related mycotoxin i.e. aflatoxin B1. The third part deals with the establishment of proof of binding of aptamer to AFM1 in milk matrix. For this purpose the methods established in part 1 and part 2 were used. Experiments were carried out in milk matrix as well as milk extracted samples. Results of all three parts are discussed here.

4.1 To develop aptamer based fluorescent assay for detection of aflatoxin M1.

Few selective aptamers and their truncated forms developed earlier in our laboratory (Malhotra et al., 2014) were chosen for this study. The secondary structure of these aptamers were predicted by Mfold software. The truncation of aptamer was done based on the retention of structural motif (as predicted by M fold) of original aptamer. The best binding conditions of AFM1 to aptamers and their binding constants which defines the affinity of aptamers towards target molecules were already studied by Malhotra et al., (2014). In this study, efforts were made to develop an alternative sensing system for the detection of AFM1 using the selected aptamers.

Aptamer are basically oligonucleotide sequences, so they have a tendency to bind with the oligonucleotide having counter sequences. If the counter oligonucleotide is present the aptamer will bind itself with the counter oligonucleotide. In our study, a fluorophore was attached to the AFM1 specific aptamer at 5' end. Fluorophore is a compound which generates fluorescence when exposed to certain wavelength. We have modified the counter oligonucleotide at 3' end with quencher. Quencher is a compound which will retard the

fluorescence of the fluorophore compound by binding with it. Therefore, in optimal condition when aptamer and counter oligonucleotide are incubated together the fluorescence will decrease due to quenching effect. However, in presence of target, it is hypothesized that aptamer will leave the counter oligonucleotide (as aptamer has high affinity for target) and as fluorophore and quencher will be separated, the fluorescence will increase with increasing concentration of target. In the present study, 6-carboxyfluorescein (FAM) and tetramethylrhodamine (TAMRA) were used as fluorophore and quencher, respectively. AFM1 specific aptamer was modified with FAM at 5' end while counter oligonucleotide was modified with TAMRA at 3' end. The sequence of truncated AFM1 specific aptamer (AFAS3-Tr Bs) and its counter oligonucleotide (AFAS3-Tr BsR) is presented in Table 3.1. The principle of the assay is presented in Figure 4.1.

For obtaining maximum quenching, the ratio of FAM tagged aptamer and TAMRA tagged oligonucleotide was optimized using different ratios of both the oligonucleotide. The results are presented in Figure 4.2 which indicate that maximum quenching was observed when the ration of FAM tagged aptamer (AFAS3-Tr Bs) to TAMRA tagged complementary oligonucleotide (AFAS3-Tr Bs R) was 1: 3. In subsequent experiments, this ratio was maintained. The incubation time was also optimized after addition of counter oligonucleotide for obtaining desirable quenching (Figure 4.3). After taking reading at different time intervals, 1 min was found to be most suitable time for incubation as maximum quenching was observed within this time.

After optimization, the experiment was carried out with different concentrations of AFM1 and it was found that the fluorescence increases gradually from 0 to 0.5 µM concentration of AFM1 (Figure 4.4). The average increase in fluorescence was from 5000 unit at 0 µM AFM1 concentration to above 10000 unit at 0.5 µM AFM1 concentration (Figure 4.5). The rise in fluorescence was gradual and significant suggesting that addition of AFM1 was able to separate the two strands of oligonucleotide. Further, it was observed that linearity was observed only in the range of 0 to 0.5 µM AFM1 concentration (Figure 4.4 and 4.5). Experiments were done up to 1.8 µM concentration of AFM1 (Figure 4.6) indicated poor linearity.

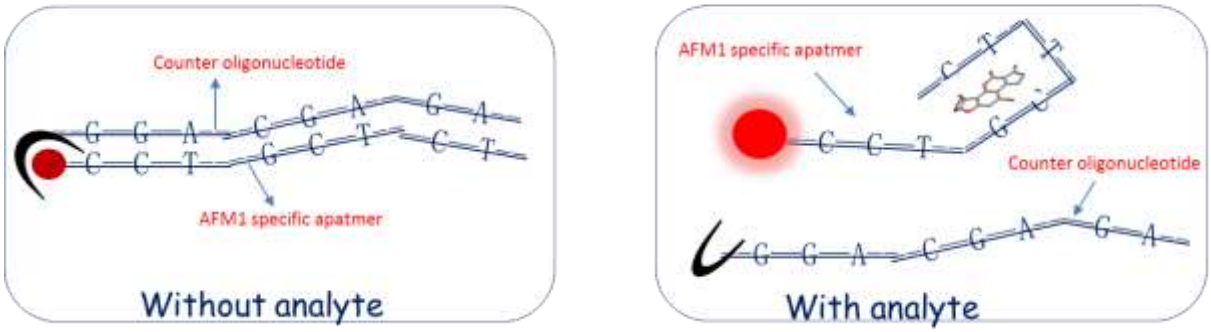

Figure 4.1: Principle used in visualization of aptamer to target binding by change in fluorescence by fluorophore tagged aptamer

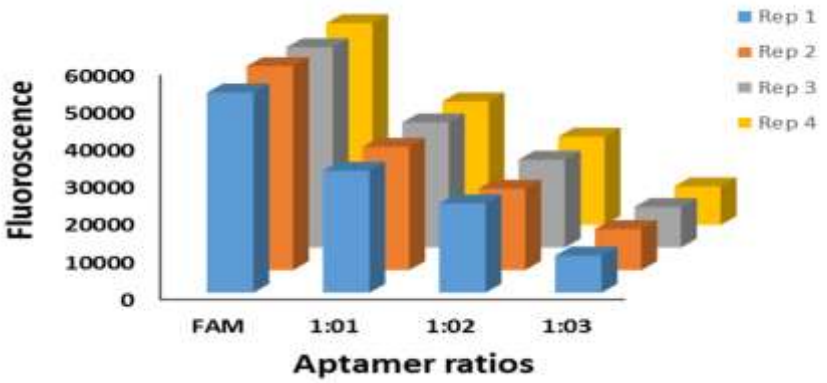

Figure 4.2: Effect of ratio of FAM tagged AFAS3-Tr Bs aptamer to TAMRA tagged complementary oligonucleotide AFAS3-Tr BsR on fluorescence

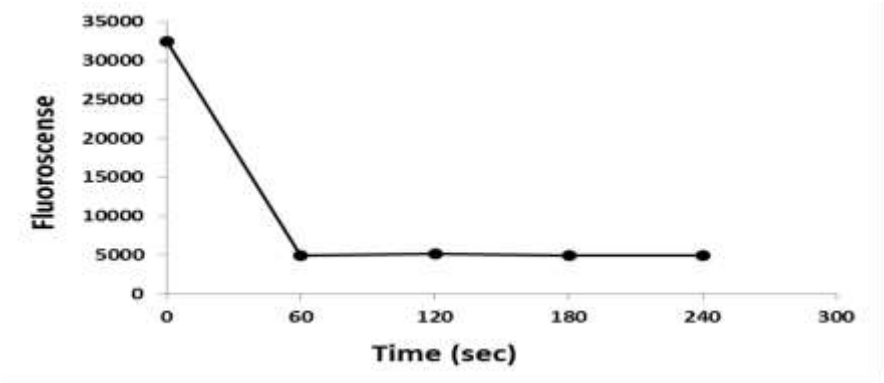

Figure 4.3: Effect of incubation time on quenching of fluorescence after mixing of FAM tagged AFAS3-Tr Bs aptamer and TAMRA tagged complementary oligonucleotide AFAS3-Tr BsR

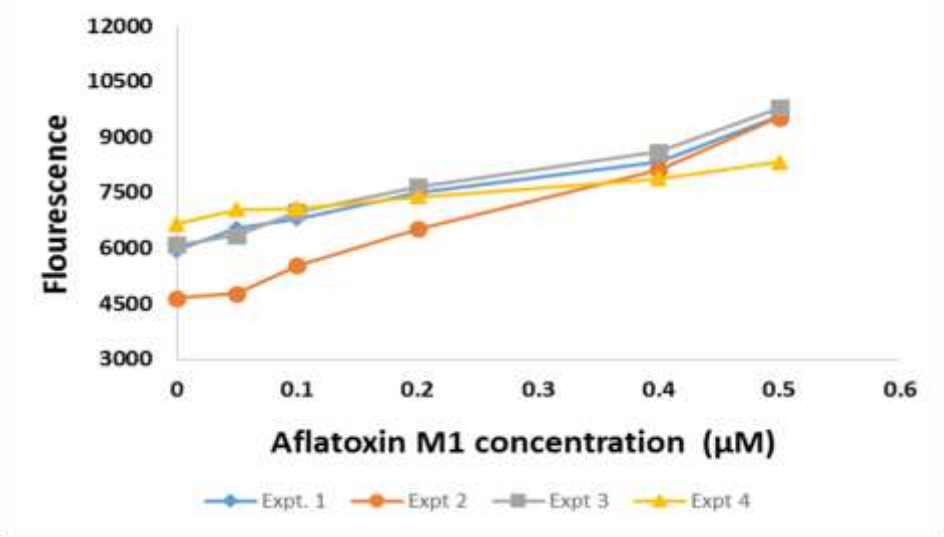

Figure 4.4: Effect of AFM1 concentration on increase in fluorescence after its addition to mixture of FAM tagged AFAS3-Tr Bs aptamer and TAMRA tagged complementary oligonucleotide AFAS3-Tr BsR. Results of five independent experiments are shown.

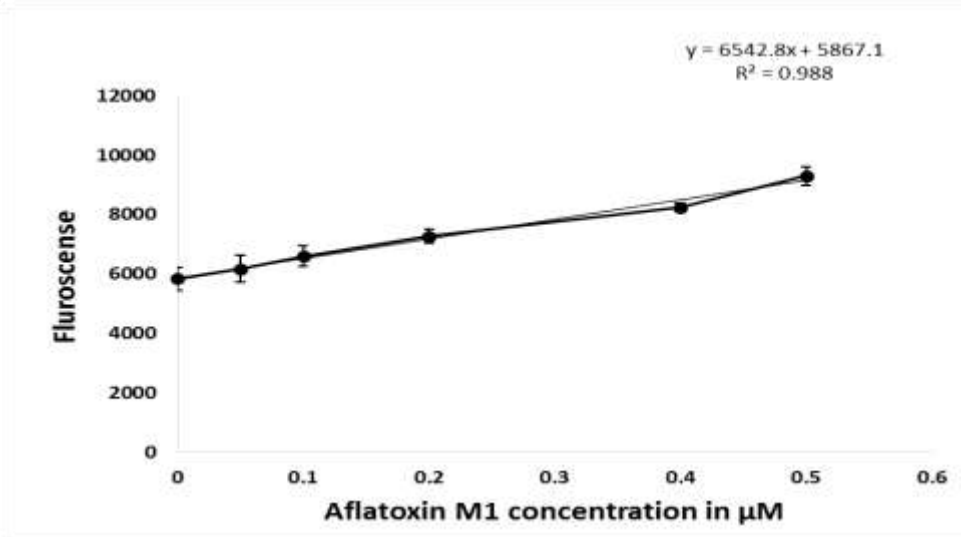

Figure 4.5: Effect of AFM1 concentration on increase in fluorescence (mean + SE) after its addition to mixture of FAM tagged AFAS3-Tr Bs aptamer and TAMRA tagged complementary oligonucleotide AFAS3-Tr BsR (n = 5).

In all the above experiments, the concentration of AFM1 specific aptamer (AFAS3-Tr Bs) used was 0.5 µM. To enhance the sensitivity of this method, 0.05 µM aptamer concentration was also tried but it resulted in either decrease or irregular quenching with increase in concentration of AFM1. Since desirable quenching was not obtained, further experiments were not done. It was also concluded that aptamer concentration should be 0.5 µM for this approach.

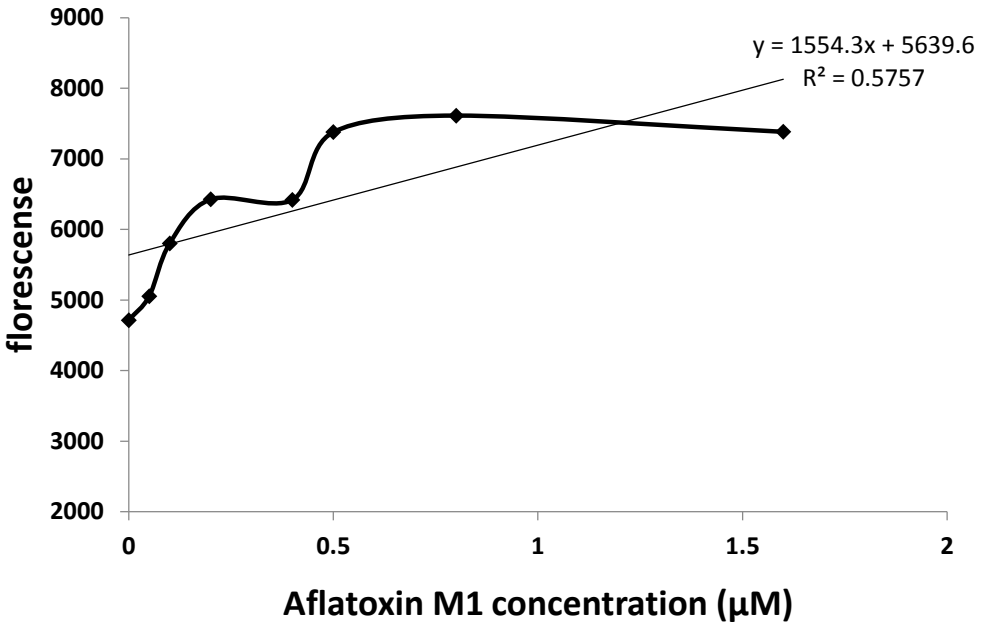

Figure 4.6: Effect of AFM1 concentration (0-1.8 µM) on fluorescence of FAM tagged AFAS3-tr in presence of TAMRA tagged complementary oligonucleotide AFAS3-Tr Bs R.

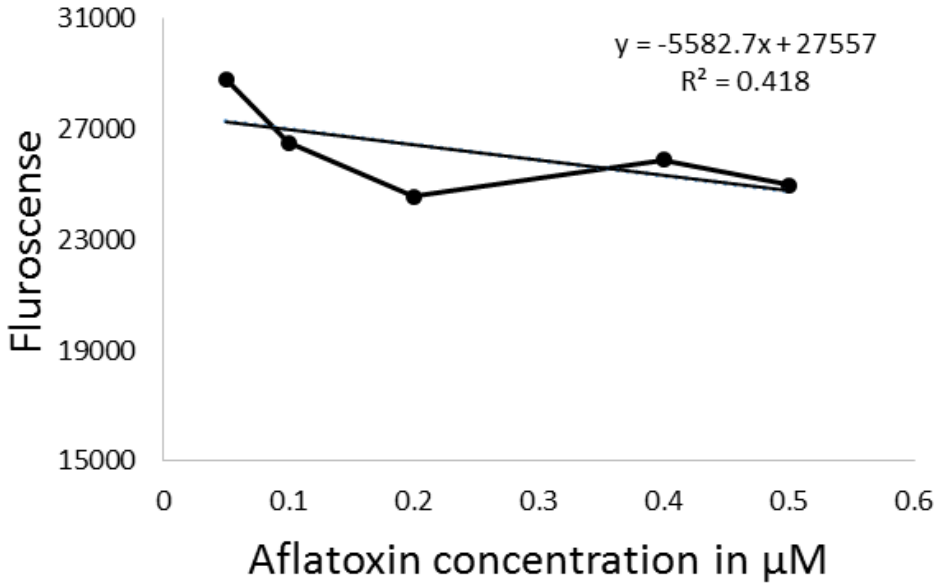

Figure 4.7: Effect of AFM1 concentration on fluorescence after its addition to mixture of FAM tagged AFAS3-Tr Bs aptamer (concentration 0.05 µM and TAMRA tagged complementary oligonucleotide AFAS3-Tr BsR (n = 2).

As this approach provided good results with AFM1, it was decided to check the cross reactivity with aflatoxin B1 (AFB1). AFB1 was used in the same concentration as that of AFM1 (0 to 0.5 µM) and results are presented in Figure 4.8. The results indicated that with increasing concentration of AFB1, there is no change in the fluorescence, thus indicating that developed system has no cross reactivity towards AFB1. Since there is minor difference in the structure of AFM1 and AFB1, the results of cross reactivity experiments indicated that (Figure 4.8) aptamer used in this approach (AFAS3-Tr) is specific to AFM1.

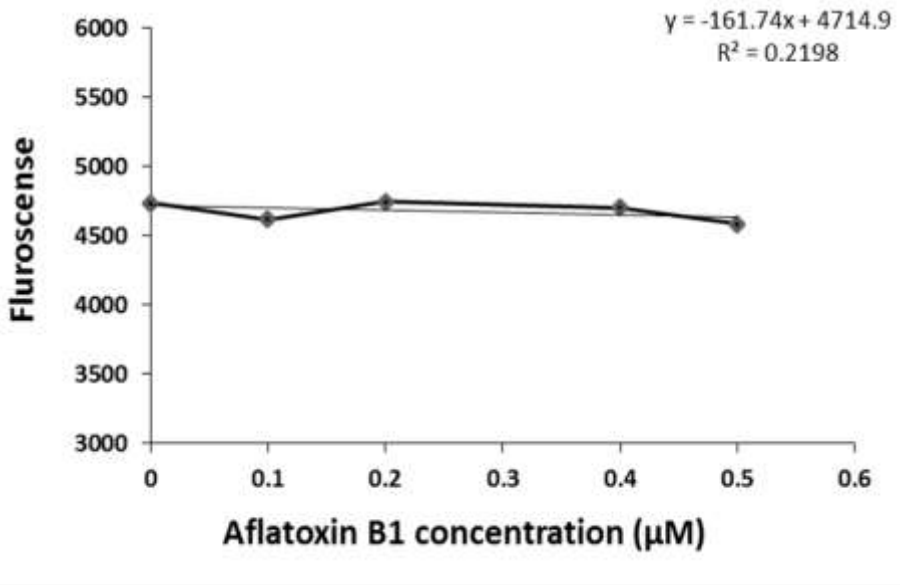

Figure 4.8: Effect of AFB1 concentration on fluorescence after its addition to mixture of FAM tagged AFAS3-Tr Bs aptamer and TAMRA tagged complementary oligonucleotide AFAS3-Tr BsR (n = 3).

4.2 Development of aptamer based DNAzyme assay for detection of AFM1.

Second approach used in fluorescent assay was of DNAzyme aptamer. Some sequences of oligonucleotides possesses enzymatic activities and the activity changes according to the changes in their structure (Jafari *et al*., 2017). Some DNAzyme have shown activity similar to horse radish peroxidase (HRP) i.e. causes oxidative changes in substrates like TMB, ABTS resulting in color changes (Yang *et al*., 2010). Complex formation of hemin and DNAzyme is necessary to show catalytic activity; hemin is an iron containing porphyrin that acts as a necessary cofactor for peroxidase activity of DNAzyme.

In present study, an oligonucleotide sequence which shows HRP activity was tagged to the AFM1 specific aptamer (Table 3.2). Total number of bases were 26 which include 8 bases of aptamer specific to AFM1 and 18 basis of DNAzyme. As mentioned earlier, the HRP activity depends on the structure of the DNAzyme and in presence of cognate target the aptamer would bind to the target resulting in changes in its 3 dimensional structure of DNAzyme which causes a quantifiable change in the catalytic activity of DNAzyme (Figure 4.9). The activity will either increase or decrease significantly compared to the initial value depending upon the concentration of target.

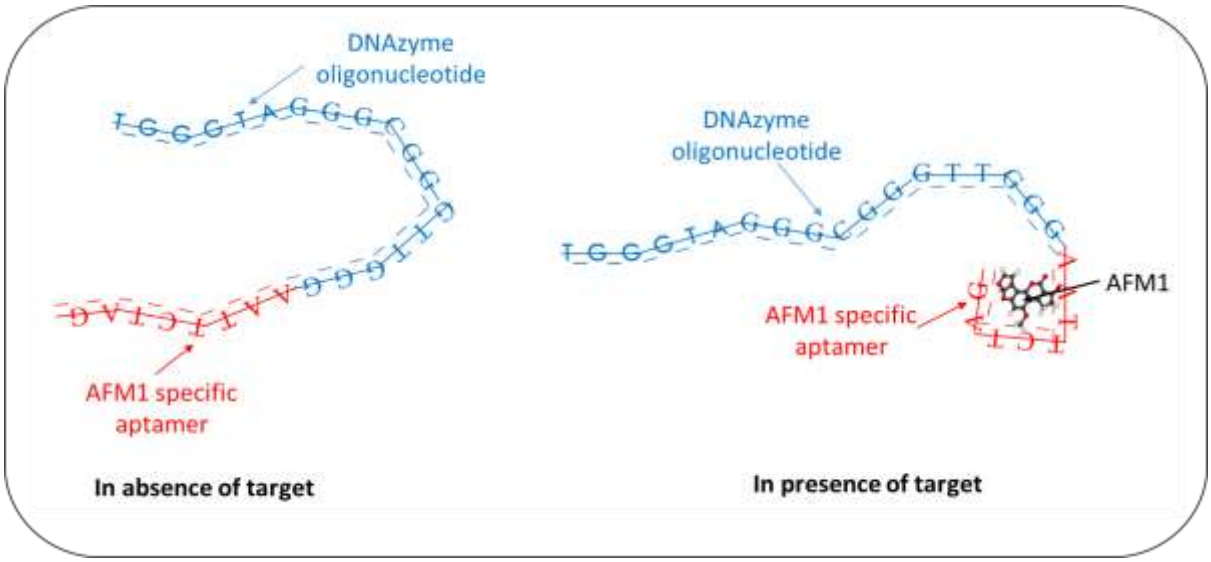

Figure 4.9: Effect of presence of cognate target on structure of aptamer tagged DNAzyme.

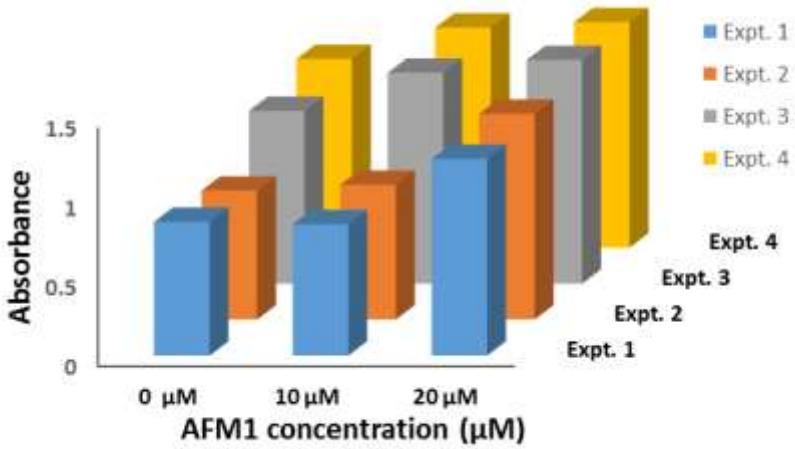

Figure 4.10: Effect on absorbance of DNAzyme aptamer at varying concentration of aflatoxin M1.

In the first step, the incubation with hemin was optimized at 30 min (Jafari *et al*., 2017) whereas incubation after addition of substrate *i.e* 3,3',5,5'-tetramethylbenzidine (TMB) was optimized to 8-10 min depending upon the development of blue color. After this incubation, the reaction was stopped by addition of dilute sulfuric acid. The plate was then read at 450 nm to measure the intensity of yellow color.

The color intensity of solution was observed to increase as compared to the intensity developed by aptamer conjugated DNAzyme only. This indicated that due to the binding of aptamer to AFM1, structural changes occurred in the DNAzyme due to which its enzyme activity increased. However it was noticed that there was variation in the initial DNAzyme activity causing variation from one experiment to other. Thus increase in the color intensity after addition of AFM1 is required to be calculated on the basis of initial intensity. The variations in initial activity of DNAzyme was may be due to slight variation in thermocycler treatment. The results of three experiments are shown in Figure 4.10 which indicates the increase in the color intensity when AFM1 was added at two different level. Figure 4.11 shows the average increase in intensity upon addition of AFM1 to DNAzyme bound aptamer with the coefficient of variation (R^2) = 0.958. Statistical studies shows that the increase in intensity was significant.

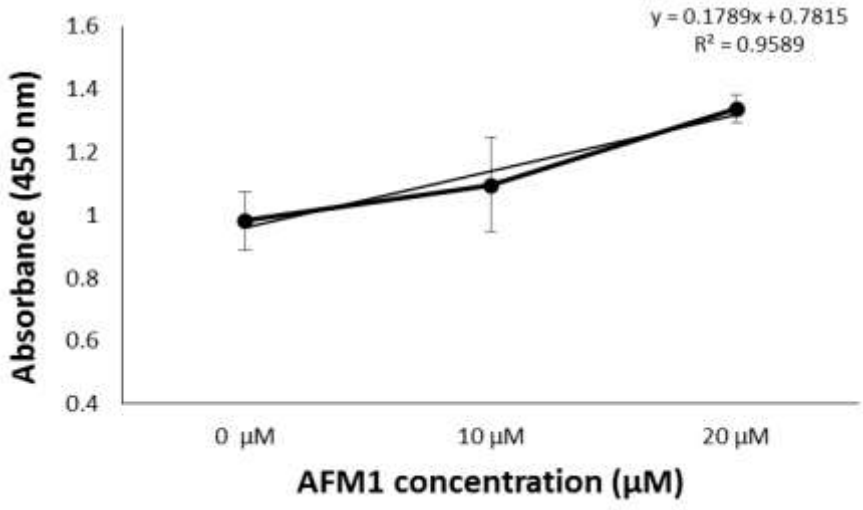

Figure 4.11: Effect of varying concentration AFM1 on absorbance (mean + SE) of DNAzyme aptamer.

The results presented in Figure 4.10 and 4.11 indicated that presence of target (AFM1) in DNAzyme bound aptamer has caused the structural changes in the DNAzyme as there was increase in DNAzyme activity. However, this change in activity was noticeable only at high concentration of AFM1. As such high concentration was not of practical importance, further experiments were not done.

4.3 Development of aptamer based electrochemical assay for the detection of AFM1.

Fluorescent based method for the detection of AFM1, though simple but lacks sensitivity. Hence the method can be used for establishing of proof of aptamer to its cognate target. However AFM1 is a class 1 carcinogen and show its carcinogenic effects even at very low concentration. Various regulatory bodies have given permissible level of AFM1 in milk and milk products, however, detection level of fluorescent assay was above the permissible level of AFM1 in milk. Hence there is need of an aptamer based method which is more sensitive and with a lower detection limit.

In our second objective, an attempt was made develop an electrochemical based assay for the detection of AFM1 using aptamers. Electrochemical assays can be done using rod electrodes as well as screen printed electrodes. In the present study, screen printed gold electrode (SPGE) were used for their higher sensitivity. SPGE essentially comprises of three electrodes: working electrode, counter electrode and reference electrode. In this approach, two AFM1 specific aptamers were used. For developing electrochemical based aptasensor, aptamer first has to be immobilized on the SPGE. The immobilization was done on working electrode of SPGE and it was done through a step wise reaction between the working gold electrode and different compounds such as dithidipropionic acid, ethanolamine and streptavidin (Figure 4.12). To immobilize the aptamer to streptavidin, AFM1 specific aptamer was modified with biotin and tetraethyleneglycol (TEG) at 5´-end of aptamer and 3´ end of AFAS3 aptamer. The modification was done at different ends of two aptamers by taking into consideration the active part of aptamers which has the potential to interact with the target. TEG was tagged as a spacer to avoid stearic hindrance during recognition.

Immobilization of various layers on SPGE such as dithiopropionic acid, streptavidin, ethanolamine and modified aptamer, the electrochemical response was checked by cyclic

voltammetry each time for ensuring proper layering over SPGE. Proper layering of each compound results in the subsequent lowering of peak current of electrode as shown in Figure 4.13

After the immobilization of aptamer over SPGE, different concentrations of AFM1 (0, 10, 100, 1000, 10000, 100000 ppt) were incubated over surface of working electrode and after each incubation, electrochemical response was taken using square wave voltammetry. The peak current of electrode was found to drop linearly with increasing concentration of AFM1 incubation. The decrease in peak current with increasing concentration of AFM1 was true for all four AFAS3 immobilized electrode and APM6-tr immobilized electrode. The decrease in peak current of AFAS3 and APM6-tr modified electrodes is shown in Figure 4.14 and 4.15, respectively. The data of AFAS3 modified 4 electrodes is plotted in Figure 4.16 and mean data of peak current drop of 4 electrodes is plotted in Figure 4.17.

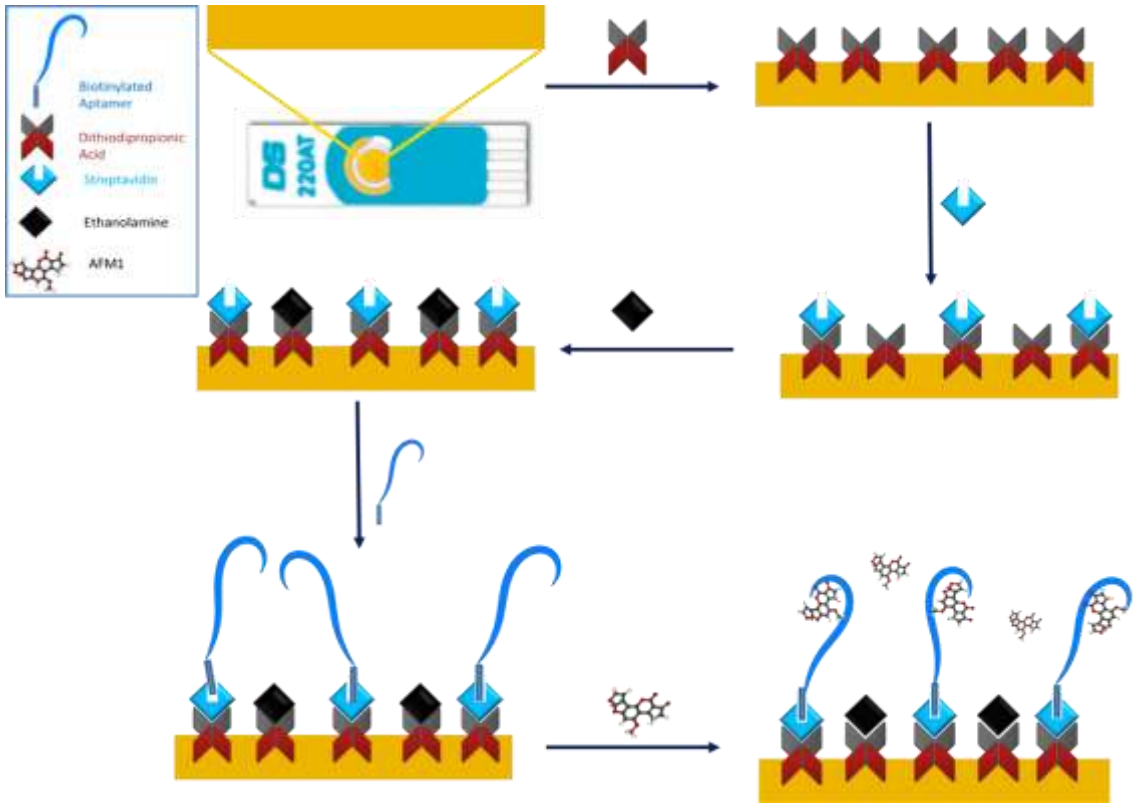

Figure 4.12: Different steps for immobilization of biotin modified aptamer over SPGE

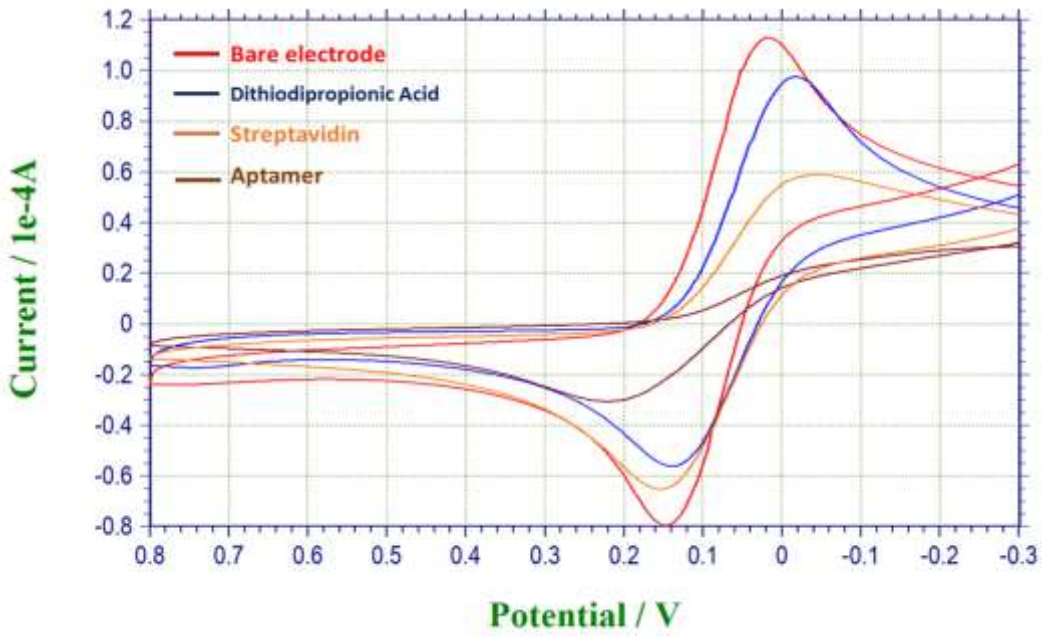

Figure 4.13: Cyclic voltammetric response of screen printed gold electrode at different steps of aptamer immobilization

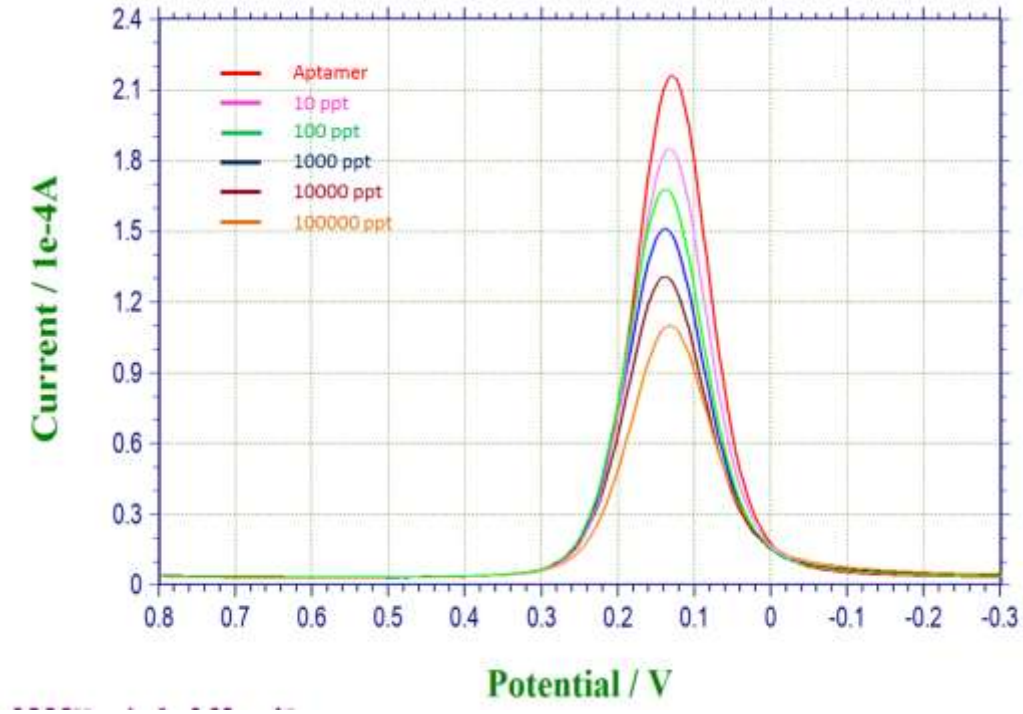

Figure 4.14: Square voltammetric response of AFAS3 immobilized screen printed gold electrode at different AFM1 concentrations.

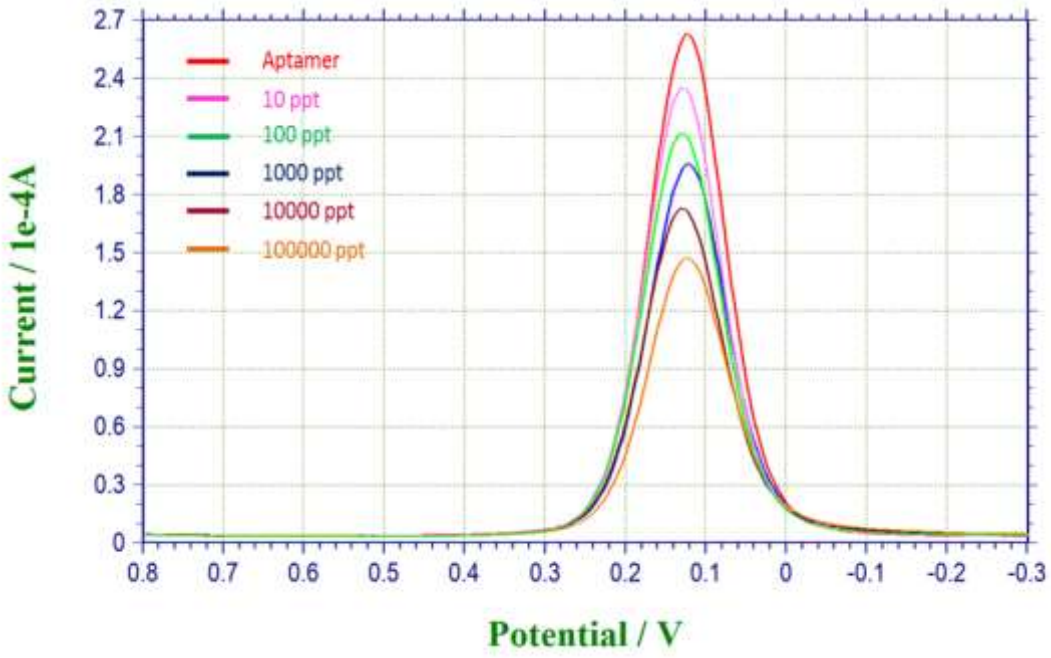

Figure 4.15: Square voltammetric response of APM6-tr immobilized screen printed gold electrode at different AFM1 concentrations

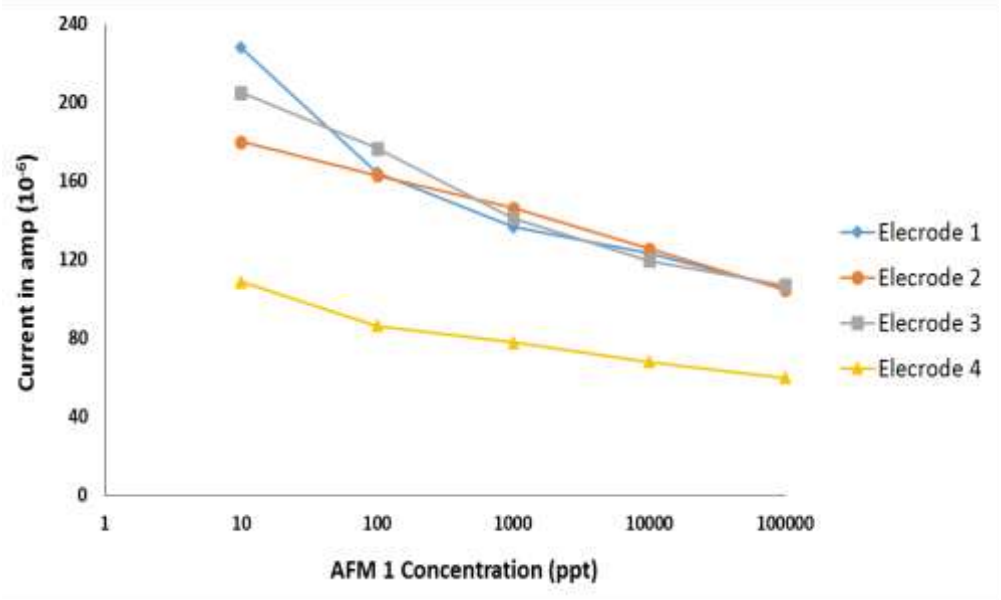

Figure 4.16: Effect of AFM1 concentration on peak current of immobilized screen printed gold electrode with AFAS3 aptamer.

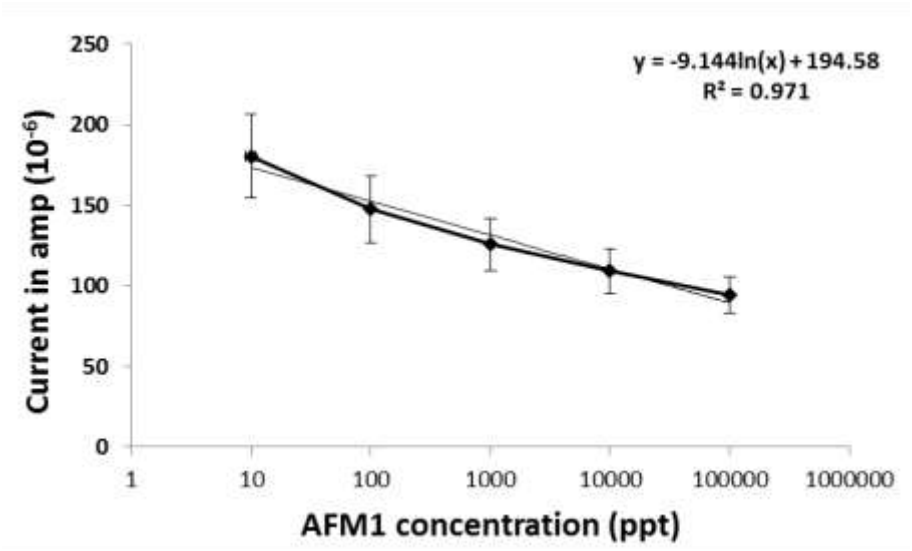

Figure 4.17: Effect of AFM1 concentration on mean peak current of immobilized screen printed gold electrode with AFAS3 aptamer. Mean has been calculated from peak current values of 4 independent screen printed gold electrodes

From the results of squares wave voltammetry (Figure 4.14 – 4.17) it can be concluded that the peak current drops linearly with increase in AFM1 concentration. When mean peak current value was calculated against logarithmic concentration of AFM1 it has shown R^2 = 0.971 (Figure: 9.17). Results of electrochemical experiments indicated that that initial peak current of each individual electrode was different. Each individual electrode behaves differently but it was noticed that decrease in peak current was same for given concentration of AFM1. Due to this, another approach of calculating the peak decrease in current in the form of percent current drop against the increasing concentration (logarithmic) of AFM1 was used. This approach showed that even if the initial peak current of individual electrode was different, the current drop in percentage after each subsequent incubation of different electrodes tends to be the same (Figure 4.18). Mean percent drop on current is plotted (figure 4.19) and it showed good linearity and value of coefficient of variation (R^2) = 0.971. Thus this approach provided satisfactory results. The peak current drop and percent current drop of APM6-tr immobilized electrode have shown similar results as AFAS3 immobilized electrode and are shown plotted in Figure 4.20 and 4.21.

The aptamer AFAS3 have shown excellent results in identifying AFM1 so we were encouraged to check its cross reactivity with other mycotoxins. For checking its specificity towards AFM1, AFB1 was chosen. The results of comparison between peak current drop and

percent current drop of AFM1 and AFB1 treated electrode is showed in Figure 4.22 and 4.23, respectively. The results indicated that AFAS3 showed no cross reactivity at low concentration however at high concentration (10^4 ppt), slight cross reactivity was noticed in peak current drop, however the value in terms of percent current drop compared with same concentration of AFM1 was negligible. These experiments proved that the aptamer AFAS3 has shown very less cross reactivity.

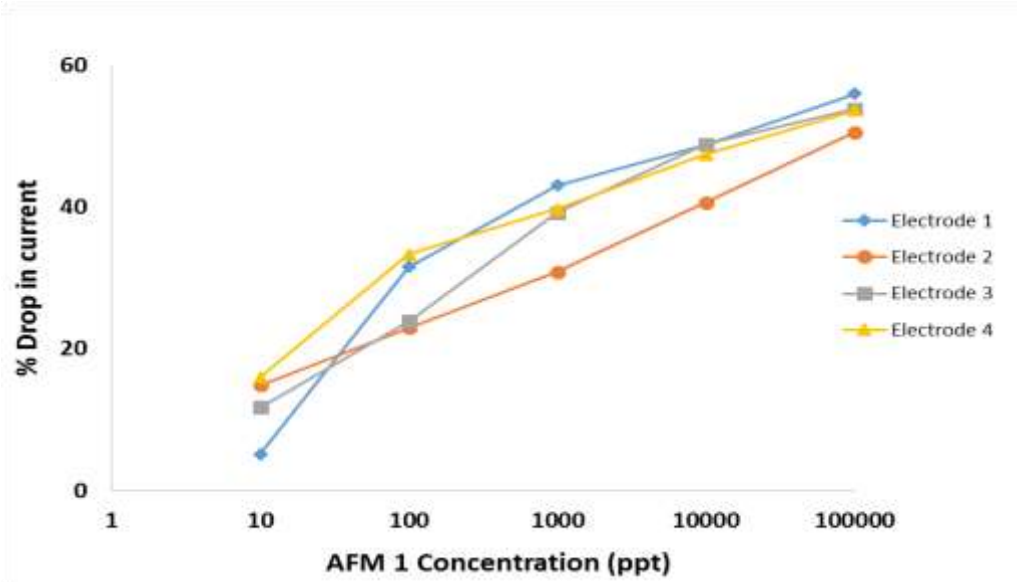

Figure 4.18: Effect of AFM1 concentration on percent drop in current of immobilized screen printed gold electrode with AFAS3 aptamer. Experiment was performed on 4 different electrodes.

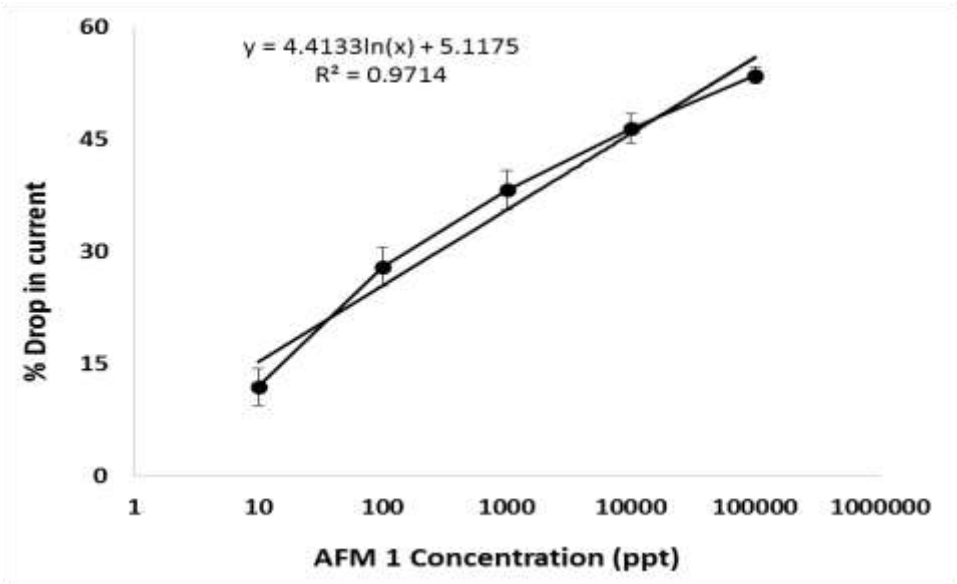

Figure 4.19: Effect of AFM1 concentration on mean percent drop in current of immobilized screen printed gold electrode with AFAS3 aptamer. Mean % drop in current has calculated from % current values of 4 independent electrodes

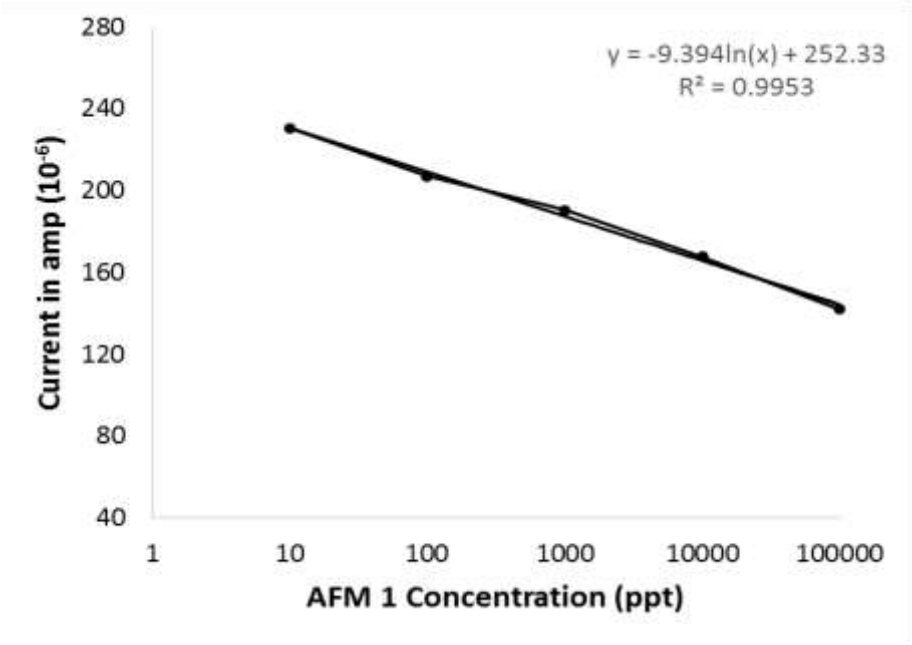

Figure 4.20: Effect of AFM1 concentration on peak current of immobilized screen printed gold electrode with APM6-tr aptamer.

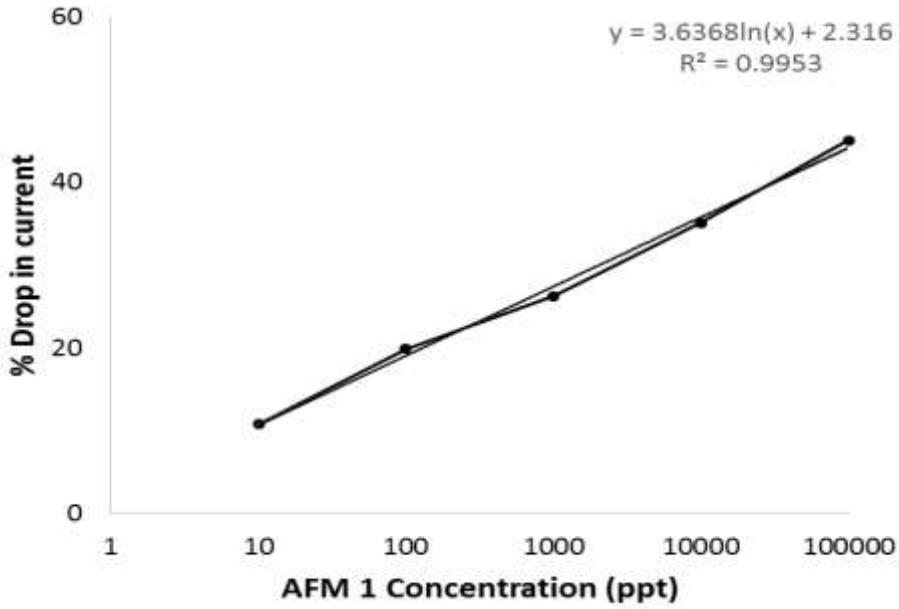

Figure 4.21: Effect of AFM1 concentration on percent drop in current of immobilized screen printed gold electrode with APM6-tr aptamer.

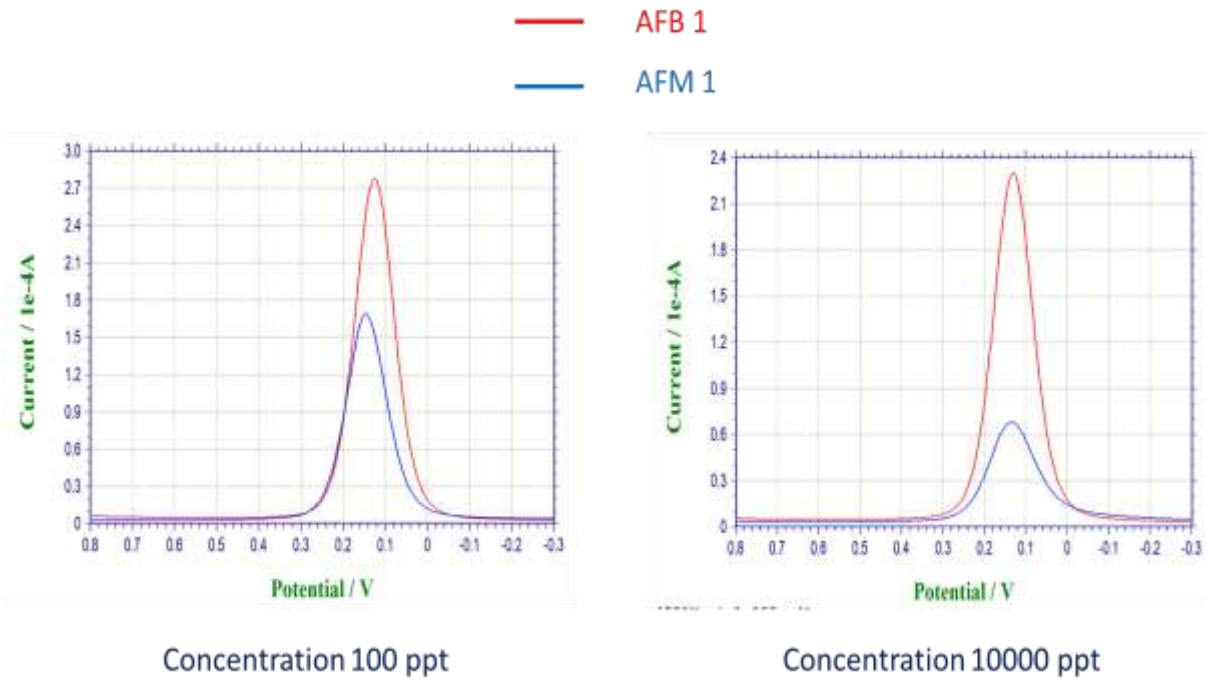

Figure 4.22: Square wave voltammetric response of AFAS3 immobilized electrodes using same concentration of AFM1 and AFB1

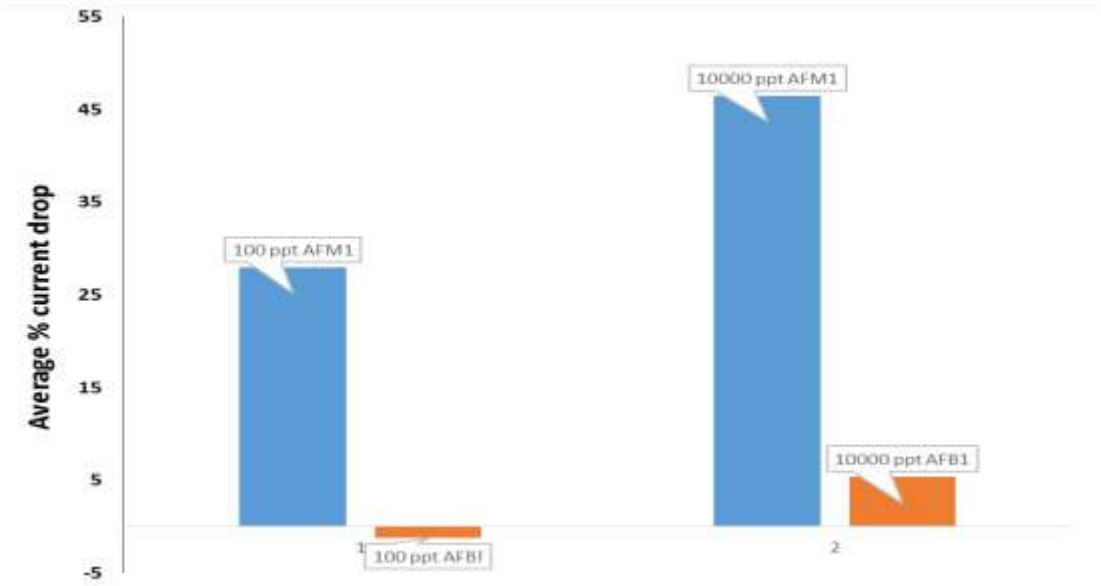

Figure 4.23: Effect of AFM1 and AFB1 concentration on percent drop in current of immobilized screen printed gold electrode with AFAS3 aptamer.

4.4 Establishment of proof of binding of aptamer in milk matrix.

We have got good results from both fluorescent assay and electrochemical assay. The results established that the aptamer binds to AFM1 in buffer matrix and the binding can be visualized through fluorescent assay as well as electrochemical assay. This encouraged us to prove the binding of aptamer to AFM1 in milk matrix. For this purpose several approaches were used using fluorophore as well as electrochemical assay we omitted DNAzyme assay for this approach due to its lack of sensitivity. Various experiments are conducted which are discussed in this part.

4.4.1 Detection of AFM1 in milk matrix using aptamer based fluorescent assay

For fluorescent approach using AFAS3-Tr Bs and counter oligonucleotide AFAS3-Tr Bs R milk sample were spiked with AFM1 and the experiments were carried out as optimized protocol of fluorescent assay. Results showed that the milk matrix interfered with the fluorescence and the fluorescence was dropped by a significant level compared to buffer.

To overcome the drop in fluorescence milk sample was heavily spiked with AFM1 then diluted with buffer and then experiments are carried out. As shown in figure 4.24 the results interpret that even after dilution of milk sample with buffer still the interference of the milk matrix was not totally nullified. Dilution didn't bought the fluorescence up to desired level. Still experiments were carried out and the results (Figure 4.25) shows that there is no significant increase in the fluorescence as the concentration of AFM1 increases in milk. Hence there was need to eliminate the interference caused by the milk matrix

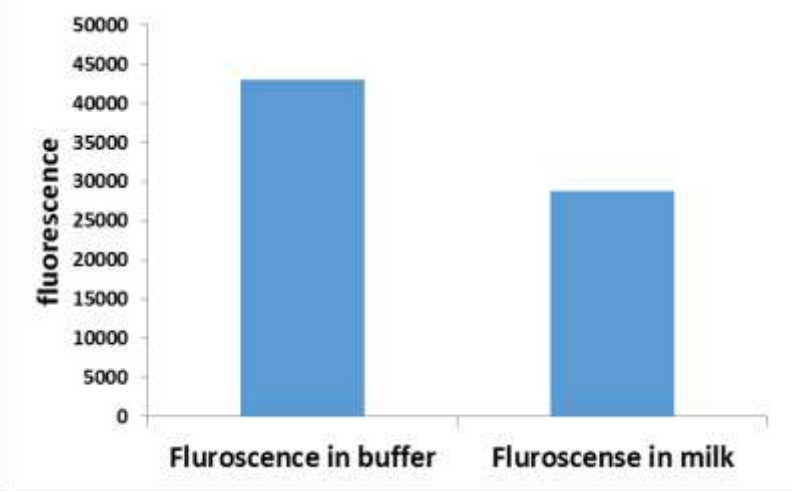

Figure 4.24: Comparison of fluorescence of FAM tagged AFAS3-Tr Bs aptamer in buffer and in milk matrix

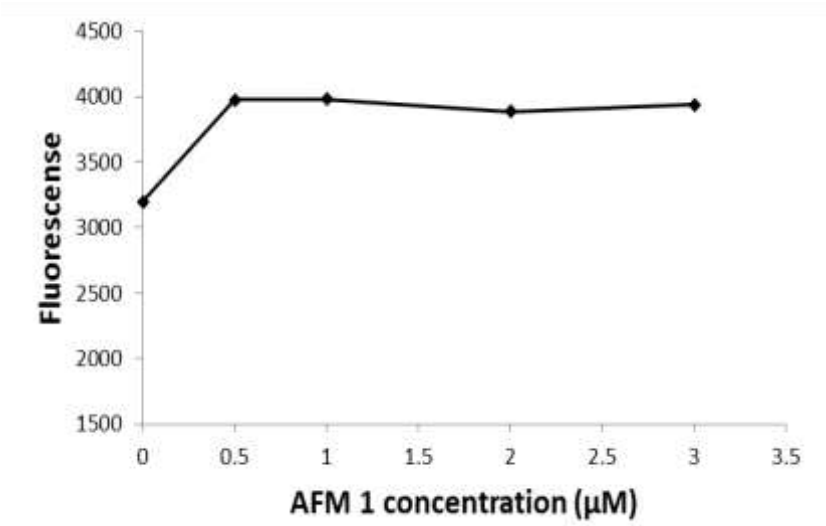

Figure 4.25: :Effect of AFM1 concentration on fluorescence of FAM tagged AFAS3-Tr Bs in presence of TAMRA tagged complementary oligonucleotide AFAS3-Tr Bs R in milk matrix

Since the fluorescence produced by the fluorophore tagged aptamer was not fully recovered in the milk matrix, so we tried another approach of extracting the AFM1 from the spiked milk sample itself with the help of chloroform and then evaporate the solvent and making up the volume with buffer. Then the same protocol developed for fluorescent based assay was followed to check the increase in fluorescence with increasing AFM1 concentration. The results shown in figure 4.26 indicates that with this approach the fluorescent of the FAM tagged aptamer was regained in the extracted milk matrix. On carrying out experiments with varying concentration of AFM1 results showed that the quenching by the counter oligonucleotide was also got interference by the milk matrix since the quenching effect after incubation of TAMRA tagged oligonucleotide is decreased by a significant level. Effect of varying concentration of AFM1 on fluorescence when checked doesn't show any significant difference as shown in figure 4.27. In this approach even if there is binding of aptamer to the cognate target is occurring then also its visualization is not possible because milk matrix is interfering with fluorescence and quenching properties of tagged molecules and this assay is based upon the fluorescence change in the presence of target molecule

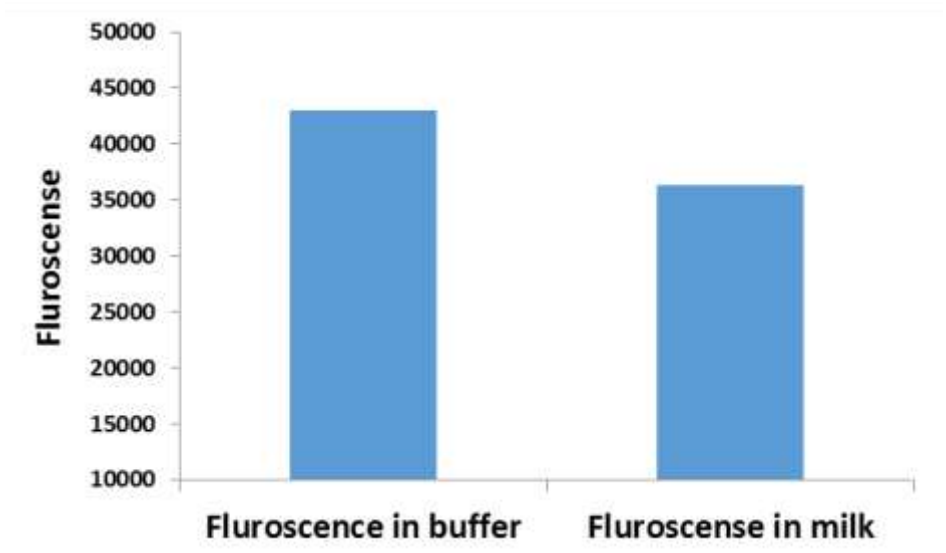

Figure 4.26: Comparison of fluorescence of FAM tagged AFAS3-Tr Bs aptamer in buffer and in milk which was chloroform extracted, evaporated and then bought in buffer

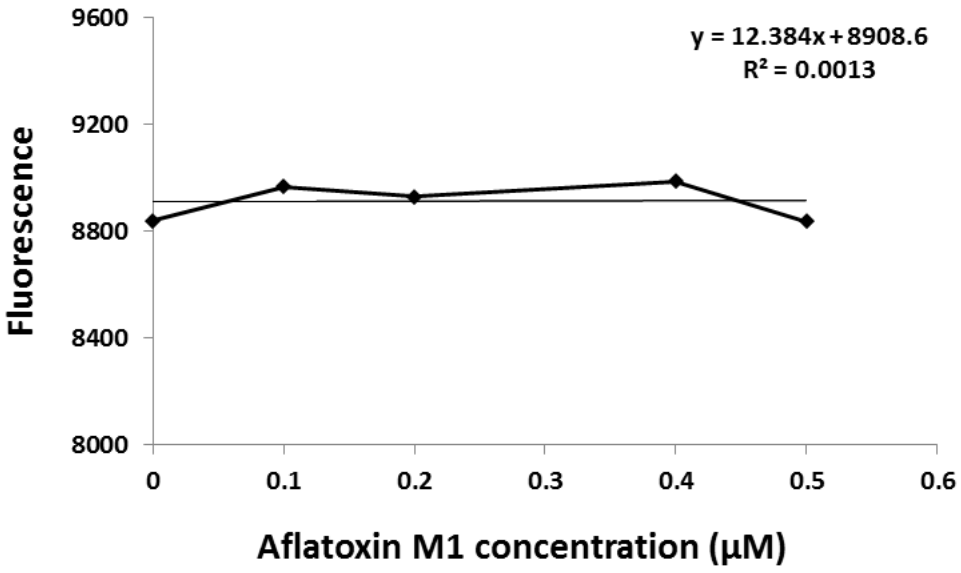

Figure 4.27: Effect of AFM1 concentration on fluorescence of FAM tagged AFAS3-Tr Bs in presence of TAMRA tagged complementary oligonucleotide AFAS3-Tr Bs R in milk which was chloroform extracted, evaporated and then bought in buffer

4.4.2 Detection of AFM1 in milk matrix using aptamer based electrochemical assay

Electrochemical assay have shown excellent results in buffer. This assay have given a significant response even at a small concentration of AFM1 such as 10 ppt. The linearity obtained in buffer was excellent and this draws our attention to try this assay on milk matrix. The aptamer immobilization over SPGE which is major part of this assay includes of layering over SPGE. One of this layering was of streptavidin, since there were chances of interference from biotin present in milk in the immobilization procedure we preferred extraction of AFM1 from milk sample after spiking and then carrying out the experiments as in objective 2. The effects of incubation with different concentration of AFM1 (extracted from spiked milk sample) on the peak current value of modified electrodes immobilized with AFAS3 and APM6-tr aptamers are discussed in this part.

Five electrodes were modified with AFAS3 and then incubated with different concentration of AFM1 extracted from spiked milk. After each incubation square wave voltammetric response was taken. The results of peak current drop are as shown in figure 4.28. The peak current drop of five different electrodes is shown in figure 4.29. The mean peak current drop of five different electrodes is shown in figure 4.30.

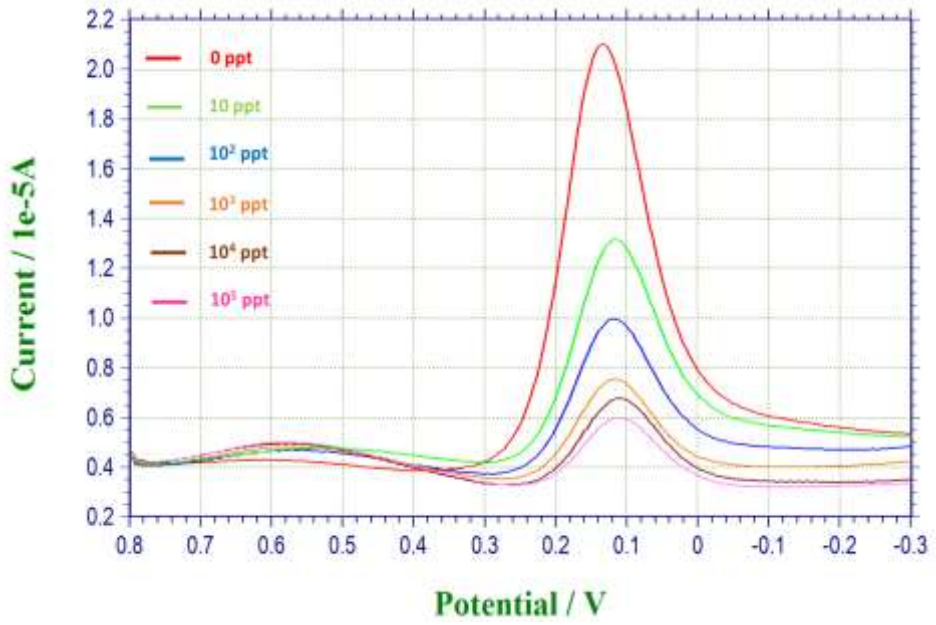

Figure 4.28: Square voltammetric response of AFAS3 immobilized screen printed gold electrode at different AFM1 concentrations extracted from milk sample

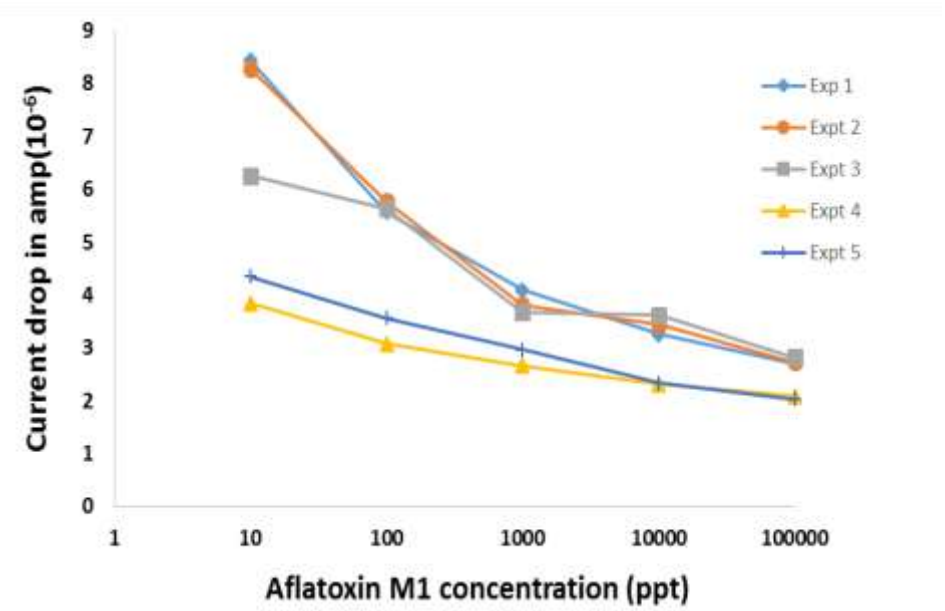

Figure 4.29: Effect of AFM1 concentration extracted from spiked milk sample on peak current of immobilized screen printed gold electrode with AFAS3 aptamer. Experiment was carried out on 5 independent screen printed gold electrodes

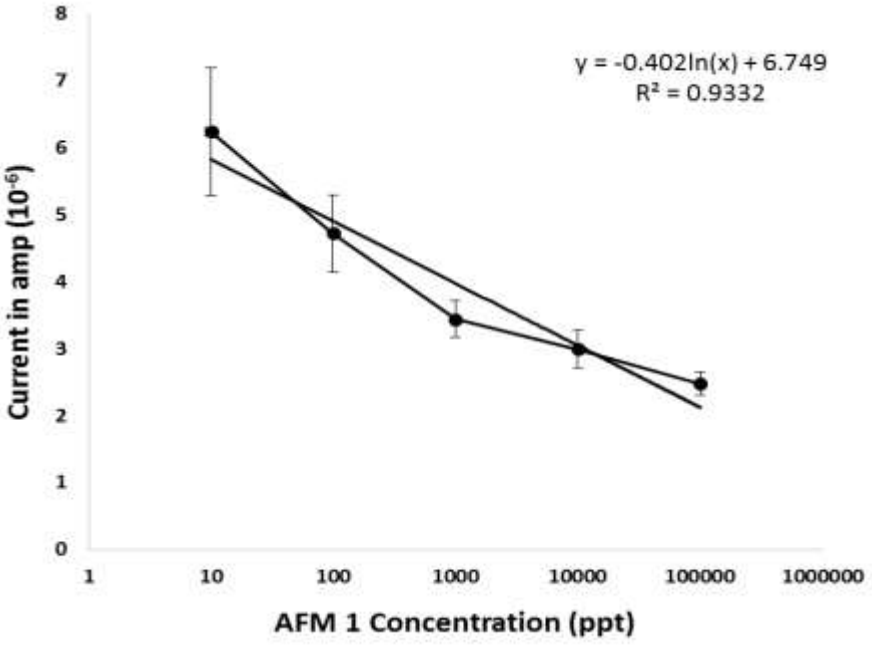

Figure 4.30: Effect of AFM1 concentration extracted from spiked milk sample on mean peak current of immobilized screen printed gold electrodes with AFAS3 aptamer. Experiment was carried out on 5 independent screen printed gold electrodes

From the figures it can be concluded that the peak current drops linearly with increase in concentration as in case of buffer. The linearity in drop of current can be seen, however values of peak current are different for each individual electrode. When mean peak current drop of 5 individual electrodes were taken, the trend line was linear and it showed coefficient of variation (R^2) = 0.933. Hence from this approach it can be concluded that the aptamer can bind to AFM1 extracted from milk matrix and its visualization can also be done using this technique. However as mentioned earlier the initial peak response of each electrode varies as every electrode is individual. Hence as in case of buffer we have also used approach of measuring percent current drop of individual electrode since this approach have provided better results in case of buffer. Percent current drop of five individual electrode is shown in figure 4.31. From figure 4.31 we can conclude that even if the individual electrode is showing variations in the peak current at a particular concentration of AFM1 the percent drop is almost same and more linearity can be seen using this approach. The mean of percent current drop in five electrodes is shown in figure 4.32. The mean is calculated from the percent drop in current of five individual electrode. The results have shown good linearity and the coefficient of variation (R^2) = 0.932.

Same approach was used using another AFM1 specific aptamer (APM6-Tr) immobilized electrode and good results were obtained. The peak drop in current after incubation of each subsequent concentration of AFM1 extracted from milk is shown in figure 4.33. The current drop of APM6-tr immobilized electrode was plotted in figure 4.34. It have shown good linearity and as in case of AFAS3 immobilized electrode another approach for correlating the change in current v/s AFM1 concentration i.e. percent drop in current was also tried for APM6-tr immobilized electrode and the similar results were obtained as in case of AFAS3 immobilized electrode showing good linearity (figure 4.35)

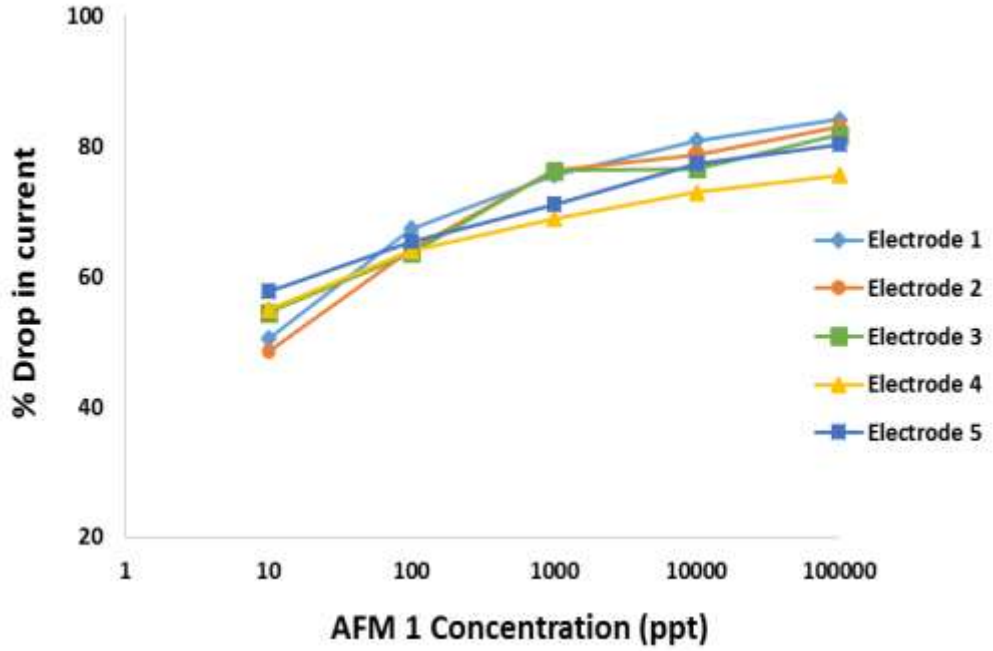

Figure 4.31: Effect of AFM1 concentration extracted from milk sample on percent drop in current of immobilized screen printed gold electrode with AFAS3 aptamer. Experiment was performed on 5 different electrodes

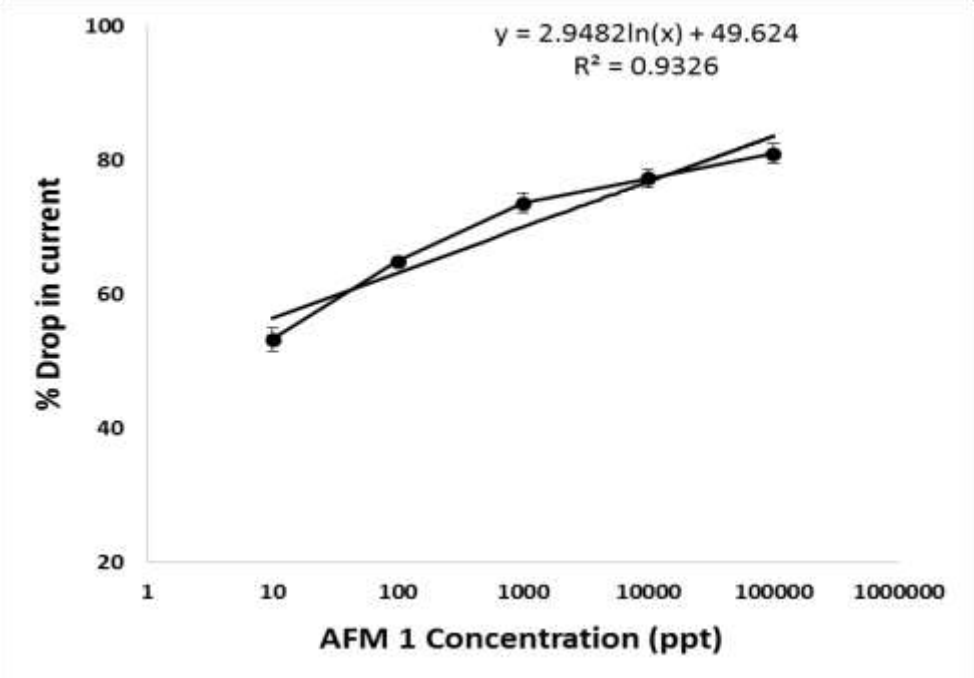

Figure 4.32: Effect of AFM1 concentration extracted from milk sample on mean percent drop in current of immobilized screen printed gold electrode with AFAS3 aptamer. Mean % drop in current has calculated from percent current values of 5 independent electrodes

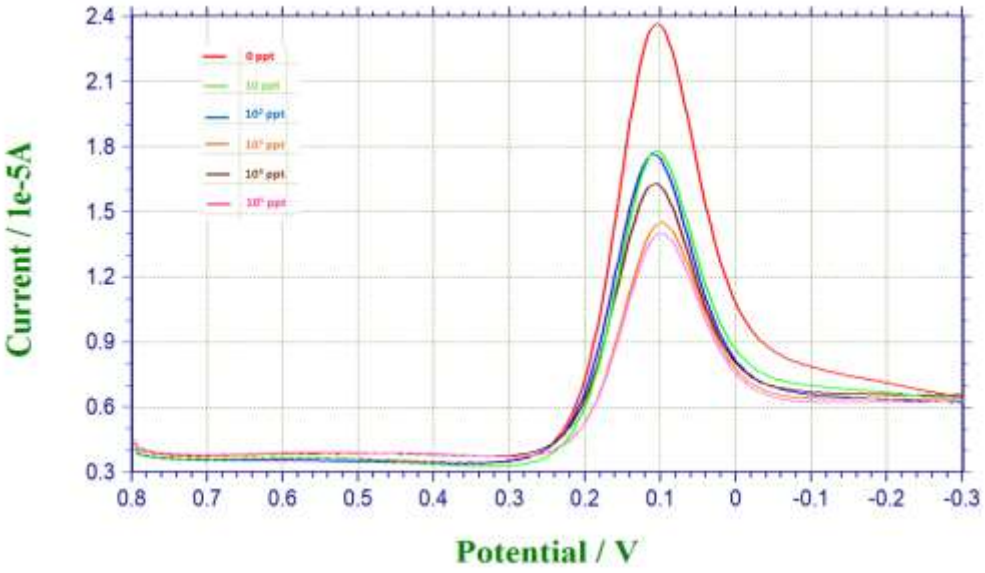

Figure 4.33: Square voltammetric response of APM6-tr immobilized screen printed gold electrode at different AFM1 concentrations extracted from milk sample

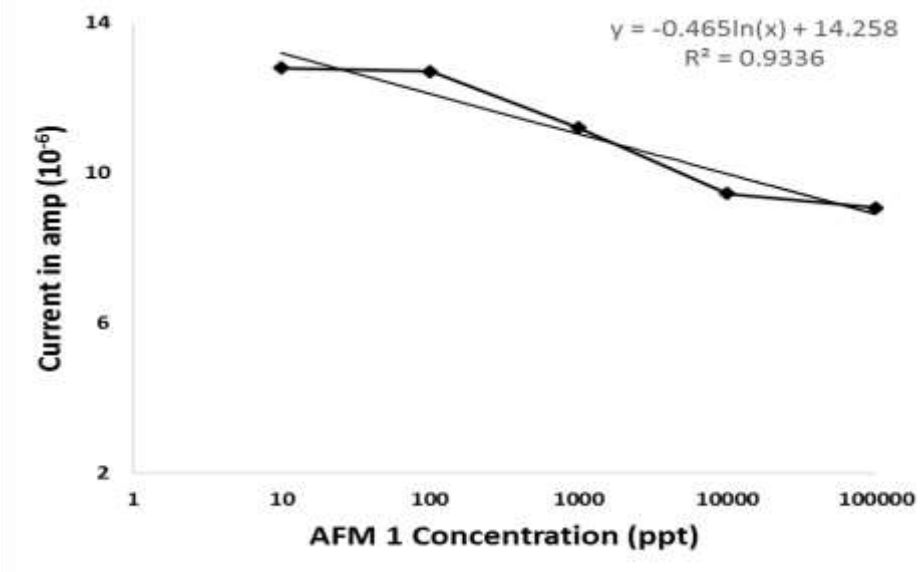

Figure 4.34: Effect of AFM1 concentration extracted from spiked milk sample on peak current of immobilized screen printed gold electrode with APM6-tr aptamer.

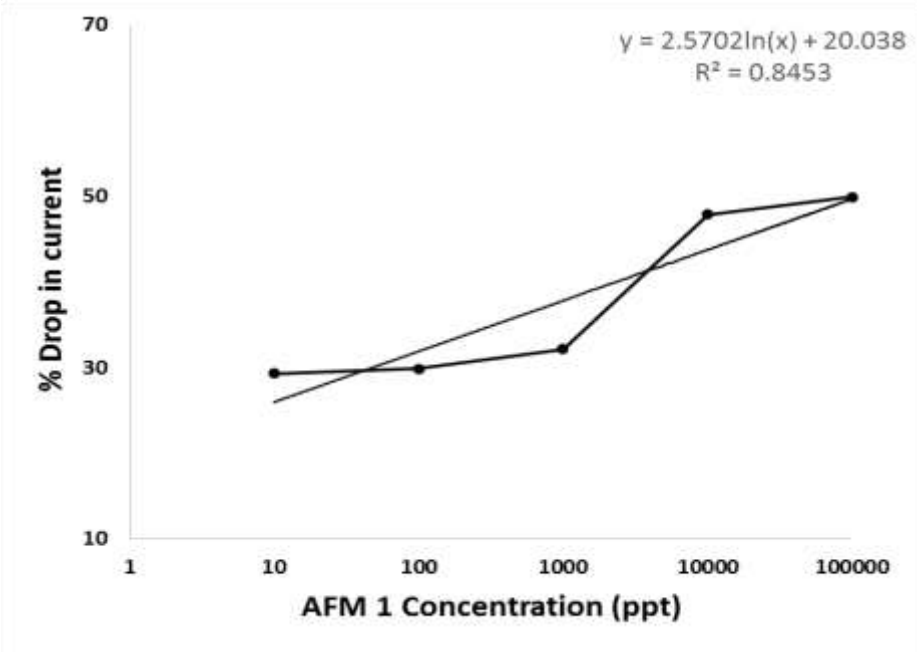

Figure 4.35: Effect of AFM1 concentration extracted from milk sample on mean percent drop in current of immobilized screen printed gold electrode with AFAS3 aptamer.

4.5 Validation using ROSA assay

Both spiked milk and extracted milk samples were analyzed using ROSA for checking the recovery of extraction method and for validation of electrochemical workstation approach. Milk sample spiked with 1000 ppt concentration of AFM1 was first analyzed in electrochemical work station and then diluted so that it will fit in range of detection limit of ROSA. The results have shown good recovery of AFM1 through the extraction protocol and electrochemical assay was validated.

Figure 4.44 : Comparison between ROSA assay between spiked milk sample and extracted milk sample

CHAPTER – 5

SUMMARY AND CONCLUSION

SUMMARY & CONCLUSION

Aflatoxin M1 (AFM1) is a hydroxylated derivative of aflatoxin B1 (AFB1). It is known as a potent carcinogen. Due to its toxicity, permissible level of AFM1 in milk is very low *i.e.* 500 ng/L. Various detection techniques are available for AFM1, but all of them have their disadvantages. AOAC approved method for detection of AFM1 is HPLC based and is expensive. Hence there is need of rapid and robust method for detection of AFM1. ELISA based assay are also used for detection of AFM1 which uses antibodies as a capturing agents. Aptamers are ssDNA or RNA molecule having strong affinity towards their cognate target and are generated through a complex procedure called SELEX. Generation of aptamer is a cumbersome process, but once generated they have wide range of applications. Aptamers have shown various advantages over antibodies and have potential to replace antibodies in target capturing assays. Further aptamers can be immobilized over different surfaces to develop aptasensing assays. In this dissertation one full length aptamer, its truncated form and another truncated aptamer having specificity towards AFM1 which are already developed by previous students were used to develop fluorescent assay and electrochemical assay. Their binding to AFM1 in milk matrix was checked and cross reactivity with AFB1 was also checked. The results of these experiments have been summarized under following subheadings.

Development of aptamer based fluorescent assay for the detection of AFM1

- Truncated version of AFAS3 aptamers (AFAS3-tr8 and AFAS3-Tr-Bs) were used for the study.
- Several modifications were done on aptamers. AFAS3-tr8 was tagged with DNAzyme at 5'-end. Whereas AFAS3-Tr-Bs was modified with FAM at 5'-end and its counter oligonucleotide was modified with TAMRA at 3'-end.
- Two approaches were used for developing aptamer based fluorescent assay. In First approach oligonucleotide showing enzymatic activity (DNAzyme) was tagged to AFM1 specific aptamer. In second approach fluorophore (FAM) tagged to AFM1 specific aptamer and quencher tagged to counter oligonucleotide were used.
- DNAzyme approach gave results but at a very high AFM1 concentration. Hence this approach can be used for visualization of AFM1 to aptamer binding but it is of no practical use since permissible limit of AFM1 is far below the range of this assay.

- Interaction of fluorophore (FAM) modified aptamer (AFAS3-tr8) with quencher (TAMRA) modified counter strand in absence and presence of target provided meaning results. There was significant increase in fluorescence as the concentration of the target gradually increases.
- Linear increase in fluorescence was observed in tris buffer over concentration range of 0 – 0.5 µM. It was also found that aptamer concentration should be minimum 0.5 µM for this assay to work since lower concentration have declining effect over fluorescence of FAM tagged aptamer.
- Attempts were also made to check cross reactivity of AFAS3-tr8 aptamer with AFB1. Since no increase in fluorescence occurred as concentration of AFB1 increased hence it can be interpreted that the aptamer is specific to AFM1 and does not show any cross reactivity.

Development of aptamer based electrochemical assay for detection of aflatoxin M1

- For this purpose, Screen Printed Gold Electrode (SPGE) was used which comprises of working gold electrode, counter gold electrode and silver reference electrode. This assay required immobilization of modified aptamers over working electrode of SPGE. Two aptamers were used; full version of aptamer AFAS3 and truncated aptamer APM6-tr. These aptamers were modified with TEG-Biotin at 3' and 5' end, respectively. Modifications in aptamers were done to felicitate there immobilizing on SPGE.
- The immobilization was achieved through sequential layering of dithidipropionic acid, streptavidin and aptamer. Biotin tagged aptamer attaches itself to streptavidin which in turn is attached with the gold surface. The immobilization step was monitored by taking cyclic electrochemical response of the electrodes after each step of layering. The peak current of the electrode drops as the layering of dithiodipropionic acid, streptavidin and aptamer progresses. The current drop suggested the layering of three layers on electrode was properly done.
- After immobilization of aptamers, different concentrations of AFM1 ranging from 10-100000 ppt were incubated over working electrode. After each subsequent incubation, electrochemical response was taken using square wave voltammetry. The results indicated that the peak current of the electrode was inversely proportional to the logarithmic concentration of AFM1. Better linearity was obtained when the results

were calculated as percent current drop. The coefficient of variation (R^2) = 0.971 indicates that the sensor can be used for AFM1 estimation.

- Cross reactivity of AFAS3 aptamer was also checked using electrochemical assay. Same concentrations of AFB1 were used for checking cross reactivity. Negligible drop in peak current of electrode suggests no cross reactivity occurred at low concentration, however at high concentration slight cross reactivity was observed.

Establishment of proof of binding of aptamer to aflatoxin M1 in milk matrix.

- To check binding of AFM1 to aptamer in milk matrix, interaction of fluorophore (FAM) modified aptamer (AFAS3-tr8) with quencher (TAMRA) modified counter oligonucleotide approach and electrochemical approach were used.
- When AFM1 spiked milk sample was as such used in fluorescent assay the fluorescence of FAM tagged aptamer decreased significantly. Even in the diluted sample of milk, the fluorescence was hampered significantly. Results showed that the fluorophore assay does not work with milk probably due to unknown interference from milk matrix.
- AFM1 was extracted from spiked milk using chloroform and finally AFM1 was brought in buffer.
- Electrochemical response of SPGE immobilized with AFAS3 was monitored using square wave voltammetry at various concentration of AFM1. The results indicated that the peak current is inversely related to the logarithmic concentration of AFM1 present in milk. The method is capable of measuring AFM1 in milk in the range of 0 to 10^5 ppt.
- The results were validated using Rosa Reader assay.

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