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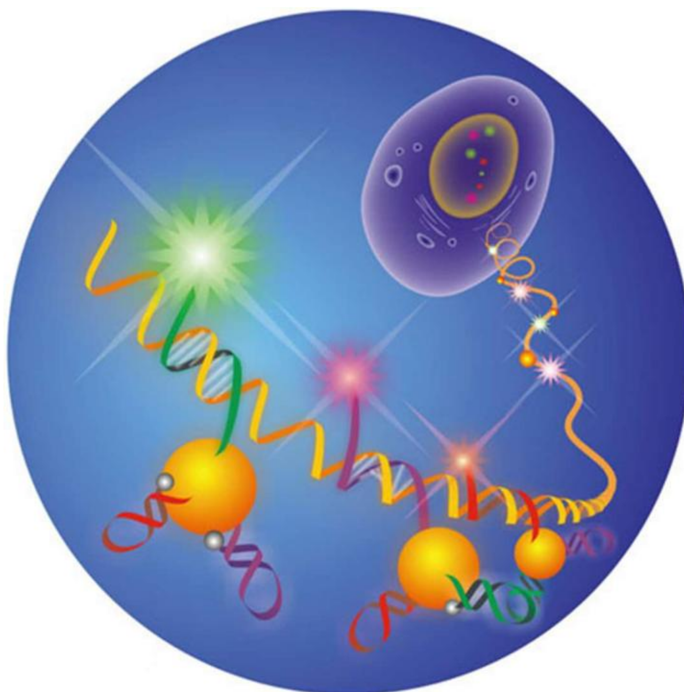
Development of novel aptasensors for the detection of mycotoxins

Xiaodong GUO



Promotors: Prof. Marie-Laure Fauconnier
Prof. Jiaqi Wang

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DEVELOPMENT OF NOVEL APTASENSORS FOR THE DETECTION OF MYCOTOXINS

Xiaodong GUO

Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

Promoteurs: Prof. Marie-Laure Fauconnier
Prof. Jiaqi Wang

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Résumé

Xiaodong Guo. (2020). Développement de nouveaux aptasenseurs pour la détection des mycotoxines (Thèse de doctorat en anglais). Gembloux, Belgique, Gembloux Agro-Bio Tech, Université de Liège, 107 p., 11 tableaux, 29 fig.

Résumé—Les contaminations par des mycotoxines, un défi mondial important, attirent de plus en plus l'attention du Centre international de recherche sur le cancer (CIRC), de l'Organisation mondiale de la santé (OMS) et des scientifiques en sécurité alimentaire. De nombreux pays et organisations ont établi les limites maximales de contamination des principales mycotoxines à des valeurs très faibles. Les stratégies analytiques traditionnelles sont principalement basées sur des méthodes quantitatives instrumentales et des tests immunologiques. Les aptamères sont un nouvel élément de reconnaissance de molécules similaires aux anticorps mais potentiellement plus performant, ils attirent de plus en plus l'attention des scientifiques. Par conséquent, des aptamères spécifiques pourraient être utilisés pour construire des biocapteurs pour la détection des mycotoxines. L'objectif de cette thèse est de développer de nouveaux biocapteurs à base d'aptamères pour la détermination sensible des traces de mycotoxines et de fournir une application prometteuse de ces aptasenseurs pour plus de facteurs de risque dans les sciences de la sécurité alimentaire.

Le contenu de la thèse et ses principaux résultats sont les suivants:

(1) Biocapteur à base d'aptamères pour la détection des mycotoxines

Les mycotoxines sont une grande famille de métabolites secondaires synthétisés par des champignons, elles présentent un grand danger pour l'homme et les animaux. Une majorité de pays et d'organismes décisionnels, tels que l'Union européenne, ont établi une série d'exigences et fixé les niveaux maximaux tolérés. De ce fait, le développement d'une plateforme analytique hautement sensible et spécifique pour les mycotoxines est très demandé. En raison de leur simplicité, de leur coût et de leur rapidité, les biocapteurs à base d'aptamères ont été développés avec succès pour la détection de diverses mycotoxines avec une sensibilité et une sélectivité élevées par rapport aux méthodes instrumentales traditionnelles et aux approches immunologiques. Dans ce travail, nous discutons et analysons le développement d'aptasenseurs pour la détection des mycotoxines dans les produits alimentaires et agricoles au cours des onze dernières années et couvrons la littérature depuis le premier rapport en 2008 jusqu'à aujourd'hui. Sont également résumés les défis et les tendances futures pour la sélection des aptamères spécifique de divers mycotoxines et aptasenseurs pour l'analyse multi-mycotoxines. Compte tenu du développement prometteur et de l'application potentielle d'aptasenseurs, les futures recherches seront le témoin de la grande potentialité du biocapteur à base d'aptamères pour le domaine de la sécurité alimentaire.

(2) Un aptasensor qPCR pour la détection sensible de l'aflatoxine M1

L'aflatoxine M1 (AFM1), l'une des mycotoxines les plus toxiques, présente de graves risques pour la santé. L'AFM1 avait précédemment été classée comme

cancérogène du groupe 2B (CIRC, 1993) et a été classé comme cancérogène du groupe 1 par le Centre international de recherche sur le cancer (CIRC) de l'Organisation Mondiale de la Santé (OMS) (CIRC, 2002). La détection de l'AFM1 joue donc un rôle important pour le contrôle de la qualité et de la sécurité sanitaire des aliments. Dans ce travail, un aptasensor sensible et fiable a été développé pour la détection de l'AFM1. L'immobilisation de l'aptamère par une forte interaction avec la biotine – streptavidine a été utilisée comme élément de reconnaissance moléculaire, et son ADNsb complémentaire a été utilisé comme modèle dans l'utilisation de Real Time, - Quantitative Polymerase Chain Reaction (RT-qPCR). Dans des conditions d'essai optimisées, une relation linéaire (allant de 1,0 × 10-4 à 1,0 µg L-1) a été obtenue avec une limite de détection (LOD) jusqu'à 0,03 ng L-1. De plus, l'aptasensor développé ici présente une sélectivité élevée pour l'AFM1 par rapport aux autres mycotoxines et de petits effets de réaction croisée avec des analogues structuraux. La méthode proposée ici a été appliquée avec succès à la détection quantitative de l'AFM1 dans des échantillons de céréales de riz pour nourrissons et de lait en poudre pour nourrissons. Les résultats ont démontrés que l'approche actuelle est potentiellement utile pour la sécurité sanitaire des aliments et qu'elle pourrait être étendue à un grand nombre de produits cibles.

(3) Un nouvel aptasenseur à base d'oxyde de graphène pour la détection par fluorescence de l'aflatoxine M1 dans le lait en poudre

Dans cet article, un aptasensor fluorescent rapide et sensible pour la détection de l'aflatoxine M1 (AFM1) dans le lait en poudre a été développé. De l'oxyde de graphène (GO) a été utilisé pour inhiber la fluorescence de l'aptamère marqué à la carboxyfluorescéine et empêcher le clivage de l'aptamère par la nucléase. Lors de l'ajout d'AFM1, une formation de complexe AFM1 / aptamère entraine le détachement de celle-ci de la surface du GO, l'aptamère est ensuite clivé par la DNase I et l'AFM1 cible est libéré pour un nouveau cycle, ce qui a conduit à une grande amplification du signal et une haute sensibilité. Dans des conditions optimisées, la détection basée sur le GO de l'aptasensor présente une réponse linéaire à AFM1 dans une plage dynamique de 0,2 à 10 µg / kg, avec une limite de détection (LOD) de 0,05 µg / kg. De plus, l'aptasensor développé a montré une haute spécificité envers AFM1 sans interférence avec d'autres mycotoxines. En outre, la technique a été appliquée avec succès pour la détection de l'AFM1 dans des échantillons de lait en poudre pour nourrissons. Cet aptasensor proposé ici offre une technologie prometteuse pour la sécurité alimentaire et peut être étendu à différent produits cibles.

(4) Aptasensor fluorescent entraîné par oxyde de graphène pour la détection de la fumonisine B1

La fumonisine B1 (FB1), est également une toxine très toxique, elle a été désignée comme cancérogène possible du groupe 2B par le Centre international de recherche sur le cancer (CIRC) en 2002. Par conséquent, la demande pour des approches simples, sensibles et spécifiques pour la détection du FB1est élevée. Dans cette étude, un nouvel aptasenseur a été introduit pour l'analyse de la FB1 basé sur l'oxyde de graphène (GO) et l'amplification du signal DNase I. Le GO a été adopté comme

extincteur de fluorescence contre les aptamères modifié par ROX et comme protecteur de l'aptamère contre le clivage par la DNase I ainsi qu'en amont des cycles permettant la détection d'amplification de signal. Cette stratégie de détection proposée a montré une bonne linéarité pour la détermination de FB1 dans la gamme dynamique de 0,5 à 20 ng mL-1 avec une bonne corrélation de R2 = 0,995. Sa détection de limite a été établie à 0,15 ng mL-1 (S / N = 3). L'analyse spécifique a indiqué que cet aptasenseur était sélectif pour FB1 ainsi que pour d'autres mycotoxines. De plus, l'application pratique dans des échantillons réels de cet aptasenseur pour la détection de FB1 a été étudiée. La plateforme de détection proposée ici sera utile pour une application dans le domaine de la sécurité alimentaire en vue l'analyse des mycotoxines.

(5) Articles faits saillants et perspectives d'avenir

En utilisant les nouveaux biocapteurs à base d'aptamères, nous avons développé plusieurs approches afin de détecter les mycotoxines les plus toxiques pour la sécurité alimentaire. De plus, cette stratégie de détection pourrait être appliquée pour une plus grande détermination des composés toxiques par simple remplacement des aptamères spécifiques. Bien que l'applicabilité, la faisabilité et la précision de ces aptasenseurs ai fait l'objet d'études sur des échantillons artificiellement contaminés, des recherches complémentaires étaient nécessaires pour une validation de ces aptasenseurs en conditions réelles afin de déterminer les performances telles que limite de détection et de quantification, la précision, la justesse, l'exactitude, etc. Grâce à la validation de cette méthode, ces aptasenseurs pourront être largement utilisés pour la détection des mycotoxines. Les perspectives se concentreront sur la simplification du principe et des dispositifs analytiques et sur la combinaison de nouveaux aptamères avec de nouveaux matériaux et techniques pour améliorer les performances analytiques et l'aspect pratique du marché des aptasenseurs.

Mots-clés: mycotoxines, aflatoxine, fumonisine, aptamère, biocapteur, RT-qPCR, oxyde de graphène, DNase I, détection, sécurité alimentaire.

Abstract

Xiaodong Guo. (2020). Development of novel aptasensors for the detection of mycotoxins (PhD Dissertation in English). Gembloux, Belgium, Gembloux Agro-Bio Tech, University of Liège, 107 p., 11 table, 29 fig.

Abstract — Mycotoxins contaminants, one of the most serious global challenges, have been attracted more and more attention from International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) and scientists in food safety sciences. Many countries and organizations have established the maximum contamination level of the main mycotoxins at very low values. Traditional analytic strategies are mainly based on instrumental quantitative method and immunoassays approaches. Aptamer, a novel molecules recognition element like or even superior to antibodies, has attracted more and more attentions for scientists. Therefore, specific aptamers could be employed to construct biosensors for the detection of mycotoxins. The objective of this thesis is to develop novel aptamer-based biosensors for sensitive determination of trace levels of mycotoxins and to provide a promising application of these aptasensors for more hazard factors in food safety sciences.

The main contents and results are as follows:

(1) Aptamer-based biosensor for detection of mycotoxins

Mycotoxins are a large types of secondary metabolites appeared by fungi, they pose a great hazard and toxic reactions to human and animals. A majority of countries and regulators, such as European Union, have established series of requirements and set the maximum tolerated levels. The development of high sensitive and specific analytical platform for mycotoxins is much in demand to address new challenges for food safety in worldwide. Due to the superiority of simple, rapid, and low-cost characteristics, aptamer-based biosensors are successfully developed for the detection of various mycotoxins with high sensitivity and selectivity compared with traditional instrumental methods and immunological approaches. In this article, we discuss and analyze the development of aptasensors for mycotoxins determination in food and agricultural products during the last eleven years and cover the literatures from the first report in 2008 until today. In addition, challenges and future trends for the selection of aptamers towards various mycotoxins and aptasensors for multi-mycotoxins analysis are summarized. Given the promising development and potential application of aptasensors, the future researches will witness the great practicability of aptamer-based biosensor for food safety field.

(2) A qPCR aptasensor for sensitive detection of aflatoxin M1

Aflatoxin M1 (AFM1), one of the most toxic mycotoxins, imposes serious health hazards. AFM1 had previously been classified as a group 2B carcinogen (IARC, 1993) and has been classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) (IARC, 2002). Determination of AFM1 thus plays an important role for quality control of

food safety. In this work, a sensitive and reliable aptasensor was developed for the detection of AFM1. The immobilization of aptamer through a strong interaction with biotin–streptavidin was used as a molecular recognition element, and its complementary ssDNA was employed as the template for a real-time quantitative polymerase chain reaction (RT-qPCR) amplification. Under optimized assay conditions, a linear relationship (ranging from 1.0×10^{-4} to 1.0 μg L^{-1}) was achieved with a limit of detection (LOD) down to 0.03 ng L^{-1}. In addition, the aptasensor developed here exhibits high selectivity for AFM1 over other mycotoxins and small effects from cross-reaction with structural analogs. The method proposed here has been successfully applied to quantitative determination of AFM1 in infant rice cereal and infant milk powder samples. Results demonstrated that the current approach is potentially useful for food safety analysis, and it could be extended to a large number of targets.

(3) A novel graphene oxide-based aptasensor for amplified fluorescent detection of aflatoxin M1 in milk powder

In this paper, a rapid and sensitive fluorescent aptasensor for the detection of aflatoxin M1 (AFM1) in milk powder has been developed. Graphene oxide (GO) was employed to quench the fluorescence of carboxyfluorescein-labelled aptamer and protect the aptamer from nuclease cleavage. Upon the addition of AFM1, a formation of AFM1/aptamer complex resulted in the aptamer detached from the surface of GO, then the aptamer was cleaved by DNase I and the target AFM1 was released for a new cycle, which led to a great signal amplification and high sensitivity. Under optimized conditions, the GO-based detection of the aptasensor exhibited a linear response to AFM1 in a dynamic range from 0.2 to 10 μg/kg, with a limit of detection (LOD) of 0.05 μg/kg. Moreover, the developed aptasensor showed a high specificity towards AFM1 without interference from other mycotoxins. In addition, the technique has been successfully applied for detection of AFM1 in infant milk powder samples. This aptasensor proposed here offers a promising technology for food safety and can be extended to various targets.

(4) Graphene oxide driven fluorescent aptasensor for the detection of fumonisin B1

Fumonisin B1 (FB1), one of the most toxic mycotoxins, has been designated as possible 2B group carcinogen by the International Agency for Research on Cancer (IARC) in 2002. Therefore, simple, sensitive and specific approaches for the detection of FB1 are much in demand. In this study, a novel aptasensor was introduced for FB1 analysis based on graphene oxide (GO) and DNase I signal amplification. GO was adopted as a fluorescence quencher against ROX-modified aptamer and a protectant for the aptamer from cleavaging by DNase I for subsequent target cycling and signal amplification detection. This proposed sensing strategy exhibited a good linearity for FB1 determination in the dynamic range from 0.5 to 20 ng mL^{-1} with a good correlation of R^2 = 0.995. Its detection of limit was established at 0.15 ng mL^{-1} (S/N = 3). The specific analysis indicated that this aptasensor was selective for FB1 other than other mycotoxins. In addition, the

practical application in real samples of this aptasensor for the detection of FB1 was investigated. The sensing platform proposed here was useful for a potential application in the field of food safety for mycotoxins analysis.

(5) Articles highlights and future perspective

By using the novel aptamer-based biosensors, we developed several approaches for the detection of the most toxic mycotoxins for food safety. In addition, these sensing strategies could be applied for more hazard factors determination by simple replacement of the specific aptamers. More importantly, though the practical applicability, feasibility, and accuracy of these proposed aptasensors were investigated and evaluated through the analysis of the spiked samples experiments, the future's researches were needed for a validation of these aptasensors with real contaminated samples to determine the performances such as limit of detection and quantification, precision, trueness, accuracy, etc. Through the method validation, these aptasensors will be widely used for the detection of mycotoxins. In addition, future direction will focus on the simplification of analytic principle and devices and the combination of novel aptamers with new materials and techniques to improve the analytical performance and market practicality of aptasensors.

Keywords: mycotoxins, aflatoxin, fumonisin, aptamer, biosensor, RT-qPCR, graphene oxide, DNase I, detection, food safety.

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Xiaodong Guo
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List of Abbreviations

AF	aflatoxins
AFB1	aflatoxin B1
AFB2	aflatoxin B2
AFG1	aflatoxin G1
AFG2	aflatoxin G2
AFM1	aflatoxin M1
AFM2	aflatoxin M2
AFM	atomic force microscopy
AEGIS	artificially expanded genetic information system
AgNCs	silver-nanocluster
AuNPs	gold nanoparticles
ADANRs	AuNR@DNTB@Ag nanorods
CV	Cyclic Voltammetry
CRET	Chemiluminescence resonance energy transfer
$CaCl_2$	anhydrous calcium chloride
CE	capillary electrophoresis
$C_6H_5Na_3O_7$	sodium citrate
Ct	cycle threshold number
DPV	Differential Pulse Voltammetry
ds DNA	double-stranded DNA
ds RNA	double-stranded RNA
EC	European Commission
ELISA	enzyme-linked immune sorbent assay
EXO	exonuclease
EIS	Electrochemical Impedance Spectroscopy
ECL	electrochemiluminescence
EDTA	ethylenediaminetetraacetic acid
EU	European Union
FAM	carboxyfluorescein
FAO	Food and Agriculture Organization of the United Nations
F	fumonisins

FB1	Fumonisin B1
FB2	Fumonisin B2
FB3	Fumonisin B3
Fe_3O_4/PANi	Fe3O4 incorporated polyaniline
FLD	fluorescence detection
FDA	Food and Drug Administration
GC–MS	gas chromatography–mass spectrometry
GO	graphene oxide
HPLC	high-performance liquid chromatography
HRPzyme	HRP-mimicking DNAzyme
HT	high-throughput
HCR	hybridization chain reaction
IARC	International Agency for Research on Cancer
IP	immunoprecipitation
Ir	iridium
Kd	dissociation constant
KCl	potassium chloride
LC–MS	liquid chromatography–mass spectrometry
LSV	Linear Sweep Voltammetry
LOD	limit of detection
MBs	magnetic beads
MB	methylene blue
MRR	microring resonators
MRLs	maximum residue levels
NA	Nucleic acid
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
Na_2CO_3	sodium carbonate
OT	ochratoxins
OTA	Ochratoxin A
OTB	Ochratoxin B
PDDA	Poly diallyldimethylammonium chloride
pg	pictogram
QDs	quantum dots

RCA	rolling circle amplification
ROX	carboxyl-X-rhodamine
RSD	relative standard deviation
RT-qPCR	real-time quantitative polymerase chain reaction
SWV	Square Wave Voltammetry
SiON	Silicon oxynitride
SAMs	self-assembled monolayers
SERS	Surface Enhance Raman Scattering
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
ss DNA	single-stranded DNA
ss RNA	single-stranded RNA
SWNTs	single-walled carbon nanotubes
SDs	Standard deviations
S/N	signal/noise
TLC	thin layer chromatography
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
USA	United States of America
WHO	World Health Organization
ZEN	zearalenone
α-ZOL	α-zearalenin
VOC	volatile organic compound
mVOCs	microbial volatile organic compounds

1

General introduction: Aptamer-based biosensor for detection of mycotoxins

In this article, we discuss and analyze the development of aptasensors for mycotoxins determination in food and agricultural products during the last eleven years and cover the literatures from the first report in 2008 until today. In addition, challenges and future trends for the selection of aptamers towards various mycotoxins and aptasensors for multi-mycotoxins analysis are summarized. Given the promising development and potential application of aptasensors, the future researches will witness the great practicability of aptamer-based biosensor for food safety field.

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Abstract: Mycotoxins are a large types of secondary metabolites appeared by fungi, they pose a great hazard and toxic reactions to human and animals. A majority of countries and regulators, such as European Union, have established series of requirements and set the maximum tolerated levels. The development of high sensitive and specific analytical platform for mycotoxins is much in demand to address new challenges for food safety in worldwide. Due to the superiority of simple, rapid, and low-cost characteristics, aptamer-based biosensors are successfully developed for the detection of various mycotoxins with high sensitivity and selectivity compared with traditional instrumental methods and immunological approaches. In this article, we discuss and analyze the development of aptasensors for mycotoxins determination in food and agricultural products during the last eleven years and cover the literatures from the first report in 2008 until today. In addition, challenges and future trends for the selection of aptamers towards various mycotoxins and aptasensors for multi-mycotoxins analysis are summarized. Given the promising development and potential application of aptasensors, the future researches will witness the great practicability of aptamer-based biosensor for food safety field.

Keywords: aptamer, biosensor, mycotoxin, detection, food safety.

1 Introduction

There are about 25% of crops in worldwide that are contaminated with mycotoxins (FAO, 2004). Mycotoxins contaminants, one of the most serious global challenges, have been attracted more and more attention from International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) and scientists in food safety sciences. Mycotoxins, one of the most important and toxic contaminants in food and agricultural products, are secondary metabolites produced mainly by various molds (Atar et al., 2015; Mata et al., 2015; Zhu et al., 2015). There are hundreds of mycotoxins that were identified mainly aflatoxins (AF), ochratoxins (OT), fumonisins (F), and zearalenone (ZEN) as seen in Figure 1-1.

Mycotoxins, a kind of great hazard contaminants for human and animals health, have been a part of food chain. High exposure of mycotoxins in food and feed can lead to mycotoxicosis on human and animals with acute and chronic effects, which mainly has an effect on the kidney, liver, endocrine, nervous and immune systems (Cigic and Prosen, 2009).

Aflatoxin B1 is present in a variety of feed and food, including cereals, maize, nuts, and fruits (Chen et al., 2014; Iqbal et al., 2014; Zhang et al., 2016). AFB1 is the most toxic mycotoxin which mainly causes liver cancer. Its metabolite aflatoxin M1 can appear in milk when dairy cows consumed the feed contaminated with AFB1 (Guo et al., 2014; Guo et al., 2016). AFM1 occurs mainly in dairy products such as raw milk, heat-treated milk, processing milk, and milk-based products. Therefore, mycotoxins pose a common threat in both feed and food products industry and their processing products industry. Ochratoxin A is responsible for the mutagenic, teratogenic and immunosuppressive effects, which occurs in various feed and food including cereals, meats, milk, grape, coffee, beer and wine, etc. Fumonisin B1, produced by *Fusarium moniliforme*, can cause oesophagal cancer, liver tumor, and kidney tumor via the contamination of maize and maize products, as well as animal feeds (Scott, 2012).

The certain mycotoxin toxicity and the general exposure of the population determins the severity and attention of this kind of mycotoxin. In industrialized countries, the chronic mycotoxicosis effects and diseases are largely more than that of the acute issues as a result of low level exposure of the population. In contrast, in developing countries, it is common that the control of mycotoxin exposure is difficult in both agricultural activities and regulations. Mycotoxin exposure of the population is generally high in these areas (Sanzani and Ippolito, 2014). China is an developing country, which is also a great agriculture-based country. In addition, it has posed an important role in food supply chain worldwide. Therefore, China government and Ministry of Health have established the maximum limits against mycotoxins in food and agricultural products for food safety. The aflatoxins levels presented in infant cereals, raw milk and edible oils are much lower than the regulation limit of China. It is first reported that aflatoxins occurred in lotus seeds.

No AFM1 appeared in infant milk powder as a result of the strict control and requirement of China for human and infants health. The levels of ochratoxin A occurred in wine were below the permissible limits established by European Union. In addition, no permissible limits for fumonisins have been regulated in China. However, due to the common occurrence of fumonisins in maize and maize products, the contamination state of fumonisins should be attentioned, and the permissible limits should be set in the future (Selvaraj et al., 2015). As seen in table 1-1, international legislation on mycotoxins was summarized with maximum admissible levels in certain commodities and products.

Table 1-1: Summary of the international legislation on mycotoxins (Anfossi et al., 2016).

Mycotoxins	Products	Maximum tolerable levels (μg/kg)	Countries and Organizations
AF	Oil seeds, nuts, dried fruits, cereals, spices	4–15 (2–12 for AFB1)	EU
		(15 for AFB1)	Australia, Canada, Nigeria, New Zeland, South Africa
		20	USA, Brazil, MERCOSUL
		30	India
AFM1	Milk and infant formula	0.25–0.05	EU, Turkey, South Africa
		0.5	Argentina, China, India, Kenya, Mexico, Uruguay, USA
		0.5–5	Brazil, MERCOSUL
OTA	Cereals, dried fruits, coffee, cocoa, wine, beer, grape juice, spices, liquorice, blood products	2–10	EU, Egypt,
		5	China, Kenya, Nigeria, Russia
		20	India
		2–30	Brazil
		50	Uruguay
F	Maize	800–4000	EU, Turkey, Norway, Switzerland
		2000–4000	USA
		2000–5000	Brazil
ZEN	Cereals, bakery products, maize oil	75–400	EU
		200–1000	Brazil
		200,000	China, Russia, Chile

Aflatoxin B1 and aflatoxin M1, the most toxic mycotoxins, have been designated as group 1 carcinogen by the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO), and ochratoxin A is classified as group 2B carcinogen by IARC (IARC, 1993; IARC, 2002; O'Brien and Dietrich, 2005). Given of the serious toxicity effect of mycotoxins on animals and human, the European Commission set the maximum contamination AFB1 level to 2 μg kg^{-1} for all cereals and cereal-derived products for food safety (Commission, 2010). In

addition, European Union has regulated a maximum tolerated level for AFM1 to 0.050 µg kg^{-1} for adult, and lower level for AFM1 to 0.025 µg kg^{-1} for children and infants consumption (Commission, 2006). Taking the high toxicity and low permissible limits into consideration, rapid, low-cost, sensitive analytical strategies for the detection of mycotoxins are vitally important and required.

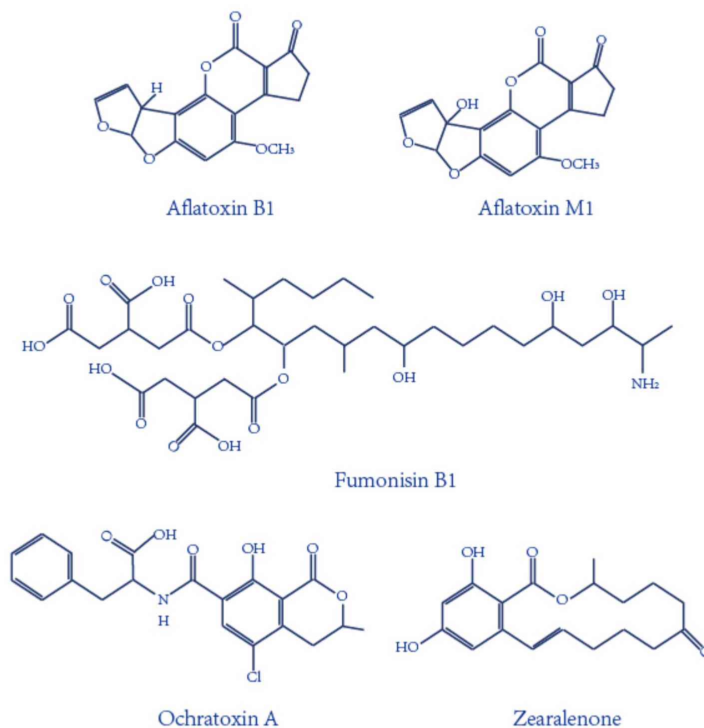

Figure 1-1: Chemical structures of the important mycotoxins.

Confirmatory and quantitative approaches for detection of mycotoxins are mainly thin layer chromatography (TLC) (Var et al., 2007), high-performance liquid chromatography (HPLC) (Mao et al., 2015; Wang et al., 2012; Lee et al., 2015; Pietri et al., 2016; Herzallah, 2009; Yazdanpanah et al., 2013), and liquid chromatography coupled with mass spectrometry (LC–MS) (Corcuera et al., 2011; Abia et al., 2013; Warth et al., 2013). However, expensive and special instruments, complicated pretreatment and professional personnel are required in these typical equipment methods (Shim et al., 2007). In the meantime, antibody-based immunoassays were developed for mycotoxin detection, including enzyme-linked immune sorbent assay (ELISA) and immunosensors methods (Li et al., 2009; Kav et al., 2011; Anfossi et al., 2015; Parker et al., 2009; Bacher et al., 2012; Vdovenko et al., 2014; Park et al., 2013; Piermarini et al., 2007; Mozaffari Nejad et al., 2014; Sheng et al., 2014; Xu et al., 2014). Though the immunoassays has the advantage of

high specificity, the high-cost and storage stability of antibody limits the application of these rapid analysis procedures. Aptamers, an alternative molecule recognition element to antibodies, are single-stranded (ss) DNA or RNA oligonucleotides that can form aptamer/target complex with very strong affinity and specificity via the conformational change. The advantages of aptamer were compared to antibody in table 1-2. With these advantages, aptamer-based biosensors were widely introduced for the detection of mycotoxins like AFB1 (Castillo et al., 2015; Evtugyn et al., 2013; Seok et al., 2015; Shim et al., 2014; Wang et al., 2016), AFM1 (Nguyen et al., 2013; Istamboulie et al., 2016), OTA (Guo et al., 2011; Kuang et al., 2010; Yang et al., 2013), FB1 (Wu et al., 2012; Wu et al., 2013), especially based on fluorescent, colorimetric and electrochemical aptasensors (Figure 1-2). However, the ultrasensitive approaches are difficult to develop via a simple aptasensor recognition. Therefore, a series of novel aptasensors with signal amplification and enhancement have been introduced for mycotoxins (Yang et al., 2007; Weizmann et al., 2006; Patolsky et al., 2002; Wu et al., 2017; Deng et al., 2009; Pavlov et al., 2004; Guo et al., 2014), which can meet the requirement of the low maximum contamination level set by many countries and organizations.

Table 1-2: Comparison of the properties between antibody and aptamer (Zhuo et al., 2017).

No.	Antibody	Aptamer
1	High molecular weight	Low molecular weight
2	Screened under physiological conditions	Screened and chemical synthesis in vitro
3	Sensitive to temperature, short storage time	Stored and transported at room temperature
4	Temperature-induced denaturation is irreversible	Temperature-induced denaturation is reversible
5	Long preparation time with high cost	Short preparation time with low cost
6	Strongly immunogenic	No obvious immunogenicity
7	Limited target substances	Wide range of target substances
8	Lose affinity to target with labels	Keep original biological activity with labels
9	Unable to separate cross-reactive substances	Can separate structural analogues or cross-reactive substances

2 Aptamers selection

Aptamers, an alternative target recognition probe to antibodies, are ssDNA or ssRNA oligonucleotides that can bind to targets with high affinity and specificity through the dimensional structure change after the formation of target/aptamer complex. At the first time, systematic evolution of ligands by exponential enrichment (SELEX) was introduced to obtain RNA sequence against T4 DNA

polymerase, which is performed in a random sequences pool. In this study, nitrocellulose filter was adopted to obtain RNA sequence, and filter binding methods was employed to recognize the process of SELEX selection. It was predicted that this SELEX strategy could obtain strong affinity and specificity ligands for any target (Tuerk and Gold, 1990).

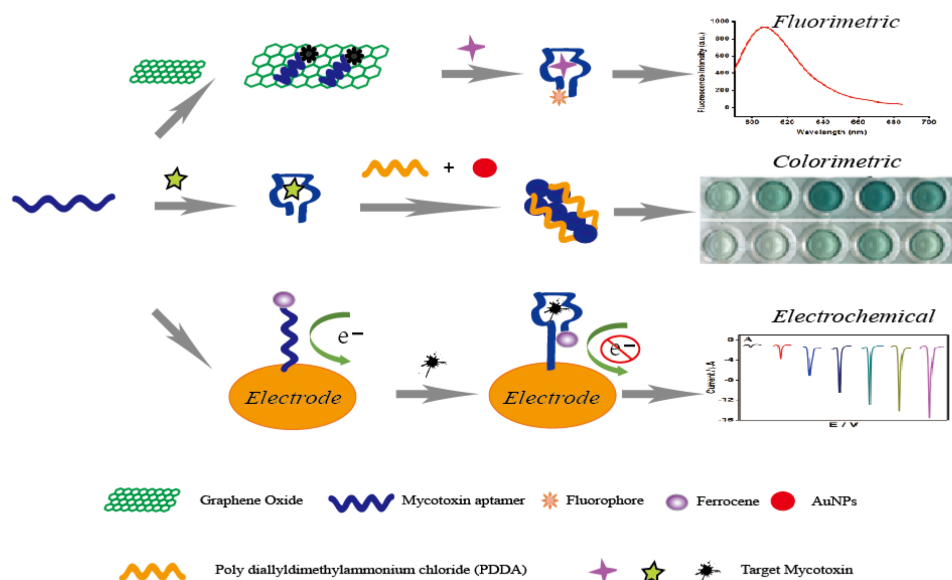

Figure 1-2: Principle illustration of fluorescence, colorimetric and electrochemical aptasensor for the detection of small molecule mycotoxins.

In the meantime, another RNA sequence specific to organic dyes was first selected based on affinity chromatography column. There is a ligand binding site of this RNA sequence towards organic dyes. This RNA sequence was defined as aptamers (Ellington and Szostak, 1990), which indicated that the aptamer was the first time successfully selected. Two years later, ssDNA aptamer of human thrombin was successfully obtained with the dissociation constant from 25 to 200 nM (Bock et al., 1992). From then on, the RNA and ssDNA sequences libraries were simultaneously employed for the process of aptamers selection (Darmostuk et al., 2015). Aptamers have many advantages, such as small molecular, screened and chemical synthesis in vitro, stored and transported at room temperature, reversible temperature-induced denaturation, short preparation time with low cost, no obvious immunogenicity, wide range of target substances, keep original biological activity with labels, separate structural analogues or cross-reactive substances. Therefore, aptamer and aptamer-based biosensor was widely applied for the detection of various targets with promising future.

In the beginning 18 years from the finding of first aptamer, the selected aptamers and novel aptamer-based biosensors were mainly used in the field of medicine, biology, chemistry and bioinformatics (Wang et al., 2019; Tan et al., 2019; Zhang et al., 2019; Xiong et al., 2019). For food safety sciences, especially in mycotoxins detection for food safety, the first aptamer specific to OTA has been reported by Cruz-Aguado in 2008 (Cruz-Aguado and Penner, 2008).In recent years, a series of SELEX-based techniques have been developed for the selection of aptamers against various targets, mainly including cell-SELEX, capillary electrophoresis-SELEX (CE-SELEX), immunoprecipitation-coupled SELEX (IP-SELEX), atomic force microscopy SELEX (AFM-SELEX), and artificially expanded genetic information system-SELEX (AEGIS-SELEX). As shown in table 1-3, the characteristics, advantages and disadvantages are summarized and discussed.

Table 1-3: Comparison of the current SELEX-based aptamer selection techniques (Zhang et al., 2019).

SELEX	Key Points	Advantages	Disadvantages
Cell-SELEX	Aptamers selection for whole live cells	Aptamers for molecules in their native state. Cell surface has many potential targets. No protein purification	Costly. For cell surface targets. Time consuming. Post SELEX identification of the target required
CE-SELEX	Electrophoretic mobility based ions separation	Rapid. Only 1–4 rounds of selection. Reduced non-specific binding. No target immobilization	Not suitable for small molecules. Expensive equipment required
IP-SELEX	Includes immunoprecipitation	For proteins. Increased affinity and specificity	Time-consuming
AFM-SELEX	Employs AFM for 3D image of sample surface	Rapid. Only 3–4 rounds required. Increased aptamer affinity	Expensive equipment required. Target and aptamers immobilization required
M-SELEX	Microfluidic system-based SELEX	Rapid. Automatable. For small molecules. Highly efficient (required only small amounts of reagents)	Low purity of aptamers. Target immobilization required
AEGIS-SELEX	Libraries with the artificially expanded genetic code	Increased aptamers specificity	Poor recognition of the unnatural bases by natural DNA polymerases
Capture-SELEX	Library is immobilized on a support	For small molecules. For structure-switching aptamers. No target immobilization	Partial oligonucleotides from the library might be not selected

3 Aptasensor for the analysis of ochratoxin A

Ochratoxins are one important type of mycotoxins, which are mainly produced by several fungi, such as *Aspergillus ochraceus* and *Penicillium verrucosum* (Liu et al., 2015). Of the several subtypes of ochratoxins, ochratoxin A (OTA) is the very common one, and is designated as a possible human carcinogen by IARC (Lv et al., 2016). Researches have paid much attention to the studies on OTA in recent years owing to its widespread occurrence and extraordinary toxic reactions on animals and human. The first aptamer, the minimal one of the selected sequences, has the highest affinity to OTA. The dissociation constant is 200 nM. Since this aptamer specific to OTA has been reported by Cruz-Aguado in 2008 (Cruz-Aguado and Penner, 2008), large numbers of novel aptasensors were developed for OTA analysis in various food products, including fluorescenct, colorimetric and electrochemical aptasensors, as well as some methods based on nanomaterials. The recent literatures of aptasensors for the analysis of ochratoxin A for food safety are illustrated in table 1-4. In addition, these articles are analyzed in more details for each group of the targets.

Table 1-4: Summary of aptasensor for the analysis of ochratoxin A.

Mycotoxins	Method	Principle	Detection range	LOD	Sample	Reference
OTA	Fluorescent aptasensor	single-walled carbon nanotubes (SWNTs) as quencher	25-200 nM	24.1 nM	Beer	(Guo et al., 2011)
OTA	Fluorescent aptasensor	PVP-protected graphene oxide as quencher	50-500 nM	21.8 nM	Red wine	(Sheng et al., 2011)
OTA	Fluorescent aptasensor	target-induced Conformational change signaling aptamer	1-100 ng mL^{-1}	0.8 ng mL^{-1}	Corn	(Chen et al., 2012)
OTA	Fluorescent aptasensor	fluorescent DNA and silver-nanocluster (AgNCs)	0.01-0.3 ng mL^{-1}	2 pg mL^{-1}	Wheat	(Chen et al., 2014)
OTA	Fluorescent aptasensor	Carboxy-modified fluorescent Particles	0.1-150 nM	0.005 nM	Beer	(Hayat et al., 2015)
OTA	Fluorescent aptasensor	aptamer-conjugated magnetic beads (MBs) and CdTe quantum dots (QDs)	0.015-100 ng mL^{-1}	5.4 pg mL^{-1}	Peanut	(Wang et al., 2015)
OTA	Fluorescent aptasensor	hybridization chain reaction (HCR)	1.0-20 pM	0.1 pM	Corn	(Wang et al., 2016)
OTA	Colorimetric aptasensor	unmodified gold nanoparticles (AuNPs) indicator	20-625 nM	20 nM	-	(Yang et al., 2011)
OTA	Colorimetric aptasensor	target-reactive aptamer-cross-linked hydrogel	0-100 nM	1.27 nM	Beer	(Liu, et al., 2015)
OTA	Colorimetric aptasensor	cationic polymer and AuNPs	0.05-50 ng mL^{-1}	0.009 ng mL^{-1}	liquor	(Luan et al., 2015)
OTA	Colorimetric aptasensor	aptamercontrolled growth of Au NPs	-	1 nM	Red wine	(Soh et al., 2015)
OTA	Colorimetric aptasensor	Au@Fe3O4 NPs	0.5-100 ng mL^{-1}	30 pg mL^{-1}	Peanut	(Wang et al., 2016)
OTA	Electrochemilumin	N-(4-aminobutyl)-N-ethylisoluminol	0.02-3.0 ng	0.007	Wheat	(Wang et al., 2010)

Mycotoxins	Method	Principle	Detection range	LOD	Sample	Reference
	escent biosensor	and AuNP-modifiedgold electrode	mL^{-1}	ng mL^{-1}		
OTA	Electrochemical aptasensor	aptamer modified gold electrode	0.1-1000 pg mL^{-1}	0.095 pg mL^{-1}	Red wine	(Wu et al., 2012)
OTA	Electrochemical aptasensor	gold electrode coupled with silver nanoparticles	0.3-30 nM	50 pM	Beer	(Evtugyn et al., 2013)
OTA	Electrochemical aptasensor	rolling circle amplification (RCA)	0.1-5000 pg mL^{-1}	0.065 pg mL^{-1}	Wine	(Huang et al., 2013)
OTA	Electrochemical aptasensor	Au NPs and methylene blue	2.5-2500 pM	0.75 pM	Red wine	(Yang et al., 2014)
OTA	Electrochemical aptasensor	exonuclease-induced recycling amplification	0.01-1.0 ng mL^{-1}	0.004 ng mL^{-1}	Corn and Oat	(Tan et al., 2015)
OTA	Electrochemical aptasensor	nanocomposites of AuNPs	0.2-4000 pg mL^{-1}	0.07 pg mL^{-1}	-	(Hao et al., 2016)
OTA	Chemiluminescence aptasensor	HRP-mimicking DNAzyme (HRPzyme)	0.1-100 ng mL^{-1}	0.22 ng mL^{-1}	Coffee beans	(Jo et al., 2016)
OTA	Electrochemical aptasensor	exonuclease (Exo) III-assisted recycling amplification	0.001-0.5 ng mL^{-1}	0.58 pg mL^{-1}	Wheat	(Liu et al., 2016)

3.1 Fluorescent aptasensor for OTA

First, Guo et al, developed a sensitive and selective aptasensor for fluorescent detection of OTA. The single-walled carbon nanotubes (SWNTs) were employed to quench the fluorescence signal produced by carboxyfluorescein-labelled aptamer. Upon the presence of OTA, the spatial structure change of the specific aptamer leads to the separation of the SWNTs with the aptamer, the fluorescence is therefore detected. The fluorescence signal has a good linear relationship with concentrations in a range from 25 to 200 nM, with a detection limit of 24.1 nM (Guo et al., 2011). In addition, this aptasensor is successfully used for OTA determination in real beer samples. In the same year, another fluorescent aptasensor was introduced by the same research team via the graphene oxide as a fluorescence quencher (Sheng et al., 2011). In this sensing platform, a linear response of 50-500 nM was obtained between the fluorescence intensity and OTA levels, with the detection limit of 21.8 nM. More importantly, the limit detection could be lowered by two orders of magnitude by using PVP-coated graphene oxide. Similarly, this current aptasensor was also validated for OTA detection in red wine samples.

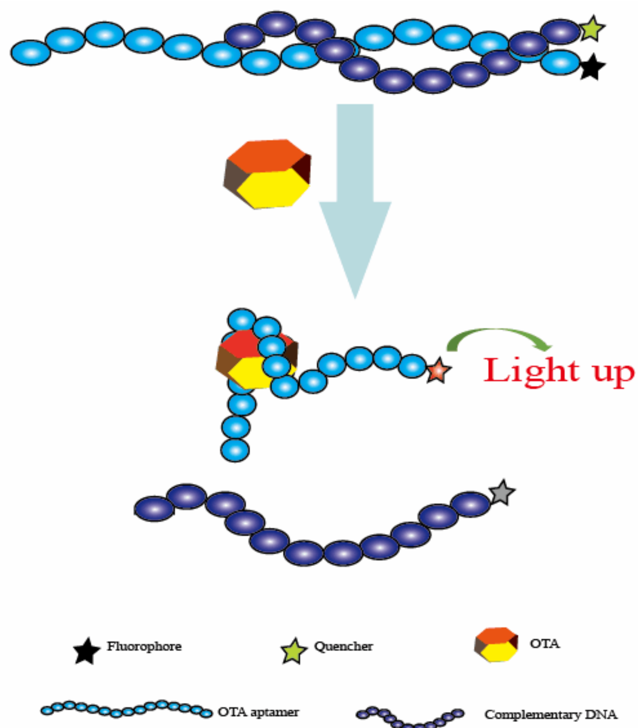

Figure 1-3: Schematic representation of the fluorescent aptasensor for OTA determination based on the conformational of aptmer.

Based on the similar fluorescence response and fluorescence quench schemes, Chen et al, reported a rapid and feasible aptasensor for OTA determination in corn samples (Chen et al., 2012). In this sensing platform (Figure 1-3), the fluorophore was used to label the aptamer while the quencher moiety was employed to label the complementary DNA to quench the fluorescence. When there was no OTA, the hybridization reaction between the complementary DNA and the aptamer led to the close distance of these two moieties, the fluorescence signal was effectively quenched. Upon OTA addition, the produce of aptamer/OTA complex resulted in the separation of the complementary DNA, and the fluorescence was thus recovered. A good linear response between the fluorescent change and OTA levels was obtained ranged from 1 to 100 ng mL^{-1}, with the detection limit of 0.8 ng mL^{-1}. More importantly, the whole analysis process of this method was within 1 min. Therefore, this was another fluorescent aptasensor for rapid determination of OTA with high efficiency.

Nanomaterials were also used to construct biosensors with several modifications to produce fluorescence. One of those aptasensors was introduced for the detection of OTA. In this research, magnetic beads, the fluorescence characterization of DNA silver nanocluster, and spatial conformational change of the aptamer was adopted. An ultrasensitive method was achieved with the limit of detection (2 pg mL^{-1}). Additionally, the practical analysis was successfully completed on wheat samples for OTA detection. This aptasensor might provide a promising sensing platform for OTA due to its unique advantages of low-cost, rapid, portable, selective, and ultrasensitive (Chen et al., 2014). Based on the same magnetic beads separation method, Akhtar et al, reported a generic fluorescent aptasensor to detect OTA. In this sensing strategy, carboxy-labelled aptamer was adopted to signal produce element, the magnetic beads were employed to separate unbound portions. A high sensitivity of this aptasensor was determined with a limit of detection (0.005 nM). Moreover, this proposed method was successfully applied on beer samples for the analysis of OTA (Hayat et al., 2015).

Additionally, another one-step fluorescent aptasensor was illustrated for OTA determination. The specific aptamer for OTA was employed as a target recognition probe, CdTe quantum dots (QDs) was used as a label, and magnetic beads were acted as the separation support. Upon OTA addition, the produce of aptamer/OTA complex and magnetic separation resulted in a significant fluorescence intensity enhancement. More importantly, a wide range response between the fluorescence signal and OTA concentrations (from 15 pg mL^{-1} to 100 ng mL^{-1}) was achieved, and the limit of detection was 5.4 pg mL^{-1}. Therefore, this developed sensing platform might represent a potential strategy for OTA routine controls for food safety(Wang et al., 2015). Finally, hybridization Chain Reaction (HCR), an important signal enhancement strategy, has been widespreadly applied for the analysis of DNA, proteins, metal ions, virus, and cancer cells, as well as the mycotoxins. An ultrasensitive aptasensor was developed for OTA determination via HCR technique. In this sensing platform, perylene derivative was conjugated and employed as the fluorescence element. DNA concatamers were obtained through the HCR strategy,

which caused the aggregation of perylenediimide probe and subsequent signal enhancement. Under the optimized conditions, fluorescence signal was established to have a good linear relationship with the targeted OTA concentrations. It ranged from 1.0 to 20 pM with a detection limit of 0.1 pM, indicating that this current aptasensor was highly sensitive for the analysis of OTA. Moreover, the practicality of this sensing strategy was validated through the successful detection of OTA levels in corn samples (Wang et al., 2016).

3.2 Colorimetric aptasensor for OTA

In addition to fluorescent aptasensors, colorimetric aptasensors are also widespread techniques for the analysis of mycotoxins because of their simple and rapid uses, without the requirement of complicated instruments. A color change was produced as a result of the aggregation of gold nanoparticals (AuNPs) in the salt presence. Yang et al. developed a colorimetric aptasensor for OTA determination, which employed the advantages of the specific aptamer and AuNPs (Yang et al., 2011). Upon the OTA addition, structural change to G-quadruplex of the aptamer from random coil to G-quadruplex resulted in the separation of the aptamer to AuNPs, leading to AuNPs aggregation under the addition of salt and subsequent color change. The absorbance values presented a good linear relationship with OTA concentrations ranged from 20 to 625 nM. Its detection limit is 20 nM. Using the same AuNPs colorimetric indicators, a novel visual aptasensor was introduced for visual analysis of OTA via the synthesis of DNA hydrogels. This technology has been widely applied for the analysis of proteins (Zhang et al., 2013), ions (Dave et al., 2010; Guo et al., 2014; Lin et al., 2011) and nucleic acids (Sun et al., 2014), as well as small molecules (Yan et al., 2013; Zhu et al., 2010). In this design, as depicted in Figure 1-4, the DNA hydrogels were obtained through linkage between the aptamer of OTA and two polymer single-stranded DNAs. With the addition of OTA, the aptamer/OTA complex formation induced dissociation of the DNA hydrogels. Subsequently, the AuNPs were released for the colorimetric detection of OTA levels. Its detection limit was determined to be 1.27 nM by signal amplification strategy. Therefore, this proposed sensing strategy represented a novel and portable method for the analysis of OTA to ensure food safety (Liu, et al., 2015).

Moreover, Luan et al. reported a sensitive and selective aptasensor for OTA determination via aggregation of AuNPs as the colorimetric generator induced by poly diallyldimethylammonium chloride (PDDA). Upon the optimal conditions, its detection limit was obtained down to 0.009 ng mL^{-1}, which demonstrated that this sensing platform provided a promising simple and sensitive platform for rapid analysis of OTA (Luan, et al., 2015).

Under the normal circumstances, the coexistence of multiple mycotoxins is a very common phenomenon in food and agricultural products. High-throughput screening and analysis of multiple mycotoxins will play an important role for food

safety. Based on the recognition reaction of aptamer and the colorimetric response of AuNPs, a rapid and sensitive aptasensor for the analysis of multiple small molecules including OTA was introduced. Upon the addition of target, the interactions between aptamer and targets caused the dissociation of the aptamer from the surface of AuNPs, leading to the color change of AuNPs. the limits of detection were 1 nM for OTA, 0.2 nM for 17β-estradiol, and 1 nM for cocaine, respectively. Therefore, the novel aptasensor became a potential strategy for high-throughput application for multiple mycotoxins detection by simple replacement of aptamer for different mycotoxins (Soh, et al., 2015).

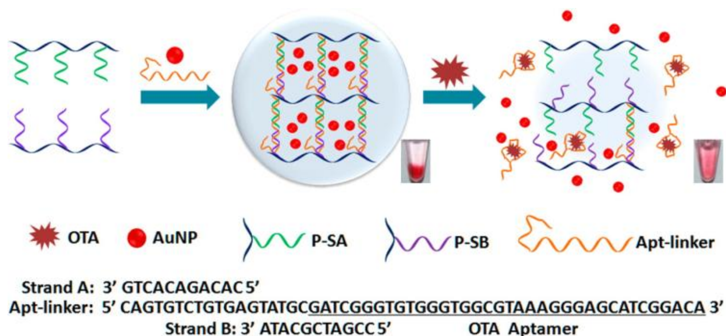

Figure 1-4: Principle illustration of colorimetric aptasensor for detection of OTA via AuNPs encapsulated DNA hydrogel. Reprinted from Liu et al. (2015) with permission.

In addition, Au@Fe_3O_4 NPs were obtained through AuNPs being functionalized by Fe_3O_4 NPs, and the Au@Fe_3O_4 NPs activity was thus increased. The aptamer of OTA was labelled to the magnetic beads while its complementary single-stranded DNA was modified on Au@Fe_3O_4 NPs. Upon the addition of OTA, the recognition between the aptamer and OTA caused the release of ssDNA-labelled Au@Fe_3O_4 NPs, which could exert the color change to a blue solution. Its detection limit was down to 30 pg mL^{-1}, demonstrating that this sensing strategy was a novel and sensitive approach for OTA determination for food safety via the application of peroxidase-like activity of AuNPs (Wang et al., 2016).

3.3 Electrochemical aptasensor for OTA

The immobilization of aptamers on the surface of transducer substrates exerted an important function on the construction of electrochemical aptasensors. The transducer substrates mainly consisted of gold and carbon-based electrodes. A series of electrochemical aptasensors were developed for OTA analysis in food and agricultural products, including Electrochemical Impedance Spectroscopy (EIS), Differential Pulse Voltammetry (DPV), Cyclic Voltammetry (CV), Linear Sweep Voltammetry (LSV), Square Wave Voltammetry (SWV), Field Effect Transistor and potentiometry. Electrochemical aptasensors attracted great interest for researchers owing to the advantages of rapid, portable, low-cost, and high sensitivity and selectivity, as well as the high efficient properties. Firstly, based on the

AuNPs-modified gold electrode and luminescence-labelled specific aptamer, Wang et al. successfully carried out an electrochemiluminescent aptasensor method for OTA determination in wheat samples. The luminescence-labelled aptamer was employed as the indicator of the sensor, and the complementary DNA modified to gold electrode was hybridized with the aptamer. Upon the addition of OTA, the structural change of the aptamer caused the separation of luminescence-labelled aptamer from complementary DNA-modified gold electrode, resulting in the decrease of electrochemiluminescence (ECL) signals. In optimized conditions, a good linear response was observed between the ECL signals and OTA concentrations ranging from 0.02 to 3.0 ng mL^{-1}, and its detection limit was 0.007 ng mL^{-1}. Therefore, this current method offered a promising sensing strategy for the analysis of OTA for food safety (Wang et al., 2010). Then, by using the same gold electrode, an electrochemical aptasensor with high sensitivity was introduced for OTA detection (Wu et al., 2012). The aptamer labeled with thiol- and methylene blue- was used as the molecule recognition probe. Under the addition of target OTA, aptamer/OTA complex was formed owing to the interaction of the aptamer and OTA, which suppressed the transfer of the electron. In the optimized conditions, the change of the electrochemical signal had a good linear response when compared to the OTA levels ranging from 0.1 to 1000 pg mL^{-1}. Its detection limit was determined to 0.095 pg mL^{-1}. Moreover, the proposed electrochemical aptasensor was successfully used for the OTA analysis in red wine samples with satisfying recoveries. This rapid approach represented a potential tool for on-site analysis of OTA.

Similarly, based on gold electrode and recognition of aptamer, another electrochemical aptasensor was presented for OTA determination (Evtugyn et al., 2013). In this study, the gold electrode was modified using silver nanoparticles and electropolymerized neutral red, and the aptamer was thiolated to the surface of the silver nanoparticles. When in the addition of OTA, aptamer/OTA complex was produced, that caused the change of charge transfer resistance, which was subsequently determined via EIS in the addition of ferricyanide ions. The limit of detection was calculated to be 0.05 nM. In addition, the method validation was confirmed through the analysis of this electrochemical aptasensor for OTA analysis in beer samples with satisfactory recoveries.

It’s important to note that many government regulators and countries have set a very low level of maximum residue of OTA in food and agricultural products due to its serious toxicity to human and animals. The signal enhancement techniques are a requirement when using this technology. Rolling circle amplification (RCA) technique was used to improve the method sensitivity of electrochemical sensors. Huang et al. developed an electrochemical aptasensor for OTA determination on the basis of specific recognition of the aptamer and the signal amplification of RCA (Figure 1-5). The RCA primer was consisted of two DNA sequences, including the aptamer and the complementary DNA modified on surface of electrode. With OTA

presence, RCA was suppressed because of the recognition between OTA and its aptamer, leading to a decreasing signal of the electrochemical aptasensor. In optimized conditions, its detection limit was obtained at 0.065 pg mL^{-1}. In addition, the feasibility and practicability have been validated for OTA determination in wine samples (Huang et al., 2013).

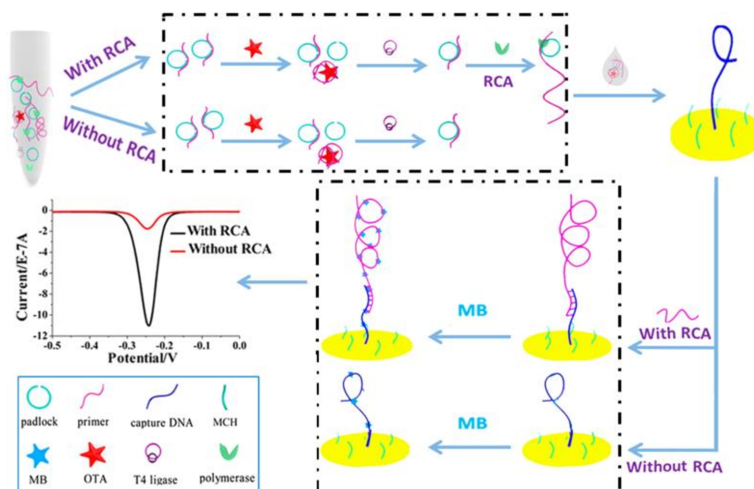

Figure 1-5: Principle diagram of electrochemical aptasensor for OTA analysis based on RCA signal amplification. Reprinted from Huang et al. (2013) with permission.

To improve detection sensitivity, a novel duplicate and signal enhanced electrochemical aptasensor was presented for OTA detection in red wine samples (Yang et al., 2014). In this research, four DNA sequences were designed and synthesized. DNA3-GNPs/DNA2/DNA1-Au were modified to surface of electrode via strand hybridization for the first signal enhancement in addition of methylene blue. Meanwhile, the DNA4, partial complementary with DNA3, offered a large amount of anchoring sites for MB due to its rich G bases. The duplicate signal amplification was thus obtained. A good electrochemical signal response was achieved against the concentrations of OTA in the 2.5 pM to 2.5 nM range, with detection limit of 0.75 pM.

However, the novel electrochemical aptasensors were also achieved via immobilization-free aptamers to the surface of electrode. The complementary DNA, labelled with MB, was adopted as the probe DNA of the electrochemical aptasensor. Since DNA contains negative charges, the hybridization duplex of aptamer and its complementary DNA was not diffused to the surface of negative charged electrode due to the repulsion reaction. Upon the presence of OTA, the conformational changes of the aptamer led to release of DNA probe subsequently digested by the exonuclease. As the DNA probe contains little negative charges, it spreads to surface of electrode, and the electrochemical signal is thus increased. On the other hand, the

target OTA was released for the next cycle to obtain the detection sensitivity. Based on the electrochemical method, an ultrasensitive aptasensor was introduced with detection limit of 0.004 ng mL^{-1} (Tan et al., 2015), demonstrating that this aptasensor approach provided a very potential analysis strategy for various contaminations. Meanwhile, Hao et al. reported an electrochemical aptasensor for OTA analysis via AuNPs functionalized iron oxide magnetic nanomaterials and graphene/AuNPs nanoparticles. A good linear response was obtained between the electrochemical aptasensor and the target OTA levels in the range of 0.2 pg mL^{-1}- 4 ng mL^{-1}. Its detection limit was established at 0.07 pg mL^{-1} (Hao et al., 2016). The proposed aptasensor offered a promising strategy for the analysis of mycotoxins for food safety.

In addition, chemiluminescence techniques were also widely employed for the food safety research owing to its simple, low cost and sensitive characteristics. Chemiluminescence resonance energy transfer (CRET) is a process of energy conversion between a chemiluminescent donor and an acceptor. Since no external light source was needed, low background signals were produced in the chemiluminescence methods. By taking advantages of chemiluminescence, Jo et al. introduced a chemiluminescent aptasensor for OTA determination in coffee. In this study, the specific aptamer to OTA was synthesized with the 5'-modification of DNAzyme and 3'-modification of dabcyl, which was used as the quencher of the CRET aptasensor. Upon the OTA and hemin addition, the complex produce of G-quadruplex/OTA led to the approaching of aptamer to the hemin, and CRET caused the quenching of the chemiluminescence signals between luminol and dabcyl. The chemiluminescence signals had a good response versus the OTA concentrations ranging from 0.1 to 100 ng mL^{-1}. Its detection limit was established to be 0.22 ng mL^{-1} (Jo et al., 2016).

Recently, a series of researches focused on the enhancement of methods detection sensitivity via application of exonuclease III (Exo III), which can effectively digest double-stranded DNA. The recycling amplification was therefore achieved without any specific recognition elements. A rapid and simple electrochemical aptasensor with high sensitivity was introduced for OTA determination in wheat samples. In this study, the specific aptamer was adopted as a molecule recognition sequence and Exo III was employed to achieve the recycling amplification. With the addition of target OTA, the recognition between aptamer and OTA resulted in the release of complementary DNA and the subsequent digestion by Exo III. A good linear response was detected between the electrochemical signals and OTA concentrations in the range of 0.001 to 0.5 ng mL^{-1} with a detection limit of 0.58 pg mL^{-1} (Liu et al., 2016). Based on the same exonuclease signal amplification technique, another research group developed an electrochemical aptasensor for sensitive detection of OTA in red wine. In this study, as shown in Figure 1-6, complementary DNA (cDNA) of the OTA aptamer was modified with methylene blue (MB). The aptamer was hybridized with cDNA to dsDNA complex.

In OTA presence, the aptamer/OTA complex was formed with G-quadruplex structure and the cDNA was then released from the dsDNA to the surface of the electrode. Subsequently, the aptamer was digested partially by RecJf exonuclease. Thus, the target OTA was released for the next cycle. Ultimately, an exonuclease signal amplification aptasensor was achieved with satisfied detection range (from 10 pg mL^{-1} to 10 ng mL^{-1}) and LOD (3 pg mL^{-1}) (Wang et al., 2019).

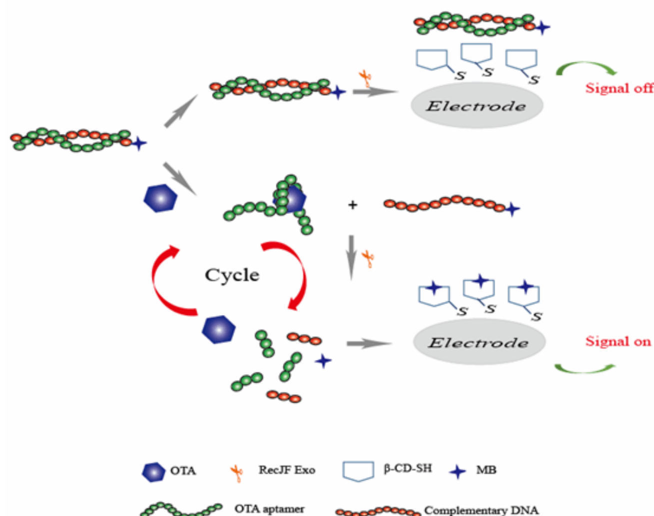

Figure 1-6: Sensing strategy of electrochemical aptasensor for detection of OTA based on exonuclease-assistant signal amplification.

4 Aptasensor for the analysis of aflatoxins

Aflatoxins (AFs), one of the most toxic mycotoxins, are secondary metabolites produced by a variety of molds which mainly include *Aspergillus flavus* and *Aspergillus parasiticus*. AFs occur massively in feeds and agricultural products, like peanuts, cereals and corn, as well as the tree nuts. Among the several kinds of AF (including B1, B2, M1, M2, G1, and G2), AFB1 and AFM1 are the most toxic ones and have been classified as group 1 carcinogenic compound by IARC (IARC, 2002). Therefore, many countries and organizations have established a maximum contamination level of these toxic mycotoxins for food safety (Commission, 2010). In 2012, the high affinity aptmer to AFB1 was first selected by Neoventures Biotechnology Inc. (Canada) (Patent: PCT/CA2010/001292). This aptamer specific to AFM1 was selected and characterized by Malhotra et al. (Malhotra et al., 2014). With the advantages of these aptamers for aflatoxins, aptamer-based biosensors were successfully developed for aflatoxins determination and have been studied in depth in the literature (table 1-5).

Table 1-5: Summary of aptasensor for aflatoxins analysis and fumonisins and zearalenone.

Mycotoxin	Method	Principle	Detection range	LOD	Sample	Reference
AFB1	Fluorescent aptasensor	CdTe quantum dots and graphene oxide	3.2 nM-320 µM	1.0 nM	Peanut oil	(Lu et al., 2014)
AFB1	Fluorescent aptasensor	nanographene oxide and nuclease	1.0-100 ng mL^{-1}	0.35 ng mL^{-1}	Corn	(Zhang et al., 2016)
AFB1	Colorimetric aptasensor	peroxidase mimicking DNAzyme activity	0.1-10000 ng mL^{-1}	0.1 ng mL^{-1}	Corn	(Seok et al., 2015)
AFB1	SERS aptasensor	electrochemical impedance spectroscopy and SERS	1×10^{-6}-1 ng mL^{-1}	0.4 fg mL^{-1}	Peanut	(Li et al., 2017)
AFB1	SERS aptasensor	magnetic-beads (CSFe3O4) as enrichment nanoprobe and AuNR@DNTB@Ag nanorods (ADANRs)	0.01-100 ng mL^{-1}	3.6 pg mL^{-1}	Peanut oil	(Chen et al., 2018)
AFM1	Electrochemical aptasensor	Fe3O4 incorporated polyaniline (Fe3O4/PANi) film	6-60 ng L^{-1}	1.98 ng L^{-1}	-	(Nguyen et al., 2013)
AFM1	Electrochemical aptasensor	carbon screen-printed electrode and ferri/ferrocyanide redox probe	2-150 ng L^{-1}	1.15 ng L^{-1}	Milk	(Istamboulie et al., 2016)
AFM1	Microring Resonators aptasensor	Silicon oxynitride (SiON) microring resonators	-	5 nM	-	(Chalyan et al., 2017)
AFM1	Fluorescent aptasensor	RT-qPCR amplification	1.0×10^{-4}-1.0 µg L^{-1}	0.03 ng L^{-1}	rice cereal, milk powder	(Guo et al., 2016)
AFM1	Fluorescent aptasensor	Graphene oxide (GO) and nuclease amplification	0.2-10 µg kg^{-1}	0.05 µg kg^{-1}	milk powder	(Guo et al., 2019)

Mycotoxin	Method	Principle	Detection range	LOD	Sample	Reference
FB1	Electrochemilum	gold nanoparticles (Au NPs) and ionic	0.5-50 ng mL^{-1}	0.27 ng	Wheat	(Zhao et al., 2014)

	inescence aptasensors	iridium complex		mL^{-1}	flour	
FB1	Microcantilever array aptasensor	array with self-assembled monolayers (SAMs) functionalized sensing cantilevers	0.1-40 μg mL^{-1}	33 ng mL^{-1}	-	(Chen et al., 2015)
ZEN	Fluorescent aptasensor	upconverting nanoparticles	0.05-100 μg L^{-1}	0.126 μg kg^{-1} / 0.007 μg L^{-1}	Corn/Beer	(Wu et al., 2017)

4.1 Aptasensor for AFB1

Simple fluorescent aptasensors have been reported for AFB1 determination based on the target-induced structure of switchable aptamer and high distance-dependent fluorescence quenching property of GO. First, Lu et al. reported a fluorescent aptasensor using quantum dots and GO for fluorescence quenching (Lu et al., 2014). A thiol-functionalized aptamer was modified at the CdTe quantum dots surface via ligand exchange. In the absence of AFB1, the fluorescence signal of the Q-dots was dramatically quenched by GO. Subsequently, fluorescence was recovered upon the presence of AFB1 ranging from 3.2 nM to 320 μM, its detection limit was 1.0 nM. Based on the similar principle, Zhang et al. introduced an amplified fluorescence aptasensor for AFB1 determination. The aptasensor adopted the ability of GO for aptamer protection from nuclease cleavage and used the nanometer size performance of GO to improve sensitivity. Three detection ranges (12.5 to 312.5 ng mL^{-1}, 5.0 to 50 ng mL^{-1}, and 1.0 to 100 ng mL^{-1}) were achieved with the detection limit of 10.0 ng mL^{-1}, 15.0 ng mL^{-1} and 0.35 ng mL^{-1}, respectively (Zhang et al., 2016). Furthermore, a colorimetric method was carried out for AFB1 determination using the aptamer and two split DNAzyme halves. A good linear response was achieved between the colorimetric signal and AFB1 concentrations ranging from 0.1 to 1 ng mL^{-1}, its detection limit was 0.054 ng mL^{-1}. This described aptasensor demonstrated high specificity for AFB1 and could be applied for AFB1 determination in corn (Seok et al., 2015).

Surface Enhance Raman Scattering (SERS) is an important fingerprint analytical technique, which is widely used to analyze a large amount of contaminated agricultural products. First, Li et al. reported an aptasensor for AFB1 determination based on SERS signal amplification (Li et al., 2017). In this study, under AFB1 addition, aptamer/AFB1 complex was formed, resulting in the dissociation of complementary DNA, which was as a signal enhancement element for the next cycle. Under the DNA hybridization, SERS tag was conjugated on the surface of AuNPs. A good linear response was achieved between the electrochemical signal and AFB1 levels in the range of 1×10^{-6} to 1 ng mL^{-1}, with detection limit of 0.4 fg mL^{-1}. Recently, based on the same SERS technique, an ultrasensitive aptasensor for AFB1 determination was introduced using CS-Fe_3O_4 nano-beads signal enrichment. Under the addition of AFB1, the release of SH-DNA2-ADANRs from the surface of CS-Fe_3O_4 was induced as a result of the competitively binding reaction between AFB1 and NH2-DNA1-CS-Fe_3O_4, the signal of SERS was thus decreased. The electrochemical signal was a linear relationship with AFB1. The concentrations ranged between 0.01 and 100 ng mL^{-1} with detection limit of 0.0036 ng mL^{-1} (Chen et al., 2018). The aptasensors based on the SERS technique took the advantages of greatly improving the detection stability and sensitivity.

Hybridization chain reaction (HCR), a signal amplification strategy, has been widely used to construct biosensors for targets determination. In 2019, Yao et al. developed a chemiluminescent aptasensor via HCR for the detection of AFB1 with

high sensitivity (Yao et al., 2019). In this design (Figure 1-7), the aptamer was hybridized with the probe to form dsDNA on the surface of magnetic beads (MBs). When AFB1 existed, the formation of aptamer/AFB1 complex led to the release of the probe on MBs after magnetic separation. Therefore, HCR signal enhancement and HRP catalysis were achieved with high AFB1 detection sensitivity, its detection limit was established at 0.2 ng mL^{-1}.

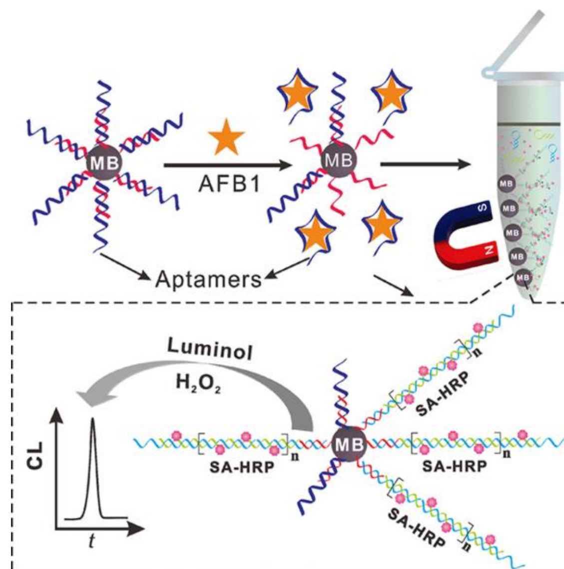

Figure 1-7: Schematic illustration of chemiluminescent aptasensor for AFB1 determination by using HCR signal amplification. Reprinted from Yao et al. (2019) with permission.

Based on the similar competitively binding of aptamer between target and cDNA, for more sensitive method to meet the requirement of low MRL, Wang et al. introduced a simple electrochemical aptasensor for AFB1 determination with high sensitivity (LOD = 2 nM). The aptamer was labelled with methylene blue (MB) and immobilized on gold electrode. In the presence of AFB1, the comformational change of aptamer resulted in the close of MB to the surface of the electrode, which caused the increase of the current (Figure 1-8). More importantly, a rapid aptasensor was achieved since only 5 min was required to complete the sample incubation (Wang et al., 2019). In order to develop more rapid even on-site sensing strategy for AFB1 determination, Xia et al. reported an ultrafast aptasensor for the detection of AFB1 based on enzyme-free signal amplification and special design of the aptamer (Xia et al., 2019). In this work, the aptamer was adopted as dual-terminal proximity structures with two fluorophores. These two fluorophores lighted up when the aptamer rapidly recognized with one molecule AFB1. This approach requires only 1 min to complete the analysis process, which was the fastest method for the AFB1 detection until now. Therefore, the proposed aptasensor has a promising application for on-site detection of mycotoxins.

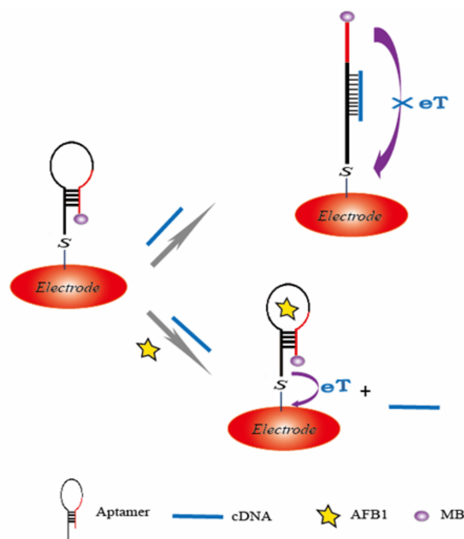

Figure 1-8: Sensing illustration of electrochemical aptasensor for detection of AFB1 based on the conformational change of aptamer.

4.2 Aptasensor for AFM1

The aptamer of AFM1 was first produced using SELEX technique, which was obtained from the institute of biotechnology, Vietnam academy of science and technology. Based on this specific aptamer, Nguyen et al. reported an electrochemical aptasensor for AFM1 determination, which combined the unique recognition ability of aptamers with the signal enhancement function of Fe_3O_4 magnetic nanomaterials. Under the condition of this amplification method, a detection limit (1.98 ng L^{-1}) was obtained in the detection range of 6-60 ng L^{-1}. However, the practical application of this aptasenor for matrices has not been validated; further studies are thus required on practical samples and broaden to a large number of mycotoxins for food safety (Nguyen et al., 2013). Nevertheless, an electrochemical impedance biosensor was described for AFM1 determination using the same specific aptamer, and this validation method in milk samples was investigated (Istamboulie et al., 2016). With the produce of aptamer/AFM1 complex, the electrochemical signal was enhanced with the increase of AFM1 concentrations ranging from 2 to 150 ng L^{-1} in buffer, and its detection limit was 1.15 ng L^{-1}. In addition, the current method was shown to detect AFM1 in a range comprise beteween 20 and 1000 ng kg^{-1} in milk, demonstrating that the developed aptasensor

was an effective sensing strategy for the analysis of AFM1. Then, optical biosensor based on microring resonators (MRR) have been also used for biomolecular analysis in food safety. A functionalized silicon oxynitride MRR based biosensor was reported for AFM1 determination. The binding of AFM1 on the functionalized microring surface led to the effective refractive index change, then the resonance shift in the MRR transmission was detected using off-chip silicon photodetectors. In addition, this approach was demonstrated for AFM1 determination specifically with a detection limit of 5 nM (Chalyan et al., 2017).

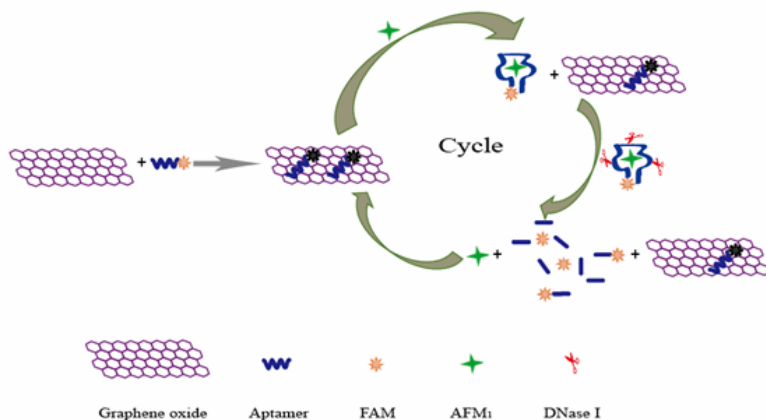

Figure 1-9: Schematic illustration of fluorescent aptasensor for detection of AFM1 by using graphene oxide signal amplification. Reprinted from Guo et al. (2019) with permission.

Recently, a new aptamer specific to AFM1 was selected by traditional SELEX technique with the dissociation constant of 35 nM (Malhotra et al., 2014). Based on the high-affinity aptamer, a real-time quantitative polymerase chain reaction (RT-qPCR) sensor was introduced for the detection of AFM1 in our research using target-induced strand displacement (Guo et al., 2016). In this detection method, the aptamer was adopted to recognize target AFM1 while its complementary ssDNA was used as template of RT-qPCR for signal amplification. A good linear response was observed between cycle threshold values and AFM1 levels in detection range from 1.0×10^{-4} to 1.0 ng L^{-1}, and its detection limit was 0.03 ng L^{-1}. However, complicated instruments and procedures were required in this aptasensor. In order to overcome these difficulties, a rapid fluorescent aptasensor was developed for AFM1 determination in further studies (Guo et al., 2019), which combined the advantages of fluorescence quenching ability of graphene oxide and target-cycled signal amplification induced by DNase I (Figure 1-9). In optimized conditions, this amplified aptasensor showed a good detection range of 0.2-10 µg kg^{-1} for AFM1, the detection limit was calculated at 0.05 µg kg^{-1}. More importantly, the feasible analysis of this method was successfully carried out in milk powder samples for the

detection of AFM1, this aptasensor is a potential good analytical tool for various mycotoxins for food safety.

5 Aptasensors for other mycotoxins

Fumonisin B1 (FB1), the most common one mycotoxin in cereals, could lead to serious threats such as cancer on human and animals. The aptamer specific to FB1 was first selected by the research team in Canada (McKeague et al., 2010). The dissociation constant was established to be 100 nM, after the selection and characteristic of the specific aptamer to FB1, an electrochemiluminescence (ECL) aptasensor was introduced for FB1 determination with good sensitivity and high accuracy via the unique recognition between FB1 and the aptamer and excellent conductivity of AuNPs. In this study, the ionic iridium (Ir) complex improves the ECL signal. Its detection limit was calculate at 0.27 ng mL^{-1} (Zhao et al., 2014).

Microcantilever method was used to construct biosensor for mycotoxins detection. Chen et al. provided an array sensor functionalized by self-assembled monolayers (SAMs) and the thiolated aptamer (Chen et al., 2015). Nonspecific response could cause the deflection. To prevent the interference from the environment, 6-mercapto-1-hexanol SAMs were adopted to modify reference cantilevers. The differential cantilevers signals showed a good linear response with FB1 levels in the range of 0.1-40 μg mL^{-1} and a detection limit of 33 ng mL^{-1}.

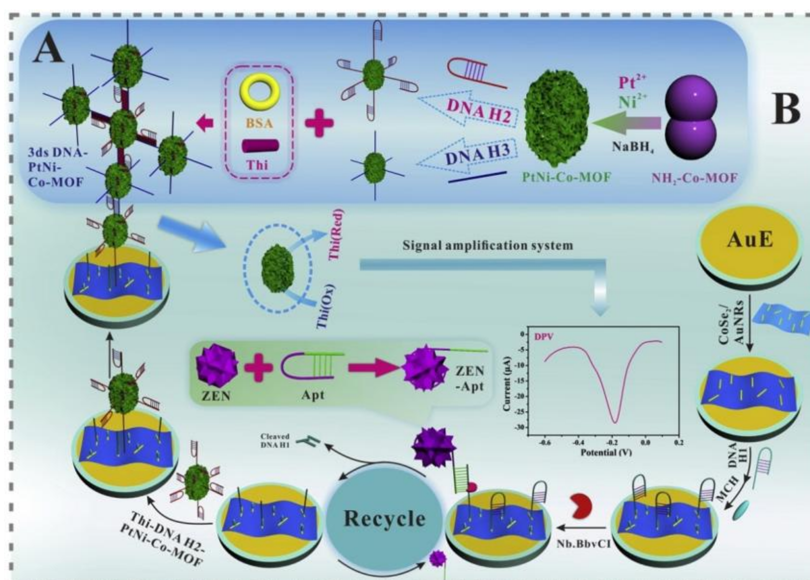

Figure 1-10: (A) Diagram of the construction of 3 ds DNA-PtNi@Co-MOF networks. (B) Schematic illustration of the proposed aptasensor for the detection of ZEN. Reprinted from He et al. (2020).

Zearalenone (ZEN) was one of the nonsteroidal estrogenic mycotoxins, ZEN and its metabolites were confirmed to have competitive reactions with estrogen receptors. Due to its widespread existence in corn, ZEN posed great hazards to human and animals and affected the quality of corn products (Yazar and Omurtag, 2008). The specific aptamer to ZEN was selected and characterized by Wang's research team (Chen et al., 2013). Up to now, there were few literatures on aptasensor for ZEN determination. Nevertheless, in 2017, a fluorescent aptasensor through upconversion nanoparticles was successfully developed for ZEN determination in corn and beer (Wu et al., 2017). The specific aptamer to ZEN was employed as a recognition probe while the complementary strand was adopted as a signal probe. Luminescence signal was achieved with a linear relationship versus the ZEN concentrations ranging from 0.05 to 100 μg L^{-1}. Its detection limit was determined at 0.126 μg kg^{-1} in corn and 0.007 μg L^{-1} for beer, demonstrating that the developed aptasensor offered a novel approach for the application analysis of ZEN for food safety. Very recently, an ultrasensitive electrochemical aptasensor was achieved for the detection of ZEN with higher sensitivity (LOD = 1.37 fg mL^{-1}). CoSe2/AuNRs, PtNi@Co-MOF and nicking enzyme were adopted to construct the sensing platform (Figure 1-10). Through this experimental design, significant signal amplification was obtained with good stability and specificity, which indicated this proposed aptasensor strategy offered a promising application to quantify even trace levels of ZEN for food safety (He et al., 2020).

6 Aptasensors for simultaneous multi-mycotoxins analysis

Hundreds of mycotoxins occur and are identified in food and agricultural products (Deng et al., 2013; Silvério et al., 2010), and the coexistence of multiple mycotoxins is a complex phenomenon. However, numerous articles published before are mainly aiming towards the single detection of mycotoxin. Therefore, the development of novel aptasensors for simultaneous analysis of multi-mycotoxins is very important in mycotoxins analysis for food safety.

Recently, a novel and rapid electrochemical aptasensor for simultaneous analysis two mycotoxins (OTA and FB1) was developed via the specific recognition of aptamer and targets (Wang et al., 2017). In this work, CdTe and PbS QDs were acted as the marks while these complementary DNA sequences were employed as the interaction probes. Upon the addition of target OTA and FB1, the interaction of aptamer and targets caused the release of QDs. After magnetic separation, good linear responses were determined between the electrochemical signals and target concentrations in dynamic range of 0.01-10 ng mL^{-1} for OTA and 0.05-50 ng mL^{-1} for FB1, respectively. In addition, this electrochemical aptasensor method has been

successfully used for these two targets in maize samples. Therefore, the proposed method offered a promising strategy for multiple mycotoxin analysis for food safety.

Moreover, in addition to quantum dots (QDs) labelled electrochemical method, fluorescence aptasensors based on photonic crystal microsphere suspension technique could also apply to the multiplex mycotoxin detection. Yang et al. introduced a potential fluorescent aptasensor for high-throughput detection of multiple mycotoxins via PHCM suspension array. In target mycotoxins addition, the conformation of aptamer/target complex resulted in changes of fluorescent signals, which had a linear response with targets levels in same range of 0.1 pg mL^{-1} to 0.1 ng mL^{-1} for OTA and AFB1, and 0.1 to 10 ng mL^{-1} for FB1. The limits of detection were established to be 3.96 fg mL^{-1} for OTA, 15.96 fg mL^{-1} for AFB1 and 11.04 pg mL^{-1} for FB1 (Yang et al., 2017).

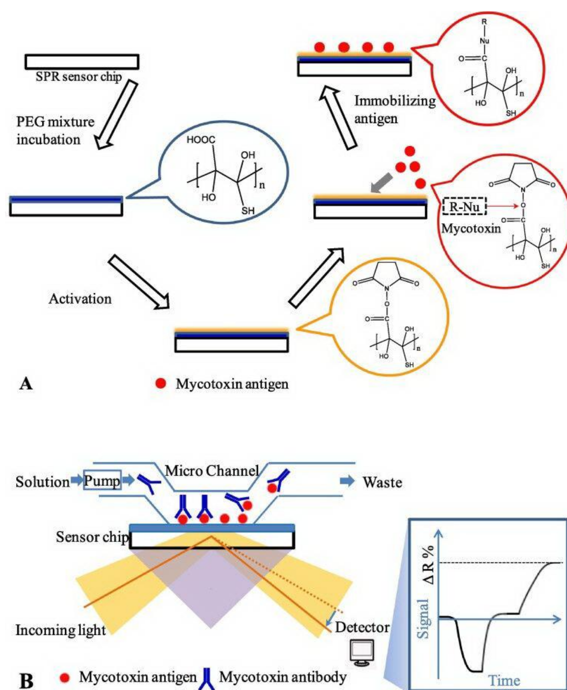

Figure 1-11: (A) Principle diagram of SPR aptasensor platform. (B) Principle diagram of sensor chip and optical setup in SPR. Reprinted from Wei et al. (2019) with permission.

In addition, DNA-scaffolded silver nanocluster and magnetic separation could also be used for simultaneous determination of multi-mycotoxins. Zhang et al, introduced a highly sensitive aptasensor for simultaneous analysis of AFB1 and OTA. In this design, two ssDNA were hybridized with two aptamers specific to

these two mycotoxins, which were employed for the synthesis of silver nanocluster. Upon the addition of OTA and AFB1, the release of these two probes was induced owing to the structure change of aptamers. After magnetic separation and the subsequent synthesis of silver nanocluster, the significantly increased fluorescence intensity was achieved to be a strong linear relationship for both these two mycotoxins in same range of 0.001-0.05 ng mL^{-1}, and its detection limit of AFB1 and OTA were established at 0.3 pg mL^{-1} and 0.2 pg mL^{-1}, respectively. Moreover, the practical application of this fluorescence aptasensor was investigated via the analysis of real cereal samples, demonstrating that the proposed approach might represent a potential sensing strategy for multiple mycotoxins determination (Zhang et al., 2016).

Finally, surface plasmon resonance (SPR), an important label-free analytical strategy, could be applied for simultaneously sensitive detection of multiple targets with superiorities such as good specificity, real-time monitoring, and high throughput detection (Patel et al., 2017). More recently, Wei et al. developed a SPR-based aptasensor chip for simultaneous determination of four mycotoxins with low crossreactivity (Figure 1-11), which has been successfully used for practical analysis in wheat and corn. The detection limit for AFB1, OTA, ZEN and deoxynivalenol (DON) were established at 0.59 ng mL^{-1}, 1.27 ng mL^{-1}, 7.07 ng mL^{-1} and 3.26 ng mL^{-1}, respectively (Wei et al., 2019).

7 Microbial volatile organic compounds (mVOCs)-based biosensors for potential application of mycotoxins determination

A new approach recently emerged for the development of a non-destructive method to analyze mycotoxin contaminations. It is well known that the majority of mycotoxins are non-volatile compounds; nevertheless fungi are notably referenced for emitting volatile organic compounds named microbial volatile organic compounds (mVOCs) (Bennett et al., 2015; Lemfack et al., 2018). The mVOCs profile depends on a lot of environmental factor such as the substrate and the temperature (Gao et al., 2002; Misztal et al., 2018).

The challenge is thus to identify and quantify specific m-VOCS emitted by the fungi in a concomitant way to the mycotoxin production and establish a clear correlation between both phenomena (Gao et al., 2002). Once this challenge is overcome, it paves the way to the development of a specific and sensitive VOC sensor adapted to the indirect detection of contamination by mycotoxins. Molecular imprinting polymer technologies are potential opportunities to reach the specificity and sensibility required in such an application (Wang et al., 2017; Stahl et al., 2017; Zhang et al., 2017). The main advantages of a VOCs sensor technology for mycotoxin contamination detection are the reduced processing time and the non-destructive side as no sample preparation is required.

8 Challenges and limitations of aptasensors

Although those promising advantages of rapid, portable, low-cost, specific and sensitive characteristics. Nucleic acid aptamer-based biosensors still have the following key scientific limitations and challenges, (i) as small molecules, the screening of nucleic acid aptamers specific to mycotoxins is a complex and time-consuming process. There are many factors that influence aptamer selection efficiency. Therefore, the successful selection of aptamer is difficult. Nowadays, only a few nucleic acid aptamers against mycotoxins such as OTA (Cruz-Aguado and Penner, 2008), AFB1 (Patent: PCT/CA2010/001292) and fumonisin B1 (McKeague et al., 2010) were successfully screened and applied, and the aptamer of ZEN (Chen et al., 2014) was screened in 2013 and almost had not yet applied for practical analysis. There are hundreds of mycotoxins have been identified, demonstrating that the available aptamers are far from meeting the requirement of mycotoxin detection. (ii) Some mycotoxins have various structural analogs, such as aflatoxin, which contains B1, B2, M1, M2, G1, G2 et al. It is more difficult to select the high specific aptamers for each analogue. How to identify and distinguish similar mycotoxins is also important. Therefore, the bottleneck problem on the development of mycotoxin aptasensors is how to identify and detect multiple mycotoxins using the limited variety of mycotoxin aptamers, especially for structurally similar mycotoxins.

9 Conclusions and future trends

In this review paper, it was demonstrated that a series of aptasensors were successfully used for determination of small molecules mycotoxins. Current reports illustrated here indicated the high promising benefits of the aptamer-based biosensors for mycotoxins determination owing to their rapid and sensitive analysis in foods and agricultural products. In comparison with traditional instrumental approaches including HPLC with fluorescence detection (FLD) and HPLC-MS, aptasensor techniques take the advantages of rapid, portable, low-cost targets analysis, as well as the potential field determination and high-throughput identification of multiple mycotoxins. In addition, taking mycotoxins hazard into consideration, aptasensor strategies provides high sensitivity and selectivity for mycotoxins determination to meet the requirement of maximum limits set by many countries and regulators.

There are hundreds of mycotoxins occurring in food and agricultural commodities, and the coexistence of multiple mycotoxins is a very common phenomenon. However, numerous researches in the present literatures focus on the single analysis of OTA and AF detection. Therefore, future research will be required for the successful selection of more specific aptamers against other mycotoxins and develop novel aptasensors and sensor array for multi-mycotoxin analysis. In the future, aptamer-based biosensors will greatly contribute to the safety control in food

and agricultural products.

Author contributions

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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References

Abia, W. A., Warth, B., Sulyok, M., Krska, R., Tchana, A. N., Njobeh, P. B., et al. (2013). Determination of multi-mycotoxin occurrence in cereals, nuts and their products in Cameroon by liquid chromatography tandem mass spectrometry (LC-MS/MS). *Food Control*. 31, 438–453. doi: 10.1016/j.foodcont.2012.10.006

Anfossi, L., Di Nardo, F., Giovannoli, C., Passini, C., Baggiani, C. (2015). Enzyme immunoassay for monitoring aflatoxins in eggs. *Food Control*. 57, 115–121. doi:10.1016/j.foodcont.2015.04.013

Anfossi, L., Giovannoli, C., and Baggiani, C. (2016). Mycotoxin detection. *Current Opinion in Biotechnology*. 37, 120–126.

Atar, N., Eren, T., and Yola, M. L. (2015). A molecular imprinted SPR biosensor for sensitive determination of citrinin in red yeast rice. *Food Chem*. 184, 7–11. doi: 10.1016/j.foodchem.2015.03.065

Bacher, G., Pal, S., Kanungo, L., Bhand, S. (2012). A label-free silver wire based impedimetric immunosensor for detection of aflatoxin M1 in milk. *Sensor Actuat B-Chem*. 168, 223–230. doi:10.1016/j.snb.2012.04.012

Bock, L.C., Grifn, L.C., Latham, J.A., Vermaas, E.H., Toole, J.J., 1992. Selection of single-stranded DNA molecules that bind and inhibit human thrombin. Nature 355, 564–566.

Castillo, G., Spinella, K., Poturnayová, A., Šnejdárková, M., Mosiello, L., and Hianik, T. (2015). Detection of aflatoxin B1 by aptamer-based biosensor using PAMAM dendrimers as immobilization platform. *Food Control*. 52, 9–18. doi: 10.1016/j.foodcont.2014.12.008

Chalyan, T., Pasquardini, L., Gandolfi, D., Guider, R., Samusenko, A., Zanetti, M., et al.

(2017). Aptamer-and Fab'-Functionalized Microring Resonators for Aflatoxin M1 Detection. *IEEE J Sel Top Quant Electron.* 23, 1-8. doi: 10.1109/JSTQE.2016.2609100

Chen, J., Fang, Z., Liu, J., and Zeng, L. (2012). A simple and rapid biosensor for ochratoxin A based on a structure-switching signaling aptamer. *Food Control.* 25, 555-560. doi: 10.1016/j.foodcont.2011.11.039

Chen, J., Zhang, X., Cai, S., Wu, D., Chen, M., Wang, S., et al. (2014). A fluorescent aptasensor based on DNA-scaffolded silver-nanocluster for ochratoxin A detection. *Biosens. Bioelectron.* 57, 226-231. doi: 10.1016/j.bios.2014.02.001

Chen, Q., Yang, M., Yang, X., Li, H., Guo, Z., and Rahma, M. H. (2018). A large Raman scattering cross-section molecular embedded SERS aptasensor for ultrasensitive Aflatoxin B1 detection using CS-Fe3O4 for signal enrichment. *Spectrochim Acta A Mol Biomol Spectrosc.* 189, 147-153. doi: 10.1016/j.saa.2017.08.029

Chen, R., Ma, F., Li, P. W., Zhang, W., Ding, X. X., Zhang, Q., et al. (2014). Effect of ozone on aflatoxins detoxification and nutritional quality of peanuts. *Food Chem.* 146, 284–288.

Chen, X., Huang, Y., Duan, N., Wu, S., Ma, X., Xia, Y., et al. (2013). Selection and identification of ssDNA aptamers recognizing zearalenone. *Anal Bioanal Chem.* 405, 6573-6581. doi: 10.1007/s00216-013-7085-9

Chen, X., Bai, X., Li, H., and Zhang, B. (2015). Aptamer-based microcantilever array biosensor for detection of fumonisin B-1. *RSC Adv.* 5, 35448-35452. doi: 10.1039/C5RA04278J

Cigic, I.K., Prosen, H. (2009). An Overview of Conventional and Emerging Analytical Methods for the Determination of Mycotoxins. *International Journal of Molecular Sciences*. 10, 62–115.

Commission Regulation (EC). (2006). Setting maximum levels for certain contaminants in foodstuffs. *Off J Eur Union*. 364, 5–24.

Commission, T. E. (2010). Commission Regulation (EU) No 165/2010 of 26 February 2010 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. *Off J Eur Union*. L50/58-L50/12.

Corcuera, L. A., Ibáñez-Vea, M., Vettorazzi, A., González-Peñas, E., and Cerain, A. L. D. (2011). Validation of a UHPLC-FLD analytical method for the simultaneous quantification of aflatoxin B1 and ochratoxin a in rat plasma, liver and kidney. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 879, 2733–2740. doi: 10.1016/j.jchromb.2011.07.039

Cruz-Aguado, J. A., and Penner, G. (2008). Determination of ochratoxin A with a DNA aptamer. *J Agric Food Chem.* 56, 10456-10461. doi: 10.1021/jf801957h

Darmostuk, M., Rimpelova, S., Gbelcova, H., Ruml, T., 2015. Current approaches in SELEX: an update to aptamer selection technology. Biotechnol. Adv. 33, 1141–1161.

Dave, N., Chan, M. Y., Huang, P.-J. J., Smith, B. D., and Liu, J. (2010). Regenerable DNA-Functionalized Hydrogels for Ultrasensitive, Instrument-Free Mercury(II) Detection and Removal in Water. *J. Am. Chem. Soc.* 132, 12668-12673. doi: 10.1021/ja106098j

Deng, C. Y., Chen, J. H., Nie, Z., Wang, M. D., Chu, X. C., Chen, X. L., et al. (2009). Impedimetric aptasensor with femtomolar sensitivity based on the enlargement of surface-charged gold nanoparticles. *Anal. Chem.* 81, 739–745. doi: 10.1021/ac800958a

Deng, G., Xu, K., Sun, Y., Chen, Y., Zheng, T., and Li, J. (2013). High Sensitive Immunoassay for Multiplex Mycotoxin Detection with Photonic Crystal Microsphere Suspension Array. *Anal. Chem.* 85, 2833-2840. doi: 10.1021/ac3033728

Ellington, A.D., Szostak, J.W. (1990). In vitro selection of RNA molecules that bind specifc ligands. *Nature*. 346, 818–822. doi: 10.1038/346818a0

Evtugyn, G., Porfireva, A., Sitdikov, R., Evtugyn, V., Stoikov, I., Antipin, I., et al. (2013). Electrochemical Aptasensor for the Determination of Ochratoxin A at the Au Electrode Modified with Ag Nanoparticles Decorated with Macrocyclic Ligand. *Electroanal.* 25, 1847–1854. doi: 10.1002/elan.201300164

FAO, Worldwide regulations for mycotoxins in food and feed in 2003. Rome, Italy: FAO. Food and nutrition paper 81, 2004.

Gao, P., Martin, J., (2002). Volatile metabolites produced by three strains of Stachybotrys chartarum cultivated on rice and gypsum board. *Appl. Occup. Environ. Hyg*. 17 (6), 430-436.

Gao, P., Korley, F., Martin, J., et al. (2002). Determination of Unique Microbial Volatile Organic Compounds Produced by Five Aspergillus Species Commonly Found in Problem Buildings. *AIHA Journal*. 63, 135–140.

Guo, W., Qi, X. J., Orbach, R., Lu, C. H., and Willner, I. (2014). Reversible Ag^{+}-crosslinked DNA hydrogels. *Chem. Commun.* 50, 4065-4068. doi: 10.1039/c3cc49140d

Guo, X., Wen, F., Zheng, N., Luo, Q., Wang, H., Wang, H., Li, S., Wang, J. (2014). Development of an ultrasensitive aptasensor for the detection of aflatoxin B1. *Biosens. Bioelectron*. 56, 340–344. doi: 10.1016/j.bios.2014.01.045

Guo, X., Wen, F., Zheng, N., Li, S., Fauconnier, M.-L., and Wang, J. (2016). A qPCR aptasensor for sensitive detection of aflatoxin M1. *Anal Bioanal Chem.* 408, 5577-5584. doi: 10.1007/s00216-016-9656-z

Guo, X., Wen, F., Qiao, Q., Zheng, N., Saive, M., Fauconnier, M. L., et al. (2019). A Novel Graphene Oxide-Based Aptasensor for Amplified Fluorescent Detection of Aflatoxin M1 in Milk Powder. *Sensors (Basel).* 19. 3840. doi: 10.3390/s19183840

Guo, Z., Ren, J., Wang, J., and Wang, E. (2011). Single-walled carbon nanotubes based quenching of free FAM-aptamer for selective determination of ochratoxin A. *Talanta*. 85, 2517–2521. doi: 10.1016/j.talanta.2011.08.015

Hao, N., Jiang, L., Qian, J., and Wang, K. (2016). Ultrasensitive electrochemical Ochratoxin A aptasensor based on CdTe quantum dots functionalized graphene/Au

nanocomposites and magnetic separation. *J Electroanal Chem.* 781, 332-338. doi: 10.1016/j.jelechem.2016.09.053

Hayat, A., Mishra, R. K., Catanante, G., and Marty, J. L. (2015). Development of an aptasensor based on a fluorescent particles-modified aptamer for ochratoxin A detection. *Anal Bioanal Chem.* 407, 7815-7822. doi: 10.1007/s00216-015-8952-3

He, B., Yan, X. (2020). Ultrasensitive electrochemical aptasensor based on CoSe2/AuNRs and 3D structured DNA-PtNi@Co-MOF networks for the detection of zearalenone. Sensor Actuat B-Chem. 306, 127558. doi.org/10.1016/j.snb.2019.127558

Herzallah, S. M. (2009). Determination of aflatoxins in eggs, milk, meat and meat products using HPLC fluorescent and UV detectors. *Food Chem*. 114, 1141–1146. doi: 10.1016/j.foodchem.2008.10.077

Huang, L., Wu, J., Zheng, L., Qian, H., Xue, F., Wu, Y., et al. (2013). Rolling chain amplification based signal-enhanced electrochemical aptasensor for ultrasensitive detection of ochratoxin A. *Anal. Chem.* 85, 10842-10849. doi: 10.1021/ac402228n

International Agency for Research on Cancer (IARC). (1993). Some naturally occurring substances: Food items and constituents heterocyclic aromatic amines and mycotoxins. IARC monographs on the evaluation of carcinogenic risk to humans. 56, 489–551.

International Agency for Research on Cancer (IARC). (2002). Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. 82, 171–230.

Iqbal, S. Z., Rabbani, T., Asi, M. R., and Jinap, S. (2014). Assessment of aflatoxins, ochratoxin A and zearalenone in breakfast cereals. *Food Chem*. 157, 257–262.

Istamboulie, G., Paniel, N., Zara, L., Granados, L. R., Barthelmebs, L., Noguer, T. (2016). Development of an impedimetric aptasensor for the determination of aflatoxin M1 in milk. *Talanta*. 146, 464–469. doi: 10.1016/j.talanta.2015.09.012

Jo, E. J., Mun, H., Kim, S. J., Shim, W. B., and Kim, M. G. (2016). Detection of ochratoxin A (OTA) in coffee using chemiluminescence resonance energy transfer (CRET) aptasensor. *Food Chem.* 194, 1102-1107. doi: 10.1016/j.foodchem.2015.07.152

Kav. K., Col, R., Kaan, T. K. (2011). Detection of aflatoxin M1 levels by ELISA in white-brined Urfa cheese consumed in Turkey. *Food Control*. 22, 1883–1886. doi:10.1016/j.foodcont.2011.04.030

Kuang, H., Chen, W., Xu, D., Xu, L., Zhu, Y., Liu, L., et al. (2010). Fabricated aptamer-based electrochemical ''signal-off'' sensor of ochratoxin A. *Biosens. Bioelectron*. 26, 710–716. doi: 10.1016/j.bios.2010.06.058

Lee, D., Lee, K. G. (2015). Analysis of aflatoxin M1 and M2 in commercial dairy products using high-performance liquid chromatography with a fluorescence detector. *Food Control*. 50, 467–471. doi:10. 1016/j.foodcont.2014.09.020

Li, P., Zhang, Q., Zhang, W., Zhang, J., Chen, X., Jiang, J., et al. (2009). Development of a class-specific monoclonal antibody-based ELISA for aflatoxins in peanut. *Food*

Chem. 115, 313–317. doi:10.1016/j.foodchem.2008.11.052

Li, Q., Lu, Z., Tan, X., Xiao, X., Wang, P., Wu, L., et al. (2017). Ultrasensitive detection of aflatoxin B1 by SERS aptasensor based on exonuclease-assisted recycling amplification. *Biosens. Bioelectron.* 97, 59-64. doi: 10.1016/j.bios.2017.05.031

Lin, H., Zou, Y., Huang, Y., Chen, J., Zhang, W. Y., Zhuang, Z., et al. (2011). DNAzyme crosslinked hydrogel: a new platform for visual detection of metal ions. *Chem. Commun.* 47, 9312. doi: 10.1039/c1cc12290h

Liu, C., Guo, Y., Luo, F., Rao, P., Fu, C., and Wang, S. (2016). Homogeneous Electrochemical Method for Ochratoxin A Determination Based on Target Triggered Aptamer Hairpin Switch and Exonuclease III-Assisted Recycling Amplification. *Food Anal Method.* 10, 1982-1990. doi: 10.1007/s12161-016-0771-4

Liu, R., Huang, Y., Ma, Y., Jia, S., Gao, M., Li, J., et al. (2015). Design and synthesis of target-responsive aptamer-cross-linked hydrogel for visual quantitative detection of ochratoxin A. *ACS Appl Mater Inter.* 7, 6982-6990. doi: 10.1021/acsami.5b01120

Lu, Z., Chen, X., Wang, Y., Zheng, X., and Li, C. M. (2014). Aptamer based fluorescence recovery assay for aflatoxin B1 using a quencher system composed of quantum dots and graphene oxide. *Microchim Acta.* 182, 571-578. doi: 10.1007/s00604-014-1360-0

Luan, Y., Chen, J., Li, C., Xie, G., Fu, H., Ma, Z., et al. (2015). Highly Sensitive Colorimetric Detection of Ochratoxin A by a Label-Free Aptamer and Gold Nanoparticles. *Toxins (Basel).* 7, 5377-5385. doi:10.3390/toxins7124883

Lv, L., Cui, C., Liang, C., Quan, W., Wang, S., and Guo, Z. (2016). Aptamer-based single-walled carbon nanohorn sensors for ochratoxin A detection. *Food Control.* 60, 296-301. doi: 10.1016/j.foodcont.2015.08.002

Joan W. Bennett, and Arati A. Inamdar, 2015, Are Some Fungal Volatile Organic Compounds (VOCs) Mycotoxins? Toxins, 7, 3785-3804.

Malhotra, S., Pandey, A. K., Rajput, Y. S., Sharma, R. (2014). Selection of aptamers for aflatoxin M1 and their characterization. *J Mol Recognit*. 27, 493–500. doi: 10.1002/jmr.2370

Mao, J., Lei, S., Liu, Y., Xiao, D., Fu, C., Zhong, L., et al. (2015). Quantification of aflatoxin M1 in raw milk by a core-shell column on a conventional HPLC with large volume injection and step gradient elution. *Food Control*. 51, 156–162. doi:10.1016/j.foodcont.2014.11.022

Marie C. Lemfack, Bjoern-Oliver Gohlke, Serge M. T. Toguem, Saskia Preissner, Birgit Piechulla and Robert Preissner, 2018, mVOC 2.0: a database of microbial volatiles, Nucleic Acids Research, 46, Database issue.

Mata, A. T., Ferreira, J. P., Oliveira, B. R., Batoreu, M. C., Barreto Crespo, M. T., Pereira, V. J., et al. (2015). Bottled water: analysis of mycotoxins by LC-MS/MS. *Food Chem*. 176, 455–464. doi: 10.1016/j.foodchem.2014.12.088

McKeague, M., Bradley, C. R., De Girolamo, A., Visconti, A., Miller, J. D., and Derosa, M. C. (2010). Screening and initial binding assessment of fumonisin B1 aptamers. *Int J Mol Sci.* 11, 4864-4881. doi: 10.3390/ijms11124864

Misztal, Pawel K ; Lymperopoulou, Despoina S ; Adams, Rachel I ; Scott, Russell A ; Lindow, Steven E ; Bruns, Thomas ; Taylor, John W ; Uehling, Jessie ; Bonito, Gregory ; Vilgalys, Rytas ; Goldstein, Allen H, 2018, Emission Factors of Microbial Volatile Organic Compounds from Environmental Bacteria and Fungi, Environmental science & technology, 52(15), 8272-8282.

Mozaffari Nejad, A. S., Sabouri Ghannad, M., and Kamkar, A. (2014). Determination of aflatoxin B1 levels in Iranian and Indian spices by ELISA method. *Toxin Rev*. 33, 151–154. doi.org/10.3109/15569543.2014.942319

Nguyen, B. H., Tran, L. D., Do, Q. P., Nguyen, H. L., Tran, N. H., and Nguyen, P. X. (2013). Label-free detection of aflatoxin M1 with electrochemical Fe3O4/polyaniline-based aptasensor. *Mater Sci Eng C Mater Biol Appl.* 33, 2229-2234. doi: 10.1016/j.msec.2013.01.044

O'Brien, E., Dietrich, D. R. (2005). Ochratoxin A: The Continuing Enigma. *Crit Rev Toxicol*. 35, 33-60. doi: 10.1080/10408440590905948

Park, J. H., Kim, Y. P., Kim, I. H. (2014). Rapid detection of aflatoxin B1 by a bifunctional protein crosslinker-based surface plasmon resonance biosensor. *Food Control*. 36, 183-190. doi: 10.1016/j.foodcont.2013.08.038

Parker, C. O., Tothill, I. E. (2009). Development of an electrochemical immunosensor for aflatoxin M1 in milk with focus on matrix interference. *Biosens. Bioelectron*. 24, 2452–2457. doi:10.1016/j. bios.2008.12.021

Patent:PCT/CA2010/001292,DNA Ligands for Aflatoxin and Zearalenone, NeoVentures Biotechnology Inch

Patel, K., Halevi, S., Melman, P., Schwartz, J., Cai, S., Singh, B. R. (2017). A novel surface plasmon resonance biosensor for the rapid detection of botulinum neurotoxins. *Biosensors*. 7, 32–47. doi-org-s.caas.cn/10.3390/bios7030032

Patolsky, F., Katz, E., Willner, I. (2002). Amplified DNA detection by electrogenerated biochemiluminescence and by the catalyzed precipitation of an insoluble product on electrodes in the presence of the doxorubicin intercalator. *Angew. Chem. Int. Ed*. 41, 3398–3402.doi:10.1002/1521-3773(20020916)41:18<3398::aid-anie3398>3.0.co;2-w

Pavlov, V., Xiao, Y., Shlyahovsky, B., Willner, I. (2004). Aptamer-Functionalized Au nanoparticles for the amplified optical detection of thrombin. *J. Am. Chem. Soc*. 126, 11768–11769. doi: 10.1021/ja046970u

Piermarini, S., Micheli, L., Ammida, N. H. S., Palleschi, G., Moscone, D., 2007. Electrochemical immunosensor array using a 96-well screen-printed microplate for aflatoxin B1 detection. *Biosens. Bioelectron*. 22, 1434. doi: 10.1016/j.bios.2006.06.029

Pietri, A., Fortunati, P., Mulazzi, A., Bertuzzi, T. (2016). Enzymeassisted extraction for the HPLC determination of aflatoxin M1 in cheese. *Food Chem*. 192, 235–241. doi:10.1016/j. foodchem.2015.07.006Sanzani, S. M., and Ippolito, A. (2014). Mycotoxin Contamination on Harvested Commodities and Innovative Strategies for

Their Detection and Control. *Acta Horticulturae*. 1053, 123–132.

Scott, P.M. (2012). Recent research on fumonisins: a review. *Food Additives and Contaminants*. 29, 242–248.

Selvaraj, J. N., Wang, Y., Zhou, L., Zhao, Y., Xing, F., Dai, X., and Liu, Y. (2015). Recent mycotoxin survey data and advanced mycotoxin detection techniques reported from China: a review. *Food Additives and Contaminants: Part A*. 32, 440–452.

Seok, Y., Byun, J. Y., Shim, W. B., and Kim, M. G. (2015). A structure-switchable aptasensor for aflatoxin B1 detection based on assembly of an aptamer/split DNAzyme. *Anal Chim Acta*. 886, 182–187. doi: 10.1016/j.aca.2015.05.041

Sheng, L., Ren, J., Miao, Y., Wang, J., and Wang, E. (2011). PVP-coated graphene oxide for selective determination of ochratoxin A via quenching fluorescence of free aptamer. *Biosens. Bioelectron.* 26, 3494-3499. doi: 10.1016/j.bios.2011.01.032

Sheng, Y. J., Eremin, S., Mi, T. J., Zhang, S. X., Shen, J. Z., and Wang, Z. H. (2014). The development of a fluorescence polarization immunoassay for aflatoxin detection. *Biomed Environ Sci*. 27, 126–129. doi: 10.3967/bes2014.027

Shim, W. B., Kim, M. J., Mun, H., and Kim, M. G. (2014a). An aptamer-based dipstick assay for the rapid and simple detection of aflatoxin B1. *Biosens. Bioelectron*. 62, 288–294. doi: 10.1016/j.bios.2014.06.059

Shim, W. B., Yang, Z. Y., Kim, J. S., Kim, J. Y., Kang, S. J., Woo, G. J., et al. (2007). Development of immunochromatography strip-test using nanocolloidal gold-antibody probe for the rapid detection of aflatoxin B1 in grain and feed samples. *J Microbiol Biotechn*. 17, 1629–1637. doi: 10.1016/j.mimet.2007.08.002

Silvério, A., Lopes, M. M., and Freitas, G. d. (2010). Determination of Fungal Contamination, Aflatoxin B1 and Ochratoxin A Content in Peanuts Available in Portuguese Market. *J Biotechn*. 150, 305-305. doi: 10.1016/j.jbiotec.2010.09.272

Soh, J. H., Lin, Y., Rana, S., Ying, J. Y., and Stevens, M. M. (2015). Colorimetric Detection of Small Molecules in Complex Matrixes via Target-Mediated Growth of Aptamer-Functionalized Gold Nanoparticles. *Anal. Chem.* 87, 7644-7652. doi: 10.1021/acs.analchem.5b00875

Stahl, U., Voigt, A., Dirschka, M., Barié, N., Richter, C., Waldbaur, A. et al. (2017), Long-Term Stability of Polymer-Coated Surface Transverse Wave Sensors for the Detection of Organic Solvent Vapors, *Sensors*, 17, 2529

Sun, L., Hu, N., Peng, J., Chen, L., and Weng, J. (2014). Ultrasensitive Detection of Mitochondrial DNA Mutation by Graphene Oxide/DNA Hydrogel Electrode. *Adv Funct Mater.* 24, 6905-6913. doi:10.1002/adfm.201402191

Tan, K.X., Pan, S., Jeevanandam, J., Danquah, M.K., 2019. Cardiovascular therapies utilizing targeted delivery of nanomedicines and aptamers. Int. J. Pharm. 558, 413–425.

Tan, Y., Wei, X., Zhang, Y., Wang, P., Qiu, B., Guo, L., et al. (2015). Exonuclease-Catalyzed Target Recycling Amplification and Immobilization-free Electrochemical Aptasensor. *Anal. Chem.* 87, 11826-11831. doi: 10.1021/acs.analchem.5b03314

Tuerk, C., Gold, L. (1990). Systematic evolution of ligands by exponential enrichment -Rna

ligands to bacteriophage-T4 DNA-polymerase. *Science*. 249, 505–510. doi: 10.1126/science.2200121

Var, I., Kabak, B., Gök, F. (2007). Survey of aflatoxin B1 in helva, a traditional Turkish food, by TLC. *Food Control*. 18, 59–62. doi: 10.1016/j.foodcont.2005.08.008

Vdovenko, M. M., Lu, C. C., Yu, F. Y., Sakharov, I. Y. (2014). Development of ultrasensitive direct chemiluminescent enzyme immunoassay for determination of aflatoxin M1 in milk. *Food Chem*. 158, 310–314. doi: 10.1016/j.foodchem.2014.02.128

Wang, B., Chen, Y., Wu, Y., Weng, B., Liu, Y., Lu, Z., et al. (2016). Aptamer induced assembly of fluorescent nitrogen-doped carbon dots on gold nanoparticles for sensitive detection of AFB1. *Biosens. Bioelectron*. 78, 23–30. doi: 10.1016/j.bios.2015.11.015

Wang, B., Wu, Y., Chen, Y., Weng, B., Xu, L., and Li, C. (2016). A highly sensitive aptasensor for OTA detection based on hybridization chain reaction and fluorescent perylene probe. *Biosens. Bioelectron.* 81, 125-130. doi: 10.1016/j.bios.2016.02.062

Wang, C, Li, Y., Zhao, Q. (2019). A signal-on electrochemical aptasensor for rapid detection of aflatoxin B1 based on competition with complementary DNA. *Biosens. Bioelectron*. 144, 111641. doi.org/10.1016/j.bios.2019.111641

Wang, C., Qian, J., An, K., Huang, X., Zhao, L., Liu, Q., et al. (2017). Magneto-controlled aptasensor for simultaneous electrochemical detection of dual mycotoxins in maize using metal sulfide quantum dots coated silica as labels. *Biosens. Bioelectron.* 89, 802-809. doi: 10.1016/j.bios.2016.10.010

Wang, C., Qian, J., Wang, K., Wang, K., Liu, Q., Dong, X., et al. (2015). Magnetic-fluorescent-targeting multifunctional aptasensorfor highly sensitive and one-step rapid detection of ochratoxin A. *Biosens. Bioelectron.* 68, 783-790. doi: 10.1016/j.bios.2015.02.008

Wang, C., Qian, J., Wang, K., Yang, X., Liu, Q., Hao, N., et al. (2016). Colorimetric aptasensing of ochratoxin A using Au@Fe3O4 nanoparticles as signal indicator and magnetic separator. *Biosens. Bioelectron.* 77, 1183-1191. doi: 10.1016/j.bios.2015.11.004

Wang, M., Wang, X., Moni, P., Liu, A., Kim, D., Jo, W., et al. (2017). CVD Polymers for Devices and Device Fabrication. *Adv. Mater*. 1604606.

Wang, T., Chen, C., Larcher, L.M., Barrero, R.A., Veedu, R.N., 2019. Three decades of nucleic acid aptamer technologies: lessons learned, progress and opportunities on aptamer development. Biotechnol. Adv. 37, 28–50.

Wang, Y., Liu, X., Xiao, C., Wang, Z., Wang, J., Xiao, H., et al. (2012). HPLC determination of aflatoxin M1 in liquid milk and milk powder using solid phase extraction on OASIS HLB. *Food Control*. 28, 131–134. doi:10.1016/j.foodcont.2012.04.037

Wang, Y., Ning, G., Wu, Y., Wu, S., Zeng, B., Liu, G., et al. (2019). Facile combination of beta-cyclodextrin host-guest recognition with exonuclease-assistant signal amplification for sensitive electrochemical assay of ochratoxin A. *Biosens.*

Bioelectron. 124–125, 82–88. doi.org/10.1016/j.bios.2018.10.007

Wang, Z., Duan, N., Hun, X., and Wu, S. (2010). Electrochemiluminescent aptamer biosensor for the determination of ochratoxin A at a gold-nanoparticles-modified gold electrode using N-(aminobutyl)-N-ethylisoluminol as a luminescent label. *Anal Bioanal Chem.* 398, 2125-2132. doi: 10.1007/s00216-010-4146-1

Warth, B., Sulyok, M., and Krska, R. (2013). LC-MS/MS-based multibiomarker approaches for the assessment of human exposure to mycotoxins. *Anal Bioanal Chem*. 405, 5687–5695. doi: 10.1007/s00216-013-7011-1

Wei, T., Ren, P., Huang, L., Ouyang, Z., Wang, Z., Kong, X., et al. (2019). Simultaneous detection of aflatoxin B1, ochratoxin A, zearalenone and deoxynivalenol in corn and wheat using surface plasmon resonance. *Food Chem*. 300, 125–176. doi.org/10.1016/j.foodchem.2019.125176

Weizmann, Y., Beissenhirtz, M. K., Cheglakov, Z., Nowarski, R., Kotler, M., Willner, I. (2006). A virus spotlighted by an autonomous DNA machine. *Angew. Chem. Int. Ed. Engl*. 45, 7384–7388. doi: 10.1002/ange.200602754

Wu, J., Chu, H., Mei, Z., Deng, Y., Xue, F., Zheng, L., et al. (2012). Ultrasensitive one-step rapid detection of ochratoxin A by the folding-based electrochemical aptasensor. *Anal Chim Acta.* 753, 27-31. doi: 10.1016/j.aca.2012.09.036

Wu, S., Duan, N., Li, X., Tan, G., Ma, X., Xia, Y., et al. (2013). Homogenous detection of fumonisin B1 with a molecular beacon based on fluorescence resonance energy transfer between NaYF4: Yb, Ho upconversion nanoparticles and gold nanoparticles. *Talanta*. 116, 611–618. doi: 10.1016/j.talanta.2013.07.016

Wu, S., Duan, N., Ma, X., Xia, Y., Wang, H., Wang, Z., et al. (2012). Multiplexed fluorescence resonance energy transfer aptasensor between upconversion nanoparticles and graphene oxide for the simultaneous determination of mycotoxins. *Anal. Chem*. 84. 6263–6270. doi: 10.1021/ac301534w

Wu, Z., Xu, E., Chughtai, M. F. J., et al. (2017). Highly sensitive fluorescence sensing of zearalenone using a novel aptasensor based on upconverting nanoparticles. *Food Chem*. 230, 673-680. doi: 10.1016/j.foodchem.2017.03.100

Xia, X., Wang, Y., Yang, H., Dong, Y., Zhang, K., Lu, Y., et al. (2019). Enzyme-free amplified and ultrafast detection of aflatoxin B1 using dualterminal proximity aptamer probes. *Food Chem*. 283, 32–38. doi.org/10.1016/j.foodchem.2018.12.117

Xiong, H.G., Yan, J., Cai, S.W., He, Q.F., Peng, D.X., Liu, Z., et al., 2019. Cancer protein biomarker discovery based on nucleic acid aptamers. Int. J. Biol. Macromol. 132.

Xu, W., Xiong, Y., Lai, W., Xu, Y., Li, C., and Xie, M. (2014). A homogeneous immunosensor for AFB1 detection based on FRET between different-sized quantum dots. *Biosens. Bioelectron*. 56, 144–150. doi: 10.1016/j.bios.2014.01.007

Yan, L., Zhu, Z., Zou, Y., Huang, Y., Liu, D., Jia, S., et al. (2013). Target-Responsive "Sweet" Hydrogel with Glucometer Readout for Portable and Quantitative Detection of Non-Glucose Targets. *J. Am. Chem. Soc.* 135, 3748-3751. doi: 10.1021/ja3114714

Yang, C., Lates, V., Prieto-Simón, B., Marty, J. L., and Yang, X. (2013). Rapid high

throughput analysis of ochratoxin A by the self-assembly of DNAzyme–aptamer conjugates in wine. *Talanta*. 116, 520–526. doi: org/10.1016/j.talanta.2013.07.011

Yang, C., Wang, Y., Marty, J. L., and Yang, X. (2011). Aptamer-based colorimetric biosensing of Ochratoxin A using unmodified gold nanoparticles indicator. *Biosens. Bioelectron.* 26, 2724-2727. doi:10.1016/j.bios.2010.09.032

Yang, L. T., Fung, C. W., Cho, E. J., Ellington, A. D. (2007). Real-Time rolling circle amplification for protein detection. *Anal. Chem*. 79, 3320–3329. doi: 10.1021/ac062186b

Yang, X., Qian, J., Jiang, L., Yan, Y., Wang, K., Liu, Q., et al. (2014). Ultrasensitive electrochemical aptasensor for ochratoxin A based on two-level cascaded signal amplification strategy. *Bioelectrochem.* 96, 7-13. doi: 10.1016/j.bioelechem.2013.11.006

Yang, Y., Li, W., Shen, P., Liu, R., Li, Y., Xu, J., et al. (2017). Aptamer fluorescence signal recovery screening for multiplex mycotoxins in cereal samples based on photonic crystal microsphere suspension array. *Sensor Actuat B-Chem.* 248, 351-358. doi: 10.1016/j.snb.2017.04.004

Yao, Y., Wang, H., Wang, X., Wang, X., Li, F. (2019). Development of a chemiluminescent aptasensor for ultrasensitive and selective detection of aflatoxin B1 in peanut and milk. *Talanta*. 201, 52–57. doi.org/10.1016/j.talanta.2019.03.109

Yazar, S., and Omurtag, G. Z. (2008). Fumonisins, Trichothecenes and Zearalenone in Cereals. *Int J Mol Sci.* 9, 2062. doi: 10.3390/ijms9112062

Yazdanpanah, H., Zarghi, A., Shafaati, A. R., Foroutan, S. M., Aboul-Fathi, F., Khoddam, A., et al. (2013). Analysis of aflatoxin B1 in Iranian foods using HPLC and a monolithic column and estimation of its dietary intake. *Iran J Pharm Res*. 12, 81–87. doi: 10.1016/j.jpba.2013.07.024

Zhang, J., Li, Z., Zhao, S., and Lu, Y. (2016). Size-dependent modulation of graphene oxide–aptamer interactions for an amplified fluorescence-based detection of aflatoxin B1 with a tunable dynamic range. *Analyst.* 141, 4029-4034. doi: 10.1039/c6an00368k

Zhang, J., Xia, Y.-K., Chen, M., Wu, D.-Z., Cai, S.-X., Liu, M.-M., et al. (2016). A fluorescent aptasensor based on DNA-scaffolded silver nanoclusters coupling with Zn(II)-ion signal-enhancement for simultaneous detection of OTA and AFB1. *Sensor Actuat B-Chem.* 235, 79-85. doi: 10.1016/j.snb.2016.05.061

Zhang, L., Lei, J., Liu, L., Li, C., and Ju, H. (2013). Self-Assembled DNA Hydrogel as Switchable Material for Aptamer-Based Fluorescent Detection of Protein. *Anal. Chem.* 85, 11077-11082. doi: 10.1021/ac4027725

Zhang, X., Li, C. R., Wang, W. C., Xue, J., Huang, Y. L., Yang, X. X., et al. (2016). A novel electrochemical immunosensor for highly sensitive detection of aflatoxin B1 in corn using single-walled carbon nanotubes/chitosan. *Food Chem*. 192, 197–202

Zhang, Y., Lai, B., Juhas, M. (2019). Recent Advances in Aptamer Discovery and

Applications. *Molecules*. 24, 941. doi:10.3390/molecules24050941

Zhang, Y., Zhang, J., and Liu, Q., (2017). Gas Sensors Based on Molecular Imprinting Technology. *Sensors*. 17, 1567

Zhao, Y., Luo, Y., Li, T., and Song, Q. (2014). Au NPs driven electrochemiluminescence aptasensors for sensitive detection of fumonisin B1. *RSC Adv.* 4, 57709-57714. doi: 10.1039/c4ra10350e

Zhu, R., Feussner, K., Wu, T., Yan, F., Karlovsky, P., and Zheng, X. (2015). Detoxification of mycotoxin patulin by the yeast Rhodosporidium paludigenum. *Food Chem*. 179, 1–5. doi: 10.1016/j.foodchem.2015.01.066

Zhu, Z., Wu, C. C., Liu, H. P., Zou, Y., Zhang, X. L., Kang, H. Z., et al. (2010). An Aptamer Cross-Linked Hydrogel as a Colorimetric Platform for Visual Detection. *Angew. Chem. Int. Ed. Engl.* 122, 1052-1056. doi: 10.1002/ange.200905570

2

Objectives and thesis structure

Given the great hazard of mycotoxins to human and animals, and the low permissible limits established by countries and organizations in the world, simple, rapid and low-cost aptasensors with high sensitivity and selectivity are vitally important. This thesis focus on the development of novel aptamers for the detection of mycotoxins. First of all, the previously reported aptasensor methods were summarized, discussed and analyzed. In this part, a review paper titled "Aptamer-based biosensor for the detection of mycotoxins" has been published in the journal of Frontiers in Chemistry. Secondly, by using RT-qPCR signal amplification technology and the specific recognition of aptamer to AFM1, a research paper titled "A qPCR aptasensor for sensitive detection of aflatoxin M1" has been published in the journal of Analytical and Bioanalytic Chemistry. Third, combined the graphene oxide fluorescence quenching ability with nuclease-induced target signal amplification strategy, a research paper titled "A Novel Graphene Oxide-Based Aptasensor for Amplified Fluorescent Detection of Aflatoxin M1 in Milk Powder" has been published in the journal of Sensors. In addition, the graphene oxide-based sensing platform was also used for the detection of fumonisin B1. A research paper titled "Graphene oxide driven fluorescent aptasensor for the detection of fumonisin B1" is to be submitted to the journal of agriculture and food chemistry. Finally, a general discussion, conclusion and future prospects was analyzed, discussed, and summarized.

The objectives of this thesis are: (1) to analyze and summarize the current research status of aptamer-based biosensors for the detection of mycotoxins for food safety (Chapter 1); (2) to develop sensitive and selective aptasensor for the detection of aflatoxin M1 via RT-qPCR signal amplification technology (Chapter 3); (3) to establish a novel aptasensor for rapid AFM1 determination based on graphene oxide and DNase I signal enhancement strategy (Chapter 4); (4) to develop a rapid and sensitive aptasensor for fluorescent detection of fumonisin B1 based on above signal enhancement platform (Chapter 5).

This thesis started with an general review on the current aptasensors for the detection of mycotoxins for food safety, we first discuss and analyze the aptasensors for mycotoxins determination in the period of the last eleven years and cover the literatures from the first report in 2008 until today. In addition, challenges and future trends for the selection of aptamers towards various mycotoxins and aptasensors for multi-mycotoxins analysis are summarized. Given the promising development and potential application of aptasensors, the future researches will witness the great practicability of aptamer-based biosensor for food safety field (Chapter 1).

Aflatoxin M1 (AFM1), one of the most important and toxic contaminants in dairy products, is a severe safety factor for human, especially for infants and children. Real-time quantitative polymerase chain reaction (RT-qPCR) based on fluorescence detection is an important, ultrasensitive and precise technique. The presence of AFM1 induced the release of complementary ssDNA because of forming an aptamer/AFM1 complex, leading to the reduction in the amount of PCR template and the increase in cycle numbers. Quantification of AFM1 has been achieved

according to the linear relationship between the change of the PCR amplification signal and AFM1 levels (Chapter 3).

A sensitive aptasensor based on real-time quantitative polymerase chain reaction (RT-qPCR) for AFM1 was developed. However, the preparation of the qPCR-based aptasensor requires a tedious and time-consuming process with long incubation periods in rigorous conditions. Therefore, there is a demand for aptasensors that could be applied for rapid and real-time analysis of AFM1. Graphene oxide (GO) could use as a fluorescent quencher and protect DNA aptamers from nuclease cleavage. The presence of AFM1 induced the release of the aptamer from the surface of GO because of the formation of AFM1/aptamer complex, which resulted in the cleavage of aptamer by DNase I and the release of AFM1 for a new cycle. Therefore, cycling signal amplification was achieved to improve detection sensitivity. A good linear relationship was measured between the change of the fluorescence intensity signal and AFM1 levels (Chapter 4).

Fumonisin B1 (FB1) is the most toxic and the most common mycotoxin among all fumonisins, which accounts for 70% in total fumonisins. a novel graphene oxide-based fluorescent aptasensor for the amplified detection of FB1 has been introduced. Upon the presence of FB1, the formation of FB1/aptamer complex led to the separation of aptamer from the surface of GO. The aptamer was then cleavaged by DNase I and release FB1, which was the target for the next cycle. As a consequence, the fluorescence signal was determined to be a linear relationship versus the concentration of FB1. More importantly, this sensing strategy was simple, rapid, easy to use, and low cost with high sensitivity and selectivity (Chapter 5).

Ultimately, all the results above are analyzed, summarized, and discussed. The conclusion and future prospects are introduced (Chapter 6).

3

A qPCR aptasensor for sensitive detection of aflatoxin M1

In this study, a new aptamer-based biosensor to sensitively and selectively detect AFM1 was developed, combining the advantages of strong recognition ability of the aptamer to AFM1 and excellent amplification efficiency of the RT-qPCR technique to improve sensitivity. Six complementary ssDNA fragments were designed to explore the binding sites between the specific aptamer and AFM1. The presence of AFM1 induced the release of complementary ssDNA because of forming an aptamer/AFM1 complex, leading to the reduction in the amount of PCR template and the increase in cycle numbers. Quantification of AFM1 has been achieved according to the linear relationship between the change of the PCR amplification signal and AFM1 levels.

From Xiaodong Guo, Fang Wen, Nan Zheng, Songli Li, Marie-Laure Fauconnier, Jiaqi Wang, A qPCR aptasensor for sensitive detection of aflatoxin M1, Analytical and Bioanalytical Chemistry, 2016, 408: 5577–5584. doi: 10.1007/s00216-016-9656-z.

Abstract: Aflatoxin M1 (AFM1), one of the most toxic mycotoxins, imposes serious health hazards. AFM1 had previously been classified as a group 2B carcinogen (IARC, 1993) and has been classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) (IARC, 2002). Determination of AFM1 thus plays an important role for quality control of food safety. In this work, a sensitive and reliable aptasensor was developed for the detection of AFM1. The immobilization of aptamer through a strong interaction with biotin–streptavidin was used as a molecular recognition element, and its complementary ssDNA was employed as the template for a real-time quantitative polymerase chain reaction (RT-qPCR) amplification. Under optimized assay conditions, a linear relationship (ranging from 1.0×10^{-4} to 1.0 μg L^{-1}) was achieved with a limit of detection (LOD) down to 0.03 ng L^{-1}. In addition, the aptasensor developed here exhibits high selectivity for AFM1 over other mycotoxins and small effects from cross-reaction with structural analogs. The method proposed here has been successfully applied to quantitative determination of AFM1 in infant rice cereal and infant milk powder samples. Results demonstrated that the current approach is potentially useful for food safety analysis, and it could be extended to a large number of targets.

Key words: aflatoxin M1; aptamer; RT-qPCR; food safety

1 Introduction

Aflatoxin M1 (AFM1), one of the most toxic contaminants in dairy products, is a metabolite produced by dairy cows as a result of being fed with feeds contaminated with Aflatoxin B1 (AFB1) (Pei et al., 2009; Anfossi et al., 2013; Liu et al., 2015). Once present in dairy products, AFM1 poses a hazard to humans (especially infants) who consume them (Mao et al., 2015). Taking this threat into consideration, many countries have set maximum limits for AFM1 and established various regulations (FAO, 2004; Hoyos Ossa et al., 2015). The European Union (EU) set a maximum tolerated level of AFM1 to 0.050 μg/kg for adult consumption and subsequently more restrictively to 0.025 μg/kg for food for infants and children (EC, 2006). In China and the United States, the maximum limit for AFM1 is 0.5 μg/kg in milk (Mao et al., 2015; Busman et al., 2015). Hence, the development of simple, sensitive and selective methods to determine the presence and level of AFM1 is much in demand for food safety organizations to implement regulatory requirements.

Approaches have been developed in recent years for quantitative determination of AFM1 including high-performance liquid chromatography (HPLC) with fluorescence detection (FLD) (Mao et al., 2015; Wang et al., 2012; Lee et al., 2015; Pietri et al., 2016), high-performance liquid chromatography (HPLC) combined with mass spectrometry (MS) (Hoyos Ossa et al., 2015; Beltrán et al., 2011; Wang et al., 2015). However, the quantitative approaches require complicated pretreatment, professional operators and expensive instruments. Some immunological methods like enzyme-linked immune sorbent assay (ELISA) (Li et al., 2009; Kav et al., 2011; Anfossi et al., 2015) and immunosensors (Parker et al., 2009; Bacher et al., 2012; Vdovenko et al., 2014) have also been reported for AFM1 detection. There are disadvantages in both preparation of the antibody and its stability and this limits its application in the field. Recently, application of aptamer-based biosensors for mycotoxins has shown a remarkable potential with the advantages of low cost, high stability, easy synthesis and ease of modification compared with antibodies. Since the report of an aptamer for ochratoxin A (OTA) in 2008 (Cruz-Aguado et al., 2008), many aptasensors for OTA and AFB1 have been developed for feed and food safety (Barthelmebs et al., 2011; Bonel et al., 2011; Guo et al., 2014; Shim et al., 2014; Wang et al., 2015; Zhao et al., 2013; De Girolamo et al., 2012). In our previous study (Guo et al., 2014), an aptasensor based on qPCR was successfully designed for AFB1 determination with high sensitivity. Aptasensors were developed for the detection of AFM1 using electrochemical methods and impedance spectroscopy techniques (Nguyen et al., 2013; Istamboulie et al., 2016). However, the selectivity of the aptamer for AFM1 was not clear since only irrelevant OTA was chosen as interference to study the cross-reaction. Cross-activity tests between other toxins (AFB1, AFB2, AFG1 and AFG2) should be carried out to answer the question whether the aptasensor is suitable for quantifying the AFM1 concentration in real samples (Nguyen et al., 2013). Recently, an aptamer specific to AFM1 with a dissociation constant (Kd) value of 35 nM has been reported (Malhotra et al., 2014). To the best of our knowledge, aptasensor based on this aptamer for AFM1 detection

have not been reported.

In this study, a new aptamer-based biosensor to sensitively and selectively detect AFM1 was developed, combining the advantages of strong recognition ability of the aptamer to AFM1 and excellent amplification efficiency of the RT-qPCR technique to improve sensitivity. Six complementary ssDNA fragments were designed to explore the binding sites between the specific aptamer and AFM1. The presence of AFM1 induced the release of complementary ssDNA because of forming an aptamer/AFM1 complex, leading to the reduction in the amount of PCR template and the increase in cycle numbers. Quantification of AFM1 has been achieved according to the linear relationship between the change of the PCR amplification signal and AFM1 levels.

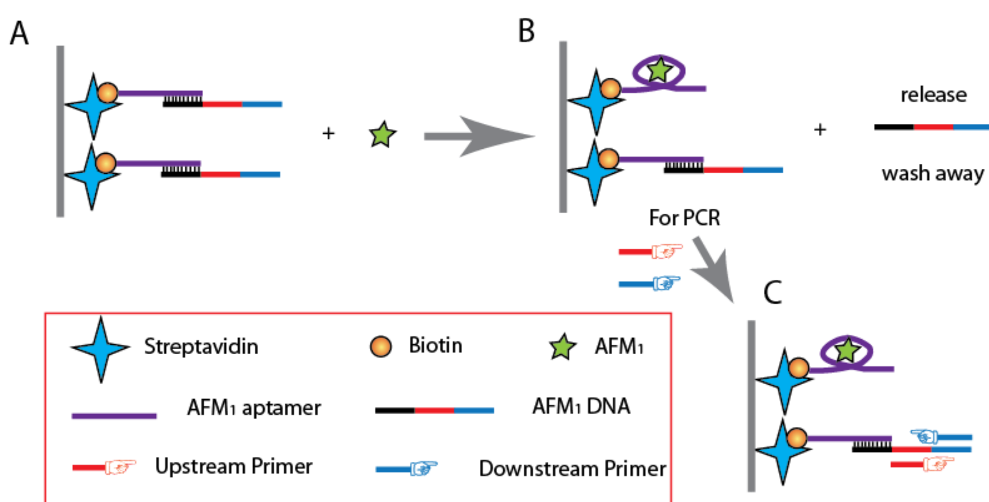


Figure 3-1: Schematic to illustrate the aptasensor for detection of Aflatoxin M_1

2 Materials and methods

2.1 Methods

The aim of this work was to develop an aptasensor for the detection of AFM1. The schematic of the sensing method is described in Figure 3-1. The aptasensor is based on conformational change of the aptamer owing to the formation of AFM1/aptamer complex and release of complementary ssDNA and signal amplification by RT-qPCR. Firstly, a strong interaction of biotin–streptavidin (Figure 3-1A) results in the immobilization of the aptamer on the surface of streptavidin-coated PCR tubes. The complementary ssDNA, as the amplification template of PCR, is partly hybridized with the single-strand aptamer to form dsDNA, which is stable in the absence of AFM1, resulting in no obvious changes of the amount of the complementary ssDNA as the template for RT-qPCR amplification. Upon the

addition of AFM1, a binding event between the aptamer and AFM1 (Figure 3-1B) induces a conformational change in the aptamer that leads to the release of the complementary ssDNA, with the result that the amount of template was reduced (Figure 3-1C). As a consequence, for the aptasensor, a strong signal change in PCR amplification was observed, which can be used for the quantification of the concentration of AFM1.

2.2 Materials and reagents

Aflatoxin M1 (AFM1) was purchased from Sigma-Aldrich (USA). Aflatoxin B1 (AFB1) was obtained from the National Standard Reference Center（Beijing, China). Ochratoxin A (OTA), zearalenone (ZEN), aflatoxin B2 (AFB2), and fumonisin (FB1) were purchased from Pribolab Co. Ltd (Singapore). Streptavidin was obtained from Sangon Biotechnology Co. Ltd. (Shanghai, China). Other chemicals such as sodium bicarbonate ($NaHCO_3$), anhydrous calcium chloride ($CaCl_2$), sodium carbonate (Na_2CO_3), ethylenediaminetetraacetic acid (EDTA), sodium chloride (NaCl), potassium chloride (KCl), 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris), , and sodium citrate ($C_6H_5Na_3O_7$) were purchased from Shanghai Chemical Reagent Company (Shanghai, China). Water was purified with a Milli-Q purification system.

The SYBR® Premix Ex Taq™ II (includes SYBR® Premix Ex Taq® (2×)(SYBR® Premix Ex Taq™ II (Perfect Real Time)) & ROX Reference Dye II (50×)) were purchased from Takara Bio Co. Ltd. (Dalian, China). In order to explore the binding sites between the specific aptamer and AFM1, the complementary DNA fragment of the aptamer was designed with six alternative sequences. The aptamer with 3′-terminal biotin groups was chemically synthesized by Genecreate Biological Co. Ltd. (Wuhan, China), and the complementary DNA fragments were chemically synthesized by Sangon Biotechnology Co. Ltd. (Shanghai, China) and purified by HPLC. Their sequences are as follows:

AFM1 Aptamer (Malhotra et al., 2014):

5′-ATCCGTCACACCTGCTCTGACGCTGGGGTCGACCCGGAGAAATGCAT TCCCCTGTGGTGTTGGCTCCCGTAT-3′

Complementary DNA (AFM1 DNA):

AFM1 DNA1:

5′-GGTGTGACGGATAATCTGGTTTAGCTACGCCTTCCCCGTGGCGATGTT TCTTAGCGCCTTAC-3′

AFM1 DNA2:

5′-AGCGTCAGAGCAAATCTGGTTTAGCTACGCCTTCCCCGTGGCGATGT TTCTTAGCGCCTTAC-3′

AFM1 DNA3:

5′-CGGGTCGACCCCAATCTGGTTTAGCTACGCCTTCCCCGTGGCGATGTT TCTTAGCGCCTTAC-3′

AFM1 DNA4:

5′-AATGCATTTCTCAATCTGGTTTAGCTACGCCTTCCCCGTGGCGATGTTT

CTTAGCGCCTTAC-3′

AFM1 DNA5:

5′-ACACCACAGGGGAATCTGGTTTAGCTACGCCTTCCCCGTGGCGATGTTTCTTAGCGCCTTAC-3′

AFM1 DNA6:

5′-ATACGGGAGCCAAATCTGGTTTAGCTACGCCTTCCCCGTGGCGATGTTTCTTAGCGCCTTAC-3′

Upstream primer: 5′-AATCTGGTTTAGCTACGCCTTC-3′

Downstream primer: 5′-GTAAGGCGCTAAGAAACATCG-3′

2.3 Immobilization of the aptamer

Based on our previous studies (Guo et al., 2014), we carried out immobilization of the aptamer with some modifications. The details are as follows. PCR tubes were treated with 50 μL 0.8% glutaraldehyde solution at 37°C for 5 h in order to improve their stability. After washing three times with ultrapure water, 50 μL of streptavidin dissolved in 0.01 M carbonate buffer solution was added and incubated at 37 ℃ for 2 h. Next, the tubes were washed twice with PBST (10 mM PBS, pH 7.2, 0.05% Tween-20). The aptamer and its complementary ssDNA were mixed sufficiently in a hybridization buffer (750 mM NaCl, 75 mM $C_6H_5Na_3O_7$, pH 8.0) in the ratio 1:1 (v/v), and 50 μL of the mixture was added to each tube and incubated at 37°C for 1 h. Then the tubes were subsequently washed three times with hybridization buffer to remove the uncombined DNA fragments.

2.4 RT-qPCR measurements for AFM1

50 μL of AFM1 standard solution was added and incubated with Tris buffer (10 mM Tris, 120 mM NaCl, 5 mM KCl, 20 mM $CaCl_2$, pH 7.0) at 45 ℃ for 1 h. Then all PCR tubes were washed three times with the Tris buffer to remove the not interacted AFM1 and the released complementary ssDNA.

In the next step, RT-qPCR was carried out using the ABI 7500 Real-Time PCR System (USA). The 50 μL PCR mixture consisted of 2 μL of 10 μM upstream and downstream primers, respectively, 25 μL SYBR® Premix Ex Taq® (2×), 1 μL of ROX Reference Dye II (50×) and 20 μL water. The reaction conditions of real-time PCR were as follows: an initial denaturation for 30 s at 95 ℃, followed by 40 cycles of denaturation for 5 s at 95 ℃, and annealing for 34 s at 60 ℃. The amplification efficiency (E) of RT-qPCR was determined using the formula, $E=10^{(-1/slope)}-1$, where the slope is estimated from the standard curve (Guo et al., 2014; Babu et al., 2011). Fluorescence measurements were taken after each annealing step. A melting curve analysis was performed from 60 ℃ to 95 ℃ to detect potential nonspecific products with the following conditions: an initial denaturation for 15 s at 95 ℃, followed by 40 cycles of denaturation for 1 min at 60 ℃, and annealing for 15 s at 95 ℃.

2.5 Specificity analysis

In order to assess the selectivity of this aptasensor and investigate whether presence of other mycotoxins could interfere with the detection of AFM1, the following mycotoxins, including OTA, ZEN, FB1, AFB1 and AFB2, were applied to the aptasensor. These mycotoxins were used at the same concentration of 1 ng mL^{-1}. All other experimental conditions were identical to that for AFM1 determination, and the change in cycle number among these mycotoxins was compared.

2.6 Method validation

Application of the method to infant rice cereal samples and infant milk powder was conducted to validate the AFM1 determination. 2.5 mL of AFM1 standard solutions in 100% methanol at 5×10^{-4}, 5×10^{-3} and 0.05 ng mL^{-1} (3 replicates per treatment) were added into 0.5 g infant rice cereal samples to make spiked infant rice cereal samples at final concentrations of 0.0025, 0.025 and 0.25 μg/kg. 2.5 mL of AFM1 standard solutions in 100% methanol at 5×10^{-4}, 5×10^{-2} and 0.1 ng mL^{-1} (3 replicates per treatment) were added into 0.5 g infant milk powder samples to make spiked infant milk powder samples at final concentrations of 0.0025, 0.25 and 0.50 μg/kg. Then, 2.5 mL of 70% methanol in water was added to extract AFM1 from the sample. The entire mixture was vortexed for 5 min using Vortex-Genie 2 (Scientific Industries, USA) and subsequently centrifuged at 10,000 g for 10 min. The supernatant was collected and concentrated to 0.5 mL under a nitrogen stream. Finally, each residue was re-dissolved in 2 mL of aqueous methanol solution (5%) and subjected to RT-qPCR.

2.7 Statistical analysis

Each analysis (aflatoxin calibration curve standards and test samples) was performed in triplicate. Amplification curves for AFM1 were plotted with Origin 8.0 software (OriginLab Corporation, Northampton, MA, USA). Simple linear regression analysis of the cycle threshold number (Ct) values on log concentrations of AFM1 were carried out using Microsoft Excel. Standard deviations (SDs) and means for Ct values were obtained from three replicates.

3 Results and discussion

3.1 Optimization of the amplification of complementary ssDNA

The complementary ssDNA is applied as the subsequent PCR template. The melting curve in this step is a key factor which affects amplification efficiency and primer specificity. The concentration of the complementary ssDNA and specificity of the primer should thus be optimized. Amplification curves are shown in Fig. S3-1(A). As the concentration of complementary ssDNA decreases over the range of 1×10^{-3} to 10 nM the cycle number (Ct) increased. Corresponding to the amplification curve the standard curve relating the cycle number threshold (Ct) and the complementary ssDNA in the range of 1×10^{-3} to 10 nM is shown in Fig. S3-1(B), demonstrating the sensitive and quantitative detection of the complementary ssDNA

with high amplification efficiency (103.1%) and a good linear relationship with a high correlation coefficient (0.995). The linear regression equation was described by Ct = -3.2495 lg C + 36.363, where Ct is cycle threshold number and C is the concentration of the complementary DNA. The optimal concentration of the complementary ssDNA was 10 nM with the lowest Ct values. The PCR melting curves are shown in Fig. S3-2, demonstrating the specificity of the PCR amplification without the appearance of primer dimers or other nonspecific DNA products since an obvious single peak was observed at 80 ℃.

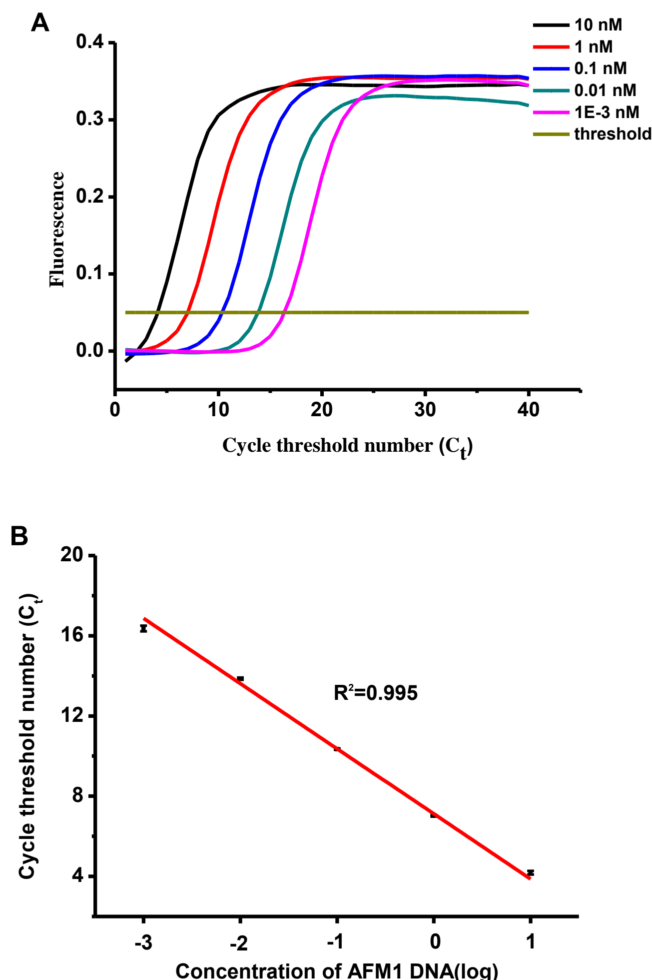


Figure S3-1: (A) The amplification curves at different concentrations of the complementary ssDNA in the range of 1×10^{-3} to 10 nM. (B) The standard curve relating and the Ct value to the complementary ssDNA concentration.

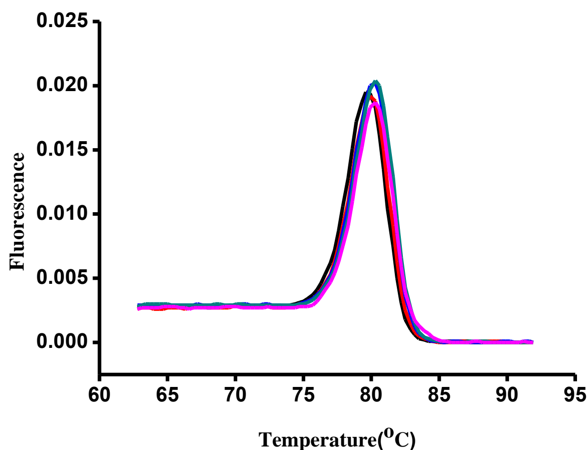


Figure S3-2: Five melting curves corresponding to the amplification curves in Fig. S3-1A for the detection of AFM_1. An obvious single peak was shown at 80 ℃.

3.2 Optimization of streptavidin and the biotinylated aptamer

Other factors would affect the performance of this method including the concentrations of streptavidin, the biotinylated aptamer and the complementary ssDNA and these all should be optimized. Under a fixed concentration of complementary ssDNA at 10 nM, the concentrations of streptavidin and the biotinylated aptamer were analyzed for the PCR amplification signal change (Fig. S3-3). The adsorptive power of streptavidin to PCR tubes and the binding ability of streptavidin-coated tubes to biotinylated aptamer was primarily detected using different concentrations of streptavidin (Fig. S3-3). This also shows that there was a clear difference of Ct values between the control group and streptavidin-coated tubes, which showed the intense adsorptive ability between biotinylated aptamer and streptavidin-coated tubes. In analysis of the Ct values at different concentrations of streptavidin, the Ct value reached the lowest level when the concentration of streptavidin was 2.5 ng mL^{-1}. This graph also shows that the Ct values decreased with the increase of the amount of aptamer when aptamer levels were below 10.0 nM andalso that the Ct values increased with the amount of aptamer above 10.0 nM mainly because of steric hindrance. Thus, 2.5 ng mL^{-1} of streptavidin and 10 nM of aptamer were the optimal conditions for RT-qPCR amplification.

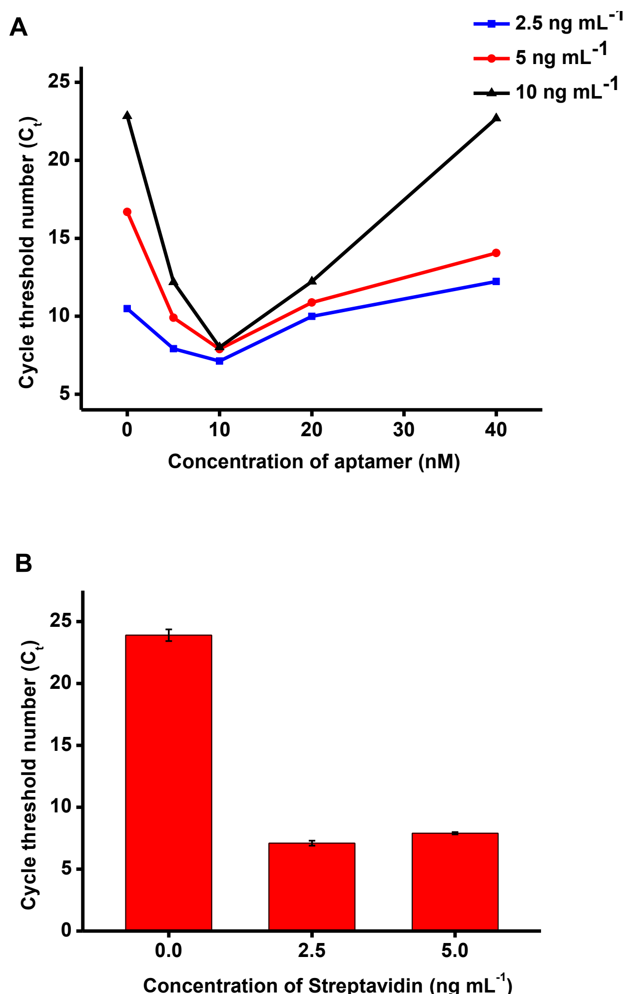


Figure S3-3: (A) Ct values change upon different concentrations of aptamer in the presence of fixed concentrations of complementary ssDNA (10 nM) and AFM1 (1 ng mL^{-1}), while the concentrations of streptavidin varied (2.5, 5 and 10 ng mL^{-1}). (B) Comparison of Ct values change at different concentrations of streptavidin in the presence of fixed concentrations of aptamer (10 nM), complementary ssDNA (10 nM) and AFM1 (1 ng mL^{-1}). Values shown are means of triplicate analyses and standard deviations are also plotted.

3.3 AFM1 determination

Under optimal conditions, the amplification curves and calibration curve of this aptasensor for different levels of AFM1 including 0.1 ng L^{-1}, 1 ng L^{-1}, 0.01 µg L^{-1}, 0.1 µg L^{-1}, and 1 µg L^{-1} were determined by RT-qPCR (Fig. 3-2). As is shown in Fig. 3-2(A), the cycle number increased with an increase in AFM1. More complementary

ssDNA would be released when more AFM1 is present in the reaction system, which leads to a decrease in the amount of the PCR template and an increase in cycle number.

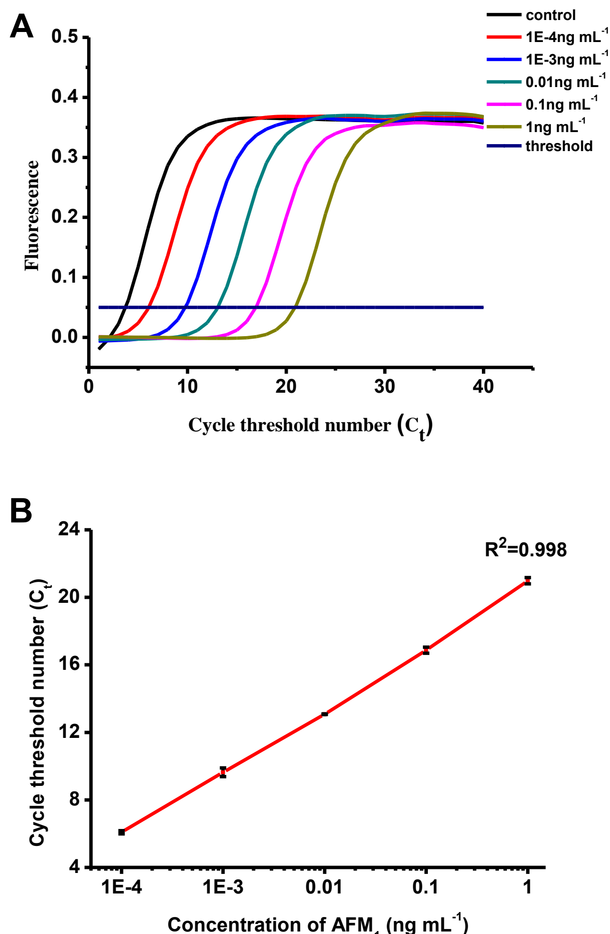
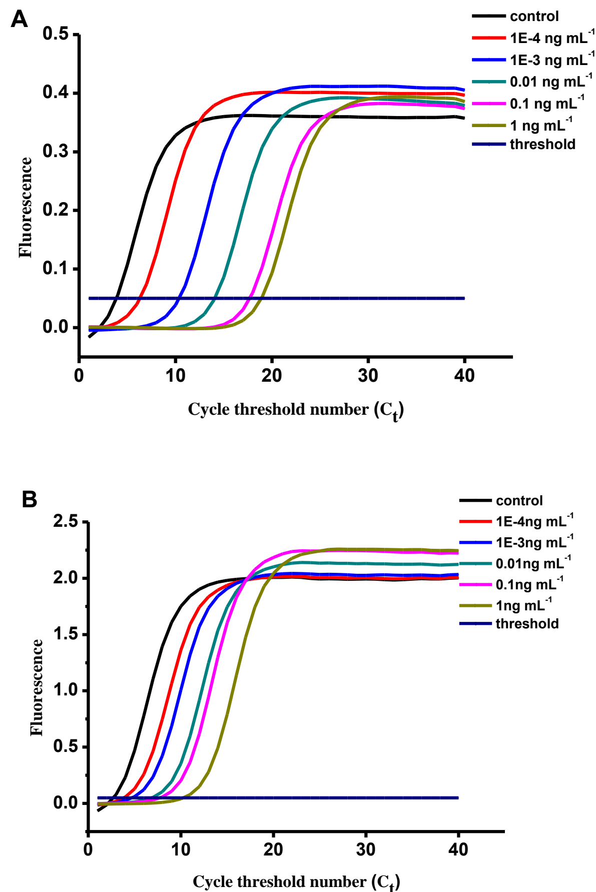
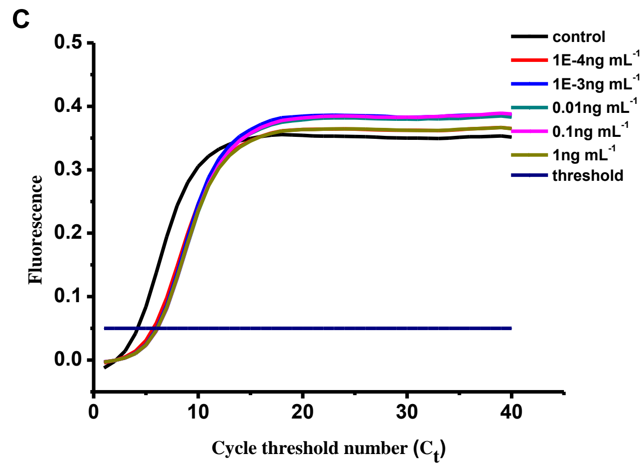


Figure 3-2: (A) The amplification curves at different concentrations of AFM1 in the range of 1×10^{-4} to 1 μg L^{-1} for the determination of AFM1 DNA1, including the negative control without AFM1. (B) The standard curves between the AFM1 concentration and the Ct value in the range of 1×10^{-4} to 1 μg L^{-1}.

The linear regression equation was described by Ct=3.703 lg C + 20.736 (R^2=0.998), where Ct is cycle threshold number and C is AFM1 concentration. A good linear relationship between Ct values and AFM1 levels in the range of 1×10^{-4} μg L^{-1} to 1 μg L^{-1} in buffer was obtained as indicated in Fig. 2(B) with the limit of detection 0.03 ng L^{-1} (S/N = 3). All the detection conditions of the other five AFM1 DNA were identical to those used in the AFM1 DNA1 procedure, allowing a

comparison of the performance of amplification curves (Fig. 3-3). Results clearly show the determination of AFM1 DNA1 has the best results with excellent amplification efficiency, indicating the binding site between the specific aptamer and AFM1 mainly exists in the position close to 5'-terminal of the aptamer. In addition, the aptasensor in this study demonstrates a high sensitivity in AFM1 determination in comparison to other current approaches (Table 3-1).

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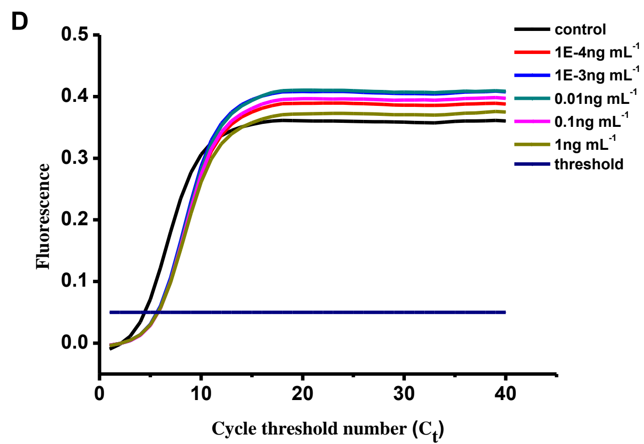
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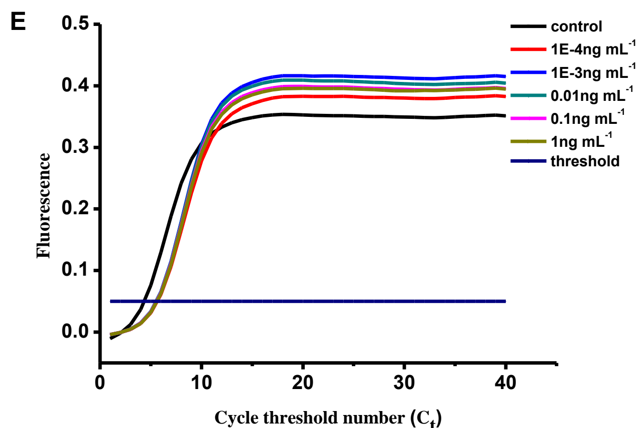

Figure 3-3: The amplification curves at different concentrations of AFM1 in the range of 1×10^{-4} to 1 μg L^{-1} for the determination of different AFM1 DNA, including the negative control without AFM1. (A) AFM1 DNA2; (B) AFM1 DNA3; (C) AFM1 DNA4; (D) AFM1 DNA5; (E) AFM1 DNA6.

Table 3-1: Comparison of the sensitivity of currently available methods for the detection of AFM_1.

No.	Method	LOD	reference
1	LC/MS/MS	0.01 ng L^{-1}	(Cavaliere et al., 2006)
2	Fluorometric sensor	25 ng L^{-1}	(Cucci et al., 2007)
3	Electrochemical immunosensors	1 ng L^{-1}	(Neagu et al., 2009)
4	Indirect competitive ELISA	0.04 μg L^{-1}	(Pei et al., 2009)
5	Impedimetric biosensor	1 μg L^{-1}	(Dinckaya et al., 2011)
6	Cellular biosensor	5 ng L^{-1}	(Larou et al., 2013)
7	Direct chemiluminescent ELISA	1 ng L^{-1}	(Vdovenko et al., 2014)
8	DART-MS	1 μg L^{-1}	(Busman et al., 2015)
9	RT-qPCR based aptasensor	0.03 ng L^{-1}	This work

3.4 Specificity analysis

The specificity of the aptasensor plays an important role in the development and practicality of this method. In order to evaluate the specificity of the detection system, the change of PCR amplification produced by other five mycotoxins (including OTA, ZEN, FB1, AFB1 and AFB2) was determined. As shown in Fig. 3-4, the detection of OTA, ZEN, FB1, as well as the control, had no obvious Ct value changes at the concentration of 1 ng mL^{-1} among the five mycotoxins. However, AFB1 and AFB2, the structural analogs of AFM1, resulted in a slight

increase of the Ct values but no significant effect (P>0.05). In addition, a similar result was detected using a mix of these five mycotoxins without AFM1 (Mix1). The corresponding Ct value of AFM1 in a mix of these five mycotoxins (Mix2) was slightly lower than the Ct value of AFM1 alone with no significant difference between Mix2 and AFM1. This sensing system thus has high specificity for the detection of AFM1 owing to the high recognition ability of the biotin-labeled aptamer to the target, and the inability of the aptamer to recognize other mycotoxins.

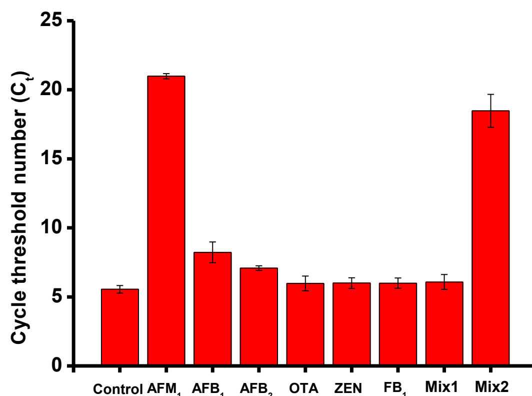

Figure 3-4: The Ct values in the absence and presence of 1 ng mL^{-1} mycotoxins including AFM1, OTA, ZEA, AFB1, AFB2, FB1, Mix1 (OTA, ZEA, AFB1, AFB2, FB1) and Mix2 (OTA, ZEA, AFB1, AFB2, FB1, and AFM1). The experiment conditions are as following: complementary ssDNA 10 nM, aptamer 10 nM, and streptavidin 2.5 ng mL^{-1}. Means and standard deviations are shown with three replicates for each treatment.

3.5 Repeatability analysis

The repeatability of this method is an important issue for the development and practical implementation of AFM1 detection, which was assessed by analyzing the Ct values of the same sample (1.0 ng mL^{-1} AFM1) five times. As indicated in Fig. S3-4, results showed a good repeatability of the measurements with a relative standard deviation (RSD) of 5.0%.

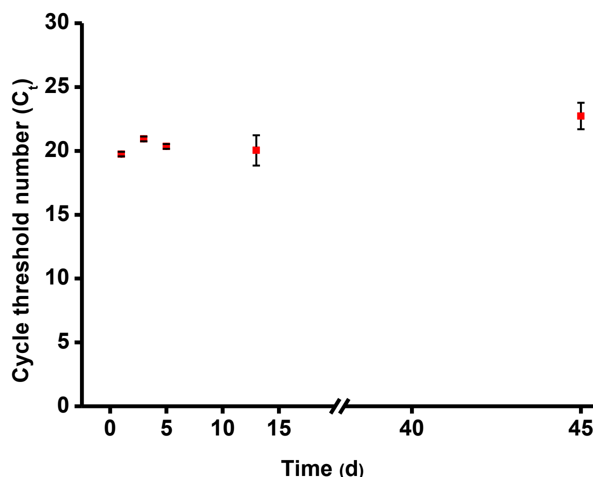

Figure S3-4: Repeatability of the proposed aptasensor for AFM1 detection. The experiment conditions are as following: AFM1 1.0 ng mL^{-1}, complementary ssDNA 10 nM, aptamer 10 nM, and streptavidin 2.5 ng mL^{-1}. Means and standard deviations are shown with three replicates at each time.

3.6 Method validation

In order to evaluate feasibility and reliability of this method, we applied it to the determination of different concentrations of AFM1 in infant rice cereal and milk powder samples. As shown in Table 2, The recoveries of the spiked infant rice cereal and milk powder samples were in the range of 84–106% and 68–80.3%, correspondingly, indicating that the proposed aptasensor was suitable for quantitative determination of mycotoxins in food samples for quality control of food safety. However, the relatively low recovery in the infant rice cereal samples might be due to sample pretreatments, and the future work would focus on improving sample pretreatments to AFM1 determination for food safety.

Table 3-2. Determination of AFM_1 spiked into infant rice cereal and milk powder samples.

Sample	Spiked concentration (μg kg^{-1})	Detected concentrations Mean[a] ±SD[b] (μg kg^{-1})	Recovery (%)
Infant rice cereal	0.25	0.225 ±0.03	90
	0.025	0.0265 ±0.0015	106
	0.0025	0.0021 ±0.0001	84
milk powder	0.50	0.4 ±0.03	80
	0.25	0.185 ±0.01	74
	0.0025	0.0017 ±0.0001	68

a. The mean of three replicates; b. SD=standard deviation

4 Conclusion

In this work, we describe a reliable and sensitive aptamer-based biosensor for determination of AFM1, which simultaneously combined the advantages of high recognition power of the aptamer to AFM1 and excellent amplification efficiency of RT-qPCR technique to improve sensitivity. Under optimal conditions, a good linear relationship existed between Ct values and AFM1 levels over the range from 1×10^{-4} to 1 μg L^{-1} with high sensitivity (LOD = 0.03 ng L^{-1}). The detection of five other mycotoxins was limited, but there may be a small cross-reaction with AFB1. This shows its value in the determination of AFM1 for food safety with an acceptable selectivity. Importantly, this method can be applied to the detection of AFM1 in infant rice cereal and milk powder samples with satisfactory recoveries. Therefore, this aptasensor has a highly potential application for biologically small molecules.

Acknowledgements

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References

Anfossi L, Baggiani C, Giovannoli C, Biagioli F, D'Arco G, Giraudi G. Optimization of a lateral flow immunoassay for the ultrasensitive detection of aflatoxin M1 in milk. Anal Chim Acta. 2013;772:75-80. doi:10.1016/j.aca.2013.02.020.

Anfossi L, Di Nardo F, Giovannoli C, Passini C, Baggiani C. Enzyme immunoassay for monitoring aflatoxins in eggs. Food Control. 2015;57:115-21. doi:10.1016/j.foodcont.2015.04.013.

Babu D, Muriana PM. Immunomagnetic bead-based recovery and real time quantitative PCR (RT iq-PCR) for sensitive quantification of aflatoxin B(1). J Microbiol Meth. 2011;86(2):188-94. doi:10.1016/j.mimet.2011.05.002.

Bacher G, Pal S, Kanungo L, Bhand S. A label-free silver wire based impedimetric immunosensor for detection of aflatoxin M1 in milk. Sensors Actuators B. 2012;168:223-30. doi:10.1016/j.snb.2012.04.012.

Barthelmebs L, Hayat A, Limiadi AW, Marty J-L, Noguer T. Electrochemical DNA aptamer-based biosensor for OTA detection, using superparamagnetic nanoparticles. Sensors Actuators B. 2011;156(2):932-7. doi:10.1016/j.snb.2011.03.008.

Beltrán E, Ibáñez M, Sancho JV, Cortés MÁ, Yusà V, Hernández F. UHPLC–MS/MS

highly sensitive determination of aflatoxins, the aflatoxin metabolite M1 and ochratoxin A in baby food and milk. Food Chem. 2011;126(2):737-44. doi:10.1016/j.foodchem.2010.11.056.

Bonel L, Vidal JC, Duato P, Castillo JR. An electrochemical competitive biosensor for ochratoxin A based on a DNA biotinylated aptamer. Biosens Bioelectron. 2011;26(7):3254-9. doi:10.1016/j.bios.2010.12.036.

Busman M, Bobell JR, Maragos CM. Determination of the aflatoxin M1 (AFM1) from milk by direct analysis in real time – mass spectrometry (DART-MS). Food Control. 2015;47:592-8. doi:10.1016/j.foodcont.2014.08.003.

Cavaliere C, Foglia P, Pastorini E, Samperi R, Lagana A. Liquid chromatography/tandem mass spectrometric confirmatory method for determining aflatoxin M1 in cow milk: comparison between electrospray and atmospheric pressure photoionization sources. J chromatogr A. 2006;1101(1-2):69-78. doi:10.1016/j.chroma.2005.09.060.

Commission Recommendation (EC). Setting maximum levels for certain contaminants in foodstuffs. Off J Eur Union. 2006;364:5-24.

Cruz-Aguado JA, Penner G. Determination of Ochratoxin A with a DNA Aptamer. J Agric Food Chem. 2008;56(22):10456-61. doi:10.1021/jf801957h.

Cucci C, Mignani AG, Dall'Asta C, Pela R, Dossena A. A portable fluorometer for the rapid screening of M1 aflatoxin. Sensors Actuators B. 2007;126(2):467-72. doi:10.1016/j.snb.2007.03.036.

De Girolamo A, Le L, Penner G, Schena R, Visconti A. Analytical performances of a DNA-ligand system using time-resolved fluorescence for the determination of ochratoxin A in wheat. Anal Bioanal Chem. 2012;403(9):2627-34. doi:10.1007/s00216-012-6076-6.

Dinckaya E, Kinik O, Sezginturk MK, Altug C, Akkoca A. Development of an impedimetric aflatoxin M1 biosensor based on a DNA probe and gold nanoparticles. Biosens Bioelectron. 2011;26(9):3806-11. doi:10.1016/j.bios.2011.02.038.

Food and Agriculture Organization of the United Nations. Worldwide regulations for mycotoxins in food and feed in 2003. Food and Nutrition Paper. 2004;81:Rome: FAO.

Guo X, Wen F, Zheng N, Luo Q, Wang H, Wang H, Li S, Wang J. Development of an ultrasensitive aptasensor for the detection of aflatoxin B1. Biosens Bioelectron. 2014;56:340-4. doi:10.1016/j.bios.2014.01.045.

Hoyos Ossa DE, Hincapié DA, Peñuela GA. Determination of aflatoxin M1 in ice cream samples using immunoaffinity columns and ultra-high performance liquid chromatography coupled to tandem mass spectrometry. Food Control. 2015;56:34-40. doi:10.1016/j.foodcont.2015.03.011.

International Agency for Research on Cancer (IARC). Some naturally occurring substances: Food items and constituents heterocyclic aromatic amines and mycotoxins. IARC monographs on the evaluation of carcinogenic risk to

humans. 1993;56:489–51.

International Agency for Research on Cancer (IARC). Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. 2002;82:171-6.

Istamboulie G, Paniel N, Zara L, Granados LR, Barthelmebs L, Noguer T. Development of an impedimetric aptasensor for the determination of aflatoxin M1 in milk. Talanta. 2016;146:464-9. doi:10.1016/j.talanta.2015.09.012.

Kav K, Col R, Kaan Tekinsen K. Detection of aflatoxin M1 levels by ELISA in white-brined Urfa cheese consumed in Turkey. Food Control. 2011;22(12):1883-6. doi:10.1016/j.foodcont.2011.04.030.

Larou E, Yiakoumettis I, Kaltsas G, Petropoulos A, Skandamis P, Kintzios S. High throughput cellular biosensor for the ultra-sensitive, ultra-rapid detection of aflatoxin M1. Food Control. 2013;29(1):208-12. doi:10.1016/j.foodcont.2012.06.012.

Lee D, Lee K-G. Analysis of aflatoxin M1 and M2 in commercial dairy products using high-performance liquid chromatography with a fluorescence detector. Food Control. 2015;50:467-71. doi:10.1016/j.foodcont.2014.09.020.

Li P, Zhang Q, Zhang W, Zhang J, Chen X, Jiang J, Xie L, Zhang D. Development of a class-specific monoclonal antibody-based ELISA for aflatoxins in peanut. Food Chem. 2009;115(1):313-7. doi:10.1016/j.foodchem.2008.11.052.

Liu D, Huang Y, Wang S, Liu K, Chen M, Xiong Y, Yang W, Lai W. A modified lateral flow immunoassay for the detection of trace aflatoxin M1 based on immunomagnetic nanobeads with different antibody concentrations. Food Control. 2015;51:218-24. doi:10.1016/j.foodcont.2014.11.036.

Malhotra S, Pandey AK, Rajput YS, Sharma R. Selection of aptamers for aflatoxin M1 and their characterization. J Mol Recognit. 2014;27(8):493-500. doi:10.1002/jmr.2370.

Mao J, Lei S, Liu Y, Xiao D, Fu C, Zhong L, Ouyang H. Quantification of aflatoxin M1 in raw milk by a core-shell column on a conventional HPLC with large volume injection and step gradient elution. Food Control. 2015;51:156-62. doi:10.1016/j.foodcont.2014.11.022.

Neagu D, Perrino S, Micheli L, Palleschi G, Moscone D. Aflatoxin M1 determination and stability study in milk samples using a screen-printed 96-well electrochemical microplate. Int Dairy J. 2009;19(12):753-8. doi:10.1016/j.idairyj.2009.06.004.

Nguyen BH, Tran LD, Do QP, Nguyen HL, Tran NH, Nguyen PX. Label-free detection of aflatoxin M1 with electrochemical Fe3O4/polyaniline-based aptasensor. Mater Sci Eng C Mater Biol Appl. 2013;33(4):2229-34. doi:10.1016/j.msec.2013.01.044.

Parker CO, Tothill IE. Development of an electrochemical immunosensor for aflatoxin M1 in milk with focus on matrix interference. Biosens Bioelectron. 2009;24(8):2452-7. doi:10.1016/j.bios.2008.12.021.

Pei SC, Zhang YY, Eremin SA, Lee WJ. Detection of aflatoxin M1 in milk products from China by ELISA using monoclonal antibodies. Food Control. 2009;20(12):1080-5. doi:10.1016/j.foodcont.2009.02.004.

Pietri A, Fortunati P, Mulazzi A, Bertuzzi T. Enzyme-assisted extraction for the HPLC determination of aflatoxin M1 in cheese. Food Chem. 2016;192:235-41. doi:10.1016/j.foodchem.2015.07.006.

Shim WB, Kim MJ, Mun H, Kim MG. An aptamer-based dipstick assay for the rapid and simple detection of aflatoxin B1. Biosens Bioelectron. 2014;62:288-94. doi:10.1016/j.bios.2014.06.059.

Vdovenko MM, Lu CC, Yu FY, Sakharov IY. Development of ultrasensitive direct chemiluminescent enzyme immunoassay for determination of aflatoxin M1 in milk. Food Chem. 2014;158:310-4. doi:10.1016/j.foodchem.2014.02.128.

Wang R, Xiang Y, Zhou X, Liu L-h, Shi H. A reusable aptamer-based evanescent wave all-fiber biosensor for highly sensitive detection of Ochratoxin A. Biosens Bioelectron. 2015;66:11-8. doi:10.1016/j.bios.2014.10.079.

Wang X, Li P. Rapid screening of mycotoxins in liquid milk and milk powder by automated size-exclusion SPE-UPLC-MS/MS and quantification of matrix effects over the whole chromatographic run. Food Chem. 2015;173:897-904. doi:10.1016/j.foodchem.2014.10.056.

Wang Y, Liu X, Xiao C, Wang Z, Wang J, Xiao H, Cui L, Xiang Q, Yue T. HPLC determination of aflatoxin M1 in liquid milk and milk powder using solid phase extraction on OASIS HLB. Food Control. 2012;28(1):131-4. doi:10.1016/j.foodcont.2012.04.037.

Zhao Q, Geng X, Wang H. Fluorescent sensing ochratoxin A with single fluorophore-labeled aptamer. Anal Bioanal Chem. 2013;405(19):6281-6. doi:10.1007/s00216-013-7047-2.

4

A novel graphene oxide-based aptasensor for amplified fluorescent detection of aflatoxin M1 in milk powder

In this study, a new graphene oxide-based aptasensor for specific detection of AFM1 was developed, which combined the ability of GO to protect the aptamer from nuclease cleavage with DNase I to cleave the aptamer for a target cycling signal amplification. The presence of AFM1 induced the release of the aptamer from the surface of GO because of the formation of AFM1/aptamer complex, which resulted in the cleavage of aptamer by DNase I and the release of AFM1 for a new cycle. Therefore, a cycling signal amplification was achieved to improve detection sensitivity. A good linear relationship was measured between the change of the fluorescence intensity signal and AFM1 levels.

From Xiaodong Guo, Fang Wen, Qinqin Qiao, Nan Zheng, Matthew Saive, Marie-Laure Fauconnier, Jiaqi Wang, A Novel Graphene Oxide-Based Aptasensor for Amplified Fluorescent Detection of Aflatoxin M1 in Milk Powder, Sensors, 2019, 19: 3840. doi:10.3390/s19183840.

Abstract: In this paper, a rapid and sensitive fluorescent aptasensor for the detection of aflatoxin M1 (AFM1) in milk powder has been developed. Graphene oxide (GO) was employed to quench the fluorescence of carboxyfluorescein-labelled aptamer and protect the aptamer from nuclease cleavage. Upon the addition of AFM1, a formation of AFM1/aptamer complex resulted in the aptamer detached from the surface of GO, then the aptamer was cleaved by DNase I and the target AFM1 was released for a new cycle, which led to a great signal amplification and high sensitivity. Under optimized conditions, the GO-based detection of the aptasensor exhibited a linear response to AFM1 in a dynamic range from 0.2 to 10 μg/kg, with a limit of detection (LOD) of 0.05 μg/kg. Moreover, the developed aptasensor showed a high specificity towards AFM1 without interference from other mycotoxins. In addition, the technique has been successfully applied for detection of AFM1 in infant milk powder samples. This aptasensor proposed here offers a promising technology for food safety and can be extended to various targets.

Keywords: Aflatoxin M1, Aptamer, Graphene oxide, DNase I, Food safety

1 Introduction

Aflatoxin M1 (AFM1), one of the most toxic mycotoxins, has been designated from a group 2B to a group 1 carcinogen by the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) (IARC, 1993; IARC, 2002). AFM1 can be finally encountered in dairy products as a hydroxylate metabolite through feeding dairy cows aflatoxin B1 contaminated feeds (Pei et al., 2009; Anfossi et al., 2013; Liu et al., 2015). Since dairy products are an important nutrient for humans, especially for infants, the presence of AFM1 in dairy products is one of the most serious hazards for food safety (Mao et al., 2015). To protect humans from this health threat, many regulatory agencies have defined maximum residue levels (MRLs) for AFM1 in dairy products (FAO, 2004; Hoyos Ossa et al., 2015). In Brazil, China and USA, the maximum level of AFM1 in milk has been fixed to 0.5 μg/kg (Busman et al., 2015; Istamboulie et al., 2016). European Commission Regulation has set much more restrictive limits, 0.05 μg/kg in milk products for adults, and this level is lowered to 0.025 μg/kg in babies and infants products (EC, 2006). Considering the severe toxicity and low permitted level of AFM1, a simple, rapid, and inexpensive diagnostics with high sensitivity and specificity are vitally required for food safety.

Analytical methods such as high-performance liquid chromatography (HPLC) with fluorescent detectors (Wang et al., 2012; Lee et al., 2015; Pietri et al., 2016) and high-performance liquid chromatography coupled with mass spectrometric detectors (Beltrán et al., 2011; Wang et al., 2015) have been developed for quantitative detection of AFM1. All these procedures rely on expensive instruments, qualified staff and complicated time-consuming pretreatments. Meanwhile, enzyme-linked immune sorbent assays (ELISA) (Li et al., 2009; Kav et al., 2011; Anfossi et al., 2015) have gained great attention for AFM1 analysis owing to the advantages of rapid, low-cost and high-throughput application. However, expensive, time-consuming and laborious antibody production and its instability during storage limit the practical applications. In this case, the design of a simple, cheap and sensitive method for rapid detection of AFM1 has become a research hotspot.

Aptamer, a single stranded DNA or RNA oligonucleotides, has been widely applied for recognizing of targets such as proteins, nucleic, cells, tissues, and small molecules with strong affinity and high specificity like or even superior to antibodies (Zhang et al., 2016; Lv et al., 2017). Up to now, a series of aptamer based biosensors for the detection of several mycotoxins including ochratoxin A (OTA), AFB1 and AFM1 have been developed (Barthelmebs et al., 2011; Bonel et al., 2011; Guo et al., 2014; Shim et al., 2014; Wang et al., 2015; Guo et al., 2016). In these homogeneous methods, the recognition reaction between the aptamer and target was based on the single-site binding, which might limit the method sensitivity. Thus, development of aptasensors coupled with signal amplification strategies for AFM1 is an on-going challenge. In our previous study (Guo et al., 2016), a sensitive aptasensor based on real-time quantitative polymerase chain reaction (RT-qPCR) for AFM1 was developed. However, preparation of the qPCR-based aptasensor requires

tedious and time-consuming process with long incubation period in rigorous conditions. Therefore, there is a demand for aptasensor that could be applied for rapid and real-time analysis of AFM1.

Graphene oxide (GO), a two-dimensional nanomaterial, is a very promising tool for construction of biosensors due to the advantages of its extraordinary electrical, thermal, and mechanical properties (Li et al., 2008; Rao et al., 2009; Chen et al., 2012; Cheng et al., 2014). The predominantly distance-dependent fluorescence quenching ability makes GO a highly efficient fluorescence quencher (Zhang et al., 2014; Tang et al., 2015; Pei et al., 2012). In addition, the previous research demonstrated that single-stranded DNA could significantly interacts with GO through π stacking between DNA bases and hexagonal cells of GO (He et al., 2010). Importantly, GO could protect DNA aptamers from nuclease cleavage as a result of the hydrophobic stacking interactions between nucleobases and GO (Lu et al., 2010; Pu et al., 2011; Tang et al., 2011). To the best of our knowledge, graphene oxide-based nuclease signal amplification aptasensors for AFM1 determination have not been reported.

In this study, a new graphene oxide-based aptasensor for specific detection of AFM1 was developed, which combined the ability of GO to protect the aptamer from nuclease cleavage with DNase I to cleave the aptamer for a target cycling signal amplification. The presence of AFM1 induced the release of the aptamer from the surface of GO because of the formation of AFM1/aptamer complex, which resulted in the cleavage of aptamer by DNase I and the release of AFM1 for a new cycle. Therefore, a cycling signal amplification was achieved to improve detection sensitivity. A good linear relationship was measured between the change of the fluorescence intensity signal and AFM1 levels.

2 Experimental

2.1 Materials and reagents

Aflatoxin M1 (AFM1) was purchased from Sigma-Aldrich (USA). Aflatoxin B1 (AFB1) was obtained from the National Standard Reference Center（Beijing, China). Ochratoxin A (OTA), zearalenone (ZEA), and α- zearalenin (α-ZOL) were purchased from Pribolab Co. Ltd (Singapore). Graphene oxide was purchased from Sangon Biotechnology Co. Ltd. (Shanghai, China). DNase I (RNase-free) was obtained from Takara Bio Co. Ltd. (Dalian, China). Other chemicals such as anhydrous calcium chloride ($CaCl_2$), sodium chloride (NaCl), potassium chloride (KCl), and 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris) were purchased from Shanghai Chemical Reagent Company (Shanghai, China). All other chemicals were analytical grade and were used as received without further purification. Water was purified with a Milli-Q purification system. DNA oligonucleotides were chemically synthesized by Sangon Biotechnology Co. Ltd. (Shanghai, China) and purified by HPLC. The sequence of AFM1 aptamer had been optimized according to our previous study (Guo et al., 2016), and was modified by FAM (carboxyfluorescein). The sequence of the FAM-labelled AFM1 aptamer was as

follows:

5′ -FAM-ATCCGTCACACCTGCTCTGACGCTGGGGTCGACCCG-3′

2.2 Fluorescent response of the amplified aptasensor for AFM1

In this amplification strategy, the FAM-labelled AFM1 aptamer was diluted to 200 nM in Tris buffer (10 mM Tris, 120 mM NaCl, 5 mM KCl, 20 mM $CaCl_2$, pH 7.0), and 20 μg mL^{-1} of GO was added to the working solution for 15 min at room temperature to form aptamer/GO complex and quench the fluorescence. Subsequently, different concentrations of AFM1 and DNase I (200 U) solutions were simultaneously added to the aptamer/GO solutions and incubated at room temperature for 1 h. Afterwards, the fluorescence intensities of the mixture were recorded using F-7000 fluorophotometer (Hitachi, Japan). The emission spectra were measured in the range of 510 to 630 nm with the excitation wavelength at 480 nm and slit widths for both of the excitation and emission were set at 10 nm.

2.3 Specificity analysis

To evaluate the specificity of this aptasensor for AFM1 over other mycotoxins, four different mycotoxins (including AFB1, OTA, ZEA and α-ZOL) were measured at the same concentration of 4 ng mL^{-1}. The other experimental procedures were the same as AFM1 determination, and the changes of fluorescence intensity among these mycotoxins were compared.

2.4 Method validation

The feasibility and practicability of this sensing platform was verified by quantitative detection of AFM1 in infant milk powder samples. The samples were spiked with 2.5 mL of AFM1 at 0, 1.5, 2.5 and 5 μg/kg (3 replicates per treatment). Each sample was accurately weighed (0.5 g) into 10 mL centrifuge tubes. Then, 2.5 mL of extraction solution (70% methanol in water) was added to extract AFM1 from the samples. The entire mixture was vortexed using Vortex-Genie 2 (Scientific Industries, USA) for 5 min and then centrifuged at 10,000 g for 10 min. The supernatant was obtained and concentrated to 0.5 mL under a nitrogen stream. Subsequently, each residue was re-dissolved in 2 mL of aqueous methanol solution (5% methanol in water). Finally, the extracts were measured by the fluorescence signal amplification experiment.

2.5 Statistical analysis

Fluorescence-emission spectra curves for AFM1 were plotted using Origin 8.0 software (OriginLab Corporation, Northampton, MA, USA). Linear regression analysis of the fluorescence intensity on concentrations of AFM1 was carried out with Microsoft Excel. Each analysis including AFM1 calibration curve standards and test samples was performed in triplicate. Standard deviations (SDs) and means for fluorescence intensity were achieved from three replicates.

3 Results and discussion

3.1 Design strategy for AFM1 detection based on graphene oxide sensing platform

GO has many advantages with unique properties, including its great binding ability to single-stranded DNA (such as aptamers) through π stacking interactions between nucleobases and GO nanosheets, as well as the highly distance-dependent fluorescence quenching performance (He et al., 2010; Chen et al., 2015). A GO-based aptasensor for the detection of AFM1 was developed using the above advantages. Schematic illustration for the sensing platform was described in Figure 4-1. In this sensing method, when the carboxyfluorescein (FAM)-modified aptamer was incubated with GO solution, the fluorescence signal quenched dramatically, demonstrating a strong binding between aptamer and GO with a high quenching efficiency. Upon the addition of AFM1, AFM1/aptamer complex was formed, such an interaction can lead to a conformational change in the aptamer, causing a separation of the aptamer conjugated from the surface of GO. Thus, the fluorescence would be recovered since the GO could not quench the fluorescence efficiently owing to the long distance. In order to confirm that the presence of AFM1 can lead to the formation of AFM1/aptamer complex and subsequently fluorescence recovery, 10 ng mL^{-1} of AFM1 was added to a tris buffer solution that contained 200 nM AFM1 aptamer and 20 μg mL^{-1} GO. As seen from Fig. 4-2, a significant fluorescence enhancement was observed, demonstrating that the AFM1/aptamer complex was formed. More importantly, the covalently modified FAM had no impact on the recognition ability of AFM1 aptamer.

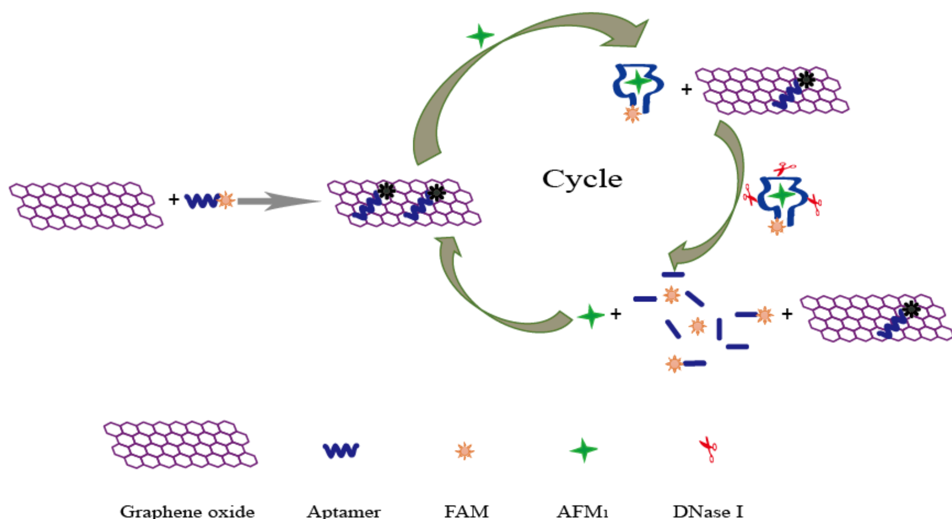

Figure 4-1: Illustration of the aptasensor for detection of aflatoxin M1.

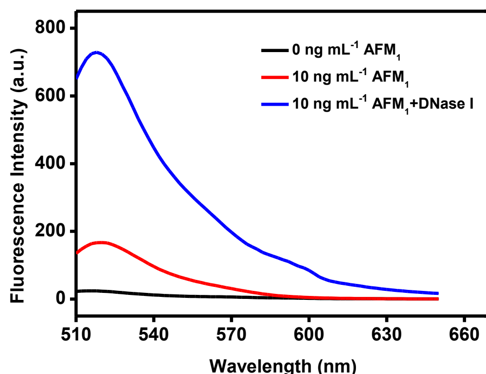

Figure 4-2: Fluorescence emission spectra of the aptasensor in the absence (0) of AFM1, presence of 10 ng mL^{-1} AFM1, and 10 ng mL^{-1} AFM1 and 200 U DNase I. Excitation wavelength (λex) is set at 480 nm. Conditions: 200 nM AFM1 aptamer, 20 μg mL^{-1} GO in Tris buffer (10 mM Tris, 120 mM NaCl, 5 mM KCl, 20 mM $CaCl_2$, pH 7.0).

The DNase I was adopted as a signal amplification strategy to improve the sensitivity of the aptasensor. As shown in Figure 4-1, upon the addition of AFM1 and DNase I, the formation of AFM1/aptamer complex caused the dissociation of the aptamer conjugate from GO, subsequently the aptamer is digested by DNase I. Once the AFM1 is released from the AFM1/aptamer complex, it is once again available to bind to another aptamer, inducing a cycle amplification of the fluorescence signal. As a consequence, the strong alteration of the fluorescence signal can be achieved for the quantification of AFM1 concentrations.

3.2 Optimization of experimental conditions

The concentration of GO would influence the fluorescence quenching efficiency. Therefore, to optimize the sensing platform, the effect of GO concentration on the change of fluorescence signal was investigated. Various concentrations of GO were added to a solution containing 200 nM AFM1 aptamer. As seen in Figure S4-1, the fluorescence intensity decreased with the increasing amount of GO, the fluorescence intensity reached the lowest level as the concentration of GO was 20 μg mL^{-1}. Thus, 20 μg mL^{-1} of GO solution was used for further sensing experiments.

To improve the signal amplification efficiency, the optimization of the concentration of DNase I is essential. In this experiment, we measured the fluorescence intensity of the complex with 10 ng mL^{-1} of AFM1. Various amounts of DNase I were added to the GO-aptamer solution containing 200 nM AFM1 aptamer and 20 μg mL^{-1} GO. As seen in Figure S4-2, the fluorescence intensity increased as the DNase I concentration increased from 0 to 200 U, and the highest level of the fluorescence was observed at 200 U of DNase I. In this case, the optimal

amount of DNase I was determined as 200 U.

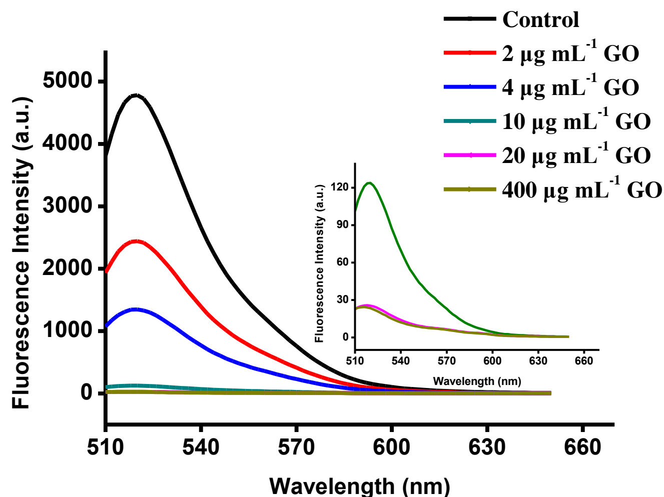

Figure S4-1: Fluorescence emission spectra of the aptasensor in the addition of GO at various concentrations. The experiment conditions are as following: λex = 480nm, 200 nM AFM1 aptamer.

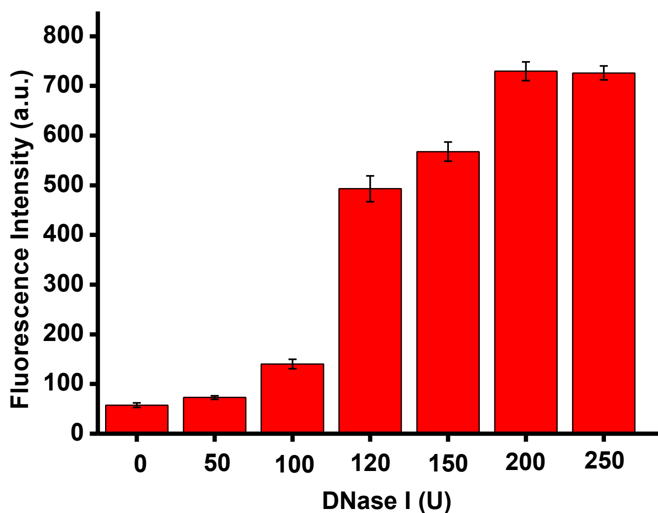

Figure S4-2: Fluorescence intensity with the addition of DNase I at various concentrations. The experiment conditions are as follows: Excitation and emission wavelength are at λex/λem = 480/520 nm, 200 nM AFM1 aptamer, 20 μg mL^{-1} GO, 10 ng mL^{-1} AFM1.

3.3 Analytical performance of the aptasensor

Under optimal conditions, the signal responses of GO-based aptasensor toward different concentrations of AFM1 were measured using DNase I induced target recycling amplification platform. The fluorescence emission spectrum was measured for AFM1 determination with excitation and emission wavelengths of 480 nm and 520 nm, respectively. As seen in Figure 4-3, the fluorescence intensity increased as the concentration of AFM1 increased from 0.2 to 10 ng mL^{-1}. The calibration curve of fluorescence intensity versus AFM1 concentrations was linear as F = 65.77 C + 46.334 (R^2 = 0.9939), in which F was the fluorescence intensity and C was AFM1 concentration. The limit of detection of the amplification aptasensor was determined to be 0.05 ng mL^{-1}, which was calculated at the signal-to-noise ratio of 3. As shown in Table 4-1, the aptasensor performed with a sensitivity for AFM1 comparable to other instrumental and rapid screening methods reported previously.

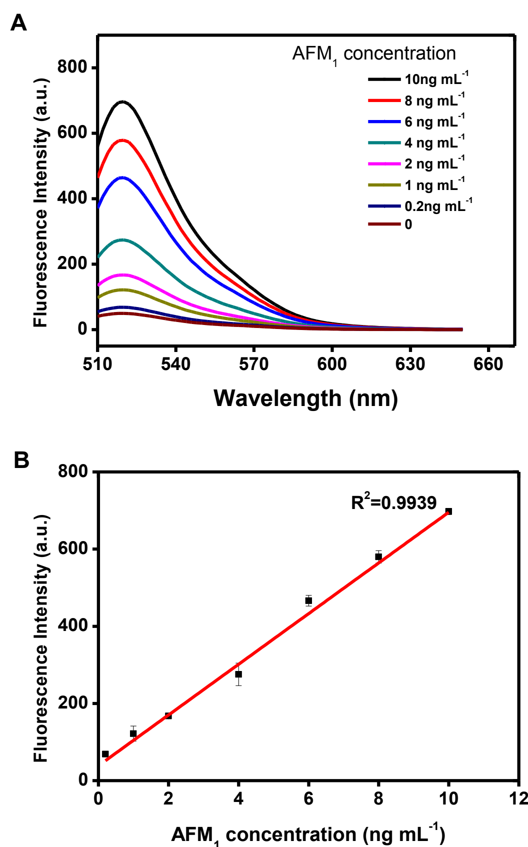

Figure 4-3: (A) Fluorescence emission spectra of the aptasensor in the addition of AFM1 at various concentrations. (B) Linear relationship between the fluorescence intensity and AFM1 concentrations in the range of 0.2 to 10 ng mL^{-1}.

Table 4-1: Comparison of the sensitivity of currently available methods for the detection of AFM_1.

No	Method	LOD	reference
1	Fluorometric sensor	0.025 μg L^{-1}	(Cucci et al., 2007)
2	Electrochemical immunosensors	0.001 μg L^{-1}	(Neagu et al., 2009)
3	Indirect competitive ELISA	0.04 μg L^{-1}	(Pei et al., 2009)
4	Impedimetric biosensor	1 μg L^{-1}	(Dinckaya et al., 2011)
5	Cellular biosensor	0.005 μg L^{-1}	(Larou et al., 2013)
6	Direct chemiluminescent ELIS	1 ng L^{-1}	(Vdovenko et al., 2014)
7	DART-MS	1 μg L^{-1}	(Busman et al., 2015)
8	Impedimetric aptasensor	1.15 ng L^{-1}	(Istamboulie et al., 2016)
9	RT-qPCR aptasensor	0.03 ng L^{-1}	(Guo et al., 2016)
10	Graphene oxide-based aptasensor	0.05 μg kg^{-1}	This work

3.4 The specificity of the aptsensor

The specificity of the aptasensor was also investigated to assess the effect of other mycotoxins. The change of the fluorescence intensity was measured under the identical experiment conditions as AFM1 with the presence of four other mycotoxins (including AFB1, OTA, ZEA and α-ZOL) at a concentration of 4 ng mL^{-1}. It can be seen that significantly higher fluorescence intensity was obtained for AFM1 determination in comparison with other mycotoxins as that of control test (Figure 4-4), which indicated that the specificity of this amplified sensing platform is suitable for AFM1 determination.

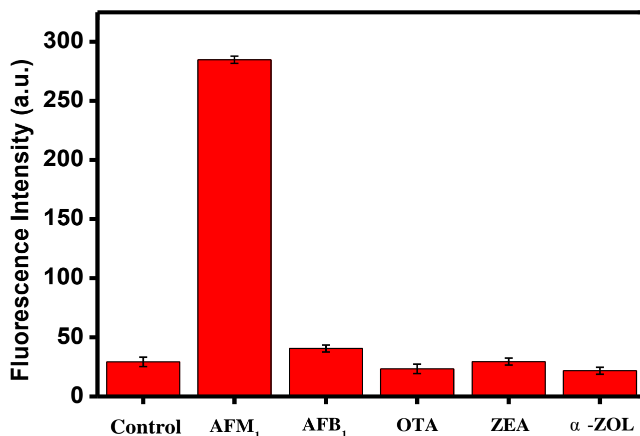

Figure 4-4: Fluorescence intensity in the absence (control) and presence of 4 ng mL^{-1} mycotoxins: AFM1, AFB1, OTA, ZEA, and α-ZOL. The experiment conditions are as follows: Excitation wavelength (λex)is set at 480 nm, 200nM AFM1 aptamer, 20 μg mL^{-1} GO, 200 U DNase I. Every data point is the mean of three replicates.

3.5 Method validation

Ultimately, the applicability and reliability of the aptasensor platform was evaluated by detecting different concentrations of AFM1 in infant milk powder samples. As indicated in Table 2, the recovery of the spiked infant milk powder samples ranged from 92% to 126%, demonstrating that the amplification strategy developed in this work can be useful as a quantitative method for AFM1 analysis in real samples for food safety.

Table 4-2: Determination of AFM_1 spiked into infant milk powder samples.

Sample	Spiked concentration (μg/kg)	Detected concentrations Mean[a] ±SD[b] (μg/kg)	Recovery (%)
Infant milk powder	0	ND[c]	-
	1.5	1.48±0.06	98
	2.5	2.3±0.42	92
	5.0	6.3±0.06	126

The mean of three replicates; b. SD = standard deviation; c. ND = not detected.

4 Conclusion

A novel graphene oxide-based aptasensor was developed for the detection of AFM1 with high sensitivity and specificity. This technique uses the properties of GO as an aptamer protector against nuclease cleavage. Thereby allowing DNase I to cleave the aptamer for a target cycling signal amplification. Under the optimal conditions, a good linear relationship was detected between fluorescence intensity and AFM1 levels in the range of 0.2 to 10 μg/kg with a detection limit of 0.05 μg/kg. Satisfactory recoveries were measured in infant milk powder samples spiked with different concentrations of AFM1. Furthermore, the aptasensor proposed in this work is rapid, simple and low-cost in comparison with other methods reported previously. This study could thus provide a very promising platform for the analysis of AFM1 in dairy products. More importantly, the aptasensor could be improved by replacing aptamer sequences for the detection of other food safety targets.

Author contributions

This work proposed in this paper was carried out in collaboration with all the authors. X.D.G. and F.W. proposed the idea of the paper, wrote the original paper and analyzed the experimental data. Q.Q.Q. and N.Z. supported the structure of the paper. M.S. and M.L.F. and J.Q.W revised the paper.

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References

Anfossi, L.; Baggiani, C.; Giovannoli, C.; Biagioli, F.; D'Arco, G.; Giraudi, G. Optimization of a lateral flow immunoassay for the ultrasensitive detection of aflatoxin M1 in milk. Anal Chim Acta. 2013, 772, 75-80.

Anfossi, L.; Di Nardo, F.; Giovannoli, C.; Passini, C.; Baggiani, C. Enzyme immunoassay for monitoring aflatoxins in eggs. Food Control. 2015, 57, 115-121.

Barthelmebs, L.; Hayat, A.; Limiadi, A.W.; Marty, J.L.; Noguer, T. Electrochemical DNA aptamer-based biosensor for OTA detection, using superparamagnetic nanoparticles. Sens. Actuator B- Chem. 2011, 156, 932-937.

Beltrán, E.; Ibáñez, M.; Sancho, J.V.; Cortés, M.Á.; Yusà, V.; Hernández, F. UHPLC–MS/MS highly sensitive determination of aflatoxins, the aflatoxin metabolite M1 and ochratoxin A in baby food and milk. Food Chem. 2011, 126, 737-744.

Bonel, L.; Vidal, J.C.; Duato, P.; Castillo, J.R. An electrochemical competitive biosensor for ochratoxin A based on a DNA biotinylated aptamer. Biosens. Bioelectron. 2011, 26, 3254-3259.

Busman, M.; Bobell, J.R.; Maragos, C.M. Determination of the aflatoxin M1 (AFM1) from milk by direct analysis in real time-mass spectrometry (DART-MS). Food Control. 2015, 47, 592-598.

Chen, D.; Feng, H.; Li, J. Graphene oxide: preparation, functionalization, and electrochemical applications. Chem Rev. 2012, 112, 6027-6053.

Chen, S.; Li, F.; Fan, C.; Song, S. Graphene-based nanoprobes for molecular diagnostics. Analyst. 2015, 140, 6439-6451.

Cheng, F.F.; Zhang, J.J.; He, T.T.; Shi, J.J.; Abdel-Halim, E.; Zhu, J.J. Bimetallic Pd–Pt supported graphene promoted enzymatic redox cycling for ultrasensitive electrochemical quantification of microRNA from cell lysates. Analyst. 2014, 139, 3860-3865.

Commission Recommendation (EC), Setting maximum levels for certain contaminants in foodstuffs. Off J Eur Union. 2006, 364, 5–24.

Cucci, C.; Mignani, A.G.; Dall'Asta, C.; Pela, R.; Dossena, A. A portable fluorometer for the rapid screening of M1 aflatoxin. Sens. Actuator B- Chem. 2007, 126, 467-472.

Dinckaya, E.; Kinik, O.; Sezginturk, M.K.; Altug, C.; Akkoca, A. Development of an impedimetric aflatoxin M1 biosensor based on a DNA probe and gold nanoparticles. Biosens. Bioelectron. 2011, 26, 3806-3811.

Food and Agriculture Organization of the United Nations, Worldwide regulations for mycotoxins in food and feed in 2003. Food and Nutrition Paper. 2004, 81, Rome: FAO.

Guo, X.; Wen, F.; Zheng, N.; Luo, Q.; Wang, H.; Wang, H.; Li, S.; Wang, J. Development of an ultrasensitive aptasensor for the detection of aflatoxin B1. Biosens. Bioelectron. 2014, 56, 340-344.

Guo, X.; Wen, F.; Zheng, N.; Li, S.; Fauconnier, M.L.; Wang, J. A qPCR aptasensor for sensitive detection of aflatoxin M1. Anal Bioanal Chem. 2016, 408, 5577-5584.

He, S.; Song, B.; Li, D.; Zhu, C.; Qi, W.; Wen, Y.; Wang, L.; Song, S.; Fang, H.; Fan, C. A graphene nanoprobe for rapid, sensitive, and multicolor fluorescent DNA analysis. Adv Funct Mater. 2010, 20, 453-459.

Hoyos Ossa, D.E.; Hincapié, D.A.; Peñuela, G.A. Determination of aflatoxin M1 in ice cream samples using immunoaffinity columns and ultra-high performance liquid chromatography coupled to tandem mass spectrometry. Food Control. 2015, 56, 34-40.

International Agency for Research on Cancer (IARC), Some naturally occurring substances: Food items and constituents heterocyclic aromatic amines and mycotoxins, IARC monographs on the evaluation of carcinogenic risk to humans. 1993, 56, 489-551.

International Agency for Research on Cancer (IARC), Some traditional herbal medicines, some mycotoxins, naphthalene and styrene, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. 2002, 82, 171-176.

Istamboulie, G.; Paniel, N.; Zara, L.; Reguillo Granados, L.; Barthelmebs, L.; Noguer, T. Development of an impedimetric aptasensor for the determination of aflatoxin M1 in milk. Talanta. 2016, 146, 464-469.

Kav, K.; Col, R.; Kaan Tekinsen, K. Detection of aflatoxin M1 levels by ELISA in white-brined Urfa cheese consumed in Turkey. Food Control. 2011, 22, 1883-1886.

Larou, E.; Yiakoumettis, I.; Kaltsas, G.; Petropoulos, A.; Skandamis, P.; Kintzios, S. High throughput cellular biosensor for the ultra-sensitive, ultra-rapid detection of aflatoxin M1. Food Control. 2013, 29, 208-212.

Lee, D.; Lee, K.G. Analysis of aflatoxin M1 and M2 in commercial dairy products using high-performance liquid chromatography with a fluorescence detector. Food Control. 2015, 50, 467-471.

Li, D.; Müller, M.B.; Gilje, S.; Kaner, R.B.; Wallace, G.G. Processable aqueous dispersions of graphene nanosheets. Nat Nanotechnol. 2008, 3, 101-105.

Li, P.; Zhang, Q.; Zhang, W.; Zhang, J.; Chen, X.; Jiang, J.; Xie, L.; Zhang, D. Development of a class-specific monoclonal antibody-based ELISA for

aflatoxins in peanut. Food Chem. 2009, 115, 313-317.

Liu, D.; Huang, Y.; Wang, S.; Liu, K.; Chen, M.; Xiong, Y.; Yang, W.; Lai, W. A modified lateral flow immunoassay for the detection of trace aflatoxin M1 based on immunomagnetic nanobeads with different antibody concentrations. Food Control. 2015, 51, 218-224.

Lu, C.H.; Lim J.; Lin, M.H.; Wang, Y.W.; Yang, H.H.; Chen, X.; Chen, G.N. Amplified Aptamer‐Based Assay through Catalytic Recycling of the Analyte. Angew Chem Int Ed. 2010, 122, 8632-8635.

Lv, L.; Li, D.; Cui, C.; Zhao, Y.; Guo, Z. Nuclease-aided target recycling signal amplification strategy for ochratoxin A monitoring. Biosens. Bioelectron. 2017, 87, 136-141.

Mao, J.; Lei, S.; Liu, Y.; Xiao, D.; Fu, C.; Zhong, L.; Ouyang, H. Quantification of aflatoxin M1 in raw milk by a core-shell column on a conventional HPLC with large volume injection and step gradient elution. Food Control. 2015, 51, 156-162.

Neagu, D.; Perrino, S.; Micheli, L.; Palleschi, G.; Moscone, D. Aflatoxin M1 determination and stability study in milk samples using a screen-printed 96-well electrochemical microplate. Int Dairy J. 2009, 19, 753-758.

Pei, H.; Li, J.; Lv, M.; Wang, J.; Gao, J.; Lu, J.; Li, Y.; Huang, Q.; Hu, J.; Fan, C. A graphene-based sensor array for high-precision and adaptive target identification with ensemble aptamers. J Am Chem Soc. 2012, 134, 13843-13849.

Pei, S.C.; Zhang, Y.Y.; Eremin, S.A.; Lee, W.J. Detection of aflatoxin M1 in milk products from China by ELISA using monoclonal antibodies. Food Control. 2009, 20, 1080-1085.

Pietri, A.; Fortunati, P.; Mulazzi, A.; Bertuzzi, T. Enzyme-assisted extraction for the HPLC determination of aflatoxin M1 in cheese. Food Chem. 2016, 192, 235-241.

Pu, Y.; Zhu, Z.; Han, D.; Liu, H.; Liu, J.; Liao, J.; Zhang, K.; Tan, W. Insulin-binding aptamer-conjugated graphene oxide for insulin detection. Analyst. 2011, 136, 4138-4140.

Rao, CeNeR.; Sood, AeK.; Subrahmanyam, KeS.; Govindaraj, A. Graphene: the new two‐dimensional nanomaterial. Angew Chem Int Ed. 2009, 48, 7752-7777.

Shim, W.B.; Kim, M.J.; Mun, H.; Kim, M.G. An aptamer-based dipstick assay for the rapid and simple detection of aflatoxin B1. Biosens. Bioelectron. 2014, 62, 288-294.

Tang, D.; Tang, J.; Li, Q.; Su, B.; Chen, G. Ultrasensitive aptamer-based multiplexed electrochemical detection by coupling distinguishable signal tags with catalytic recycling of DNase I. Anal Chem. 2011, 83, 7255-7259.

Tang, L.; Wang, Y.; Li, J. The graphene/nucleic acid nanobiointerface. Chem Soc Rev. 2015, 44, 6954-6980.

Vdovenko, M.M.; Lu, C.C.; Yu, F.Y.; Sakharov, I.Y. Development of ultrasensitive direct chemiluminescent enzyme immunoassay for determination of aflatoxin M1 in milk. Food Chem. 2014, 158, 310-314.

Wang, R.; Xiang, Y.; Zhou, X.; Liu, L.H.; Shi, H. A reusable aptamer-based evanescent wave all-fiber biosensor for highly sensitive detection of Ochratoxin A. Biosens. Bioelectron. 2015, 66, 11-18.

Wang, X.; Li, P. Rapid screening of mycotoxins in liquid milk and milk powder by automated size-exclusion SPE-UPLC-MS/MS and quantification of matrix effects over the whole chromatographic run. Food Chem. 2015, 173, 897-904.

Wang, Y.; Liu, X.; Xiao, C.; Wang, Z.; Wang, J.; Xiao, H.; Cui, L.; Xiang, Q.; Yue, T. HPLC determination of aflatoxin M1 in liquid milk and milk powder using solid phase extraction on OASIS HLB. Food Control. 2012, 28, 131-134.

Zhang, H.; Jia, S.; Lv, M.; Shi, J.; Zuo, X.; Su, S.; Wang, L.; Huang, W.; Fan, C.; Huang, Q. Size-dependent programming of the dynamic range of graphene oxide–DNA interaction-based ion sensors. Anal Chem. 2014, 86, 4047-4051.

Zhang, J.; Li, Z.; Zhao, S.; Lu, Y. Size-dependent modulation of graphene oxide–aptamer interactions for an amplified fluorescence-based detection of aflatoxin B1 with a tunable dynamic range. Analyst. 2016, 141, 4029-4034.

5

Graphene oxide driven fluorescent aptasensor for the detection of fumonisin B1

In this work, a novel graphene oxide-based fluorescent aptasensor for the amplified detection of FB1 has been introduced. Upon the presence of FB1, the formation of FB1/aptamer complex led to the separation of aptamer from the surface of GO. The aptamer was then cleavaged by DNase I and release FB1, which was the target for the next cycle. As a consequence, the fluorescence signal was determined to be a linear relationship versus the concentration of FB1. More importantly, this sensing strategy was simple, rapid, easy to use, and low cost with high sensitivity and selectivity.

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Abstract: Fumonisin B1 (FB1), one of the most toxic mycotoxins, has been designated as possible 2B group carcinogen by the International Agency for Research on Cancer (IARC) in 2002. Therefore, simple, sensitive and specific approaches for the detection of FB1 are much in demand. In this study, a novel aptasensor was introduced for FB1 analysis based on graphene oxide (GO) and DNase I signal amplification. GO was adopted as a fluorescence quencher against ROX-modified aptamer and a protectant for the aptamer from cleavaging by DNase I for subsequent target cycling and signal amplification detection. This proposed sensing strategy exhibited a good linearity for FB1 determination in the dynamic range from 0.5 to 20 ng mL^{-1} with a good correlation of R^2 = 0.995. Its detection of limit was established at 0.15 ng mL^{-1} (S/N = 3). The specific analysis indicated that this aptasensor was selective for FB1 other than other mycotoxins. In addition, the practical application in real samples of this aptasensor for the detection of FB1 was investigated. The sensing platform proposed here was useful for a potential application in the field of food safety for mycotoxins analysis.

Keywords: Aptamer; Aptasensor; Fumonisin B1; Graphene oxide; DNase I; Food safety

1 Introduction

Mycotoxins are toxic secondary metabolites produced and released by a variety of fungi, which poses serious hazards to human health via contamination of feeds and foods (Atar, Eren, and Yola, 2015; Mata et al., 2015; Zhu et al., 2015). Fumonisins, a group of carcinogenic mycotoxins, are mainly produced by *Fusarium moniliforme* and *Fusarium proliferatum* composed of various tricarballylic acid and polyhydric alcohol (Ross et al., 1990; Richard et al., 2007; Quan et al., 2006). Of the four series of fumonisins (A, B, C, and P), fumonisin B1 (FB1) is the most toxic and the most common one, which accounts for 70% (Rheeder et al., 2002; Fernández-Blanco et al., 2016; Martins et al., 2012; Krska et al., 2007). In addition, FB1 has been classified as possible 2B group carcinogen by the International Agency for Research on Cancer (IARC) in 2002 (IARC, 2002). Therefore, the United States Food and Drug Administration (FDA) has regulated the maximum limits for total fumonisin (combined FB1, FB2, and FB3) to be 2 mg kg^{-1} in degermed dry-milled corn products (Shephard et al., 2002; Li et al., 2012), while the much more restrictive limit for that value was established at 0.8 mg kg^{-1} by European Commission Regulation (Anfossi et al., 2016) Taking the low permissible limit and toxic effect into consideration, rapid, sensitive and specific analytical strategies of FB1 are required and important for food safety.

Confirmatory and quantitative approaches have been applied to FB1 determination, which mainly includes high-performance liquid chromatography (HPLC) (Martins et al., 2012; Muscarella et al., 2008), high-performance liquid chromatography combined with mass spectrometry (LC–MS) (Gazzotti et al., 2009; Ediage et al., 2012), and gas chromatography combined with mass spectrometry (GC–MS) (Plattner et al., 1991). However, they usually require expensive instruments and highly trained personnel, as well as complicated procedures. Meanwhile, researchers paid great attention to immunoassays-based rapid analysis methods for the detection of FB1, such as enzyme-linked immune sorbent assay (ELISA) (Wang et al., 2014), immunochromatographic assay (Wang et al., 2014), and electrochemical immunoassays (Masikini et al., 2016; Yang et al., 2015; Milua et al., 2015). Disadvantages like antibody produce and its stability limits the application of immunoassays in this field. Aptamers, an alternative to antibodies, are single-stranded DNA or RNA oligonucleotides selected by systematic evolution of ligands by exponential enrichment (SELEX) in vitro (Ellington et al., 1990; Tuerk et al., 1990). Due to the advantages of aptamer such as high specificity, low lost, simple synthesis, ease of modification and high stability, aptamer-based sensors have been developed for FB1 determination based on fluorescent detection (Wu et al., 2012), and electrochemical analysis (Shi et al., 2015; Zhao et al., 2014), as well as microcantilever array method (Chen et al., 2015). However, these approaches require conjugation between the aptamer and probes, and time-consuming detection procedures. Thus, simple, rapid, and cost-effective sensing strategies for FB1 determination are still challenging.

Graphene oxide (GO) is a two-dimensional nanoparticle, which has attracted great

attention to the application of sensing platforms due to the unique mechanical, electrical and thermal characteristics (Müller et al., 2008; Rao et al., 2009; Chen et al., 2012). Graphene oxide could be employed as a fluorescence quencher with high efficiency owing to its highly distance-dependent fluorescence quenching property. Single-stranded DNA aptamers are ease of modification with fluorophores, and the aptamer can bind to GO strongly via π stacking (He et al., 2010). Subsequently, the fluorescence quenching performance would be achieved. In addition, graphene oxide could protect single-stranded DNA aptamers from nuclease cleavage due to the hydrophobic stacking reactions between GO and nucleobases (Lu et al., 2010; Tang et al., 2011). To the best of our knowledge, aptasensors based on GO fluorescence-quenching and aptamer protection for FB1 amplified determination have not been reported previously.

In this work, a novel graphene oxide-based fluorescent aptasensor for the amplified detection of FB1 has been introduced. Upon the presence of FB1, the formation of FB1/aptamer complex led to the separation of aptamer from the surface of GO. The aptamer was then digested by DNase I and release FB1, which was the target for the next cycle. As a consequence, the fluorescence signal was determined to be a linear relationship versus the concentration of FB1. More importantly, this sensing strategy was simple, rapid, easy to use, and low cost with high sensitivity and selectivity.

2 Experimental

2.1 Materials and reagents

Aflatoxin B1 (AFB1), aflatoxin M1 (AFM1), ochratoxin A (OTA) and fumonisin B1 (FB1) standard substances were purchased from Sigma-Aldrich (USA). Graphene oxide and DNase I (RNase-free) were obtained from Sigma-Aldrich (USA). Other chemicals such as potassium chloride (KCl), sodium chloride (NaCl), anhydrous calcium chloride ($CaCl_2$), and 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris) were obtained from Shanghai Chemical Reagent Company (Shanghai, China). All chemicals were at least of analytical grade and were used as received without further purification. Double-distilled water was used in the whole study. The specific aptamer oligonucleotides used in this study were chemically synthesized by Sangon Biotech. Co. Ltd. (Shanghai, China) and purified by HPLC. Aptamer stock solution was prepared using Tris buffer (10 mM Tris, 120 mM NaCl, 5 mM KCl, 20 mM $CaCl_2$, pH 7.0), the aptamer sequence of the carboxyl-X-rhodamine (ROX)-labelled FB1 aptamer was as follows (McKeague et al., 2010):

5′-ROX-ATACCAGCTTATTCAATTAATCGCATTACCTTATACCAGCTTATTCAATTACGTCTGCACATACCAGCTTATTCAATTAGATAGTAAGTGCAATCT-3′

2.2 Fluorescent aptasensing towards FB1

For FB1 amplified detection, the ROX-labelled aptamer was diluted to 100 nM in

Tris buffer. 20 μg mL^{-1} of graphene oxide was mixed with the aptamer solution for 15 min at room temperature to form aptamer/GO complex (Figure 1) and the fluorescence of the aptamer was significantly quenched. Subsequently, different amounts of FB1 and DNase I (100 U) were added to the solution simultaneously. Then, the mixture was incubated for 1 h at room temperature. Ultimately, the fluorescence signals of the mixture were measured via Shimadzu RF-5301 Luminescence Spectrometer (Tokyo, Japan). The emission spectra were recorded in the wavelengths ranging from 590 to 690 nm, and the excitation wavelength was 585 nm. Slit widths for both of the excitation and emission were set at 10 nm.

2.3 Specificity analysis

Three mycotoxins such as AFB1, AFM1, and OTA were used to investigate the selectivity of this sensing strategy. Each toxin standard substance was respectively added to this experiment with the same concentration of 5 ng mL^{-1}. The detection procedures were identical to that for the analysis of FB1.

2.4 Practicability of the sensing platform

The practicability of this sensing method was evaluated for quantitative FB1 analysis in wheat flour samples. All samples were spiked with 2 mL of FB1 (0, 1.5, 8, and 15 ng mL^{-1}) and were performed in triplicate to make final concentrations of 0, 1.5, 8, and 15 μg kg^{-1} . Each sample was accurately weighed (2.00 ± 0.05 g), 2 mL of extraction solution (50% methanol in water) was then added. The entire mixture was centrifuged at 10,000 g for 10 min. Subsequently, the mixture was filtrated for three times using a syringe filter (0.45 μm). Finally, the filtrate was collected and measured using this amplified aptasensor experiments.

2.5 Statistical analysis

Standard deviations (SDs) and means for fluorescence signals were performed in triplicate. The analysis of FB1 calibration curve standards and samples were achieved from three replicates. Fluorescence-emission spectra curves for FB1 were plotted with 8.0 software (OriginLab Corporation, Northampton, MA, USA). Linear regression analysis of the fluorescence intensity versus FB1 concentrations was carried out using Microsoft Excel.

3 Results and discussion

3.1 Sensing strategy for FB1 detection

Graphene oxide has attracted much attention to the construction of biosensors due to the special electrical and thermal characteristics. More importantly, graphene oxide can bind to ssDNA like aptamers with high efficiency owing to π stacking and hydrophobic interactions. Subsequently, the fluorescence of fluorophore-labelled aptamer would be quenched by GO due to its great fluorescence quenching performance (Chen et al., 2015). In order to improve the detection sensitivity, the DNase I was employed to digest the aptamer for the release of target FB1.

Schematic introduction of this aptasensor based on GO and DNase I performance was illustrated in Figure 5-1. In this study, the aptamer was designed with ROX fluorophore modification. When the aptamer was added to GO solution, the fluorescence of the aptamer decreased dramatically, which indicated that a great adsorption of the aptamer to the surface of GO and its great fluorescence quenching performance was achieved. When in presence of FB1, structural change of aptamer was produced as a result of the formation of aptamer/FB1 complex, leading to the release of the aptamer to the solution. Then, the aptamer was digested by DNase I. The target FB1 was released from the complex and was available for binding other aptamer. Therefore, a cycle enhancement of fluorescence signal was achieved. Ultimately, a correlation response would be obtained between fluorescence intensity and the concentrations of FB1.

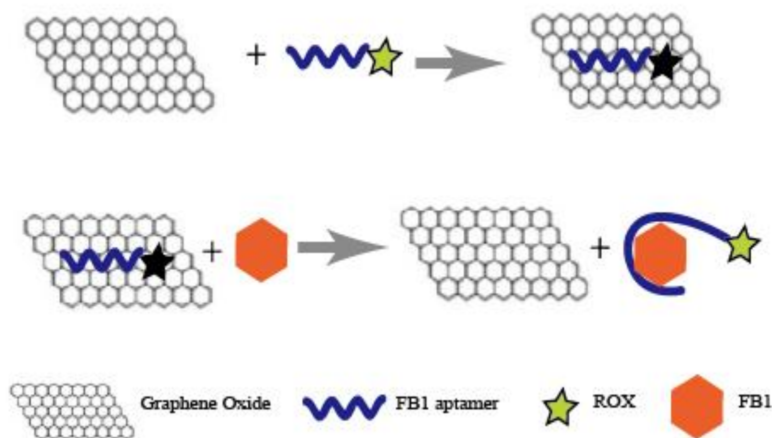

Figure 5-1: Illustration of the aptasensor for detection of fumonisin B1.

3.2 Amplified detection of FB1 with DNase I

As seen in Figure 5-2, upon the presence of GO (20 µg mL^{-1}), the fluorescence signal of the aptamer was quenched dramatically. In the addition of FB1 at the concentrations of 10 ng mL^{-1}, the fluorescence signal was recovered, indicating that the aptamer/FB1 complex was produced and the fluorescence signal of released aptamer was observed. In addition, the modification of fluorophore ROX had no influence on recognition performance of the specific aptamer. When FB1 and DNase I were added in the solution simultaneously, a significant enhancement of fluorescence was achieved, which demonstrated that the presence of DNase I induced a signal amplification of fluorescence intensity and a amplified detection of FB1.

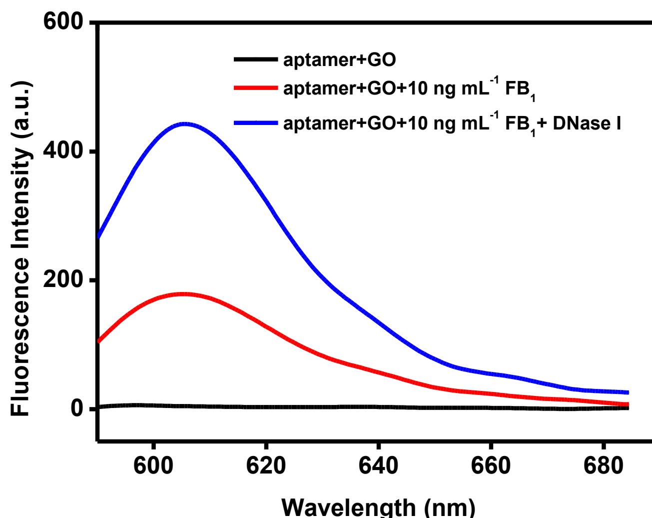

Figure 5-2: Fluorescence emission spectra of the aptasensor in the absence (0) of FB1, presence of 10 ng mL^{-1} FB1, and 10 ng mL^{-1} FB1 and 100 U DNase I. Excitation wavelength (λex)is set at 585 nm. Conditions: 100 nM FB1 aptamer, 20 μg mL^{-1} GO in Tris buffer (10 mM Tris, 120 mM NaCl, 5 mM KCl, 20 mM $CaCl_2$, pH 7.0).

3.3 Analytical performance of this sensing platform

The analytical performance of this GO and DNase I-induced amplified sensing platform was investigated via the measurement of fluorescence emission spectrum versus different concentrations of FB1. The excitation wavelength was 585 nm and the emission wavelength was 605 nm. As illustrated in Figure 5-3, the fluorescence signal enhanced with the increase of FB1 levels ranging from 0.5 to 20 ng mL^{-1}. A good linear response was achieved between the fluorescence intensity and concentrations of FB1 with the linear equation as F = 31.65 C + 126.05 and a good correlation of R^2 = 0.995, in which F was adopted as the fluorescence intensity and C was the levels of FB1. Its limit of detection (LOD) was established at 0.15 ng mL^{-1} based on the signal-to-noise ratio of 3, which indicated a wide dynamic detection range and compatible LOD for the target FB1 compared with other methods presented in the current literatures.

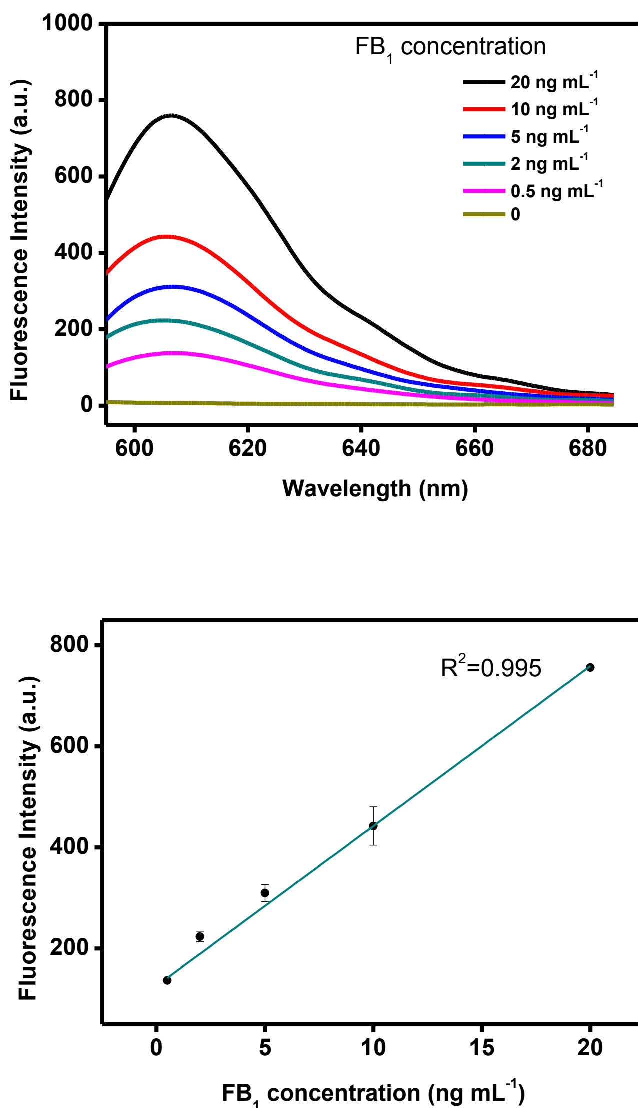

Figure 5-3: (up) Fluorescence emission spectra of the aptasensor in the addition of FB1 at various concentrations. (down) Linear relationship between the fluorescence intensity and FB1 concentrations in the range of 0.5 to 20 ng mL^{-1}.

Table 5-1: Comparison of the sensitivity of currently available methods for the detection of FB_1.

No	Method	LOD	reference
1	Chemiluminescence ELISA	0.12 ng mL^{-1}	(Shu et al., 2016)
2	Differential pulse voltammetry	2 pg mL^{-1}	(Yang et al., 2015)
3	Amperometric	0.33 μg L^{-1}	(Jodra et al., 2015)
4	ELISA	0.15 ng mL^{-1}	(Shu et al., 2015)
5	Chemiluminescence	1.7 pg mL^{-1}	(Li et al., 2016)
6	Fluorescent aptasensor	0.15 ng mL^{-1}	This work

3.4 The specificity of this sensing platform

Specificity analysis played an very important part for mycotoxins detection. In order to examine the specificity of this sensing strategy towards FB1, other mycotoxins (including AFB1, AFM1, and OTA) were employed to this analysis system at a same concentration of 5 ng mL^{-1} as that of FB1. In addition, the other experiment conditions were also identical as FB1 determination. As shown in Figure 5-4, this GO-based aptasensor exhibited high fluorescence signal in the presence of FB1 while low fluorescence signal was observed in the presence of other three mycotoxins and the control group, which attributed to the specific recognition of the aptamer for FB1. Therefore, the good specificity of this aptasensor was achieved for the detection of FB1.

3.5 Method validation

Practical application of this novel aptasensor was carried out by the analysis of FB1 in wheat flour samples. As seen in table 2, the recovery ratios of the spiked wheat flour samples were from 99% to 111%, which further indicated that this proposed sensing strategy was a promising platform for the detection of FB1 in real samples.

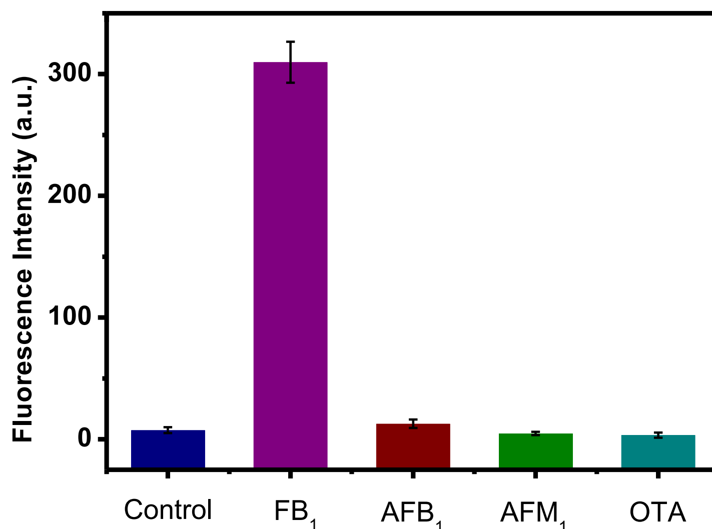

Figure 5-4: Fluorescence intensity in the absence (control) and presence of 5 ng mL^{-1} mycotoxins: FB1, AFM1, AFB1, OTA. The experiment conditions are as follows: Excitation wavelength (λex)is set at 585 nm, 100 nM FB1 aptamer, 20 μg mL^{-1} GO, 100 U DNase I. Every data point is the mean of three replicates.

Table 5-2: Determination of FB_1 from the wheat flour samples.

Sample	Spiked concentration (ng/mL)	Detected concentrations Mean[a] ±SD[b] (ng/mL)	Recovery (%)
Wheat flour	0	ND[c]	-
	1.5	1.67±0.02	111
	8	7.93±0.56	99
	15	15.47±0.68	103

a. The mean of three replicates; b. SD = standard deviation; c. ND = not detected

4 Conclusion

In this work, a novel, sensitive and accurate aptasensor for amplified and specific detection of FB1 was first introduced, which relies on the GO and DNase I-induced target cycling and signal enhancement strategies. A widely dynamic range from 0.5 to 20 ng mL^{-1} was achieved between the fluorescence intensity and concentrations of FB1, its detection of limit was determined to be 0.15 ng mL^{-1}, which is sensitive and compatible with the current methods. In addition, the specific tests and practical analysis performance were also examined by detecting different mycotoxins and real wheat flour samples. This proposed GO and DNase I driven fluorescent aptasensor offered a promising application in mycotoxin detection for food safety.

Author contributions

This work proposed in this paper was carried out in collaboration with all the authors. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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References

Anfossi, L., Giovannoli, C., and Baggiani, C. Mycotoxin detection. Current Opinion in Biotechnology. 2016, 37, 120–126.

Atar, N., Eren, T., Yola, M. L. A molecular imprinted SPR biosensor for sensitive determination of citrinin in red yeast rice. Food Chem. 2015, 184, 7–11.

Chen, D., Feng, H., Li, J. Graphene oxide: Preparation, functionalization, and electrochemical applications. Chem. Rev. 2012, 112, 6027–6053.

Chen, S., Li, F., Fan, C., Song, S. Graphene-based nanoprobes for molecular diagnostics. Analyst. 2015, 140, 6439–6451.

Chen, X. J., Bai, X. J., Li, H. Y., Zhang, B. L. Aptamer based microcantilever array biosensor for detection of fumonisin B-1. RSC Adv. 2015, 09, 55–59.

Ediage, E. N., Mavungu, J. D. D., Song, S., Wu, A., Peteghem, C. V., Saeger, S. D. A direct assessment of mycotoxin biomarkers in human urine samples by liquid chromatography tandem mass spectrometry. Anal. Chim. Acta, 2012, 741,

58–69.

Ellington, A. D., and Szostak, J. W. In vitro selection of RNA molecules that bind specifc ligands. Nature, 1990, 346, 818–822.

Fernández-Blanco, C., Frizzell, C., Shannon, M., Ruiz, M. J., Connolly, L. An in vitro investigation on the cytotoxic and nuclear receptor transcriptional activity of the mycotoxins fumonisin B1 and beauvericin. Toxicol. Lett, 2016, 257, 1–10.

Gazzotti, T., Lugoboni, B., Zironi, E., Barbarossa, A., Serraino, A., Pagliuca, G. Determination of fumonisin B1 in bovine milk by LC-MS/MS. Food Control. 2009, 20, 1171–1174.

He, S., Song, B., Li, D., Zhu, C., Qi, W., Wen, Y., Wang, L., Song, S., Fang, H., Fan, C. A graphene nanoprobe for rapid, sensitive, and multicolor fluorescent DNA analysis. Adv. Funct. Mater. 2010, 20, 453–459.

International Agency for Research on Cancer (IARC), Fumonisin B1. In IARC Monographs on the EValuation of Carcinogenic Risks to Humans, Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene, IARC: Lyon, France. 2002, 82, 301–366.

Jodra, A., Ángel López, M., Escarpa, A. Disposable and reliable electrochemical magnetoimmunosensor for fumonisins simplified determination in maize-based foodstuffs. Biosens. Bioelectron, 2015, 64, 633–638.

Krska, R., Welzig, E. and Boudra, H. Review: Analysis of Fusarium toxins in feed. Anim. Feed Sci. Technol. 2007, 137, 241–264.

Li, D., Müller, M. B., Gilje, S., Kaner, R. B., Wallace, G. G. Processable aqueous dispersions of graphene nanosheets. Nat. Nanotechnol. 2008, 3, 101–105.

Li, L., Xia, L. R., Zhao, Y. F., Wang, H. Y., Development of immune-affinity 96 spots monolith array for multiple mycotoxins detection in food samples. J. Chromatogr. B. 2016, 1029–1030, 72–80.

Li, Y. S., Zhou, Y., Lu, S. Y., Guo, D. J., Ren, H. L., Meng, X. M., Zhi, B. H., Lin, C., Wang, Z., Li, X. B., Liu, Z. S. Development of a one-step test strip for rapid screening of fumonisins B1, B2 and B3 in maize. Food Control. 2012, 24, 72–77.

Lu, C.H., Lim, J., Lin, M.H., Wang, Y.W., Yang, H.H., Chen, X., Chen, G.N. Amplified Aptamer-Based Assay through Catalytic Recycling of the Analyte. Angew. Chem. Int. Ed. 2010, 122, 8632–8635.

Martins, F. A., Ferreira, F. M. D., Bando, É., Nerilo, S. B., Hirook, E. Y., Machinski, J. M. Daily intake estimates of fumonisins in corn-based food products in the population of Parana, Brazil. Food Control, 2012, 26, 614–618.

Martins, H. M. L., Almeida, I. F. M., Camacho, C. R. L., Santos, S. M. O., Costa, J. M. G., and Bernardo, F. M. A. Rev Iberoam Micol. 2012, 29, 175–177.

Masikini, M., Williams, A.R., Sunday, C.E., Waryo, T.T., Nxusani, E., Wilson, L., Qakala, S., Bilibana, M., Douman, S., Jonnas, A., Baker, P. G. L., Iwuoha, E. I. Label free poly(2,5-dimethoxyaniline)-multi-walled carbon nanotubes

impedimetric immunosensor for fumonisin B1 detection. Materials. 2016, 9, 273–285.

Mata, A. T., Ferreira, J. P., Oliveira, B. R., Batoreu, M. C., Barreto Crespo, M. T., Pereira, V. J., and Bronze, M. R. Bottled water: analysis of mycotoxins by LC-MS/MS. Food Chemistry. 2015, 176, 455–464.

McKeague, M., Bradley, C. R., De Girolamo, A., Visconti, A., Miller, J. D., DeRosa, M. C. Screening and Initial Binding Assessment of Fumonisin B1 Aptamers. Int. J. Mol. Sci. 2010, 11, 4864–4881.

Milua, M., Stephen, N. M., Abebaw, T., Njagi, N., Kerileng, M. M., Chinwe, O. I., Christopher, E. S., Candice, R., Lindsay, W., Priscilla, G. L. B., Emmanuel, I. I. A fumonisins immunosensor based on polyanilino-carbon nanotubes doped with palladium telluride quantum dots. Sensors. 2015, 15, 529–546.

Muscarella, M., Magro, S. L., Nardiello, D., Palermo, C., Centonze, D. Development of a new analytical method for the determination of fumonisins B1 and B2 in food products based on high performance liquid chromatography and fluorimetric detection with post-column derivatization. J. Chromatogr. A. 2008, 1203, 88–93.

Plattner, R. D., Ross, P. F., Reagor, J., Stedelin, J., Rice, L. G. Analysis of corn and cultured corn for fumonisin B1 by HPLC and GC/MS by four laboratories. J. Vet. Diagn. Invest. 1991, 3, 357–358.

Quan, Y., Zhang, Y., Wang, S., Lee, N., and Kennedy, I. R. A rapid and sensitive chemiluminescence enzyme-linked immunosorbent assay for the determination of fumonisin B1 in food samples. Anal. Chim. Acta. 2006, 580, 1–8.

Rao, C. E. E. Sood, A. E. Subrahmanyam, K. E. Govindaraj, A. Graphene: The new two-dimensional nanomaterial. Angew. Chem. Int. Ed. 2009, 48, 7752–7777.

Rheeder, J. P., Marasas, W.F.O., Vismer, H.F. Production of fumonisin analogs by fusarium species. Appl. Environ. Microbiol. 2002, 68, 2101–2105.

Richard, J. L. Some major mycotoxins and their mycotoxicoses — An overview. Int. J. Food Microbiol. 2007, 119, 3–10.

Ross, P. F., Nelson, P. E., Richard, J. L., Osweiler, G. D., Rice, L. G., Plattner, R. D. Production of fumonisins by fusarium moniliforme and fusarium proliferatum isolates associated with equine leukoencephalomalacia and a pulmonary edema syndrome in swine. Appl. Environ. Microbiol. 1990, 56, 3225–3226.

Shephard, G. S., Leggott, N. L., Stockenstrom, S., Somdyala, N. I. M. and Marasas, W. F. O. Preparation of South African maize porridge: effect on fumonisin mycotoxin levels. S. Afr. J. Sci. 2002, 98, 393–396.

Shi, Z. Y., Zheng, Y. T., Zhang, H. B., He, C. H., Wu, W. D., Zhang, H. B. DNA electrochemical aptasensor for detecting fumonisins B1 based on graphene and thionine nanocomposite. Electroanalysis. 2015, 27, 1097–1103.

Shu, M., Xu, Y., Liu, X., Li, Y. P., He, Q. H., Tu, Z., Fu, J.H., Gee, S. J., Hammock, B. D. Anti-idiotypic nanobody-alkaline phosphatase fusion proteins: development of a one-step competitive enzyme immunoassay for fumonisin B1

detection in cereal. Anal. Chim. Acta. 2016, 924, 53–59.

Shu, M., Xu, Y., Wang, D., Liu, X., Li, Y. P., He, Q. H., Tu, Z., Qiu, Y. L., Ji, Y. W., Wang, X. X. Anti-idiotypic nanobody: a strategy for development of sensitive and green immunoassay for Fumonisin B1. Talanta. 2015, 143, 388–393.

Tang, D.; Tang, J.; Li, Q.; Su, B.; Chen, G. Ultrasensitive aptamer-based multiplexed electrochemical detection by coupling distinguishable signal tags with catalytic recycling of DNase I. Anal. Chem. 2011, 83, 7255–7259.

Tuerk, C. and Gold, L. Systematic evolution of ligands by exponential enrichment -Rna ligands to bacteriophage-T4 DNA-polymerase. Science, 1990, 249, 505–510.

Wang, Y. K., Wang, Y. C., Wang, H. A., Ji, W. H., Sun, J. H., and Yan, Y. X. An immunomagnetic-bead-based enzyme-linked immunosorbent assay for sensitive quantification of fumonisin B1. Food Control. 2014, 40, 41–45.

Wang, Z. H., Li, H., Li, C. L., Shen, J. Z., Saeger, S. D. Development and application of a quantitative fluorescence-based immunochromatographic assay for fumonisin B1 in maize. J. Agric. Food Chem. 2014, 62, 6294–6298.

Wu, S., Duan, N., Ma, X., Xia, Y., Wang, H., Wang, Z., and Zhang, Q. Multiplexed Fluorescence Resonance Energy Transfer Aptasensor between Upconversion Nanoparticles and Graphene Oxide for the Simultaneous Determination of Mycotoxins. Anal. Chem. 2012, 84, 6263–6270.

Yang, X. X., Zhou, X. P., Zhang, X., Qing, Y., Luo, M., Liu, X., Li, C. R., Li, Y. L., Xia, H. M., Qiu, J. F. A highly sensitive electrochemical immunosensor for fumonisin B1 detection in corn using single-walled carbon nanotubes/chitosan. Electroanalysis. 2015, 27, 2679–2687.

Yang, X. Y., Zhou, X., Zhang, X., Qing, Y., Luo, M., Liu, X., Li, C.R., Li, Y., Xia, H. M., Qiu, J. F. A highly sensitive electrochemical immunosensor for fumonisin B1 detection in corn using single-walled carbon nanotubes/chitosan. Electroanalysis. 2015, 27, 2679–2687.

Zhao, Y., Luo, Y. D., Li, T. T., Song, Q. J. AuNPs driven electrochemiluminescence aptasensors for sensitive detection of Fumonisin B1. RSC Adv. 2014, 4, 57709–57714.

Zhu, R., Feussner, K., Wu, T., Yan, F., Karlovsky, P., and Zheng, X. Detoxification of mycotoxin patulin by the yeast Rhodosporidium paludigenum. Food Chemistry. 2015, 179, 1–5.

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General discussion, conclusion and future prospects

Traditional analysis strategies for mycotoxins determination are mainly including thin layer chromatography (TLC) (Var et al., 2007), high-performance liquid chromatography (HPLC) (Mao et al., 2015; Wang et al., 2012; Lee et al., 2015; Pietri et al., 2016; Herzallah, 2009; Yazdanpanah et al., 2013), liquid chromatography coupled with mass spectrometry (LC–MS) (Abia et al., 2013; Warth et al., 2013), and gas chromatography coupled with mass spectrometry (GC–MS) (Plattner et al., 1991). Nevertheless, expensive instruments, time-consuming pretreatment and special personnel are essencial in these instrumental methods (Shim et al., 2007). At the same time, immunoassays based on antibody detection have been developed for the detection of mycotoxins, such as enzyme-linked immune sorbent assay (ELISA) and immunosensors methods (Li et al., 2009; Kav et al., 2011; Anfossi et al., 2015; Parker et al., 2009; Bacher et al., 2012; Vdovenko et al., 2014; Park et al., 2013; Piermarini et al., 2007; Mozaffari Nejad et al., 2014; Sheng et al., 2014; Xu et al., 2014). These immunoassays methods are sensitive and specific, while the selection process of antibody is high-cost and time-consuming. In addition, it is difficult to ensure the stability of the available antibody during the transportation and storage.

Aptamers are selected by systematic evolution of the ligand by the exponential enrichment process (SELEX) in vitro, which was first introduced against T4 DNA polymerase (Tuerk and Gold, 1990) and organic dyes (Ellington and Szostak, 1990). In the beginning 18 years from the finding of first aptamer, the selected aptamers and novel aptamer-based biosensors were mainly used in the field of medicine, biology, chemistry and bioinformatics (Wang et al., 2019; Tan et al., 2019; Zhang et al., 2019; Xiong et al., 2019). For food safety sciences, especially in mycotoxins detection for food safety, the first aptamer specific to OTA has been reported by Cruz-Aguado in 2008 (Cruz-Aguado and Penner, 2008). The aptamer sequence against FB1 was obtained by McKeague's group (McKeague et al., 2010). Neoventures Biotechnology Inc. (Canada) selected specific aptamers for AFB1 and ZEN (Patent:PCT/CA2010/001292). A high-affinity aptamer specific to AFM1 was successfully selected in 2014 (Malhotra et al., 2014). In the same year, the aptamer of ZEN was achieved as well (Chen et al., 2013). These specific aptamers have attracted more and more attention for researches to develop fluorescent, colorimetric and electrochemical aptasensors for the detection of these mycotoxins (Castillo et al., 2015; Evtugyn et al., 2013; Seok et al., 2015; Shim et al., 2014; Wang et al., 2016; Nguyen et al., 2013; Istamboulie et al., 2016; Guo et al., 2011; Kuang et al., 2010; Yang et al., 2013; Wu et al., 2012; Wu et al., 2013). However, simple aptasensor methods might have relatively low sensitivity. Therefore, signal amplification and enhancement sensing strategiesare much in demand to meet the requirement of the low permissible limits established by countries and organizations in the world.

Development of novel aptasensors for the detection of mycotoxins

Real-time quantitative polymerase chain reaction (RT-qPCR) based on fluorescence detection is an important, ultrasensitive and precise technique.

Scientists pay more attention to this technology for the detection of specific DNA fragments since RT-qPCR has the advantages of great amplification efficiency and quantitative determination (Kubista et al., 2006; Mestdagh et al., 2008; Schmittgen and Livak, 2008). There are many reports for the detection of macromolecules via RT-qPCR based on aptamer recognition reaction (Lee et al., 2009; Lin and McNatty, 2009; Pinto et al., 2009). However, few aptamer-based RT-qPCR biosensors were developed for small molecules mycotoxins detection.

By using this technology, we describe a reliable and sensitive aptamer-based biosensor for determination of AFM1, which simultaneously combined the advantages of high recognition power of the aptamer to AFM1 and excellent amplification efficiency of RT-qPCR technique to improve sensitivity. Under optimal conditions, a good linear relationship existed between Ct values and AFM1 levels over the range from 1×10^{-4} to 1 μg L^{-1} with high sensitivity (LOD = 0.03 ng L^{-1}). The detection of five other mycotoxins was limited, but there may be a small cross-reaction with AFB1. This shows its value in the determination of AFM1 for food safety with an acceptable selectivity. Importantly, this method can be applied to the detection of AFM1 in infant rice cereal and milk powder samples with satisfactory recoveries. Therefore, this aptasensor has a highly potential application for biologically small molecules.

In comparision with previously reported detection strategies, this proposed RT-qPCR-based aptasensor takes the superiority of low cost (several hundreds dollars), great sensitivity and selectivity. This method offers a highly promising platform for high-throughput determination of trace levels of AFM1 in dairy products. However, about 2.5 hours were required for the detection process, it is not a very rapid method. In addition, this developed aptasensor cannot be considered for on-site detection because the equipment is not portable but available for standard laboratory analysis, and the time-consuming pretreatment is needed in this analysis process. In future's research, we would focus on the development of rapid, portable and sensitive aptasensors for mycotoxins detection. Graphene oxide (GO), a two-dimensional nanomaterial, is a very promising tool for construction of biosensors due to the advantages of its extraordinary electrical, thermal, and mechanical properties (Li et al., 2008; Rao et al., 2009; Chen et al., 2012; Cheng et al., 2014). The predominantly distance-dependent fluorescence quenching ability makes GO a highly efficient fluorescence quencher (Zhang et al., 2014; Tang et al., 2015; Pei et al., 2012). In addition, the previous research demonstrated that single-stranded DNA could significantly interacts with GO through π stacking between DNA bases and hexagonal cells of GO (He et al., 2010). Importantly, GO could protect DNA aptamers from nuclease cleavage as a result of the hydrophobic stacking interactions between nucleobases and GO (Lu et al., 2010; Pu et al., 2011; Tang et al., 2011). To the best of our knowledge, graphene oxide-based nuclease signal amplification aptasensors for AFM1 determination have not been reported.

A novel graphene oxide-based aptasensor was developed for the detection of AFM1 with high sensitivity and specificity. This technique uses the properties of GO as an aptamer protector against nuclease cleavage. Thereby allowing DNase I to

cleave the aptamer for a target cycling signal amplification. Under the optimal conditions, a good linear relationship was detected between fluorescence intensity and AFM1 levels in the range of 0.2 to 10 μg/kg with a detection limit of 0.05 μg/kg. Satisfactory recoveries were measured in infant milk powder samples spiked with different concentrations of AFM1. Furthermore, the aptasensor proposed in this work is rapid, simple and low-cost in comparison with other methods reported previously. This study could thus provide a very promising platform for the analysis of AFM1 in dairy products. More importantly, the aptasensor could be improved by replacing aptamer sequences for the detection of other food safety targets.In addition, given the advantages of the graphene oxide fluorescence quenching ability and the nuclease-induced target signal enhancement strategies, a novel, sensitive and accurate aptasensor for amplified and specific detection of FB1 was first introduced, which relies on the GO and DNase I-induced target cycling and signal enhancement strategies. A widely dynamic range from 0.5 to 20 ng mL^{-1} was achieved between the fluorescence intensity and concentrations of FB1, its detection of limit was determined to be 0.15 ng mL^{-1}, which is sensitive and compatible with the current methods. In addition, the specific tests and practical analysis performance were also examined by detecting different mycotoxins and real wheat flour samples. This proposed GO and DNase I driven fluorescent aptasensor offered a promising application in mycotoxin detection for food safety.

Compared to the current approaches reported in the literatures and our previous RT-qPCR technique, this novel graphene oxide-based aptasensor has the advantages like simple experiments operation, high sensitivity and specificity, low cost (several hundreds dollars). This proposed strategy is a great potential platform for the in-field analysis since it only took 5 mins to complete this analysis detection. However, the relatively complicated instrumentation is required by using this method. In the future, the simplified and small equipment is much in demand for the real in field detection of mycotoxins for food safety.

It is difficult to achieve high sensitivity and wide dynamic response for the simple binding assay between aptamer and target. Therefore, different signal amplification strategies were introduced to these sensing platform to address this challenge. For instance, by integrating RT-qPCR signal enhancement technique, the femtogram sensitivity of aptasensors can be achieved for the detection of mycotoxins, which is one of the most sensitive methods until the present time. These kinds of aptasensors show remarkable characteristics like great sensitivity and selectivity. Therefore, if the goal and requirement of a researcher is high sensitivity and low limit of detection, the RT-qPCR signal enhancement aptasensor will be useful and helpful.

On the other side, rapid, on-site and real-time analysis platform seems to be one of the most important factors and goals for high-throughput and large-scale samples detection. In addition, relatively low detection sensitivity is also needed to meet the requirement of the low permissible limits. Thus, the nuclease-induced target signal enhancement strategies and graphene oxide-based aptasensor are suggested.

In addition, portable sensing platforms have attracted more and more attention to construct aptasensors for real in-field analysis, such as dipstick assay and

enzyme-linked aptamer assays (ELAAs). In these kinds of design, capture elements and signal detector are required. These methods have the advantages of low-cost, ease of operation and great stability, as well as the high sensitivity. Therefore, researchers can select the desirable sensing strategies for their own requirements and goals. Enzyme-linked aptamer assays (ELAAs) seem to be the simple replacement of the antibody (ELISAs) with aptamer. Aptamers have been considered to be "chemical antibodies" that are capable of recognizing and binding targets with high affinity and selectivity that are similar or even superior to antibodies. With these unique advantages, aptasensors have been widely developed for the detection of mycotoxins and have attracted more and more attention from researches. However, for practical samples, such as food and agricultural products, antibody-based immunoassays are mainly used, while the application of aptamer-based biosensors in the detection of mycotoxins is still in varying stages of scientific research. Up until now, no commercial kit based on aptamers has been produced and applied.

In our opinion, there are several reasons for this. First of all, traditional antibody preparation needs animal experiments and is thus difficult to prepare in large quantities through chemical synthesis, and antibody preparation technology can also be monopolized due to technical barriers. However, once the aptamer is screened successfully, the sequence of aptamer will be disclosed and can be prepared in large quantities through chemical synthesis, which is difficult to form technical barriers for commercial companies. Secondly, the affinity of aptamers is generally weaker than that of antibodies. In the application of complex systems, affinity and sensitivity are always a challenge. Third, commercial kits need to consider sensitivity, stability, repeatability, cost, technical barriers, and other factors. Currently, the published scientific papers on the detection of mycotoxins by aptamer biosensors may be superior to the ELISA of antibody technology in some aspects, such as sensitivity, stability, repeatability, time consuming, cost, etc., but the comprehensive performance is not necessarily superior to an ELISA. There are few articles comparing aptamer-based biosensors with a commercial ELISA kit that takes into consideration all these aspects. Limitations of aptamers are mainly, though not limit to, the requirements for sophisticated instruments, professional personnel, extraneous signal interference, time spent, as well as quality of aptasensor systems.

Via using the above aptamer-based biosensors, we developed several approaches for the detection of the most toxic mycotoxins for food safety. In addition, these sensing strategies could be applied for more hazard factors determination by simple replacement of the specific aptamers. More importantly, though the practical applicability, feasibility, and accuracy of these proposed aptasensors were investigated and evaluated through the analysis of the spiked samples experiments, the future's researches were needed for a validation of these aptasensors with real contaminated samples to determine the performances such as limit of detection and quantification, precision, trueness, accuracy, etc. Through the method validation, these aptasensors will be widely used for the detection of mycotoxins. In addition, future direction will focus on the simplification of analytic principle and devices and

the combination of novel aptamers with new materials and techniques to improve the analytical performance and market practicality of aptasensors.

Reference

Abia, W. A., Warth, B., Sulyok, M., Krska, R., Tchana, A. N., Njobeh, P. B., et al. (2013). Determination of multi-mycotoxin occurrence in cereals, nuts and their products in Cameroon by liquid chromatography tandem mass spectrometry (LC-MS/MS). Food Control. 31, 438–453. doi: 10.1016/j.foodcont.2012.10.006.

Anfossi, L., Di Nardo, F., Giovannoli, C., Passini, C., Baggiani, C. (2015). Enzyme immunoassay for monitoring aflatoxins in eggs. Food Control. 57, 115–121. doi:10.1016/j.foodcont.2015.04.013.

Bacher, G., Pal, S., Kanungo, L., Bhand, S. (2012). A label-free silver wire based impedimetric immunosensor for detection of aflatoxin M1 in milk. Sensor Actuat B-Chem. 168, 223–230. doi:10.1016/j.snb.2012.04.012.

Castillo, G., Spinella, K., Poturnayová, A., Šnejdárková, M., Mosiello, L., and Hianik, T. (2015). Detection of aflatoxin B1 by aptamer-based biosensor using PAMAM dendrimers as immobilization platform. Food Control. 52, 9–18. doi: 10.1016/j.foodcont.2014.12.008.

Chen, D.; Feng, H.; Li, J. Graphene oxide: Preparation, functionalization, and electrochemical applications. Chem. Rev. 2012, 112, 6027–6053.

Chen, X., Huang, Y., Duan, N., Wu, S., Ma, X., Xia, Y., et al. (2013). Selection and identification of ssDNA aptamers recognizing zearalenone. Anal Bioanal Chem. 405, 6573-6581. doi: 10.1007/s00216-013-7085-9.

Cheng, F.F.; Zhang, J.J.; He, T.T.; Shi, J.J.; Abdel-Halim, E.; Zhu, J.J. Bimetallic Pd–Pt supported graphene promoted enzymatic redox cycling for ultrasensitive electrochemical quantification of microRNA from cell lysates. Analyst 2014, 139, 3860–3865.

Cruz-Aguado, J. A., and Penner, G. (2008). Determination of ochratoxin A with a DNA aptamer. J Agric Food Chem. 56, 10456-10461. doi: 10.1021/jf801957h.

Ellington, A.D., Szostak, J.W., 1990. In vitro selection of RNA molecules that bind specifc ligands. Nature. 346, 818–822.

Evtugyn, G., Porfireva, A., Sitdikov, R., Evtugyn, V., Stoikov, I., Antipin, I., et al. (2013). Electrochemical Aptasensor for the Determination of Ochratoxin A at the Au Electrode Modified with Ag Nanoparticles Decorated with Macrocyclic Ligand. Electroanal. 25, 1847–1854. doi: 10.1002/elan.201300164.

Guo, Z., Ren, J., Wang, J., and Wang, E. (2011). Single-walled carbon nanotubes based quenching of free FAM-aptamer for selective determination of ochratoxin A. Talanta. 85, 2517–2521. doi: 10.1016/j.talanta.2011.08.015.

He, S.; Song, B.; Li, D.; Zhu, C.; Qi, W.; Wen, Y.; Wang, L.; Song, S.; Fang, H.;

Fan, C. A graphene nanoprobe for rapid, sensitive, and multicolor fluorescent DNA analysis. Adv. Funct. Mater. 2010, 20, 453–459.

Herzallah, S. M. (2009). Determination of aflatoxins in eggs, milk, meat and meat products using HPLC fluorescent and UV detectors. Food Chem. 114, 1141–1146. doi: 10.1016/j.foodchem.2008.10.077.

Istamboulie, G., Paniel, N., Zara, L., Granados, L. R., Barthelmebs, L., Noguer, T. (2016). Development of an impedimetric aptasensor for the determination of aflatoxin M1 in milk. Talanta. 146, 464–469. doi: 10.1016/j.talanta.2015.09.012.

Kav. K., Col, R., Kaan, T. K. (2011). Detection of aflatoxin M1 levels by ELISA in white-brined Urfa cheese consumed in Turkey. Food Control. 22, 1883–1886. doi:10.1016/j.foodcont.2011.04.030.

Kuang, H., Chen, W., Xu, D., Xu, L., Zhu, Y., Liu, L., et al. (2010). Fabricated aptamer-based electrochemical ‘‘signal-off’’ sensor of ochratoxin A. Biosens. Bioelectron. 26, 710–716. doi: 10.1016/j.bios.2010.06.058.

Kubista, M., Andrade, J.M., Bengtsson, M., Forootan, A., Jona k, J., Lind, K., Sindelka, R., Sjoback, R., Sj ¨ogreen, B., Str ¨ombom, L., 2006. Molecular Aspects of Medicine ¨27 (2), 95–125.

Lee, H.J., Kim, B.C., Kim, K.W., Kim, Y.K., Kim, J., Oh, M.K., 2009. Biosensors and Bioelectronics 24 (12), 3550–3555.

Lee, D., Lee, K. G. (2015). Analysis of aflatoxin M1 and M2 in commercial dairy products using high-performance liquid chromatography with a fluorescence detector. Food Control. 50, 467–471. doi:10. 1016/j.foodcont.2014.09.020

Li, D.; Müller, M.B.; Gilje, S.; Kaner, R.B.; Wallace, G.G. Processable aqueous dispersions of graphene nanosheets. Nat. Nanotechnol. 2008, 3, 101–105.

Li, P., Zhang, Q., Zhang, W., Zhang, J., Chen, X., Jiang, J., et al. (2009). Development of a class-specific monoclonal antibody-based ELISA for aflatoxins in peanut. Food Chem. 115, 313–317. doi:10.1016/j.foodchem.2008.11.052.

Lin, J.S., McNatty, K.P., 2009. Clinical Chemistry 55 (9), 1686–1693.

Lu, C.H.; Lim, J.; Lin, M.H.; Wang, Y.W.; Yang, H.H.; Chen, X.; Chen, G.N. Amplified Aptamer-Based Assay through Catalytic Recycling of the Analyte. Angew. Chem. Int. Ed. 2010, 122, 8632–8635.

Mao, J., Lei, S., Liu, Y., Xiao, D., Fu, C., Zhong, L., et al. (2015). Quantification of aflatoxin M1 in raw milk by a core-shell column on a conventional HPLC with large volume injection and step gradient elution. Food Control. 51, 156–162. doi:10.1016/j.foodcont.2014.11.022.

Malhotra, S., Pandey, A. K., Rajput, Y. S., Sharma, R. (2014). Selection of aptamers for aflatoxin M1 and their characterization. J Mol Recognit. 27, 493–500. doi: 10.1002/jmr.2370.

McKeague, M., Bradley, C. R., De Girolamo, A., Visconti, A., Miller, J. D., and Derosa, M. C. (2010). Screening and initial binding assessment of fumonisin

B1 aptamers. Int J Mol Sci. 11, 4864-4881. doi: 10.3390/ijms11124864.

Mestdagh, P., Feys, T., Bernard, N., Guenther, S., Chen, C., Speleman, F., Vandesompele, J., 2008. Nucleic Acids Research 36 (21) e143-e143.

Mozaffari Nejad, A. S., Sabouri Ghannad, M., and Kamkar, A. (2014). Determination of aflatoxin B1 levels in Iranian and Indian spices by ELISA method. Toxin Rev. 33, 151–154. doi.org/10.3109/15569543.2014.942319.

Nguyen, B. H., Tran, L. D., Do, Q. P., Nguyen, H. L., Tran, N. H., and Nguyen, P. X. (2013). Label-free detection of aflatoxin M1 with electrochemical Fe3O4/polyaniline-based aptasensor. Mater Sci Eng C Mater Biol Appl. 33, 2229-2234. doi: 10.1016/j.msec.2013.01.044.

Park, J. H., Kim, Y. P., Kim, I. H. (2014). Rapid detection of aflatoxin B1 by a bifunctional protein crosslinker-based surface plasmon resonance biosensor. Food Control. 36, 183-190. doi: 10.1016/j.foodcont.2013.08.038.

Parker, C. O., Tothill, I. E. (2009). Development of an electrochemical immunosensor for aflatoxin M1 in milk with focus on matrix interference. Biosens. Bioelectron. 24, 2452–2457. doi:10.1016/j. bios.2008.12.021.

Patent:PCT/CA2010/001292,DNA Ligands for Aflatoxin and Zearalenone, NeoVentures Biotechnology Inch.

Pei, H.; Li, J.; Lv, M.; Wang, J.; Gao, J.; Lu, J.; Li, Y.; Huang, Q.; Hu, J.; Fan, C. A graphene-based sensor array for high-precision and adaptive target identification with ensemble aptamers. J. Am. Chem. Soc. 2012, 134, 13843–13849.

Piermarini, S., Micheli, L., Ammida, N. H. S., Palleschi, G., Moscone, D., 2007. Electrochemical immunosensor array using a 96-well screen-printed microplate for aflatoxin B1 detection. Biosens. Bioelectron. 22, 1434. doi: 10.1016/j.bios.2006.06.029.

Pietri, A., Fortunati, P., Mulazzi, A., Bertuzzi, T. (2016). Enzymeassisted extraction for the HPLC determination of aflatoxin M1 in cheese. Food Chem. 192, 235–241. doi:10.1016/j. foodchem.2015.07.006.

Pinto, A., Redondo, M.C.B., Ozalp, V.C., O'Sullivan, C.K., 2009. Molecular Biosystems 5 (5), 548–553.

Plattner R.D., Ross, P.F. Reagor, J. Stedelin, J. Rice L.G., Analysis of corn and cultured corn for fumonisin B1 by HPLC and GC/MS by four laboratories, J. Vet. Diagn. Invest. 3 (1991) 357–358.

Pu, Y.; Zhu, Z.; Han, D.; Liu, H.; Liu, J.; Liao, J.; Zhang, K.; Tan, W. Insulin-binding aptamer-conjugated graphene oxide for insulin detection. Analyst 2011, 136, 4138–4140.

Rao, C.E.E.; Sood, A.E.; Subrahmanyam, K.E.; Govindaraj, A. Graphene: The new two-dimensional nanomaterial. Angew. Chem. Int. Ed. 2009, 48, 7752–7777.

Schmittgen, T.D., Livak, K.J., 2008. Nature Protocols 3 (6), 1101–1108.

Sheng, Y. J., Eremin, S., Mi, T. J., Zhang, S. X., Shen, J. Z., and Wang, Z. H. (2014). The development of a fluorescence polarization immunoassay for

aflatoxin detection. Biomed Environ Sci. 27, 126–129. doi: 10.3967/bes2014.027.

Shim, W. B., Yang, Z. Y., Kim, J. S., Kim, J. Y., Kang, S. J., Woo, G. J., et al. (2007). Development of immunochromatography strip-test using nanocolloidal gold-antibody probe for the rapid detection of aflatoxin B1 in grain and feed samples. J Microbiol Biotechn. 17, 1629–1637. doi: 10.1016/j.mimet.2007.08.002.

Seok, Y., Byun, J. Y., Shim, W. B., and Kim, M. G. (2015). A structure-switchable aptasensor for aflatoxin B1 detection based on assembly of an aptamer/split DNAzyme. Anal Chim Acta. 886, 182–187. doi: 10.1016/j.aca.2015.05.041.

Shim, W. B., Kim, M. J., Mun, H., and Kim, M. G. (2014a). An aptamer-based dipstick assay for the rapid and simple detection of aflatoxin B1. Biosens. Bioelectron. 62, 288–294. doi: 10.1016/j.bios.2014.06.059.

Tan, K.X., Pan, S., Jeevanandam, J., Danquah, M.K., 2019. Cardiovascular therapies utilizing targeted delivery of nanomedicines and aptamers. Int. J. Pharm. 558, 413–425.

Tang, D.; Tang, J.; Li, Q.; Su, B.; Chen, G. Ultrasensitive aptamer-based multiplexed electrochemical detection by coupling distinguishable signal tags with catalytic recycling of DNase I. Anal. Chem. 2011, 83, 7255–7259.

Tang, L.; Wang, Y.; Li, J. The graphene/nucleic acid nanobiointerface. Chem. Soc. Rev. 2015, 44, 6954–6980.

Tuerk, C., Gold, L., 1990. Systematic evolution of ligands by exponential enrichment Rna ligands to bacteriophage-T4 DNA-polymerase. Science 249, 505–510.

Var, I., Kabak, B., & Gök, F. (2007). Survey of aflatoxin B1 in helva, a traditional Turkish food, by TLC. Food Control, 18(1), 59–62.

Vdovenko, M. M., Lu, C. C., Yu, F. Y., Sakharov, I. Y. (2014). Development of ultrasensitive direct chemiluminescent enzyme immunoassay for determination of aflatoxin M1 in milk. Food Chem. 158, 310–314. doi: 10.1016/j.foodchem.2014.02.128.

Wang, C., Qian, J., Wang, K., Yang, X., Liu, Q., Hao, N., et al. (2016). Colorimetric aptasensing of ochratoxin A using Au@Fe3O4 nanoparticles as signal indicator and magnetic separator. Biosens. Bioelectron. 77, 1183-1191. doi: 10.1016/j.bios.2015.11.004.

Wang, T., Chen, C., Larcher, L.M., Barrero, R.A., Veedu, R.N., 2019. Three decades of nucleic acid aptamer technologies: lessons learned, progress and opportunities on aptamer development. Biotechnol. Adv. 37, 28–50.

Wang, Y., Liu, X., Xiao, C., Wang, Z., Wang, J., Xiao, H., et al. (2012). HPLC determination of aflatoxin M1 in liquid milk and milk powder using solid phase extraction on OASIS HLB. Food Control. 28, 131–134. doi:10.1016/j.foodcont.2012.04.037.

Warth, B., Sulyok, M., and Krska, R. (2013). LC-MS/MS-based multibiomarker

approaches for the assessment of human exposure to mycotoxins. Anal Bioanal Chem. 405, 5687–5695. doi: 10.1007/s00216-013-7011-1.

Wu, S., Duan, N., Ma, X., Xia, Y., Wang, H., Wang, Z., et al. (2012). Multiplexed fluorescence resonance energy transfer aptasensor between upconversion nanoparticles and graphene oxide for the simultaneous determination of mycotoxins. Anal. Chem. 84. 6263–6270. doi: 10.1021/ac301534w.

Wu, S., Duan, N., Li, X., Tan, G., Ma, X., Xia, Y., et al. (2013). Homogenous detection of fumonisin B1 with a molecular beacon based on fluorescence resonance energy transfer between NaYF4: Yb, Ho upconversion nanoparticles and gold nanoparticles. Talanta. 116, 611–618. doi: 10.1016/j.talanta.2013.07.016.

Xiong, H.G., Yan, J., Cai, S.W., He, Q.F., Peng, D.X., Liu, Z., et al., 2019. Cancer protein biomarker discovery based on nucleic acid aptamers. Int. J. Biol. Macromol. 132.

Xu, W., Xiong, Y., Lai, W., Xu, Y., Li, C., and Xie, M. (2014). A homogeneous immunosensor for AFB1 detection based on FRET between different-sized quantum dots. Biosens. Bioelectron. 56, 144–150. doi: 10.1016/j.bios.2014.01.007.

Yang, C., Lates, V., Prieto-Simón, B., Marty, J. L., and Yang, X. (2013). Rapid high throughput analysis of ochratoxin A by the self-assembly of DNAzyme–aptamer conjugates in wine. Talanta. 116, 520–526. doi: org/10.1016/j.talanta.2013.07.011.

Yazdanpanah, H., Zarghi, A., Shafaati, A. R., Foroutan, S. M., Aboul-Fathi, F., Khoddam, A., et al. (2013). Analysis of aflatoxin B1 in Iranian foods using HPLC and a monolithic column and estimation of its dietary intake. Iran J Pharm Res. 12, 81–87. doi: 10.1016/j.jpba.2013.07.024.

Zhang, H.; Jia, S.; Lv, M.; Shi, J.; Zuo, X.; Su, S.; Wang, L.; Huang, W.; Fan, C.; Huang, Q. Size-dependent programming of the dynamic range of graphene oxide–DNA interaction-based ion sensors. Anal. Chem. 2014, 86, 4047–4051.

Zhang, Y., Lai, B.S., Juhas, M., 2019. Recent advances in aptamer discovery and applications. Molecules (Basel, Switzerland) 24, 941.

Appendix – Publications

1. **Xiaodong Guo**, Fang Wen, Nan Zheng, Qiujiang Luo, Jiaqi Wang, Development of an ultrasensitive aptasensor for the detection of aflatoxin B1, *Biosensors and Bioelectronics*, 2014, 15; 56:340-344.

2. **Xiaodong Guo**, Fang Wen, Nan Zheng, Songli Li, Marie-Laure Fauconnier, Jiaqi Wang, A qPCR aptasensor for sensitive detection of aflatoxin M1, *Analytic and Bioanalytic Chemistry*, 2016, 408: 5577-5584.

3. **Xiaodong Guo**, Fang Wen, Qinqin Qiao, Nan Zheng, Matthew Saive, Marie-Laure Fauconnier, Jiaqi Wang, A Novel Graphene Oxide-Based Aptasensor for Amplified Fluorescent Detection of Aflatoxin M1 in Milk Powder, *Sensors*, 2019, 19: 3840.

4. **Xiaodong Guo**, Fang Wen, Nan Zheng, Marie-Laure Fauconnier, Jiaqi Wang, Aptamer-based biosensor for detection of mycotoxins. *Frontiers in Chemistry*, 2019, 8,195.

5. **Xiaodong Guo**, Marie-Laure Fauconnier, Jiaqi Wang, Aptasensors technologies for aflatoxin B1 application. *Current Trends in Veterinary and Dairy Research*. 2020. CTVDR. MS.ID.000101.

6. **Xiaodong Guo**, Fang Wen, Nan Zheng, Marie-Laure Fauconnier, Jiaqi Wang, Graphene oxide driven fluorescent aptasensor for the detection of fumonisin B1. *Journal of agriculture and food chemistry,* 2020 (to be submitted)

7. Bingyao Du, Fang Wen, **Xiaodong Guo**, Nan Zheng, Jiaqi Wang, Evaluation of an ELISA-based visualization microarray chip technique for the detection of veterinary antibiotics in milk, *Food Control*, 2019, 106, 106713. (Co-first author)

8. Lu Chen, Fang Wen, Ming Li, **Xiaodong Guo**, Songli Li, Nan Zheng, Jiaqi Wang, A simple aptamer-based fluorescent assay for the detection of Aflatoxin B1 in infant rice cereal. *Food Chemistry* 215 (2017) 377-382.

9. **Xiaodong Guo**, Fang Wen, Nan Zheng, Jiaqi Wang, Development of anultrasensitive aptasensor for the detection of aflatoxin B1, Biosens Bioelectron, *American Dairy Science Association*, 2014. (International conference poster)

10. **Xiaodong Guo**, Fang Wen, Nan Zheng, Marie-Laure Fauconnier, Jiaqi Wang, A qPCR aptasensor for sensitive detection of aflatoxin M1. Anal Bioanal Chem, *American Dairy Science Association*, 2016. (International conference poster)

11. Nan Zheng, **Xiaodong Guo**, Fang Wen, et al, A RT-qPCR method for detection of aflatoxin B1. China, *Patent*. 201410315905.8.

12. Nan Zheng, Fang Wen, **Xiaodong Guo**, et al, Aptamer, sensor, reagent kits for aflatoxin M1 determination. China, *Patent*. 201710383866.7.