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D I P L O M A R B E I T

Optimierung der DNAzyme-katalysierten H_2O_2-vermittelten Oxidation von 3,3',5,5'-Tetramethylbenzidin und deren potenziellen Anwendung für einen Aptamer basierenden Biosensor für Mykotoxine

Ausgeführt am

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Abstract

Aflatoxins are toxic natural substances and one of the most prevalent types of mycotoxin contamination. They represent a high risk to human health as they contaminate a variety of agricultural raw materials. National and international institutions and organisations such as the European Commission have set regulatory limits for major mycotoxin classes. Several different methods for the detection and quantification of aflatoxins have already been developed. On the one hand, highly sophisticated analytical reference methods like liquid chromatography coupled with mass spectrometry are applied, on the other hand, rapid screening methods based on immunochemical techniques have also been developed. Immunoassays are based on antigen-antibody interactions but the use of antibodies has many restrictions such as a low antibody stability and high costs of production. In recent years, an innovative method based on a new class of molecules, named aptamers, has been developed for analytical applications. Aptamers are specific oligonucleotides which are able to bind to a target substance with high affinity and specificity due to their three-dimensional, specific structures. Among the methodologies used for transforming aptamer-target interactions into a visible signal, the horseradish peroxidase-like DNAzyme has been used frequently to catalyse chemical reactions to amplify biosensing events. DNAzymes are catalytic nucleic acids that act like enzymes and therefore represent good alternatives to enzymes. Like the horseradish peroxidase, the HRP-mimicking DNAzyme can also catalyse the oxidation of 3,3',5,5'-Thetramethylbenzidine (TMB) by the use of H_2O_2 as oxidising agent to produce a visible signal. In the overall project, research is done on an aptamer-based assay for the detection of aflatoxin B_1 which should allow the rapid detection of this mycotoxin in maize. In this work, several factors of the DNAzyme-catalysed H_2O_2-mediated oxidation of TMB including the DNA sequence of the DNAzyme, substrate buffer, concentrations of TMB, H_2O_2 and hemin as well as the concentrations ratios and reaction time have been investigated and gradually optimised for this sensor developing purposes. The use of higher concentrated substrate and stopping solution and simultaneously less reaction volume was found to lead to higher absorbance values. Furthermore, the best results for sensor-developing purposes could be achieved when having a concentration ratio of TMB and H_2O_2 of 1:3 and DNAzyme and hemin of 1:8. Among the different substrate buffers tested, the most consistent results in the absorbance measurements with the lowest error rates could be achieved by using a substrate buffer containing 300 mM citric acid and 1 mM potassium sorbate. Moreover, acetonitrile was found to have the ability to enhance the catalytic reaction leading to higher absorbance values. Due to the optimisation of these factors, we were able to achieve a 50-times increase in sensitivity of the DNAzyme-catalysed H_2O_2-mediated oxidation of TMB compared to the beginning of the optimisation of this assay.

Zusammenfassung

Aflatoxine sind giftige natürliche Substanzen und eine der am häufigsten vorkommenden Mykotoxin-Verunreinigungen. Sie stellen ein hohes gesundheitliches Risiko für den Menschen dar, da sie eine Reihe von landwirtschaftlichen Rohstoffen kontaminieren. Nationale und internationale Institutionen und Organisationen wie die Europäische Kommission haben daher Grenzwerte für Mykotoxine erstellt. Verschiedene Methoden zur Detektion und Quantifizierung von Aflatoxinen sind bereits entwickelt worden. Einerseits werden hochentwickelte analytische Referenzmethoden wie die Flüssigchromatografie gekoppelt mit der Massenspektrometrie angewandt, andererseits wurden schnelle immunochemische Screeningverfahren entwickelt. Die Verwendung von Antikörpern hat viele Nachteile wie beispielsweise geringe Antikörperstabilität und hohe Produktionskosten. In den letzten Jahren wurde eine innovative Methode für analytische Anwendungen entwickelt, welche auf einer neuen Klasse von Molekülen basiert, sogenannten Aptameren. Aptamere sind Oligonukleotide die aufgrund ihrer dreidimensionalen Struktur mit hoher Affinität und Spezifität an eine Zielsubstanz binden. Unter den Methoden die für die Umwandlung von Aptamer-Zielmolekül-Interaktionen in ein sichtbares Signal entwickelt wurden, wurde vor allem das Meerrettichperoxidase-ähnliche DNAzyme häufig zur Katalyse von chemische Reaktionen verwendet um biosensorische Ereignisse zu verstärken. DNAzymes sind katalytische Nukleinsäuren die sich wie Enzyme verhalten. Wie die Meerrettichperoxidase, kann auch das Meerrettichperoxidase-ähnliche DNAzyme die Oxidation von 3,3',5,5'-Thetramethylbenzidin (TMB) mit H_2O_2 als Oxidationsmittel katalysieren um ein sichtbares Signal zu produzieren. Im Gesamtprojekt wird Forschung an einer Aptamer basierenden Nachweismethode für die schnelle Detektion von Aflatoxin B_1 in Mais betrieben. In dieser Arbeit wurden verschiedene Faktoren der DNAzyme-katalysierten H_2O_2-vermittelten Oxidation von TMB untersucht und schrittweise optimiert. Zu diesen Faktoren zählen die DNA Sequenz des DNAzyme, Substratpuffer, Konzentrationen von TMB, H_2O_2 und Hemin sowie Konzentrationsverhältnisse und Reaktionszeit. Die Verwendung von höher konzentrierter Substrat- und Stopplösung bei gleichzeitig weniger Reaktionsvolumen führte zu höheren Absorptionwerten. Weiters konnten die besten Resultate für Sensor-Entwicklungszwecke mit einem Konzentrationsverhältnis von TMB zu H_2O_2 von 1:3 und DNAzyme zu Hemin von 1:8 erzielt werden. Unter den getesteten Substratpuffern konnten die stabilsten Ergebnisse in der Absorptionsmessung mit einem Substratpuffer, der 300 mM Zitronesäure und 1 mM Kaliumsorbat enthält, erreicht werden. Außerdem scheint Acetonitril die katalytische Reaktion zu verstärken. Durch die Optimierung der DNAzyme-katalysierten H_2O_2-vermittelten Oxidation von TMB konnten wir eine 50-fache Sensitivitätssteigerung verglichen mit den Absorptionswerten zu Beginn der Optmierung dieser Reaktion erreichen.

Contents

1 Introduction

1.1 Mycotoxins

Fungi produce a wide array of secondary metabolites, many of which have been associated with adverse effects on the health of humans and animals. These compounds have been given the term mycotoxins. They occur in the mycelium of filamentous fungi but may also be present in the spores of these organisms. The harmful effects are referred to as mycotoxicoses. The nature of toxicity in animals and humans are as diverse as the fungal species which produce these compounds [1].

More than 300 mycotoxins of widely different chemical structures and differing modes of action - some target the kidney, liver, or immune system and some are carcinogenic - have been identified [2]. Also the economic losses due to recall of contaminated food and feed by mycotoxins make this issue a cause of global concern. The major toxic species of fungi and their mycotoxins are presented in Table 1.1.1.

Table 1.1.1: The major toxigenic species of fungi and their principal mycotoxins [1].

Fungal species	**Mycotoxins**
Aspergillus flavus, A. parasiticus	Aflatoxins
A. ochraceus, Penicillium viridicatum; P. cyclopium	Ochratoxin A
Fusarium culmorum; F. graminearum; F. sporomchioides	Deoxynivalenol
F. culmorum; F. graminearum; F. sporotrichioides	Zearalenone
F. verticillioides; F. moniliforme	Fumonisins
P. expansum	Patulin
F. sporotrichioides; F. poae	T-2 toxin
A. flavus	Cyclopiazonic acid
F. sporotrichioides; F. graminearum; F. poae	Diacetoxyscirpenol
Acremonium coenophialum	Ergopeptine alkaloids
A. lolii	Lolitrem alkaloids
Phomopsis leptostromiformis	Phomopsins
Pithomyces chartarum	Sporidesmins

1.1.1 Aflatoxin B_1

Aflatoxins are the most toxic natural substances known to human and one of the most prevalent types of mycotoxin contamination. Aflatoxins are produced by strains of *Aspergillus flavus* and *A. parasiticus*. Of the several types of aflatoxins (B_1, B_2, G_1, G_2), aflatoxin B_1 (AFB_1) is the

most toxic. When ingested, inhaled or adsorbed through the skin, aflatoxin B_1 has carcinogenic, teratogenic and mutagenic effects in human and animals even at very small concentrations. Therefore it has been classified as group 1 carcinogenic compound by the International Agency for Research on Cancer (IARC, 2002) of the World Health Organization (WHO) [3, 4]. Figure 1.1.1 shows the different types of aflatoxins.

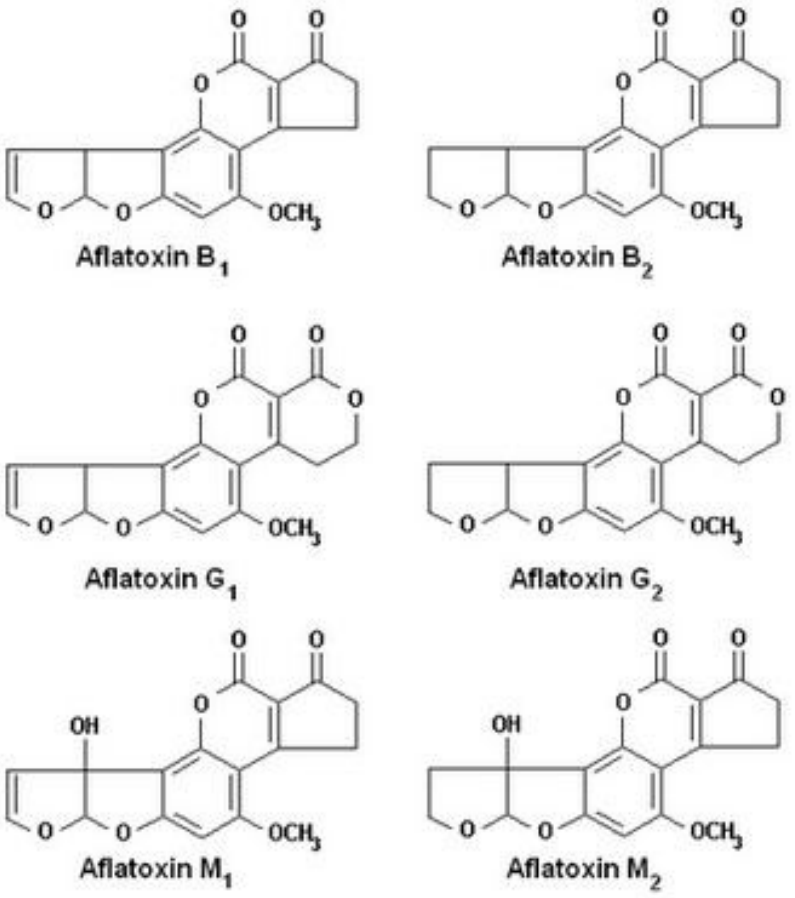

Figure 1.1.1: Aflatoxins

Source: http://en.engormix.com

Aflatoxin B_1 represents a high risk as it contaminates a variety of agricultural raw materials such as tree nuts, peanuts, corn, cereals, grains, cotton seed, milk, animal feeds, and also derived products and herbal medicines [5]. The occurrence of AFB_1 in food is therefore considered to be one of the most important global food safety issues. As a consequence, many countries have set regulations for AFB_1 or the sum of AFB_1 and other aflatoxins in foods and agricultural stuffs [6]. Therefore, the ability to detect aflatoxin B_1 is very important and numerous methods have been developed for the confirmation and determination of AFB_1.

1.2 Methods of detection

National and international institutions and organisations such as the European Commission have set regulatory limits for major mycotoxin classes such as aflatoxin B_1. The requirement to apply these regulatory limits has pushed the development of analytical methods for the identification and quantification of mycotoxins in various samples such as food, feed and other biological matrices [2]. On the one hand, this demand has led to the development of highly sophisticated analytical reference methods for example liquid chromatography coupled with mass spectrometry. On the other hand, rapid screening methods for single mycotoxins or whole mycotoxin classes mainly based on immunochemical techniques have also been developed [2].

1.2.1 Reference detection methods for mycotoxin detection

Due to the natural fluorescence of aflatoxin B_1, chromatographic techniques have been usually taken as reference methods [7]. Methods have been developed for the confirmatory and quantitative determination of aflatoxin B_1 based on thin layer chromatography (TLC) [8], high-performance liquid chromatography (HPLC) and liquid chromatography combined with either fluorescent detection (FLD) or coupled with mass spectrometry (MS) [9, 10, 11]. The instrument based methods HPLC and LC-MS have been officially accepted as reference methods for the quantification of aflatoxin B_1 [7, 12].

Although these methods are extremely sensitive, accurate and reproducible, they still have some disadvantages such as being laborious, time consuming, unsuitable for on-site detection and the requirement of expensive equipments and trained personnel. Furthermore, complicated and time consuming pretreatment procedures owing to sample preparation and pre-concentration before determination are involved in these instrumental methods. In addition, these methods are unsuitable for the routine screening of large sample numbers [12]. Hence, these methods cannot be applied for rapid and simple detection of samples.

1.2.2 Rapid immunochemical based detection methods

In addition to the classic chromatographic detection methods mentioned in 1.2.1, rapid screening methods based on immunoassays such as enzyme-linked immunosorbent assays (ELISA), immunochromatographic assays and immunosensors have been developed for aflatoxin B_1 detection [13, 14, 15]. These biochemical methods have received considerable attention owing to their low cost, simplicity, rapidity and possibility of miniaturization allowing on-site detection. Immunoassays are simpler and easy-to-use methods compared to chromatographic methods

and are being used increasingly for screening of AFB_1 in food and agricultural commodities. [16]

Especially, **ELISA** is becoming more widespread for the surveillance of aflatoxins because of the sensitivity, specificity, rapidity, simplicity, and cost-effectiveness of the method and the ability to achieve quantitative analysis. However, ELISA often requires long reaction times and involves multiple incubation and washing steps and its utilization has been restricted to well equipped laboratories [17]. These disadvantages make on-site detection difficult.

Therefore, a simple, rapid, and on-site detection method for aflatoxins testing in food and agricultural products is desired. **Immunochromatographic** and **dipstick assays** are typical on-site detection technologies and are based on a membrane containing detection and capture reagents. Hence, this immunochromatographic technology combines several benefits including a user-friendly format, short assay time, long-term stability and cost-effectiveness. These properties make it attractive for on-site screening by untrained personnel [18]. Immunochromatographic and dipstick assays for various mycotoxins including aflatoxin B_1 have been developed previously [19].

Although immunochemical methods benefit from good sensitivity with the potential for high-throughput screening, they often suffer from matrix interference and frequent false-negative or false-positive screening results [20]. Furthermore, immunoassays are based on antigen-antibody interactions and therefore these assays involve the use of antibodies specific to aflatoxin B_1 as the molecular recognition element [19]. Being produced by animal immunization, the use of antibodies has many disadvantages in terms of antibody stability during transport and storage and high costs of production [21].

To overcome the various limitations of antibodies, alternatives to antibody-based biosensors such as synthetic bioreceptors appear as promising recognition tools for analytical applications. In recent years, an innovative method based on a new class of molecules, named aptamers, has been developed in the field of analytical methods.

1.3 Aptamers

Except encoding the genetic information of all living organisms, nucleic acids have the ability to perform other interesting functions, including ligand binding and catalysis. These kinds of nucleic acids are known as functional nucleic acids (FNAs) [22]. One representative group of FNAs are aptamers.

The word aptamer is derived from the Latin word "aptus" meaning "to fit", describing a nucleic acid fragment that "fits" to its target [23]. Aptamers are artificial short single-stranded oligonucleotides, either DNA or RNA, selected from a combinatorial library of sequences according to their ability to recognize a target with high affinity and specificity. They can be generated against various targets such as proteins [24], drugs [25], organic or inorganic molecules [23]. Aptamers fold into well defined three dimensional structures and bind to their ligands by complementary shape interactions, they can incorporate small molecules into their nucleic acid structure or integrate into the structure of larger molecules [26, 27]. Figure 1.3.1 shows a simplified illustration of an aptamer/target-complex.

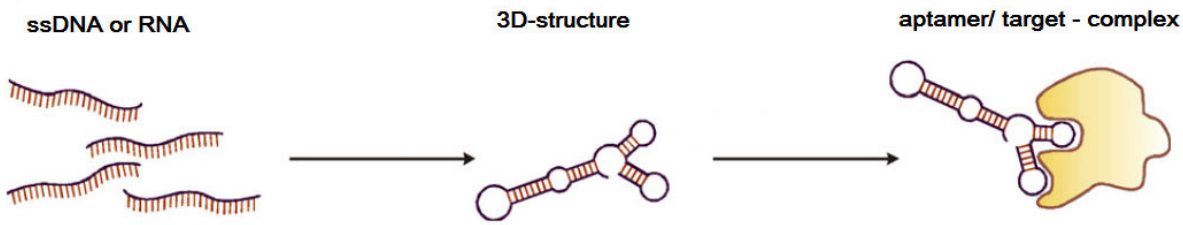

Figure 1.3.1: Aptamer/target - complex

source: http://www.anfitech.com

Aptamers are generated in vitro by a procedure called Systematic Evolution of Ligand by Exponential Enrichment (**SELEX**) from random-sequence nucleic acids libraries [23, 28]. The SELEX process is an iterative process. First, an oligonucleotide combinatorial library comprising a large sequence diversity and structural complexity is synthesized. Each oligonucleotide contains a random central region flanked by a defined primer-binding region at each end. From this DNA oligonucleotide library, only those oligonucleotides are selected and enriched during several SELEX rounds which can bind very tightly to the specific molecular target. Basic steps of the SELEX process are the binding reaction between oligonucleotides and target, washing steps to remove unbound oligonucleotides, enzymatic amplification of target-bound oligonucleotides via Polymerase Chain Reaction (PCR) and purification of the selected oligonucleotide pool to subsequently start the next selection round. The best fitting sequences survive the selection procedure and represent the target-specific aptamer pool as the result of a successful SELEX process [29]. Figure 1.3.2 shows a schematic illustration of the basic steps of the SELEX process in a simplified way.

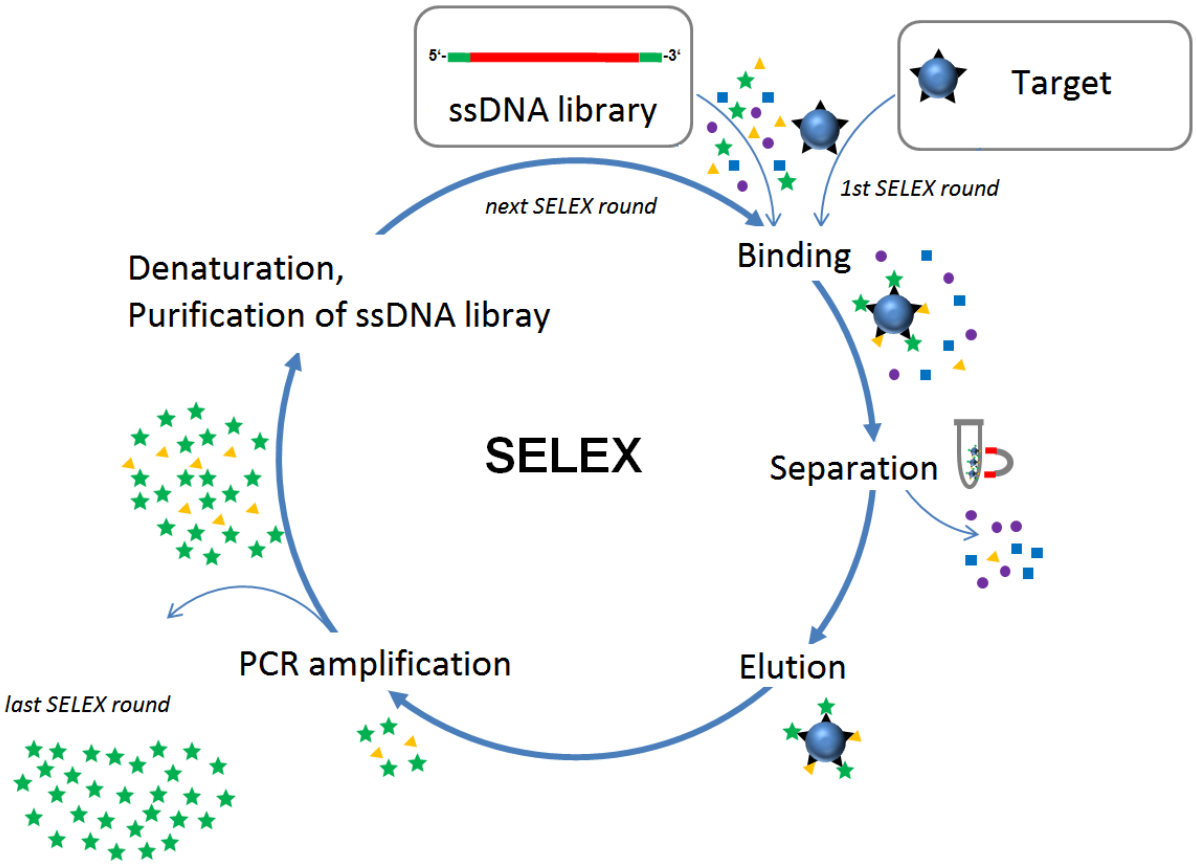

Figure 1.3.2: Systematic Evolution of Ligand by Exponential Enrichment (**SELEX**).

Aptamers have many advantages over conventional antigen-antibody technologies [27]:

- Antibodies are identified through an *in vivo* process, requiring animal immunization which is a labor-intensive process that can take several month including purification. On the contrary, aptamers can be identified completely *in vitro* without the requirement of living cells or animals allowing the selection of aptamers against toxic or poor immunogenic targets.

- The performance of antibodies can vary from batch to batch. In contrast, aptamers are chemically synthesized with great reproducibility and purified to a very high degree of purity.

- During antibody production, the target is identified by the immune system of the animal. The influence on the production of antibodies is restricted by *in vivo* parameters. On the other side, SELEX conditions can be controlled to select aptamers with the desired properties.

- Due to their protein-like nature, antibodies are very sensitive to temperature and pH variations and are easily irreversible denatured during storage. Aptamers are stable to high temperatures. They undergo reversible denaturation and can be renatured in minutes, therefore aptamers possess desirable storage properties. Furthermore, the function of immobilised aptamers is regenerable, therefore they can be reused.

- During chemical synthesis, modifications could be attached to aptamers for an easier immobilisation on transducers enabling the design of several detection methods. For example, aptamers can be easily labeled with fluorescent dyes, enzymes, biotin and DNA ligands [30].

On the other hand, aptamers have also some disadvantages over antibodies:

- Lack of literature.

- The use of aptamers as recognition elements is strongly dependent on pH and salt concentrations.

- Faster excretion than antibodies due to smaller size [31].

Recently, aptamers for several mycotoxins including the aptamer specific to ochratoxin A [32], fumonisin B_1 [33], zearalenone [34], deoxynivalenol [35] and also aflatoxin B_1 [36] have been developed.

Since aptamers specific to individual mycotoxins have been described, fundamental research efforts are directed toward the application of aptamers as recognition substances for the design of biosensors (aptasensors).

1.4 Aptamer-based biosensors

Aptamer-based sensing technologies have been designed using various strategies for transforming the aptamer-target interaction into a visible or measurable signal [37].

Especially, the aptamer specific to ochratoxin A, which was first screened and reported in 2008 [32], has been well investigated and studied for the development of various aptamer-based sensors. The signalling processes involve **calorimetric** [38, 39], **fluorescent** [40], **electrochemical** [41, 42], and **chromatographic** [43] approaches. In addition, **enzyme-linked aptamer assays** (ELAAs) for the detection of ochratoxin A have been reported with experimental procedures almost identical to those of ELISAs [44].

In the case of aptamer-based detection of aflatoxin B_1, only a few approaches to detect aflatoxin B_1 on the basis of aptamers are described in literature. Here are listed some of them:

- Guo et al.[4] describe an **ultrasensitive aptasensor** for the detection of AFB_1 where they use the aptamer specific to AFB_1 as molecular recognition probe, while its complementary DNA (AFB_1 DNA in Fig. 1.4.1) plays a role as signal generator for the amplification by real-time quantitative polymerase chain reaction (PCR). The principle behind the sensing strategy is based on a conformational change as the result of the formation of an AFB_1/aptamer complex leading to the release of the complementary DNA and consequently a reduction in the amount of the complementary DNA template for RT-qPCR amplification [4]. Figure 1.4.1 shows a schematic illustration of their proposed aptasensor for the detection of aflatoxin B_1. Being dependent on PCR, this aptasensor is not suitable for rapid on-site detection.

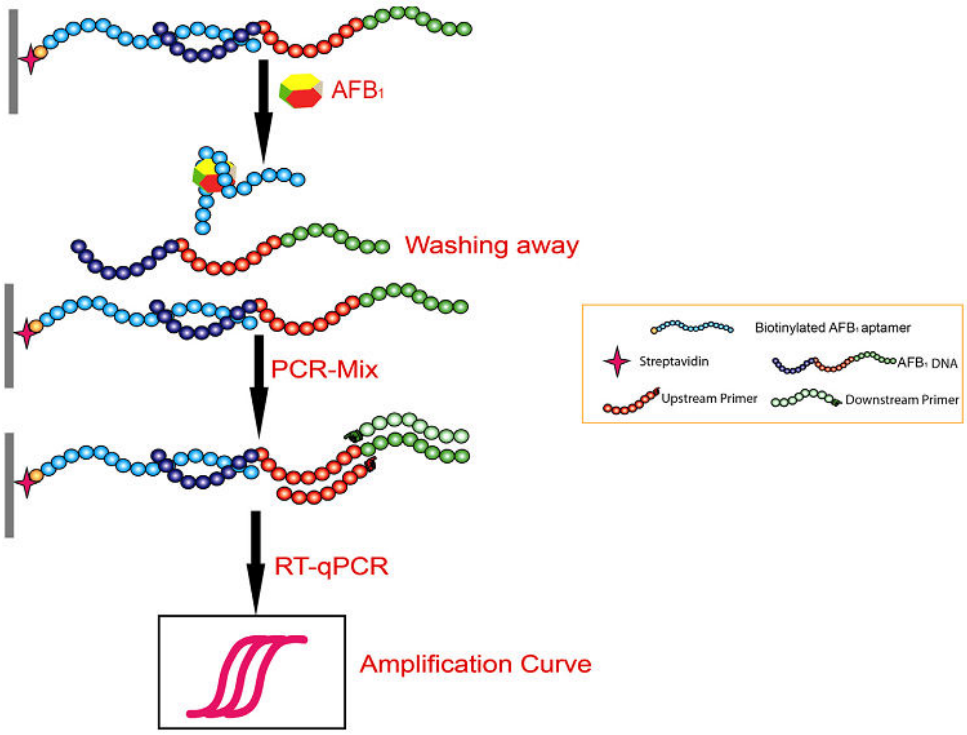

Figure 1.4.1: Schematic illustration of the aptasensor for the detection of AFB_1 [4].

- Ma et al.[45] describe the development of a **fluorescent assay** using an aptamer specific to AFB_1 which they generated through SELEX. They also use the aptamer as recognition probe, while its complementary DNA is labeled with a fluorescent dye, which is released as a result of the formation of a AFB_1/aptamer complex and consequently the fluorescence is measured [45].

- Shim et al.[6] describe a **chemiluminescence competitive aptamer assay** using a horseradish peroxidase-mimicking DNAzyme (HRP-DNAzyme) linked with an aptamer specific to AFB_1 [6]. The aptamer assay developed in this study was based on similar procedures as belongs to competitive ELISA. A schematic diagram of the aptamer assay is shown in Figure 1.4.2.

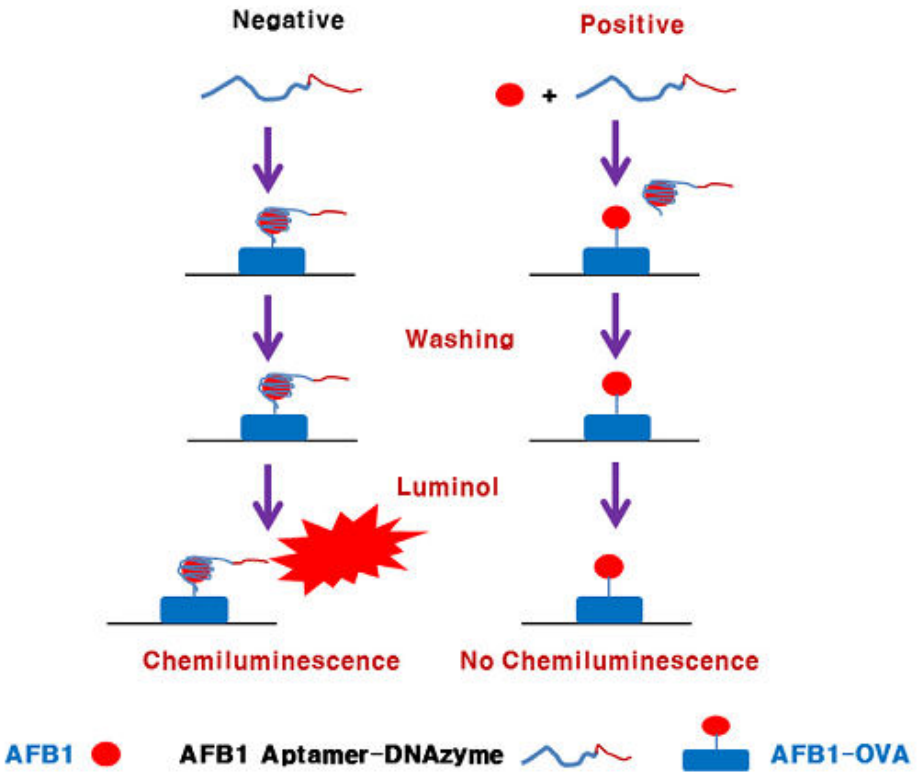

Figure 1.4.2: Schematic illustration of the chemiluminescence competitive aptamer assay for the detection of AFB_1 [6].

Signal amplification of biorecognition events, such as the formation of an aptamer-target complex, is one of the important concepts since it can allow a higher analytical sensitivity [46]. Several methods for signal amplification have been reported such as rolling circle amplification (RCA) [47], strand displacement amplification (SDA) [48], and enzyme-labeling techniques [49]. Although these signal amplification methods improve the sensitivity of aptasensors, they are expensive and laborious.

Among the methodologies used for transforming aptamer-target interactions into a visible signal, specifically, the horseradish peroxidase-like DNAzyme has been used to catalyse chemical reactions to amplify biosensing events [6, 39, 50].

DNAzymes are catalytic nucleic acids that act like enzymes and therefore represent good alternatives to enzymes in the application as amplifying labels of biorecognition events.

1.5 DNAzymes

DNAzymes are another type of functional nucleic acids. These short DNA fragments have catalytic potentials equalling protein-based enzymes. Like aptamers, catalytic nucleic acids (DNAzymes or ribozymes) are generated by the Systematic Evolution of Ligand by Exponential Enrichment (SELEX, see section 1.3) [23, 28].

DNAzymes have several advantages over typical enzymes, which make them valuable for the development of biosensors and aptasensors as amplifying labels even though there are numerous natural protein enzymes [51]:

- Although enzymes can be linked to aptamers, this is a time-consuming approach compared to the chemical synthesis of aptamer and DNAzyme which are both nucleic acids.
- Enzymes exhibit catalytic activity only in a narrow temperature range, while DNA is less susceptible to temperature variations and preserves its activity even at high temperatures.
- Proteins also require complex preparation and purification, unlike oligonucleotides, which can be easily chemically synthesized and stored for long periods in solution compared to enzymes which become unstable when stored in an aqueous solution over a longer period [52].

Among the DNAzymes currently being used, specifically, the hemin/G-quadruplex horseradish peroxidase-mimicking DNAzyme (HRP-DNAzyme) [53] has been frequently used as a catalytic nucleic acid label for **amplified biosensing** and can be used as an alternative to HRP. [54]

1.5.1 HRP-DNAzyme

G-rich oligonucleotides have the ability to self-assemble into a four-stranded structure known as G-quadruplex. The building blocks of G-quadruplexes are structures known as G-quartets, which is the association of four planar G bases via Hoogsteen hydrogen bonds (Fig. 1.5.1,B) [55].
The G-quartets are piled up helical and are stabilized by hydrophobic interactions and by the presence of monovalent cations such as K^+ and Na^+, which are placed in between the G-quadruplex plains. G-quadruplexes (Fig. 1.5.1,C) are very variously shaped and are classified

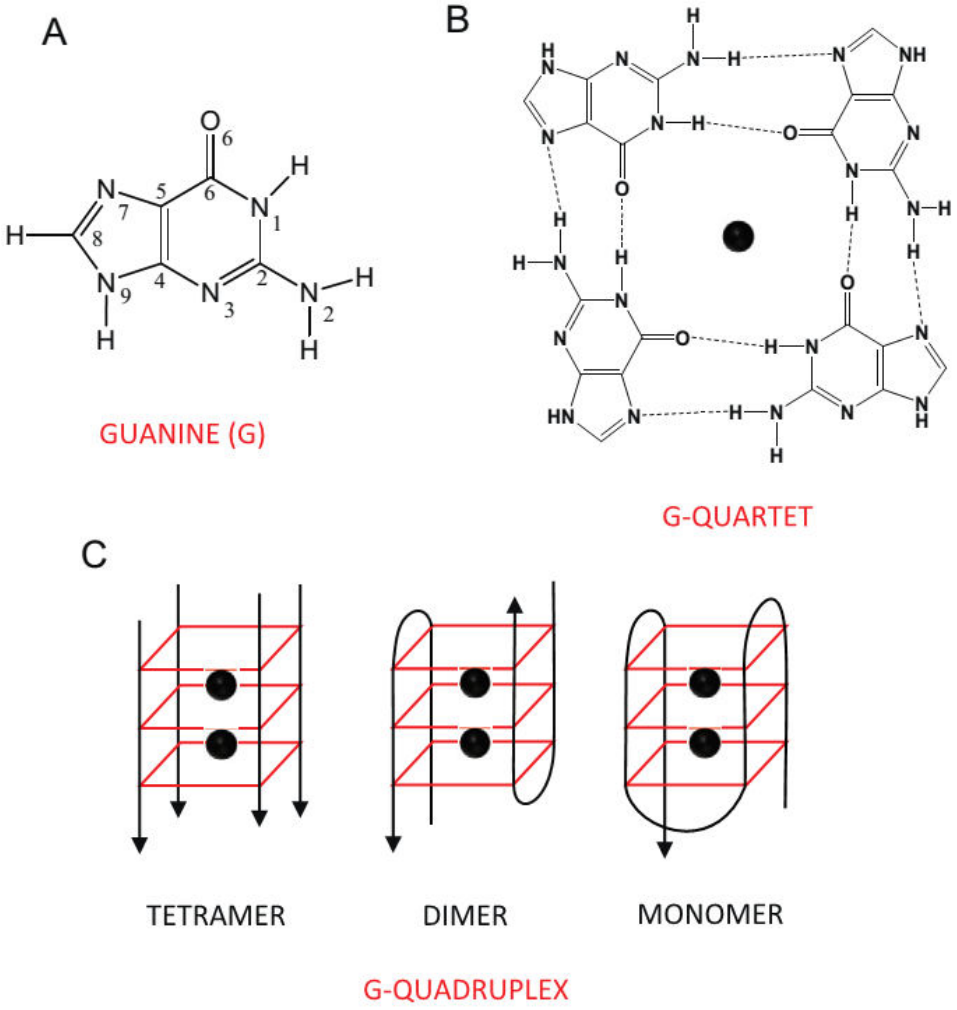

Figure 1.5.1: Schematic illustration of G-quadruplexes [55].

(A) Chemical structure of the guanine (G) base. (B) A G-quartet showing the hydrogen bonding between four planar G bases. (C) Schematic representation of tetramer, dimer and monomer G-quadruplexes composed by three staked G-quartets; the cations that stabilize the G-quadruplexes are shown as black balls.

in terms of their molecularity (monomer, dimer or tetramer quadruplexes), strand polarity (parallel or antiparallel orientations), and other varying features [55, 56, 57, 58].

Hemin, which is an iron-containing porphyrin (Fig. 1.5.2), can act as a ligand and is able to specifically bind to several G-quadruplexes with high affinity. Hemin has the function of an active co-factor which exhibits itself very low peroxidase-like activity. The DNAzyme could not only form a hemin/G-quadruplex complex but also mimic the function of a horseradish peroxidase. This HRP-mimicking DNAzyme may display a highly enhanced catalytic activity compared with hemin itself [53, 59, 60].

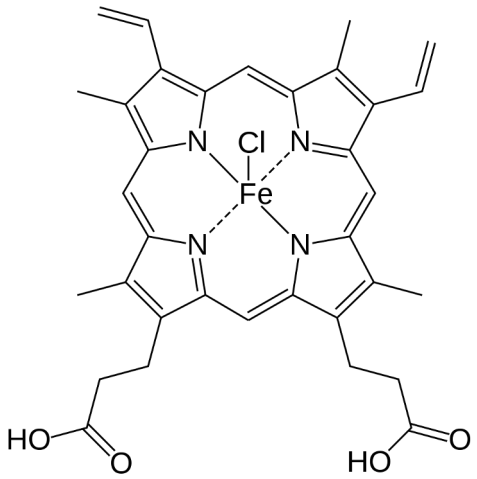

Figure 1.5.2: Chemical structure of hemin.

Based on their catalysis, detection methods such as **calorimetry** [61, 50], **electrochemistry** [46] and **chemiluminescence** [62, 63] have been reported with DNAzyme-based assays.

3,3′,5,5′-Tetramethylbenzidine (TMB) is a commonly used peroxidase substrate [64]. The HRP catalyses the H_2O_2-mediated oxidation of the colourless TMB into two coloured products (Fig. 1.5.3) [64].

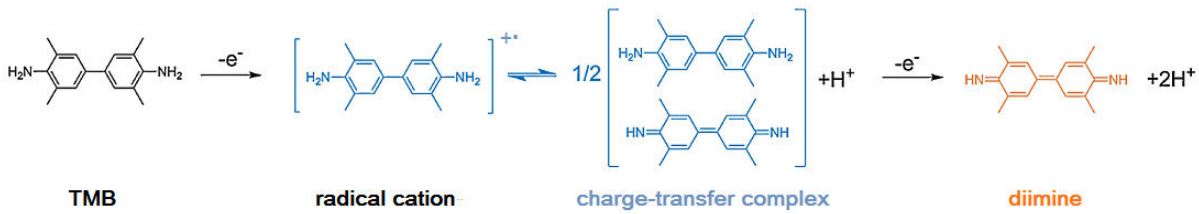

Figure 1.5.3: The structure of TMB and the catalytic reaction products.

The first product is a blue charge-transfer complex of the parent diamine and the diimine oxidation product. This species exists in rapid equilibrium with the radical cation and has its maximal absorbance at 370 nm and 652 nm. Upon the addition of sufficient acid, the blue product will be further oxidized to a yellow diimine, which is stable at acidic conditions, has its maximal absorbance at 450 nm and is therefore visible to human eyes. In summary, the blue product is a one-electron oxidation product of TMB and that the yellow product is the two-electron oxidation product (diimine) [64].

Like the HRP, the HRP-mimicking DNAzyme can also catalyse the oxidation of TMB by the use of H_2O_2 as oxidising agent to produce the two coloured products (Fig. 1.5.3) with much higher catalytic activity than hemin alone [65]. Figure 1.5.4 shows an illustration of the DNAzyme-based calorimetry.

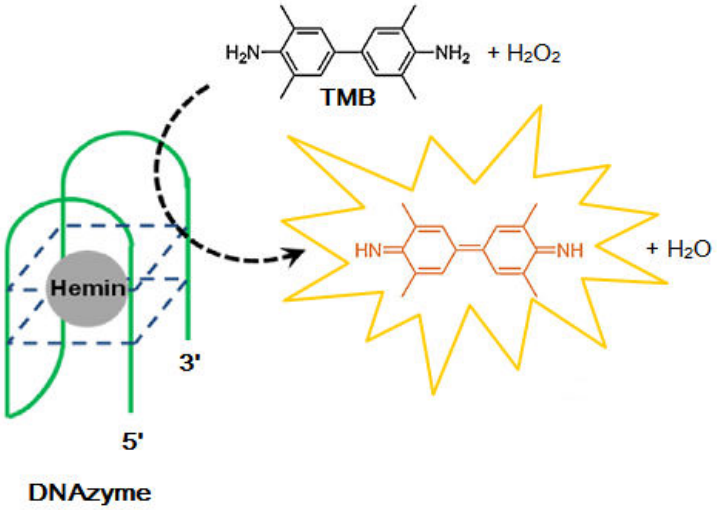

Figure 1.5.4: Schematic illustration of a DNAzyme-based calorimetric assay with TMB as substrate.

The use of functional nucleic acids for amplified biosensing can be accomplished by designing aptamer–DNAzyme conjugates that combine recognition units (aptamer) and amplifying units (DNAzyme) [66].

This combination of functional nucleic acids appears also very advantageous in terms of developing rapid, cheap and on-site detection methods for aflatoxin B_1. Moreover, the application of DNAzymes for aflatoxin B_1-detection is very beneficial because of their ability of **signal amplification**. The requirement of detecting very low amounts of aflatoxin B_1 in contaminated food and feed can only be achieved by an amplification of the initial aptamer-target binding signal.

2 Aim of this work

2.1 Overall project

In the overall project, research is done on an aptamer-based assay for the detection of aflatoxin B_1 which should allow the rapid detection of this mycotoxin in maize. The planned aptamer-based assay takes the advantage of hybridisation of a complementary DNA with the aptamer-DNA and the dehybridisation once the aptamer meets the target molecule aflatoxin B_1. The schematic illustration of the setup of the planned aptasensor is shown in Figure 2.1.1.

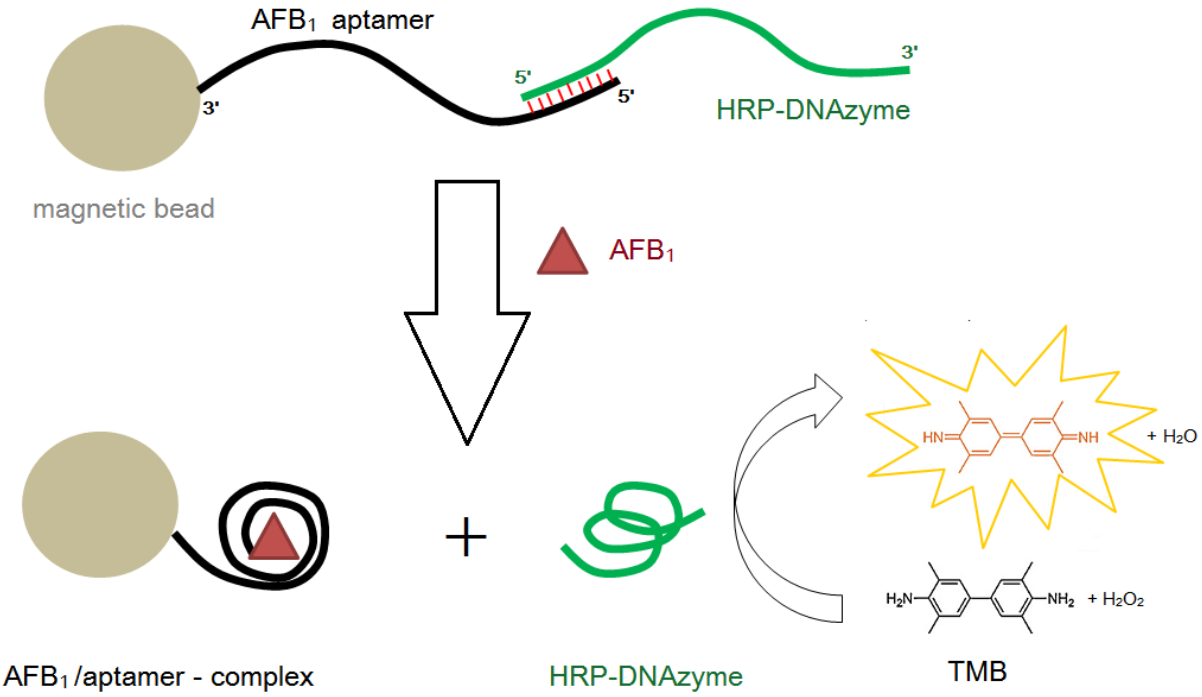

Figure 2.1.1: Schematic illustration of the planned aptamer- and DNAzyme-based detection of AFB_1.

The set-up of the aptamer-based sensors is as follows: As aptamer specific to AFB_1 serves the AFB_1 aptamer which was published by NeoVentures Biotechnology Inc. in 2012 [36]. This AFB_1 aptamer is immobilised onto magnetic beads via biotin-streptavidin coupling on its 3'-end and hybridised over a short overlap at its 5'-end with a complementary single-strand DNA. As complementary ssDNA serves a HRP-mimicking DNAzyme which consists of a short overlap at its 5'-end where the hybridisation with the AFB_1 aptamer takes place. In the presence of aflaoxin B1, the process of the formation of the AFB_1/aptamer-complex induces

the release of the HRP-DNAzyme. After magnetic separation, these HRP-DNAzymes should show chemiluminescence using TMB as substrate.

2.2 Aim of this work

In preliminary experiments, the DNAzyme-catalysed H_2O_2-mediated oxidation of TMB was testified and investigated [65]. However, these experiments showed not enough sensitivity for sensor-developing purposes. The aim of this work is to further develop and optimise the DNAzyme-catalysed H_2O_2-mediated oxidation of TMB (Fig. 1.5.4). To address this task, several factors could be examined to influence the colour-generating activity of this system. These factors include:

- DNA sequence of the DNAzyme (HRP-DNAzyme),
- substrate buffer conditions,
- concentration of substrate (TMB) and co-substrate (H_2O_2),
- concentration of co-factor (hemin) and its concentration ratio to DNAzyme,
- reaction time.

By optimising this colorimetric system, the goal is to generate the most intensive colour in the shortest period of time with the lowest concentration of DNAzyme. Furthermore, the immobilisation and the use of the AFB_1 aptamer with the hybridised DNAzyme as recognition probe is tested in first dehybridisation attempts.

In the long run, the optimisation of the colorimetric system and the investigation of dehybridisation characteristics are very important and form the basis for further research on the planned aptamer-based assay, which is shown in Figure 2.1.1, to obtain a rapid, sensitive and on-site detection method for AFB_1.

3 Materials and methods

3.1 Materials and reagents

Sodium chloride (NaCl), tris(hydroxymethyl)aminomethane (Tris), sodium hydroxide (NaOH), potassium chloride (KCl) and calcium chloride ($CaCl_2$) were purchased from Carl Roth (Austria). Acetonitrile (ACN) was purchased from J.T.Baker®. The DNA sequences for the DNAzymes and the specific 3'-terminal biotinylated aptamer used are listed in Table 3.1.1. These DNA sequences, hemin (from porcine), sulfuric acid (H_2SO_4) and 3,3',5,5'- Tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (Austria). BioMag® Streptavidin beads were purchased from Polysciences, Inc. BioMag® Streptavidin is a suspension of BioMag® particles approximately 1.5 μm in size to which streptavidin is covalently attached to the surface. Each DNA was diluted to a given concentration with Tris-HCl (pH 7), heated at 65 °C while shaking at 1400 rpm for 10 min (to dissociate any intermolecular interaction), then gradually cooled to room temperature and stored at 4 °C before use. The water used throughout all experiments was purified by a Milli-*Q*-purification system (ELGA LabWater, Purelab Ultra Analytic Water Purification System).

- **TMB stock solution**: The stock solution of TMB (52 mM) was prepared in dimethyl sulfoxide (DMSO) and methanol (1:5).
- **Hemin stock solution**: The stock solution of hemin (1 mM) was prepared in 20 mM NaOH and diluted to the required concentrations with Tris-HCl (pH 8.4).
- **Reaction solution**: The reaction solution for the DNAzyme-catalysed H_2O_2-mediated oxidation of TMB consists of DNAzyme, hemin and reaction buffer containing 10 mM Tris-HCl buffer (pH 8.4), 120 mM NaCl, 5 mM KCl and 20 mM $CaCl_2$.
- **Substrate solution**: The substrate solution was freshly prepared by adding TMB-stock and H_2O_2 (30%) into the substrate buffer, which contains citric acid and potassium sorbate (pH 4).
- **Stopping solution**: As stopping reagent for the catalytic reaction was used H_2SO_4.
- **Hybridisation buffer**: The hybridisation buffer for the AFB_1 aptamer and DNAzyme consists of 750 mM NaCl and 75 mM trisodium citrate (pH 8).
- **Hybrid stock solution**: The hybrid stock solution was prepared by addition of AFB_1 aptamer and DNAzyme 7 (Tab. 3.1.1) in equal ratio of 10 μM in hybridisation buffer, heating 5 minutes at 60 °C followed by gradually cooling to room temperature.

Table 3.1.1: Sequences of oligonucleotides used in this work.

AFB_1 aptamer	5'-**GTTGGGCACGTGT**TGTCTCTCTGTGTCTCGTGCCCTTCGCTAGGCCC-*biotin*-3'
DNAzyme 1	5'-**ACACGTGCCCAAC**TTTTGGGTAGGGCGGGTTGGG-3'
DNAzyme 2	5'-**ACACGTGCCCAAC**TTTTTGGGTAGGGCGGGTTGGG-3'
DNAzyme 3	5'-**ACACGTGCCCAAC**TTTTGGGTAGGGCGGGTTGGGTTTTGGGTAGGGCGGGTTGGG-3'
DNAzyme 4	5'-**ACACGTGCCCAAC**AAAAAGGGTAGGGCGGGTTGGGAAAAAGGGTAGGGCGGGTTGGG-3'
DNAzyme 5	5'-**ACACGTGCCCAAC**TTTTGGGTAGGGCGGGTTGGGAAAAAGGGTAGGGCGGGTTGGGAAAAAGGG TAGGGCGGGTTGGG-3'
DNAzyme 6	5'-**ACACGTGCCCAAC**AAAAAGGTAGGGCGGGTTGGGTAAAT-3'
DNAzyme 7	5'-**ACACGTGCCCAAC**AAAAAGGTAGGGCGGGTTGGGTAAATAAAAAGGGTAGGGCGGGTTGGGTAAAT-3'
DNAzyme 8	5'-**ACACGTGCCCAAC**TTTTGGTAGGGCGGGTTGGGTAAATTTTTTGGGTAGGGCGGGTTGGGTAAAT-3'
DNAzyme 9	5'-**ACACGTGCCCAAC**TTTTGTGGGTAGGGCGGGTTGG-3'
DNAzyme 10	5'-**ACACGTGCCCAAC**TTTTGTGGGTAGGGCGGGTTGGTTTTGTGGGTAGGGCGGGTTGG-3'
DNAzyme 7-1	5'-**CACGTGCCCAAC**AAAAAGGTAGGGCGGGTTGGGTAAATAAAAAGGGTAGGGCGGGTTGGGTAAAT-3'
DNAzyme 7-2	5'-**ACGTGCCCAAC**AAAAAGGTAGGGCGGGTTGGGTAAATAAAAAGGGTAGGGCGGGTTGGGTAAAT-3'
DNAzyme MM 1	5'-**ACACGTGCCCAA**A**A**AAAAAGGTAGGGCGGGTTGGGTAAATAAAAAGGGTAGGGCGGGTTGGGTAAAT-3'
DNAzyme MM 2	5'-**ACACGTGCCCA**T**C**AAAAAGGTAGGGCGGGTTGGGTAAATAAAAAGGGTAGGGCGGGTTGGGTAAAT-3'
DNAzyme MM 3	5'-**ACACG**A**GCCCAAC**AAAAAGGTAGGGCGGGTTGGGTAAATAAAAAGGGTAGGGCGGGTTGGGTAAAT-3'

Nucleotide sequences in bold are complementary bases between aptamer and DNAzymes and therefore indicate the area where the hybridisation with the aptamer takes place. Single nucleotides which are written in red (DNAzyme MM 1-3) indicate mismatches in the hybridisation area with the aptamer.

3.2 Instrumentation

The absorption spectra of the DNAzyme-catalysed H_2O_2-mediated oxidation of TMB to the diimine oxidation product were recorded on a NanoView Plus Spectrophotometer (GE Healthcare Life Sciences) at room temperature. A reaction buffer containing hemin without DNAzyme was used as the respective negative control and blank in the absorption measurements. For the dehybridisation experiments, the respective negative controls and therefore blanks in the calorimetric measurements contain the same reagents as the tested samples, but remain unheated at room temperature. The measurement of the absorbance of the particular samples was done in triplicates.

3.3 Procedures of the experiments

DNAzyme-catalysed oxidation of TMB in the prescence of H_2O_2

Due to reasons of optimisation of the DNAzyme-catalysed oxidation of TMB and H_2O_2, most concentrations of the used reagents have been altered throughout the experiments. The general procedure of testing and subsequently optimising the assay was as follows:

In a typical experiment, 50 μl of solution containing reaction buffer, DNAzyme and hemin, were added into 1.5 ml micro tubes. After that, 20 μl of substrate solution containing TMB and H_2O_2 in substrate buffer were added, stirred and incubated till such time as the reaction solution has turned visibly and clearly blue. After the first experiments and the optimisation of single parameters, the incubation time was set to 20 min for all subsequent experiments for reasons of comparability. After that, the catalytic reaction was stopped by addition of H_2SO_4. Subsequently, the absorbance was measured at a wavelength of 452 nm. The absorbance measurements were performed in triplicates. All assays were performed at room temperature.

Dehybridisation experiments of immobilised AFB_1 aptamers and hybridised DNAzymes with subsequent colorimetric detection of DNAzymes

In a typical experiment, 10 μl of BioMag® Streptavidin beads stock solution were added into 1.5 ml micro tubes and the stock buffer of the beads was discarded via magnetic separation. After that, 20 μl of hybrid stock solution (10 μM) were added to the beads. This suspension was incubated at 37 °C for one hour. Then, the beads were separated magnetically, the supernatant was discarded and the beads were washed. After that, 42 μl of reaction buffer was added to the beads. The samples were then heated several minutes on a heating block for the purpose of dehybridisation of the aptamer and DNAzyme. Meanwhile, the respective

negative controls remained unheated. After the dehybridisation step, the beads were separated via magnetic separation and the supernatant was quickly transferred into a new 1.5 ml micro tube in order to avoid re-hybridisation events. Then, 8 μl of hemin (10 μM) and 20 μl of substrate solution containing 7 mM TMB and 21 mM H_2O_2 in substrate buffer (300 mM citric acid, 1 mM potassium sorbate, pH 4) were added, stirred and incubated till such time as the reaction solution has turned visibly and clearly blue. The catalytic reaction was then stopped by addition of 5 μl of 5 M H_2SO_4. Subsequently, absorbance was measured at a wavelength of 452 nm. The absorbance measurements were performed in triplicates. All assays were performed at room temperature.

4 Results and discussion

4.1 DNAzyme-catalysed oxidation of TMB in the presence of H_2O_2

4.1.1 Comparison of the activity of different DNAzymes

Ten DNA molecules (DNAzyme 1-10 in Table 3.1.1) were used in this experiment in order to test the catalytic capability of these DNAzymes to oxidise TMB in the prescence of H_2O_2. The conditions of this experiment are given in Table 4.1.1. The molar concentrations of TMB and H_2O_2 and the substrate buffer were adopted from a typical ELISA standard protocol performed with peroxidase.

Table 4.1.1: Experimental parameters for testing the catalytic ability of 10 different DNA molecules.

Reagent	**Concentration**	**V [μl]**
reaction solution:		120
DNAzyme	100 nM	
hemin	200 nM	
substrate solution:		100
TMB	413 μM	
H_2O_2	1.9 mM	
substrate buffer	200 mM citric acid, 666 μM potassium sorbate	

It was observed that after the substrate solution (containing TMB and H_2O_2 in substrate buffer) was added, the reaction solution of some DNAzymes changed to blue gradually. Figure 4.1.1 shows the gradual formation of a blue coloured solution after 1, 3 and 24 hours. After 24 hours of incubation, nearly all DNAzyme solutions turned blue. This suggests that the charge-transfer complex is formed successfully [64]. The catalytic reaction has not been stopped by addition of H_2SO_4, because only the ability of the different DNAzymes to oxidise TMB causing a coloured solution at all was of interest at this stage of optimisation.
As shown in Figure 4.1.1, DNAzyme 5, 7 and 8 appeared to be the best performing DNAzymes of this collection and therefore have been taken for further experiments. A fast signal generation is one of the main requirements for developing a rapid detection method. Therefore, higher DNAzyme concentrations were used in the following experiments to make sure that the assay works in a short period of time (10 to 20 minutes). By optimising this catalytic system, the DNAzyme concentrations were subsequently reduced again.

DNAzyme 5, 7 and 8 were tested at a concentration of 5 and 10 μM in 50 μl reaction solution.

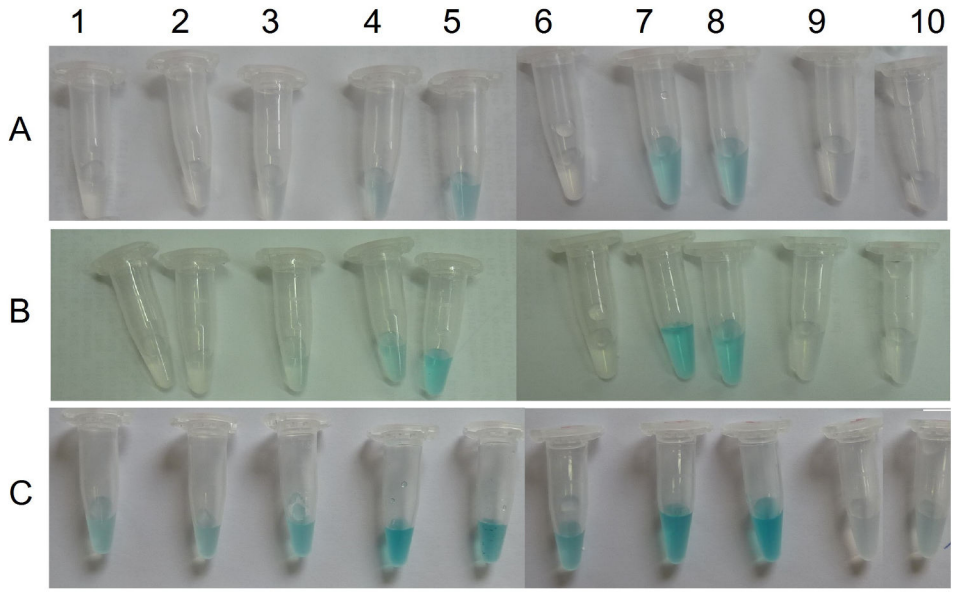

Figure 4.1.1: Testing DNAzyme 1-10 for the DNAzyme-catalysed oxidation of TMB. Photographs of H_2O_2-mediated oxidation of TMB after (A) 1-, (B) 3- and (C) 24-hours incubation.

The conditions of this experiment are given in Table 4.1.2. In less than 1 min the reaction solution turned slightly blue (Figure 4.1.2). After 10 min, the catalytic reaction was stopped by addition of H_2SO_4. As expected, as soon as H_2SO_4 was added, the colour turned yellow immediately, which suggests the formation of the diimine [64].

Table 4.1.2: Experimental parameters for testing the catalytic ability of DNAzyme 5, 7 and 8.

	Concentration		
Reagent	**1.**	**2.**	**V [μl]**
reaction solution:			50
DNAzyme	5 μM	10 μM	
hemin	10 μM	20 μM	
substrate solution:			100
TMB	413 μM		
H_2O_2	1.9 mM		
substrate buffer	200 mM citric acid, 666 μM potassium sorbate		
H_2SO_4	2 M		150

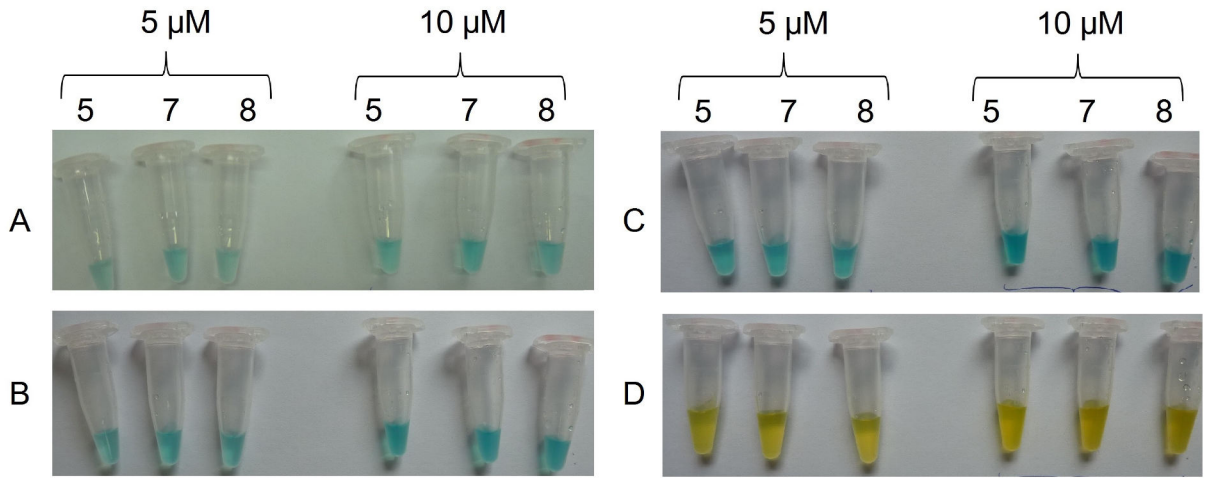

Figure 4.1.2: Testing DNAzyme 5,7 and 8 for the DNAzyme-catalysed oxidation of TMB. Photographs of H_2O_2-mediated oxidation of TMB after (A) 5-, (B) 10- and (C) 20-minutes incubation and after (D) stopping the reaction by addition of H_2SO_4.

As **DNAzyme 7** was performing slightly better than DNAzyme 5 and 8, we decided to use DNAzyme 7 in all subsequent experiments for the optimisation of the assay.

4.1.2 Effect of the concentration of the substrate and the stopping solution on the colorimetric reaction

The visible effect on the catalytic reaction of different amounts of substrate and stopping solution was measured (a-e, Table 4.1.3).

Table 4.1.3: Experimental parameters for testing the visible effect of different volumes (a-e) of substrate and stopping solution.

		V [μl]				
Reagent	**Concentration**	**a**	**b**	**c**	**d**	**e**
reaction solution:		50				
DNAzyme	5 μM					
hemin	10 μM					
substrate solution:		10	20	40	80	160
TMB	413 μM					
H_2O_2	1.9 mM					
substrate buffer	200 mM citric acid, 666 μM potassium sorbate					
H_2SO_4	2 M	60	70	90	130	210

As visible in Figure 4.1.3, the catalytic reaction performs better by addition of 160 μl (e) than 10 μl (a) substrate solution. Table 4.1.4 shows the absorbance of the samples after addition

of acid. After the addition of stopping solution, it can be noticed, that the visible difference of (B,a) and (B,e) in Figure 4.1.3 is not as obvious than (A,a) and (A,e) due to diluting effects in (B,e) after addition of 210 μl stopping solution. These findings lead to the assumption that the use of higher concentrated substrate and stopping solution but simultaneously less volumes produces more of the yellow diimine.

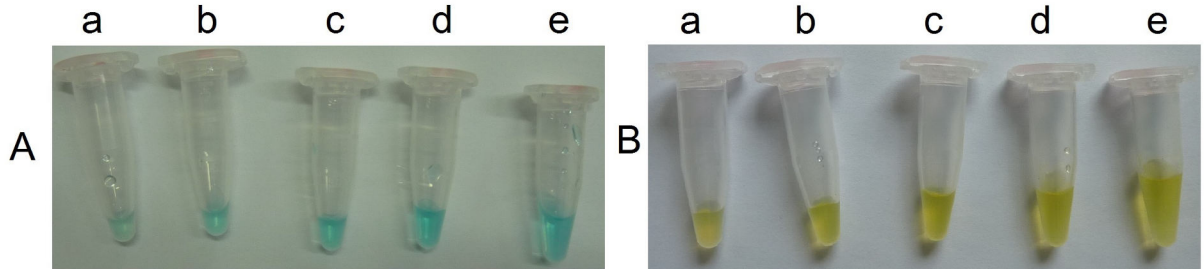

Figure 4.1.3: Testing DNAzyme 7 with different volumes of substrate and stopping solution. Photographs of oxidation of different amounts of substrate solution (a-e, Tab. 4.1.3) after (A) 6- minutes incubation and after (B) stopping the reaction after 20 min by addition of different amounts (a-e) of H_2SO_4.

Table 4.1.4: Absorbance of samples with different volumes of substrate solution 5 minutes after stopping the catalytic reaction by addition of different volumes of acid.

Absorbance				
a	**b**	**c**	**d**	**time**
0.098	0.115	0.129	0.145	5 min

Therefore, different concentrations of TMB (and therefore H_2O_2) in the substrate solution were tested (a-d, Table 4.1.5). Furthermore, the concentration of the DNAzyme was reduced to 1.5 μM. A sample containing all reagents with exception of the DNAzyme (NC, Table 4.1.5) serves as negative control. The results can be seen in Figure 4.1.4. In contrast, after 20 minutes of incubation, the hemin-only solution (NC) is colourless. Thus, compared to hemin only, the DNAzyme can indeed significantly accelerate the oxidation of TMB. The most intensive colour can be achieved with the highest concentration of TMB in the substrate solution (A,d) and (B,d) in Fig. 4.1.4. The absorbance of the samples was measured using the negative control as blank. The results can be seen in Table 4.1.6. Interestingly, some time after stopping the catalytic reaction with H_2SO_4, a loss of absorbance of the samples can be observed which is even visible for the naked eye (Fig. 4.1.4). The greatest and also fastest

loss of absorbance shows the sample with the highest concentration of TMB and H_2O_2 (d, Table 4.1.6).

Table 4.1.5: Different concetrations of TMB in the substrate solution.

	Concentration					
Reagent	**a**	**b**	**c**	**d**	**NC**	**V [μl]**
reaction solution:						50
DNAzyme			1.5 μM		0 μM	
hemin			3 μM			
substrate solution:						20
TMB	815 μM	1.3 mM	1.7 mM	3.3 mM	3.3 mM	
H_2O_2	3.9 mM	6.0 mM	7.9 mM	15.5 mM	15.5 mM	
substrate buffer	200 mM citric acid, 666 μM potassium sorbate					
H_2SO_4	5 M					10

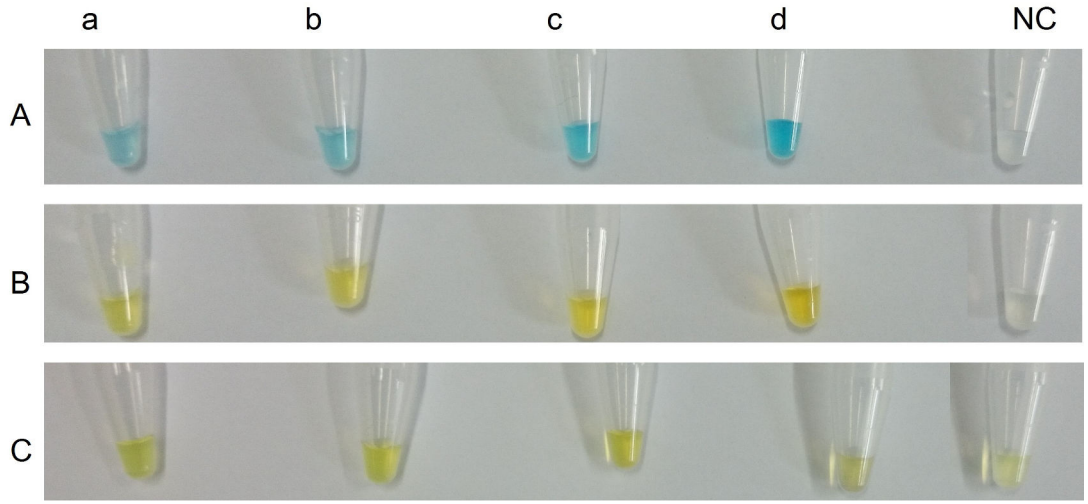

Figure 4.1.4: Different concetrations of TMB in the substrate solution. Photographs of oxidation of different concentrations of TMB (a-d, Tab. 4.1.5) in the substrate solution after (A) 20- minutes incubation, after (B) stopping the reaction after 20 min by addition of H_2SO_4 and (C) seven hours after stopping the reaction.

If the acid-treated incubation was observed for a prolonged time, e.g. seven hours (C in Fig. 4.1.4), all solutions changed their colour and even the negative control became slightly yellow. This can be explained as follows: In a conventional peroxidase system, the enzyme is usually used at very low concentrations (nM), therefore the likewise low concentrations of hemin causes no further colour change after the acid treatment. Here, in these experiments, the hemin concentrations used are very high (μM), so the remaining hemin after stopping the

Table 4.1.6: Absorbance of samples with different concentrations of TMB in the substrate solution x time after stopping the catalytic reaction by addition of acid.

Absorbance				
a	**b**	**c**	**d**	**time**
0.087	0.119	0.176	**0.229**	10 min
		0.159	**0.096**	30 min
		0.140	0.065	1.5 h

reaction continues the catalytic reaction, because hemin cannot be destroyed by strong acids. Therefore, all absorbance should be carried out immediately after addition of H_2SO_4. As the greatest and also fastest loss of absorbance occurs with the highest concentration of TMB and H_2O_2 (3.3 mM and 15.5 mM respectively), the optimal concentration ratio between TMB and H_2O_2 had to be identified in order to further optimise the assay. While optimising the whole reaction in the following experiments, the concentration of the DNAzmye was gradually reduced.

4.1.3 Effect of different ratios between TMB and H_2O_2 on the colorimetric reaction

To further optimise the DNAzyme-catalysed oxidation of TMB in the presence of H_2O_2, the optimal concentration ratio between TMB and H_2O_2 was determined. In theses experiments, the concentration of the DNAzyme used was further reduced to 1 μM. The experimental parameters for this experiment are given in Table 4.1.7.

Table 4.1.8 shows that the concentration of H_2O_2 was in (1a, 1b, 1c, 1d) **4.7**-, in (2a, 2b, 2c, 2d) **2.2**- and in (3a, 3b, 3c, 3d) **3-times** the concentration of TMB. After stopping the reaction with H_2SO_4, the absorbance of the samples was measured. The results of the absorbance measurements are given in Table 4.1.9. Figure 4.1.5 shows photographs of the samples before and after stopping the catalytic reaction with sulphuric acid as well as some time after addition of H_2SO_4. As mentioned above (section 4.1.2), a loss in the absorbance of the samples after addition of acid can be observed. In order to generate a consistent signal (which is a main requirement of a well-performing sensor), it is important to have a very low loss in the absorbance of the coloured samples.

Table 4.1.7: Experimental parameters for testing different concentration ratios between TMB and H_2O_2.

Reagent	Concentration Samples	NC	V [μl]
reaction solution:			50
DNAzyme	1 μM	0 μM	
hemin	2 μM		
substrate solution:			20
TMB	see Table 4.1.8		
H_2O_2	see Table 4.1.8		
substrate buffer	200 mM citric acid, 666 μM potassium sorbate		
H_2SO_4	5 M		5

Table 4.1.8: Different concentration ratios and concentrations of TMB and H_2O_2 in substrate buffer.

	Concentration [mM]														
	1a	**2a**	**3a**	**1b**	**2b**	**3b**	**1c**	**2c**	**3c**	**1d**	**2d**	**3d**	**1NC**	**2NC**	**3NC**
TMB	1.7		5	3.3		6	4.9		7	6.6		8	6.6		8
H_2O_2	8	3.7	15	15.5	7.3	18	23	10.8	21	31	14.5	24	31	14.5	24

When looking at the results of the absorbance measurements in Table 4.1.9, it seems that too high concentrations of H_2O_2 compared to TMB makes the results more inconsistent. For example, the H_2O_2 concentration was in samples 1a-d **4.7**- and in samples 2a-d only **2.2-times** the concentration of TMB. Compared to samples 1a-d, the absorbance of the samples 2a-d was decreasing less after the catalytic reaction had been stopped. These findings suggest that H_2O_2, when used at high concentrations, has the ability to reduce the colour of the product. Alternatively, the highest absorbance immediately after addition of acid can be generally achieved with the highest concentrations of H_2O_2 (3d in Table 4.1.9). Also, when having too high concentrations of TMB ("d" samples have the highest concentrations of TMB), a faster loss in the absorbance of the samples can be noticed. Five minutes after addition of the acid, 3d in Table 4.1.9 shows the highest absorbance of all tested samples, but a fast decrease in the absorbance can be measured after additional 5 minutes. Otherwise, it can be generally observed that the higher the concentration of TMB in the substrate solution, the higher are the measured absorbances of the samples.

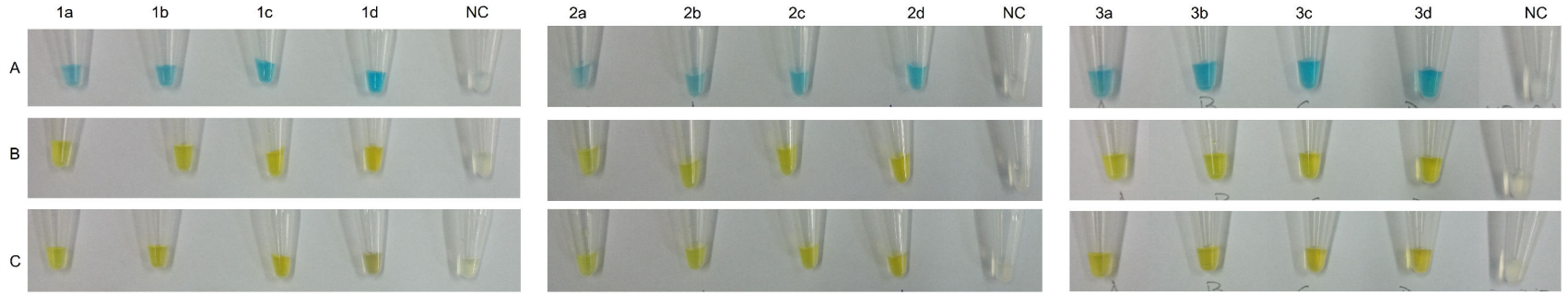

Figure 4.1.5: Different concentration ratios and concentrations of TMB and H_2O_2 in substrate buffer. Photographs of oxidation of different concentrations and ratios of TMB and H_2O_2 (1a-d, 2a-d, 3a-d, Tab. 4.1.8) in substrate buffer after (A) 15- minutes incubation, after (B) stopping the reaction after 20 min by addition of H_2SO_4 and (C) 15 minutes after stopping the reaction.

Table 4.1.9: Absorbance of samples with different ratios and concentrations of TMB/H_2O_2 in substrate buffer x time after addition of acid.

Absorbance												
1a	**1b**	**1c**	**1d**	**2a**	**2b**	**2c**	**2d**	**3a**	**3b**	**3c**	**3d**	**time**
0.119	0.193	**0.235**	**0.210**	0.087	0.145	0.161	0.185	0.179	0.200	**0.229**	**0.236**	5 min
0.117	0.164	**0.182**	**0.152**	0.084	0.137	0.153	0.171	0.173	0.195	**0.225**	**0.199**	10 min
0.112	0.148	**0.171**	**0.087**	0.080	0.135	0.142	0.165	0.173	0.184	**0.198**	**0.170**	15 min
0.105	0.140	0.163	0.075	0.078	0.131	0.135	0.162	0.168	0.179	0.185	0.149	20 min

In order to generate a colour that is reasonable consistent over a period of at least 15 minutes, a compromise between a high concentration of TMB and H_2O_2 as well as the optimal concentration ratio between the two reagents has to be found out to achieve an absorbance that is on the one hand high enough after addition of acid and on the other hand only decreases slowly in a given period of time. In these experiments, 3c in Table 4.1.9 fulfils these requirements the best. Therefore, a concentration of **7 mM TMB** and **21 mM H_2O_2** in the substrate buffer was used for subsequent experiments.

4.1.4 Effect of different concentration ratios between the DNAzyme and hemin on the colorimetric reaction

Not only the the concentrations of TMB and H_2O_2, but also the concentration of hemin plays a crucial role in order to generate a high absorbance after addition of acid. To find the optimal concentration of hemin for sensor-developing purposes, different ratios between the concentration of the used DNAzyme and hemin were tested. In these experiments, the concentration of the DNAzyme used was further reduced to 0.5 μM. Four different concentration ratios between DNAzyme and hemin were tested (DNAzyme:hemin = 1:1, 1:2, 1:4, 1:8). The respective negative controls contained all reagents except the DNAzyme. The experimental parameters are given in Table 4.1.10.

Table 4.1.10: Different concentrations of hemin in the reaction solution.

	Samples				
Reagent	**a**	**b**	**c**	**d**	**V [μl]**
reaction solution:					50
DNAzyme	0.5 μM				
hemin	0.5 μM	1 μM	2 μM	4 μM	
substrate solution:					20
TMB	7 mM				
H_2O_2	21 mM				
substrate buffer	200 mM citric acid, 666 μM potassium sorbate				
H_2SO_4	5 M				5

After stopping the reaction with H_2SO_4, the absorbance of the samples was measured. The results of the measurements are given in Table 4.1.11. Figure 4.1.6 shows photographs of the samples before and after stopping the reaction with sulphuric acid.

Table 4.1.11: Absorbance of different concentrations of hemin in the reaction solution x time after stopping the catalytic reaction by addition of acid.

Absorbance				
a	**b**	**c**	**d**	**time**
0.050	0.098	0.131	**0.201**	5 min
0.048	0.094	0.128	**0.192**	15 min
0.054	0.091	0.125	0.168	25 min

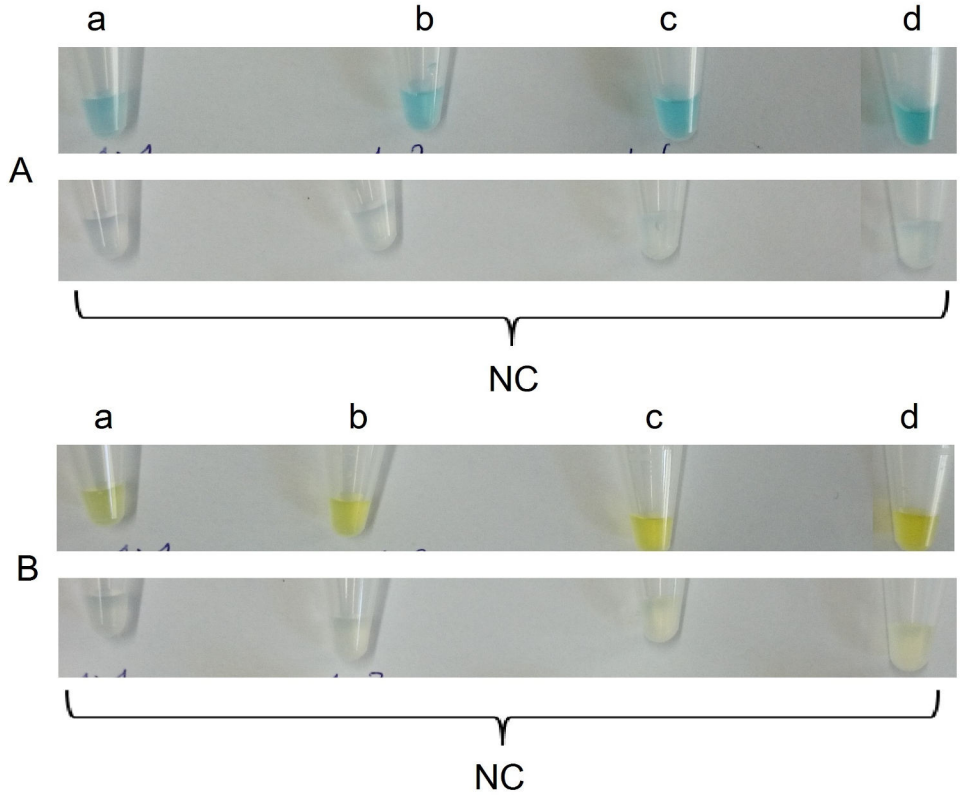

Figure 4.1.6: Different concetrations of hemin in the reaction solution. Photographs of H_2O_2-mediated oxidation of TMB using different concentrations of hemin (a-d, Tab. 4.1.10) in the reaction solution after (A) 18- minutes incubation and (B) stopping the reaction after 20 min by addition of H_2SO_4. Negative controls without DNAzyme are shown below the respective samples.

It can be assumed, that with higher hemin concentrations, a more intensive colour and hence a higher absorbance could be achieved. The disadvantage by using high concentrations of hemin is the circumstance, that also the respective negative control turns slightly more yellow (NC von B,d in Figure 4.1.6) compared to negative controls containing less hemin. However, the absorbance of the samples is measured relatively to the respective negative controls. Accordingly, the highest difference in absorbance compared to the negative control and therefore the sample with the most intensive colour is the one with the highest concentration of hemin (d, Tab. 4.1.11). It can further be noticed, that the loss in absorbance is slightly higher with higher concentrations of hemin, but in the context of designing a rapid detection method, it is not necessarily essential to generate a signal that is consistent over a long period of time. Therefore, a concentration of **hemin**, which is **eight-times the concentration of the DNAzyme** in the reaction buffer was applied for subsequent experiments.

4.1.5 Effect of the substrate buffer conditions and reproducibility tests

In order to further optimise this assay, different conditions of the substrate buffer were tested. In these experiments, the concentration of the DNAzyme was further reduced to 100 nM. Three different concentrations of citric acid and potassium sorbate in the substrate buffer were examined. For the purpose of testing the reproducibility of the assay, three exactly same experiments were performed. Table 4.1.12 shows the experimental parameters for these experiments. The catalytic reaction was stopped after 20 minutes of incubation by addition of H_2SO_4. The absorbance of the three exactly same experiments was measured five as well as ten minutes after addition of acid.

Table 4.1.12: Experimental parameters for testing different conditions of substrate buffer.

Reagent	**Samples NC**	**V [μl]**
reaction solution:		50
DNAzyme	100 nM 0 nM	
hemin	0.8 μM	
substrate solution:		20
TMB	7 mM	
H_2O_2	21 mM	
substrate buffer	Table 4.1.13	
H_2SO_4	5 M	5

The results of the absorbance measurements are given in Table 4.1.14. Figure 4.1.7 shows bar graphs of the measured absorbance of the samples. As it can be seen in Figure 4.1.7,

Table 4.1.13: Different concentrations of citric acid and potassium sorbate in the substrate buffer.

substrate buffer	citric acid [mM]	potassium sorbate [μM]
a	100	333
b	200	666
c	300	1000

the most stable results with the least errors in absorbance can be achieved by using substrate buffer c (green bar in Fig. 4.1.7). Therefore, a **substrate buffer** containing **300 mM citric acid** and **1 mM potassium sorbate** was chosen as standard substrate buffer.

Table 4.1.14: Absorbance of different substrate buffers x time after stopping the catalytic reaction by addition of acid.

	Experiments			
Substrate buffer	**1.**	**2.**	**3.**	**time**
a	0.080	0.060	0.106	5 min
	0.074	0.095	0.104	10 min
b	0.094	0.079	0.097	5 min
	0.096	0.068	0.104	10 min
c	0.093	0.094	0.099	5 min
	0.087	0.086	0.101	10 min

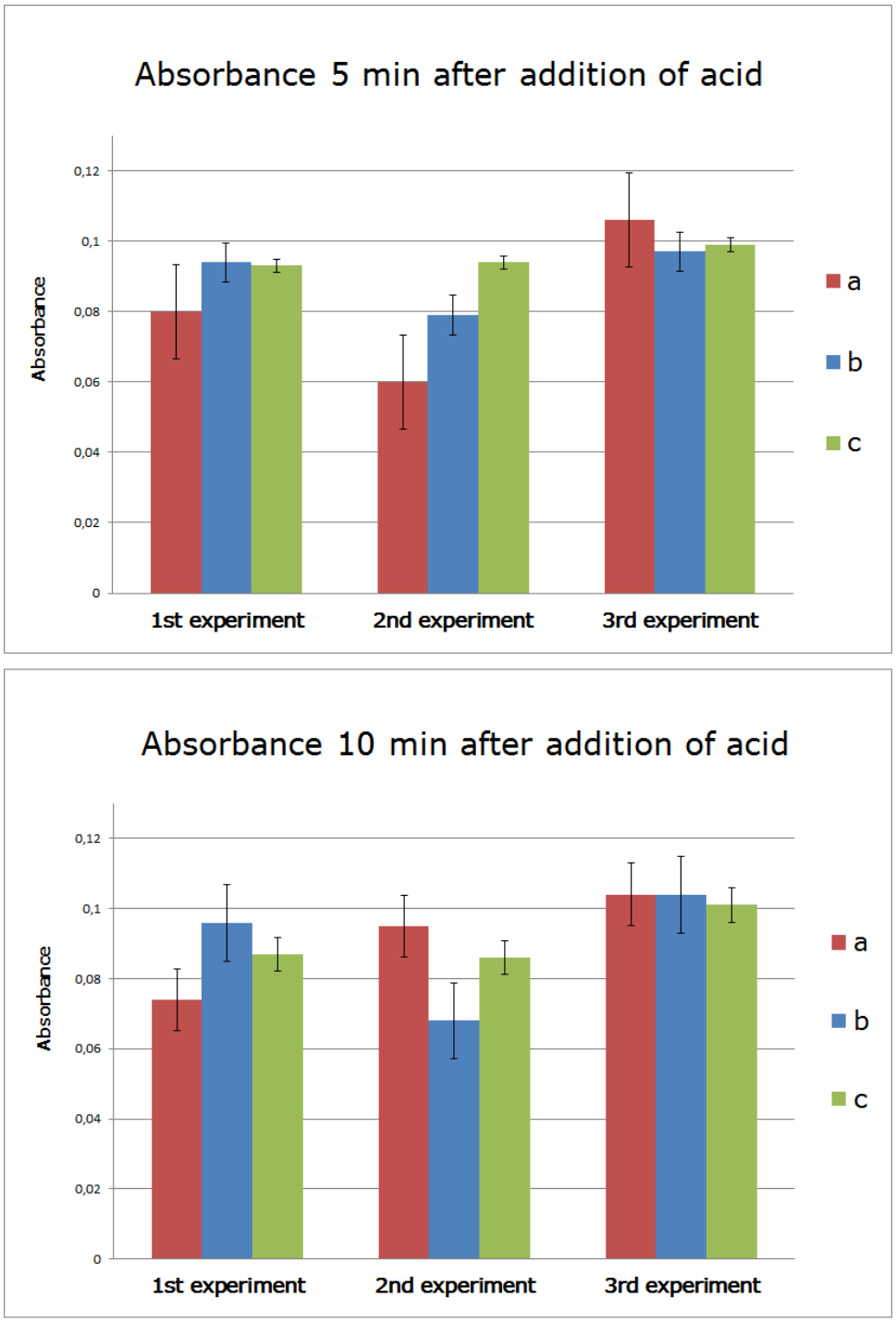

Figure 4.1.7: Reproducibility test. Bar graphs of absorbance values of the samples with different substrate buffer conditions (a, b and c) measured after 5 and 10 min. Substrate buffer contains 100 (a), 200 (b) or 300 (c) mM citric acid and 333 (a), 666 (b) or 1000 (c) μM potassium sorbate. Error bars are the standard deviation of three independent measurements of each experiment.

4.1.6 Effect of acetonitrile on the colorimetric reaction

The DNAzyme-catalysed oxidation of TMB in the presence of H_2O_2 is performed in aqueous solution (see parameters of the above experiments). In the following experiments, the effect of acetonitrile on the G-quadruplex complexes in the reaction solution was to be tested. Aflatoxin B_1 is quite stable in the organic solvent acetonitrile and therefore often stored in solutions consisting acetonitrile. Hence, it was of great interest to find out whether the DNAzyme-catalysed oxidation of TMB still works if different amounts of acetonitrile are added to the aqueous system. The effect of the organic solvent acetonitrile on the hydrogen bonds of the DNA and therefore on the DNA folding was to be examined. Table 4.1.15 shows the experimental parameters for these experiments. The catalytic reaction was stopped after 20 minutes incubation by addition of sulphuric acid.

Table 4.1.15: Experimental parameters for testing different percentages of acetonitrile in the reaction solution.

	Samples							
Reagent	**a**	**b**	**c**	**d**	**e**	**f**	**NC**	**V [μl]**
reaction solution:								50
DNAzyme	100 nM						0 nM	
hemin	0.8 μM							
acetonitrile	0%	3%	5%	10%	20%	30%	0-30%	
substrate solution:								20
TMB	7 mM							
H_2O_2	21 mM							
substrate buffer	300 mM citric acid, 1 mM potassium sorbate							
H_2SO_4	5 M							5

The absorbance of the samples was measured 5 minutes after stopping the catalytic reaction with H_2SO_4. The results of the measurements are given in Table 4.1.16. Figure 4.1.8 shows photographs of the samples before and after stopping the reaction with sulphuric acid.

Table 4.1.16: Absorbance of different percentages of acetonitrile (a-f) in the reaction solution 5 minutes after stopping the catalytic reaction by addition of acid.

Absorbance						
a	**b**	**c**	**d**	**e**	**f**	**time**
0.091	0.098	0.107	0.122	0.148	0.167	5 min

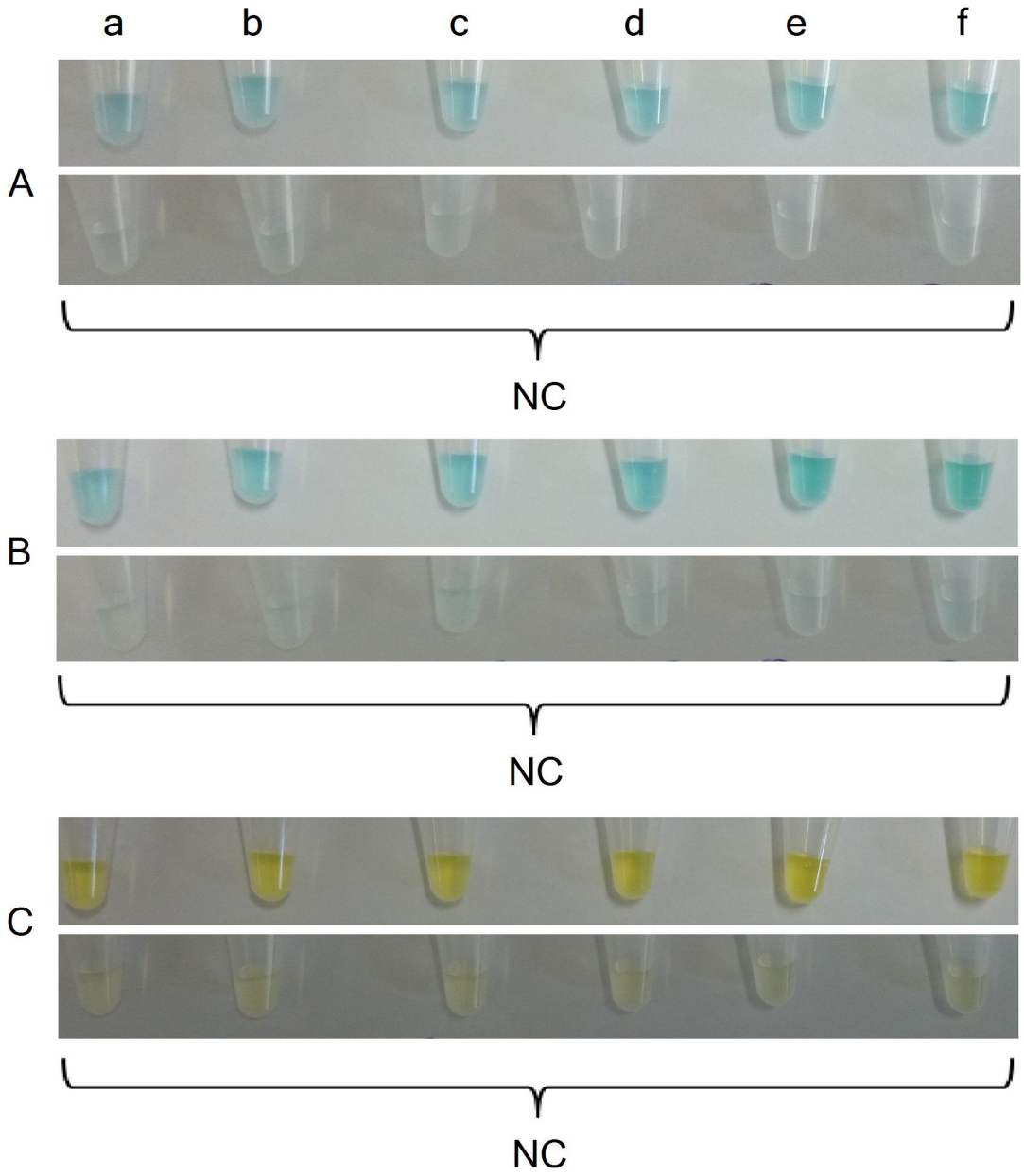

Figure 4.1.8: Different percentages of acetonitrile (a-f) in the reaction solution. Photographs of H_2O_2-mediated oxidation of TMB using different percentages of acetonitrile (a-f, Tab. 4.1.15) in the reaction solution after (A) 10- minutes and (B) 20- minutes incubation and (C) stopping the reaction after 20 minutes by addition of H_2SO_4. Negative controls without DNAzyme are shown below the respective samples.

Interestingly, acetonitrile seems to somehow stabilise and benefit the formation of the G-quadruplex complexes. As shown in Figure 4.1.8, the more acetonitrile was used in the reaction solution, the more intensive coloured products were formed (sample "f" contains the highest percentage of acetonitrile in these experiments). The Absorbance in Table 4.1.16 confirm these visible results. The sample with the highest percentage of acetonitrile ("f" contains 30% acetonitrile) shows the highest absorbance. These findings lead to the assumption that acetonitrile does not disturb the catalytic reaction of the DNAzyme in aqueous media. Quite the contrary, acetonitrile seems to enhance the catalytic reaction by stabilising the G-quadruplex complexes.

4.1.7 Final protocol of the DNAzyme-catalysed oxidation of TMB in the presence of H_2O_2

After having optimised the DNAzyme-catalysed H_2O_2-mediated oxidation of TMB, the final protocol of the experimental procedures is as follows:

1. 50 μl of solution containing DNAzyme (100 nM) and hemin (0.8 μM) in reaction buffer are added into a 1.5 ml micro tube.
2. 20 μl of substrate solution containing TMB (7 mM) and H_2O_2 (21 mM) in substrate buffer (300 mM citric acid and 1 mM potassium sorbate) are added, stirred and incubated for 20 minutes.
3. After that, the catalytic reaction is stopped by addition of 5 μl of H_2SO_4 (5 M).
4. The absorbance is measured at a wavelength of 452 nm.

4.2 Dehybridisation experiments of immobilised AFB_1 aptamers and hybridised DNAzymes with subsequent colorimetric detection of DNAzymes

In these experiments, it was tested whether the hybridisation between AFB_1 aptamer and DNAzyme 7 (Tab. 3.1.1) or the immobilisation of the formed hybrid onto the magnetic beads performs well in order to develop an aptamer-based sensor which is described in section 2 (Fig. 4.2.1). For the purpose of testing the hybridisation between aptamer and DNAzyme, the dehybridisation was to be performed in order to detect the free DNAzymes with the help of the H_2O_2-mediated oxidation of TMB. In these experiments, the dehybridisation of the aptamer and the DNAzyme was achieved through heating the formed hybrid on a heating block. There was no evaluation by addition of different amounts of aflatoxin B_1 at this stage of the research.

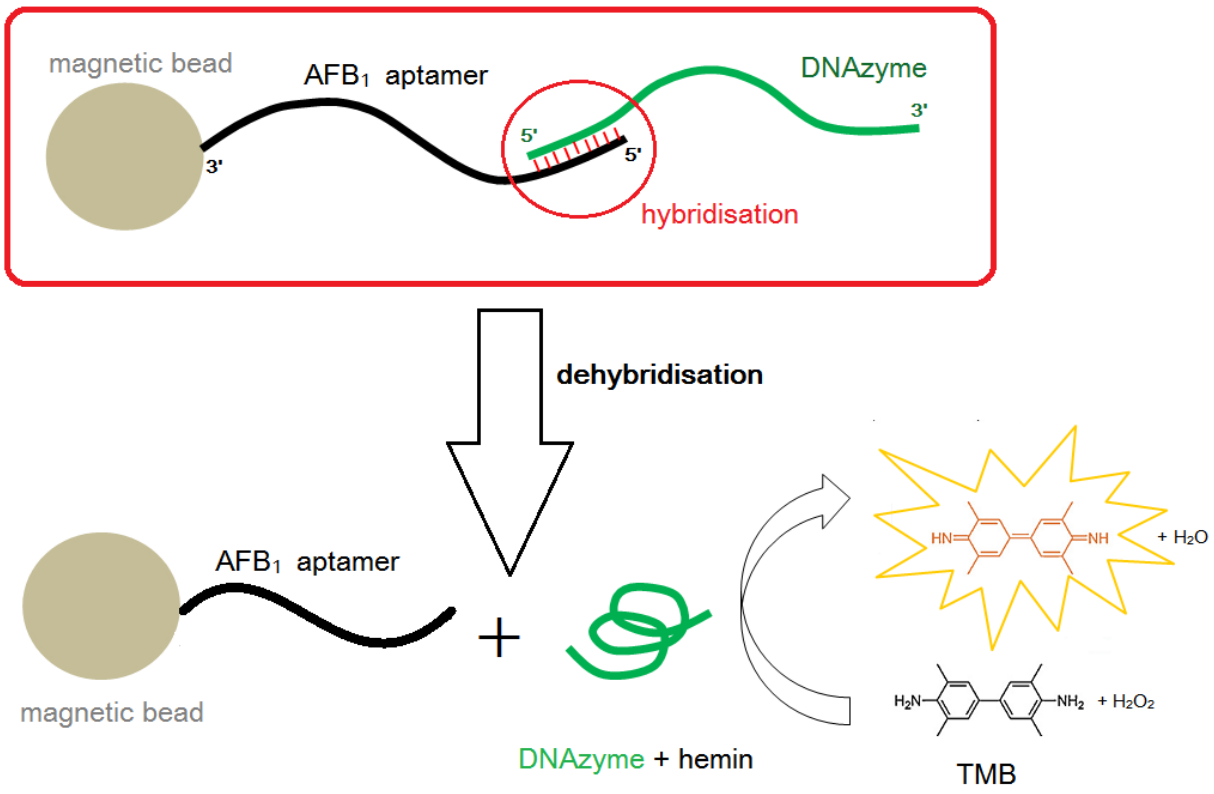

Figure 4.2.1: Schematic illustration of the dehybridisation experiments.

4.2.1 Effect of washing buffer for the beads with immobilised hybrid on the hybridisation between the aptamer and the DNAzyme

The experimental parameters for these experiments can be seen in Table 4.2.1. After incubation of the magnetic beads with the hybrid, the beads were washed with different washing buffers. Sample "a" and the respective negative control was washed three times with hybridisation buffer, whereas sample "b" and the respective negative control was washed three times with reaction buffer.

Table 4.2.1: Experimental parameters for testing different washing buffers for beads with immobilised hybrid.

	Samples				
Reagent	**a**	**NC(a)**	**b**	**NC(b)**	**V [μl]**
BioMag® Streptavidin beads					10
hybrid			10 μM		20
washing buffer	hybridisation buffer		reaction buffer		3x100
reaction buffer					42
heat treatment	5 min, 85 °C	no	5 min, 85 °C	no	
colorimetric assay:					
hemin			10 μM		8
substrate solution	7 mM TMB, 21 mM H_2O_2, 300 mM citric acid, 1 mM potassium sorbate				20
H_2SO_4			5 M		5

The dehybridisation was achieved by heating the samples for 5 minutes at 85 °C, whereas the respective negative controls remained unheated. After quick magnetic separation of the beads after heating, the H_2O_2-mediated oxidation of TMB was performed. The catalytic reaction was stopped after 30 minutes by addition of sulphuric acid. Figure 4.2.2 shows the visible results of these experiments. The results of the absorbance measurements are given in Table 4.2.2.

Table 4.2.2: Absorbance of samples with different washing buffers used for beads with immobilised hybrid 5 minutes after stopping the catalytic reaction by addition of acid.

Absorbance		
a	**b**	**time**
0.039	ND	5 min

ND = not detectable

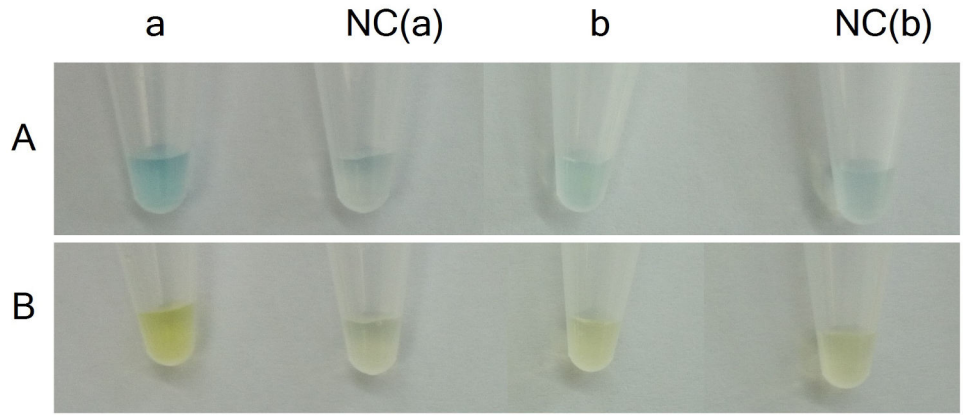

Figure 4.2.2: Different washing buffers used for beads with immobilised hybrid. Photographs of H_2O_2-mediated oxidation of TMB of samples with different washing buffers used for the beads with immobilised hybrid (a and b, Tab. 4.2.1) after (A) 30- minutes incubation and (B) stopping the reaction after 30 minutes by addition of H_2SO_4. Respective negative controls [NC(a) and NC(b)] are shown next to the samples.

The obtained results lead to the findings, that the immobilisation between bead and hybrid or more likely the hybridisation of aptamer and DNAzyme seem to dissolve if the beads with the immobilised hybrid are washed with reaction buffer. The salt concentration of the reaction buffer seems to be too low to maintain the hybridisation. Therefore the DNAzymes are washed out when washing the beads with reaction buffer. As a result, there are no DNAzymes left in the supernatant after dehybridisation at 85 °C for the H_2O_2-mediated oxidation of TMB, simply because there where certainly no DNAzymes bound any more even before the dehybridisation step. When using the hybridisation buffer instead of the reaction buffer for washing the beads with immobilised hybrid, there are still enough DNAzymes left in the supernatant after dehybridisation at 85 °C to give a coloured product when performing the H_2O_2-mediated oxidation of TMB (see (A,a) and (B,a) in Fig. 4.2.2). Also, the measurements of the absorbance of the samples (Tab. 4.2.2) are still possible. Therefore, **hybridisation buffer** was used as washing buffer for the beads with the immobilised hybrid for subsequent experiments.

4.2.2 Effect of the hybrid concentration on the intensity of the absorbance signal

In order to test the binding capacity of the BioMag® Streptavidin beads, different concentrations of the hybrid were used to be immobilised onto the magnetic beads. Aim of these experiments was to test whether a more intensive absorbance signal could be achieved by using higher concentrations of the hybrid. Table 4.2.3 shows the experimental parameters for these

experiments. The respective negative controls remained unheated at room temperature. The H_2O_2-mediated oxidation of TMB was stopped after 30 minutes by addition of sulphuric acid.

Table 4.2.3: Experimental parameters for testing different concentrations of hybrid.

	Samples			
Reagent	**a**	**b**	**c**	**V [μl]**
BioMag® Streptavidin beads				10
hybrid	10 μM	20 μM	40 μM	20
washing buffer	hybridisation buffer			3x100
reaction buffer				42
heat treatment	5 min, 85 °C			
colorimetric assay:				
hemin	10 μM			8
substrate solution	see Tab. 4.2.1			20
H_2SO_4	5 M			5

Figure 4.2.3 shows photographs of the samples before and after stopping the catalytic reaction with sulphuric acid. The absorbance of the samples was measured five minutes after addition of the acid. The results of the absorbance measurements are given in Table 4.2.4. As shown in Tab. 4.2.4, no higher absorbance could be achieved by using a higher concentration of hybrid for immobilisation onto the magnetic beads (the fourfold concentration of hybrid was used in sample "c" than in sample "a" to be immobilised onto the beads). These findings lead to the assumption that the maximum binding capacity of the beads is already defined at a hybrid concentration of 10 μM.

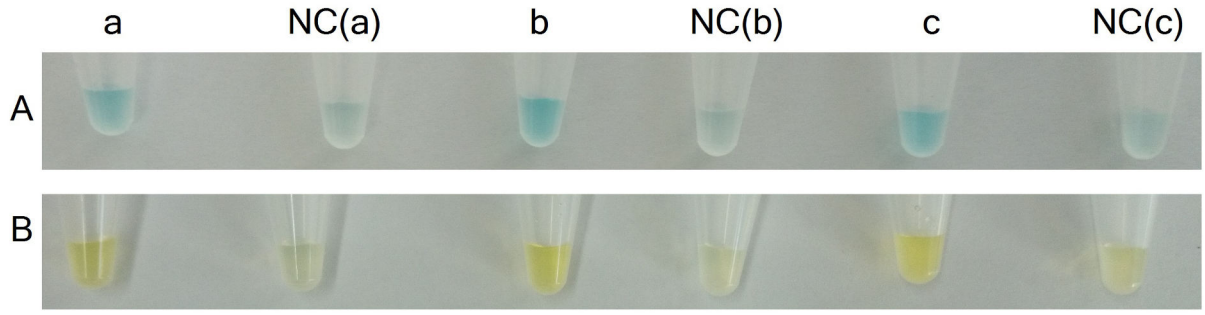

Figure 4.2.3: Different concentrations of hybrid used for immobilisation onto the beads. Photographs of H_2O_2-mediated oxidation of TMB of samples with different concentrations of immobilised hybrid [(a) 10 μM, (b) 20 μM and (c) 40 μM] after (A) 30- minutes incubation and (B) stopping the reaction after 30 minutes by addition of H_2SO_4. Respective negative controls [NC(a), NC(b) and NC(c)] are shown next to the samples.

Table 4.2.4: Absorbance of samples with different concentrations of immobilised hybrid 5 minutes after stopping the catalytic reaction by addition of acid.

Absorbance			
a	**b**	**c**	**time**
0.041	0.039	0.038	5 min

4.2.3 Effect of acetonitrile and the temperature on the dehybridisation

In these experiments, the optimal temperature for the dehybridisation between the aptamer and the DNAzyme was identified while simultaneously the optimal acetonitrile concentration in the reaction buffer was checking. These experiments serve to gain information about the strength of the hybridisation between aptamer and DNAzyme. The maximal temperature at which the base pairing between the aptamer and the DNAzyme is maintained was to be found out in order to determine the temperature at which the detection reaction of aflatoxin B_1 is to be performed at a later stage of the research. The temperature at which the hybridisation between the aptamer and the DNAzyme is just maintained would be a good working temperature for the mycotoxin detection. While adding the target molecule aflatoxin B_1, the dehybridisation will happen easily because of the formation of the aptamer/target-complex and also because of the temperature which already favours a dehybridisation.

Table 4.2.5: Experimental parameters for testing different temperatures for dehybridisation and different percentages of acetonitrile in reaction buffer.

	Samples					
Reagent	**a**	**b**	**c**	**d**	**e**	**V [μl]**
BioMag® Streptavidin beads						10
hybrid			10 μM			20
washing buffer			hybridisation buffer			3x100
acetonitrile in reaction buffer	0%	5%	10%	20%	30%	42
heat treatment	50 °C, 60 °C, 70 °C & 80 °C					
colorimetric assay:						
hemin			10 μM			8
substrate solution			see Tab. 4.2.1			20
H_2SO_4			5 M			5

For this purpose, four different temperatures for the dehybridisation step were tested with samples with five different percentages of acetonitrile in the reaction buffer. Table 4.2.5 shows

the experimental parameters for these experiments. The heat treatment for the dehybridisation lasted five minutes. The respective negative controls to the samples (a: 0%, b: 5%, c: 10%, d: 20%, e: 30% acetonitrile, see Tab. 4.2.5) remained unheated and served as blank in the absorbance measurements. The H_2O_2-mediated oxidation of TMB was stopped after 30 minutes by addition of sulphuric acid.

The absorbance of the samples was measured five minutes after addition of acid. The results of the absorbance measurements can be seen in Figure 4.2.4. The measurements reveal some kind of pattern: 10% of acetonitrile in the reaction buffer seem to be a critical percentage above which there is hardly any detectable absorbance. Interestingly, as described in section 4.1.6, acetonitrile does not disturb the catalytic reaction of the DNAzyme. It even seems to enhance the formation of a coloured product, inasmuch the more acetonitrile was used in the reaction solution, the more intensive coloured products were formed (4.1.6).

	0%	5%	10%	20%	30%
50°C	ND	ND	0,030	ND	ND
60°C	0,007	0,031	0,030	0,006	0,005
70°C	0,014	0,039	0,039	0,014	0,004
80°C	0,037	0,047	0,053	0,038	ND

Figure 4.2.4: Absorbance of samples with different concentrations of acetonitrile in reaction buffer (0-30%) and different dehybridisation temperatures (50-80 °C)

One possible explanation for 10% of acetonitrile in the reaction buffer being a critical amount in the dehybridisation experiments could be the fact that acetonitrile has a lower boiling point (82 °C) compared to water. When performing the heat treatment in order to dehybridise aptamer and DNAzyme, acetonitrile vaporises earlier than water and when used at higher percentages in reaction buffer ($\geq 10\%$), an (even visible) effect of condensation on the lids of the micro tubes could be noticed. This circumstance makes it impossible to compare the results of the absorbance measurements at a higher concentration of acetonitrile than 10%. Therefore, a **percentage of 10% of acetonitrile** in the reaction buffer was used for all subsequent experiments.

Another outcome of these experiments is the circumstance, that temperatures below 80 °C are too low to perform the dehybridisation between aptamer and DNAzyme 7 (Fig. 4.2.4).

4.2.4 Effect of truncated DNAzymes exhibiting shorter double stranded regions

In the following experiments we wanted to gain some more information about the strength of the hybridisation between aptamer and DNAzyme. Also, we wanted to see whether it is possible to conduct the dehybridisation step at a lower temperature. For this purpose, the 5'-end of DNAzyme 7 was truncated by the removal of one and two bases ("DNAzyme 7-1" and "DNAzyme 7-2" in Tab. 3.1.1). The experimental parameters for these experiments are given in Table 4.2.6. Due to shortening of the sequence of DNAzyme 7 where the hybridisation with the aptamer takes place, the strength of the hybridisation is weakened and therefore lower temperatures are supposed to be needed to achieve dehybridisation. Temperatures ranging from 30 °C to 80 °C for the dehybridisation step were tested and the heat treatment lasted 5 minutes. Respective negative controls remained unheated. Furthermore, to avoid too early dehybridisation between aptamer and the shortened DNAzyme or rather to ensure that hybridisation occurs in the first place, higher salt concentrations in the hybridisation buffer and lower temperatures for incubation of the hybrid with the beads were tested (Tab. 4.2.6).

Table 4.2.6: Experimental parameters for testing different shortenings of DNAzyme 7.

Reagent	**DNAzyme 7-1**			**DNAzyme 7-2**		**V [μl]**
	a	**b**	**c**	**d**	**e**	
BioMag® Streptavidin beads						10
hybrid			10 μM			20
hybridisation buffer:						
sodium chloride	750 mM		1 M	750 mM	1.25 M	
sodium citrate	75 mM		100 mM	75 mM	125 mM	
incubation with beads	37 °C	25 °C	20 °C	37 °C	20 °C	
washing buffer			hybridisation buffer			3x100
acetonitrile in reaction buffer			10%			42
heat treatment			30, 40, 50, 60, 70 & 80 °C			
colorimetric assay:						
hemin			10 μM			8
substrate solution			see Tab. 4.2.1			20
H_2SO_4			5 M			5

The H_2O_2-mediated oxidation of TMB was incubated for one hour and stopped by addition of sulphuric acid. The absorbance of the samples was measured five minutes after stopping the catalytic reaction and the results of the absorbance measurements can be seen in Table 4.2.7. With exception of sample "c" ("DNAzyme -1" with a hybridisation buffer containing 1 M sodium chloride and 100 mM sodium citrate, incubation of the hybrid with the beads at 20 °C and a temperature of 60 °C for dehybridisation, Tab. 4.2.6), it was not possible to

measure the absorbance of any other samples. Also, the obtained absorbance of sample "c" cannot compete with the results of the original DNAzyme 7 obtained at 80 °C (compare "c" in Tab. 4.2.7 with "c" at 80 °C heat treatment in Tab. **??**). Therefore, the truncations of the regarding DNAzyme in the dehybridisation experiments were not as promising as assumed.

Table 4.2.7: Absorbance measurement of samples with shortened DNAzyme 5 minutes after stopping the catalytic reaction by addition of acid.

	Absorbance				
heat treatment	**a**	**b**	**c**	**d**	**e**
30 °C	ND	ND	ND	ND	ND
40 °C	ND	ND	ND	ND	ND
50 °C	ND	ND	ND	ND	ND
60 °C	ND	ND	**0.031**	ND	ND
70 °C	ND	ND	ND	ND	ND
80 °C	ND	ND	ND	ND	ND

ND = not detectable

4.2.5 Effect of mismatches between the aptamer and the DNAzyme on the stability of the hybridisation

Besides truncations of the DNAzyme, also sequence changes of DNAzyme 7 which would lead to mismatches in the area of hybridisation were tested ("DNAzyme MM1", "DNAzyme MM2" and "DNAzyme MM3" in Table 3.1.1). The intention was that these mismatches should weaken the hybridisation of aptamer and DNAzyme and therefore make a dehybridisation at lower temperatures possible. Table 4.2.8 shows the experimental parameters for these experiments. Due to the resulting mismatches in the area of hybridisation, the salt concentration in the hybridisation buffer was increased in order to ensure hybridisation between aptamer and DNAzymes in the first place. The heat treatment in order to dehybridise the aptamer and the regarding DNAzyme was performed in five minutes, whereas the respective negative controls remained unheated. The H_2O_2-mediated oxidation of TMB was incubated for one hour and stopped by addition of sulphuric acid. The absorbance of the samples was measured five minutes after addition of acid. The results of the absorbance measurements are given in Table 4.2.9. Unfortunately, with exception of sample "c" ("DNAzyme MM3" and a temperature of 40 °C for dehybridisation), it was not possible to measure the absorbance of any the other samples.

Table 4.2.8: Experimental parameters for testing different alterations in the DNA sequence of DNAzyme 7.

Reagent	**DNAzyme MM1 a**	**DNAzyme MM2 b**	**DNAzyme MM3 c**	**V [μl]**
BioMag® Streptavidin beads				10
hybrid		10 μM		20
hybridisation buffer:				
sodium chloride		1 M		
sodium citrate		100 mM		
incubation with beads		20 °C		
washing buffer		hybridisation buffer		3x100
acetonitrile in reaction buffer		10%		42
heat treatment		30, 40, 50, 60, 70 & 80 °C		
colorimetric assay:				
hemin		10 μM		8
substrate solution		see Tab. 4.2.1		20
H_2SO_4		5 M		5

Even though it is a success, that with sample "c" the dehybridisation is possible at only 40 °C, the measured absorbance (0.025, Tab. 4.2.9) is very low and significantly lower the measured absorbance of the sample with the unmodified DNAzyme 7 at a dehybridisation temperature of 80 °C (0.053, Fig. 4.2.4). Therefore, the introduced mismatches in the hybridisation area of the aptamer and the DNAzyme did not lead to the same results compared to the dehybridisation experiments with the unchanged DNAzyme 7.

Table 4.2.9: Absorbance of samples with different alterations in the DNA sequence of DNAzyme 7 five minutes after stopping the catalytic reaction by addition of acid.

	Absorbance		
heat treatment	**a**	**b**	**c**
30 °C	ND	ND	ND
40 °C	ND	ND	**0.025**
50 °C	ND	ND	ND
60 °C	ND	ND	ND
70 °C	ND	ND	ND
80 °C	ND	ND	ND

ND = not detectable

4.2.6 Final protocol of the dehybridisation experiment of the immobilised AFB_1 aptamer and the hybridised DNAzyme with subsequent colorimetric detection of the DNAzyme

1. 10 μl of BioMag® Streptavidin beads stock solution are added into a 1.5 ml micro tube and the stock buffer of the beads is discarded via magnetic separation.
2. 20 μl of hybrid stock solution (10 μM) is added to the beads.
3. The beads are incubated at 37 °C for one hour.
4. The beads are separated magnetically, the supernatant is discarded and the beads are washed three times with 100 μl hybridisation buffer.
5. 42 μl of reaction buffer containing 10% acetonitrile are added to the beads.
6. The suspension is heated five minutes at 80 °C.
7. The beads are separated magnetically and the supernatant is transferred quickly to a 1.5 ml micro tube.
8. The colorimetric detection of the DNAzyme is performed (see 4.1.7).

5 Conclusion

In this work, the DNAzyme-catalysed H_2O_2-mediated oxidation of TMB was carried out, consequently investigated and optimised. The HRP-mimicking DNAzyme can catalyse the oxidation of TMB by the use of H_2O_2 as oxidising agent to produce the two coloured products:

(i) a blue charge-transfer complex and

(ii) after addition of acid the yellow diimine.

Several experimental conditions have been optimised in this catalytic system. These optimisations can be useful for further sensor-developing purposes, e.g. in terms of AFB_1 detection (see section 2):

- Different DNAzyme molecules exhibit different catalytic activities. Among the DNAzymes investigated in this work, DNAzyme 7 (Tab. 3.1.1) displayed the highest catalytic activity.
- The use of higher concentrated substrate and stopping solution and simultaneously less reaction volume lead to higher absorbance values.
- Among the different concentrations of TMB, H_2O_2, hemin and DNAzyme tested in this work, the best results for sensor-developing purposes could be achieved when having a concentration ratio of H_2O_2 and TMB of $\sim$3 and hemin and DNAzyme of $\sim$8.
- Also, the substrate buffer plays a crucial role in this catalytic system. Among the different substrate buffers tested, the most consistent results in the absorbance measurements with the lowest error rates could be achieved by using a substrate buffer containing 300 mM citric acid and 1 mM potassium sorbate.
- We further found out, that acetonitrile is able to enhance the catalytic reaction leading to higher absorbance values.

Due to optimisation of the DNAzyme-catalysed oxidation of TMB in the presence of H_2O_2, we were able to use as low as 100 nM DNAzyme, which correspond to only 5 pmol in the given reaction volume of 50 μl. At the beginning of the optimisation process (see section 4.1.2), 5 μM DNAzyme (250 pmol in 50 μl) were needed to achieve comparable absorbance values (compare Absorbance in Tab. 4.1.4,b and Tab. 4.1.14,c). This corresponds to a **50-times increase in sensitivity** of the DNAzyme-catalysed H_2O_2-mediated oxidation of TMB compared to the beginning of the optimisation of this assay. Moreover, we found out that by addition of acetonitrile to the reaction solution, an even higher sensitivity could be achieved (compare Absorbance in Tab. 4.1.14,c and Tab.4.1.16,f).

Furthermore, this colorimetric system was used in a simple biosensor model design for visual recognition of the complementary sequence of the G-quadruplex DNA. We were able to prove that the AFB_1 aptamer, which contains a complementary sequence of the used DNAzyme, was successfully immobilised onto magnetic beads. The application of an aptamer and a hybridised DNAzyme was tested and optimised in several dehybridisation model experiments:

- We found out, that the beads with the immobilised hybrid have to be washed with hybridisation buffer instead of reaction buffer. In the latter case, the salt concentrations are too low to maintain the hybridisation between aptamer and DNAzyme.
- Among the different percentages of acetonitrile tested in this work, the upper limit for acetonitrile in reaction buffer is 10%.
- We were able to show that by using a shorter hybridisation area or introducing mismatches, the temperature which is needed to perform the dehybridisation of aptamer and DNAzyme can drastically be reduced.

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List of Abbreviations

AFB_1 Aflatoxin B_1

ELAA Enzyme-linked aptamer assay

ELISA Enzyme-linked immunosorbent assay

FLD Fluorescent detection

FNA Functional nucleic acid

HPLC High-performance liquid chromatography

HRP Horseradish peroxidase

LC-MS Liquid chromatography coupled with mass spectrometry

MS Mass spectrometry

PCR Polymerase Chain Reaction

RT-qPCR Real-Time-quantitative-PCR

SELEX Systematic Evolution of Ligand by Exponential Enrichment

TMB 3,3′,5,5′-Tetramethylbenzidine