Detection of Small Molecules Using Aptamers

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Abstract

Nucleic acid aptamers are versatile molecular recognition agents that bind to their targets with high affinity and selectivity. They can be selected by an *in vitro* procedure against a broad range of targets molecules including small molecules (molecular weight <1000 g/mol). Small molecules include toxins, antibiotics, molecular markers, drugs, and heavy metals ions. The detection of small molecules is important in different areas including public health, environmental monitoring, food safety, and antiterrorism. To meet the increasing demand for small molecule detection, methods are needed that are sensitive, reliable, rapid, cost effective and simple to use. In this context, aptamer-based detection platforms are becoming a promising alternative to conventional methods for small molecule detection.

Concerning signal generation, mass-dependent detection methods, sandwich assay format and single-site binding assay formats are not always suitable for small molecule detection. The structural flexibility of aptamers enables the development of unique aptamer-based sensing platforms because aptamers fold into a well-defined three-dimensional structure upon binding to their target molecules. This specific property of aptamers allows to develop target-Induced dissociation (TID) of complementary oligonucleotide and target-induced structure switching (TISS)-based assays.

In this PhD work, a novel aptamer-based assay (Apta-qPCR) was developed, which relies on TID for the detection of small molecules originating from biological, food and environmental samples. The Apta-qPCR assay was developed and optimized for the detection of ATP, ochratoxin A, and oxytetracycline. The assays are highly sensitive and selective for the target molecules. In addition, a rapid colorimetric assay was developed based on the TISS principle, which can detect ATP and ochratoxin A in 15 minutes.

Keywords: Aptamer, qPCR, magnetic bead, small molecule detection, Target-induced dissociation, Target-induced structure switching

Kurzfassung

Nukleinsäure-Aptamere sind vielseitige molekulare Biorezeptoren, die mit hoher Affinität und Selektivität an ihre Zielmoleküle binden. Durch ein In-vitro-Verfahren können sie gegen eine breite Palette von Zielmolekülen, einschließlich kleiner Moleküle (Molekulargewicht <1000 g/mol), selektiert werden. Zu den kleinen Molekülen gehören unter anderem Toxine, Antibiotika, molekulare Marker, Wirkstoffe und Schwermetallionen. Der Nachweis von kleinen Molekülen findet unter anderem in den Bereichen der öffentlichen Gesundheit, Umweltüberwachung, Lebensmittelsicherheit und Antiterrorismus Anwendung. Um der steigenden Nachfrage zum Nachweis verschiedener kleiner Moleküle gerecht zu werden, werden Methoden benötigt, die empfindlich, zuverlässig, schnell, kostengünstig und einfach zu bedienen sind. In diesem Zusammenhang entwickeln sich Aptamer-basierte Detektionsplattformen zu einer vielversprechenden Alternative zu herkömmlichen Methoden zur Detektion von kleinen Molekülen.

Für den Nachweis von kleinen Molekülen sind massenabhängige Nachweisverfahren, Sandwich-Assays und Single-Site Binding-Assays nicht immer geeignet. Bei der Signalerzeugung ermöglicht die strukturelle Flexibilität der Aptamere die Entwicklung einzigartiger Aptamer-basierter Sensorplattformen, da die Nukleinsäure-Aptamere sich bei der Bindung an ihre Zielmoleküle in eine spezifische dreidimensionale Struktur falten. Diese besondere Eigenschaft von Aptameren ermöglicht die Entwicklung von Nachweisverfahren, die die Aptamer-spezifischen Mechanismen einer Target-induzierten Dissoziation von komplementären Oligonukleotiden (TID) und die Target-induzierte Strukturänderung (Target-Induced Structure Switch, TISS) nutzen.

In dieser Doktorarbeit wurde ein neuartiger Assay, die Apta-qPCR, basierend auf dem TID Mechanismus für den Nachweis von kleinen Molekülen aus biologischen Proben, Lebensmittel- und Umweltproben, entwickelt. Der Apta-qPCR Assay wurde für den Nachweis von ATP, Ochratoxin A und Oxytetracyclin entwickelt und optimiert. Dabei konnte eine hohe Empfindlichkeit und Selektivität für die Zielmoleküle ermittelt werden. Außerdem konnte ein kolorimetrischer Schnelltest nach dem TISS-Prinzip entwickelt werden, der ATP und Ochratoxin A innerhalb von 15 Minuten nachweisen kann.

Schlüsselwörter: Aptamer, qPCR, Magnetpartikel, Detektion von kleinen Molekülen, Target-induzierten Dissoziation, Target-induzierte Strukturänderung

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List of abbreviations

3D	Three dimensional
ABs	Antibodies
ACV	Alternating current voltammetry
Apta-beads complex	Aptamer/complementary sequence/dT beads complex
ATP	Adenosine triphosphate
AuNP	Gold nanoparticles
C	Celcius
CE	Capillary electrophoresis
cOligo	Complementary oligonucleotide
Ct value	Cycle threshold value
CV	Cyclic voltammetry
Da	Dalton
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPV	Differential pulse voltammetry
dsDNA	Double-stranded DNA
dT	Deoxythymidine
dT-beads	Magnetic beads modified with $dT_{(25)}$
e.g.	For example
ECL	Electrogenerated chemiluminescence
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
FDA	Food and drug administration
GO-SELEX	Graphene oxide-SELEX
HIV	Human immunoglobulin virus
HPLC	High-performance liquid chromatography
i.e.	id est
IgG	Immunoglobulin G
ITC	Isothermal titration calorimetry
K_d	Dissociation constant
LAMP	Loop-mediated isothermal amplification

LC	Liquid chromatography
LLC	Limited liability company
LOD	Limit of detection
MBs	Micro- or nano-sized magnetic beads
MES	2-Morpholinoethanesulfonic acid
MS	Mass spectroscopy
MST	Microscale thermophoresis
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Oxytetracycline
PCR	Polymerase chain reaction
QCM	Quartz crystal microbalance
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT	Room temperature
SELEX	Systematic evolution of ligands by exponential enrichment
SPR	Surface plasmon resonance
ssDNA	Single-stranded DNA
SWV	Squarewave voltammetry
T-Jump	Temperature jump
TID	Target-Induced dissociation
TISS	Target-induced structure switching
TRIS	2-Amino-2-(hydroxymethyl)-1,3-propanediol
UV	Ultraviolet

1. Introduction

The need to detect small molecules (molecular weight <1000 g/mol [1, 2]) such as residual antibiotics or drugs, illegal drugs, environmental toxicants, chemical warfare agents, and heavy metals, is increasingly important in aspects that include public health, environmental monitoring, food safety, and antiterrorism [3]. It is also important to detect small molecules with rapid assays and in some cases, on-site detection is required, as small molecules are not stable during longer storage and their chemical structure can be changed in different storage conditions [4-6].

Traditionally, small molecules are detected with high-performance liquid chromatography (HPLC) in combination with UV (HPLC-UV) and/or fluorescence detector [7] or with the help of liquid chromatography in combination with mass spectroscopy (LC-MS) [8, 9]. These methods are time-consuming, laborious and not feasible for developing rapid assays [10]. Conventional binding ligands such as antibodies and enzymes offer a good alternative but they come with their own limitations. For example, there are not enough suitable enzymes available for the detection of small molecules [11]. In case of antibodies, there are several analytes, such as highly toxic and non-immunogenic targets, against which antibodies are difficult to be generated [12]. In addition, to develop antibodies against small molecules, they first need to be converted into haptens (small molecule-protein conjugates) and the developed antibodies are often only able to recognize the conjugate rather than the small molecule itself [13].

The relatively new technology of aptamers can address all these challenges related to the detection of small molecules [3, 10, 11]. The smaller size of the aptamers is highly beneficial for developing detection platform for small molecules [3, 11]. Aptamers are single-stranded (ss) DNA (deoxyribonucleic acid) or RNA (ribonucleic acid) molecules, which specifically bind to their target molecules with high affinity. They are selected by an *in vitro* selection process termed systematic evolution of ligands by exponential enrichment (SELEX). In recent years, aptamers have been developed against a broad range of target molecules, including metal ions, small molecules, organic dyes, peptides and proteins, and even whole cells and microorganisms [14-22].

The *in vitro* aptamer selection process enables aptamers to be developed in various binding conditions including nonphysiological salt concentrations, temperatures and pH [23]. Moreover, aptamers can be also developed against targets having no immunogenicity and high toxicity.

The robustness of the phosphodiester backbone provides improved stability of aptamers. Importantly, aptamers can be reversibly denatured by changing the surrounding conditions which is not possible with their protein counterparts (antibodies and enzymes) [24]. Specifically, aptamers offer a huge advantage for the detection of small molecules due to possibilities to develop assays that can use target-binding induced changes in the aptamer structure [10, 25].

One of the important concerns for developing a sensing platform is the transducer for signal production. For the detection of small molecules, mass-dependent detection platforms, such as surface plasmon resonance (SPR), quartz crystal microbalance (QCM), and cantilevers, are not suitable because it is difficult to get adequate signals using these platforms. A sandwich assay format which is widely used in immunoassay is often not suitable for the detection of small molecules because these molecules normally are intercalated deeply within the structure of the aptamer, leaving minute space for the interaction with a second aptamer [11]. The nucleic acid nature of aptamers and structural flexibility enables the development of unique aptamer-based sensing platforms. Nucleic acid aptamers fold into a well-defined three-dimensional structure upon binding to their target molecules. This phenomenon is known as target-induced structure switching (TISS) [26]. Another approach is to utilize the aptamers ability to hybridize with complementary sequences. This helix structure can be easily dissociated by competitive binding of the aptamers to their targets, which is known as target-induced dissociation (TID) of complementary oligonucleotides [10, 27, 28].

2. Objectives

The aim of this research project is to develop an assay, which combines the binding abilities of aptamers, their potential to detect small molecules via TID with their ability to be easily amplified via PCR. This should result in assays with outstanding sensitivities. Moreover, the assay should be easy to use and not comprise various protocols for separation and detection. Therefore magnetic beads will be used as a platform allowing for direct detection of analytes from complex samples. As a first model system, a well-characterized aptamer against ATP will be used. To demonstrate broad applicability of the developed assay, it will be transferred to other analytes by using corresponding aptamers. The goal is to develop a universal assay platform applicable to many different small molecules.

3. Theoretical background

Detection of small molecules is very challenging especially in the context of rapid assay formats and on-site testing [29]. Currently available methods are mainly limited to benchtop methods which require large instrumentation and trained personal for the measurements. Additionally, the overall process includes several steps such as washing, purification and finally detection. During processing, a large amount of analyte can be lost and it severely limits the overall analytical performance [25]. To overcome these challenges, an aptamer-based assay called Apta-qPCR was developed, in this work, where there is no need to process the samples to purify the analyte [27, 28].

3.1. Small molecules

Small molecules are low molecular weight compounds typically less than 1000 g/mol [1, 2]. Small molecules play key roles in many biological processes due to their ability to diffuse across cell membranes [30]. These targets may be harmful, such as toxins and carcinogens, or beneficial, such as drugs or nutrients. In cells, small molecules serve as cell signalling molecules, pigments, or as part of defence mechanisms. In molecular biology, they can be used as antibiotics or other important drugs. In the food industry, small molecules are important as food-supplements or can act as pesticides. Therefore, the analysis of small molecules is of vital importance in healthcare/medical applications and numerous other commercial applications such as in the field of agriculture and environmental analysis (Figure 3.1.). Detection of small molecules is a challenge due to their smaller size and limited availability of functional groups [2, 29]. In addition, when the small molecules need to be analysed in biological samples, additional sample treatment steps may be required, such as separation and isolation, which can severely compromise the analytical performance [10, 25].

3.1.1. Detection of small molecules

Traditionally, small molecules are detected with HPLC in combination with UV and/or fluorescence detector [7]. Another popular method for the detection is liquid chromatography in combination with mass spectrometry (LC-MS) [8, 9]. In these methods, complex samples have to be subjected to sample pre-treatment to be suitable for HPLC columns. These steps are vital for the protocol and are time-consuming (takes approximately two-thirds of the total process) [31]. To detect small molecules with these methods, the samples need to be collected and sent to the laboratories. The overall process for the detection is laborious, time-consuming

and, importantly, in some cases not feasible as many small molecules are not stable during longer storage [1, 4, 25].

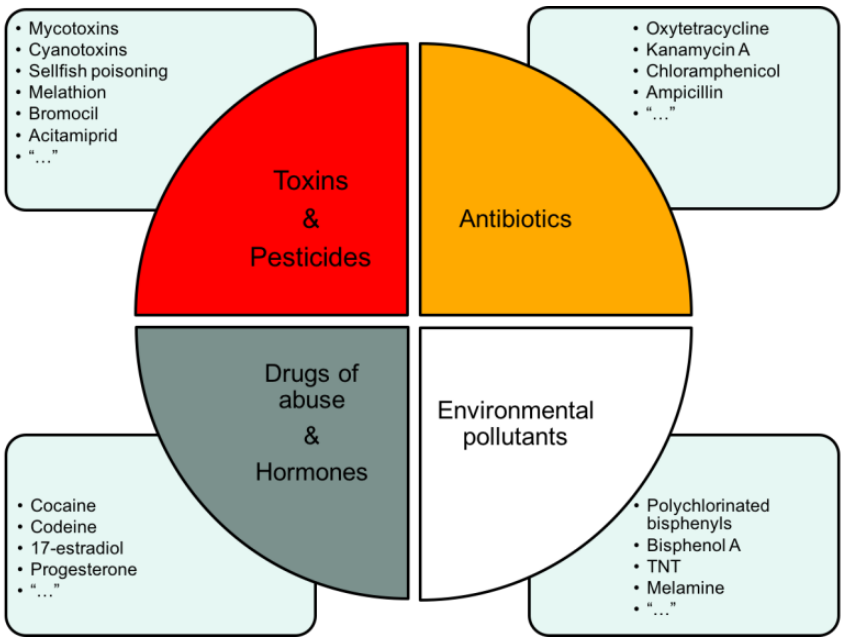

Figure 3.1. Important small molecule targets.

Due to this, there is need to develop new assays which enables to detect small molecules rapidly and, ideally, in some cases allow for on-site detection. Development of protein-based ligands such as antibodies and enzymes have been helpful in this field in past years but these ligands have some specific limitations. For example, for many small molecules enzymes are not available [11]. In case antibodies, usually haptens (small molecule-protein conjugates) are used to develop antibodies against small molecules. The protein used to prepare haptens, are chosen specifically for their ability to elicit an immune response and often antibodies recognise the conjugate, rather than the target molecule [13]. Moreover, proteins like antibodies or enzymes are sensitive to degradation, which limits the shelf-life and the use of the binding ligands under non-physiological conditions [32].

While antibodies have long been considered to be the standard in molecular recognition, the relatively new technology of aptamers offers several advantages, and addresses all the challenges associated with the detection of small molecules [3, 29]. Aptamers can form "cages" around small molecules (Figure 3.2.B) [33] and they provide opportunity to develop non-traditional assays allowing rapid detection and excellent signal-to-noise ratios [25-28].

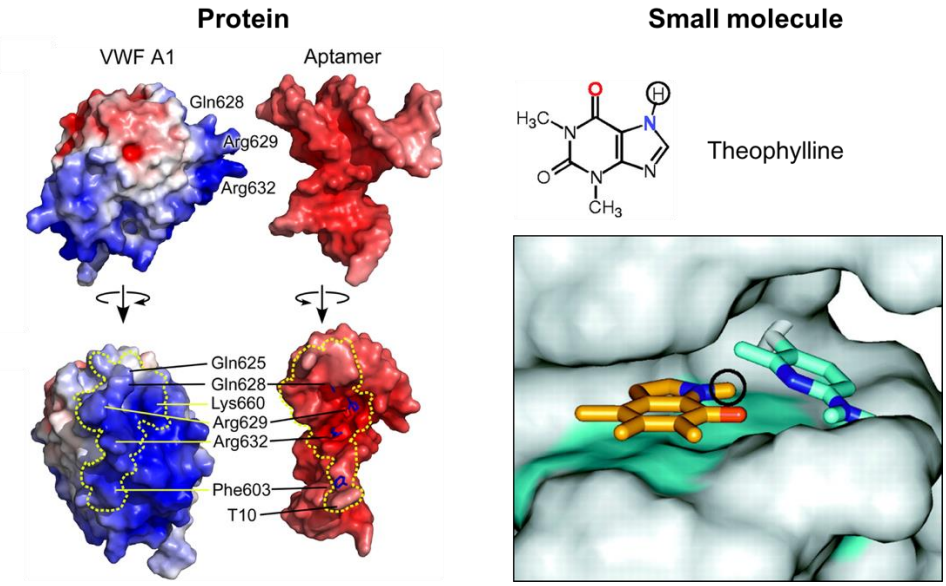

Figure 3.2. Comparison of aptamer interaction with protein (on left, Von Willebrand factor [34]) and small molecules (on right, theophylline [33]). Reproduced with permission from [33, 34]. Copyrights 2009, Elsevier; 2000, Science.

3.2. Aptamers

Historically, nucleic acids were associated with the storage of genetic information and have been thought to be less complex because of less chemical diversity than proteins. However, like proteins, nucleic acids can fold into intricate three-dimensional (3D) structures that are able to accomplish a variety of functions including gene-regulation, catalytic activity and ligand-binding. After the discovery of catalytic and binding properties of non-coding ribonucleic acids (RNAs), the field of these so-called "non-functional" nucleic acids have attracted a lot of attention. In 1990, two research groups independently developed an *in vitro* selection process, Systematic Evolution of Ligands by EXponential enrichment (SELEX), for isolating RNA sequences that could bind specifically to given target molecules [16, 35]. These RNA oligonucleotides sequences were then termed aptamers, derived from the Latin *aptus*, meaning "to fit" [16]. Consequently, DNA aptamers have been also developed, and they offer a big advantage in terms of its stability [36].

Aptamers are also termed as "chemical antibodies" because of their artificial selection process, and possibility to synthesize them chemically [37]. Aptamers are able to address specific shortcomings of antibodies (Table 1). The production of antibodies relies on induction of animal immune system and it can be difficult to develop antibodies when the target molecule is highly toxic or non-immunogenic [10, 16, 36]. The *in vitro* selection process enables aptamers to be developed virtually against all possible targets [23, 38]. After nearly 25 years of research in this

field, aptamers are available against a broad range of targets including metal ions (e.g., K^+ [39], Co^{2+} [40], Cd^{2+} [41], Hg^{2+} [42], Pb^{2+} [43] and Zn^{2+} [18]), small molecules (e.g., amino acids [44], ATP [17], antibiotics [19], food toxins [45], narcotic substances [46], organic dyes [16], and vitamins [47]), peptides [22] and proteins (e.g., thrombin [20], antibodies [48], cytokines [49], cell receptors [15], enzymes [35], and HIV-associated peptides [21]), viruses (e.g., human norovirus [50], vesicular stomatitis virus [51]) and even whole cells [52] and microorganisms (e.g., cancer cells [53], bacteria [14]). Importantly, the availability of such a large pool of aptamers makes it possible to develop novel bioassay tools covering areas that include diagnostics, anti-bioterrorism, and environmental and food analysis [37].

Being oligonucleotides, aptamers can fold in a stable 3D-structure when they come in contact with their target molecules. As example, they can incorporate small molecules into their nucleic acid structure or they interact with macromolecules such as proteins via complementarity of the surfaces of folded aptamer and target (Figure 3.2.A) [33, 34]. These properties of aptamers have made them very attractive tools for diagnostics and therapeutics (Table 3.1.). In particular, aptamer-based assays offer unprecedented advantages due to their smaller size when it comes to detection of small molecules (Figure 3.3.). It is also possible to develop non-traditional assays, which allow to develop rapid assays and to automate the whole detection process in microfluidic devices [54].

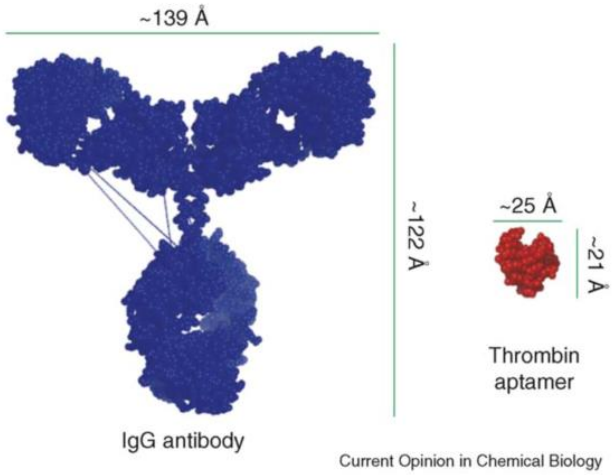

Figure 3.3. Size comparison of antibodies and aptamers. Here, Human IgG antibody is shown on the left and thrombin aptamer is shown on the right. Reproduced with permission from [55]. Copyrights 2006, Elsevier.

Table 3.1. Comparison of aptamers and antibodies [10, 56]

Property	Aptamers	Antibodies
Development	*In vitro* selection process	Animals are required
Production	Chemical synthesis possible	Cell lines required
Size	5-20 kDa	150 kDa
Affinity	Low micromolar to picomolar	Low micromolar to picomolar
Stability	Stable at high temperature	Irreversible denaturation at high temperature
Shelf life	Long-term storage at room temperature (RT)	Limited, required to be stored -20° C for longer storage
Modification	Easy chemical modification and possible to do site-specific modification	Comparatively difficult chemical modification, site-specific modification not possible
Kinetic parametrs	On/off rate can be changed on demand	Cannot be changed
Quality control	No batch-to-batch variation	Possibility to get batch-to-batch variation

3.2.1. Selection of aptamers

The concept of *in vitro* evolution of nucleic acids-based binding ligands was first reported in the 1967 where specific RNA molecules were isolated that can serve as an activator for replicases (RNA-dependent RNA polymerases), which is able to synthesise a complete virus particle [57]. Later, the sequences were also evolved having specific traits such as resistance to ethidium bromide [58]. The potential of *in vitro* evolution was not realized until the modern biotechnological advances such as the development of polymerase chain reaction (PCR), the invention of reverse transcriptase and the possibility to generate oligonucleotides using solid-phase synthesis. Equipped with these modern techniques, two separate groups independently reported *in vitro* selection and evolution of functional nucleic acids in 1990 [16, 35]. Tuerk and Gold [35] used the term SELEX, for the first time, for the process of selecting RNA ligands

against T4 DNA polymerase and Ellington and Szostak [16] introduced the term aptamers for RNA ligands selected against various organic dyes.

Since its invention, several researchers have used SELEX to develop nucleotide aptamers with a wide variety of functions. While SELEX itself has been evolved for specific purposes, the general scheme of SELEX remains the same [16, 23, 38]. Typically, SELEX starts with an initial library of random nucleic acid sequences (RNA/DNA depending on the final requirements). SELEX libraries preferably consist of 25–80 random nucleotides flanked by primer-binding sites necessary for PCR amplification. The library is then incubated with the target molecule immobilised on a solid-support matrix and several washing steps are employed to eliminate non-functional/unbound sequences. Later, the target-bound sequences are eluted and amplified using PCR (reverse transcription PCR for RNA aptamers) to amplify the library for subsequent, more stringent, selection rounds. Elution can be performed using heat, high concentrations of the target molecule, or chaotropic agents (such as urea) as the interactions between nucleotide sequences and the target are noncovalent in nature. As PCR amplification produces double-stranded DNA and aptamers are single-stranded, the DNA aptamer sequence is detached from its complementary strand using different techniques, such as streptavidin-coated magnetic beads. Single-stranded RNA aptamers are produced using *in vitro* transcription from the double-stranded DNA PCR products, for the further SELEX rounds [24].

The progress in SELEX process can be monitored by modifying the aptamer strand with a traceable label, which is very helpful to determine when more stringent conditions should be applied [59]. The enriched library after several cycles can be additionally subjected to counter selection using molecules with high molecular similarity to the target to remove the nucleotide sequences which are not target-specific. After several rounds (normally 8-12), the enriched library is cloned, sequenced and characterized to isolate aptamers with the desired properties. Once these sequences are elucidated, solid-phase chemical synthesis is used to reproducibly synthesize aptamers in large quantities [29].

3.2.1.1. Aptamer development for small molecules

Following, the success of the first *in vitro* selection experiments to small organic dyes [16], much of the original SELEX focus was on developing aptamers for small molecules. However, once it was found that aptamers could be easily selected for proteins and cells, new aptamers for small molecules became less prevalent. Macromolecules contain more functional groups and structural motifs, and provide a higher probability to develop aptamers that can interact with

the target via hydrogen bonds, electrostatic interactions, and hydrophobic interactions [23]. As can be noted from Figure 3.4., nearly a quarter of existing aptamers have been generated for small molecule targets [60].

Challenges associated with the aptamer selection against small molecules

Selection of aptamers against small molecules is accompanied by specific problems that do not occur during selections using proteins and other macromolecules. First of all, small molecules have limited functional groups that can interact with aptamers, and the need to immobilize small molecules on a solid support-matrix further reduces the probability of selecting aptamers. It is also important to consider here that the immobilisation of small molecules may generate a novel epitope that is required for aptamer interaction, which means the selected aptamer may not interact with the target in solution. So, the immobilisation of small molecules not only reduces the probability of selection of aptamers, but it can affect the aptamer performance during its application [61, 62]. This problem becomes critical when it is required to distinguish closely related molecules which differ only in one functional group especially when this group is used for immobilization of the small molecule [63].

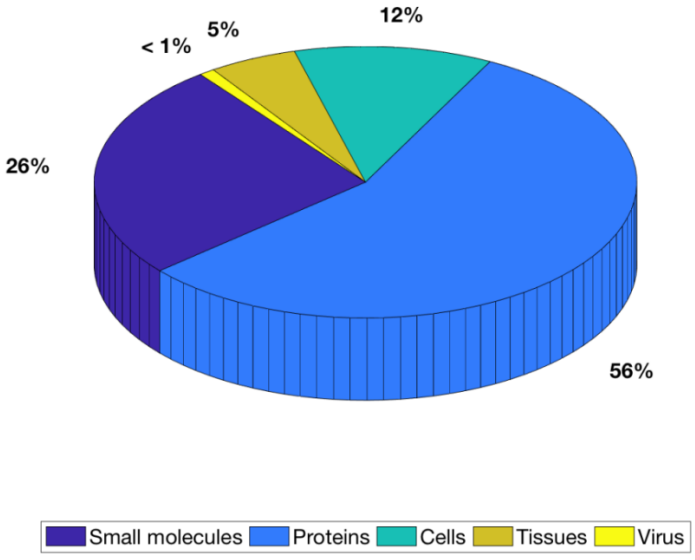

Figure 3.4. Comparison of currently existing aptamer targets. The list was obtained from an Aptamer database from Aptagen, LLC (accessed on 14/06/18) [60].

To enable aptamers to detect the targets in solution, affinity elution can provide the solution where a high target concentration is used to elute the aptamers during selection process [64]. In some cases, the solubility of the target is the limiting factor and in these cases the binding to the non-immobilized target can be inefficient.

For protein targets, the success rate of SELEX has been immensely increased using nucleobase-modified nucleic acid libraries during SELEX process [59]. As limited available functional groups are often a problem for aptamer selection, modifying nucleic acids according to the requirement of binding can provide better outcome for selecting aptamers against small molecules.

Target immobilisation-free selection process for small molecules

Whether the target is a small molecule, a macromolecule or a large cell, the important step in the selection process is the affinity separation of target-bound and target-unbound sequences [3]. When the target is large, e.g. a cell, the target-bound sequences could be separated from unbound sequences by centrifugation, which is simple and effective [14]. In case of macromolecules, such as proteins, it is easy to attach them on a solid support. Macromolecules have many functional groups and also motifs where aptamers can easily interact [65]. However, when it comes to small molecules, to attach them on a solid support can be a problem as mentioned earlier. To overcome this problem, target immobilization-free SELEX protocols have emerged, such as GO-SELEX (graphene oxide-SELEX) [66], multi-GO-SELEX [67], and capture-SELEX [68, 69] (Figure 3.5., Table 3.2.).

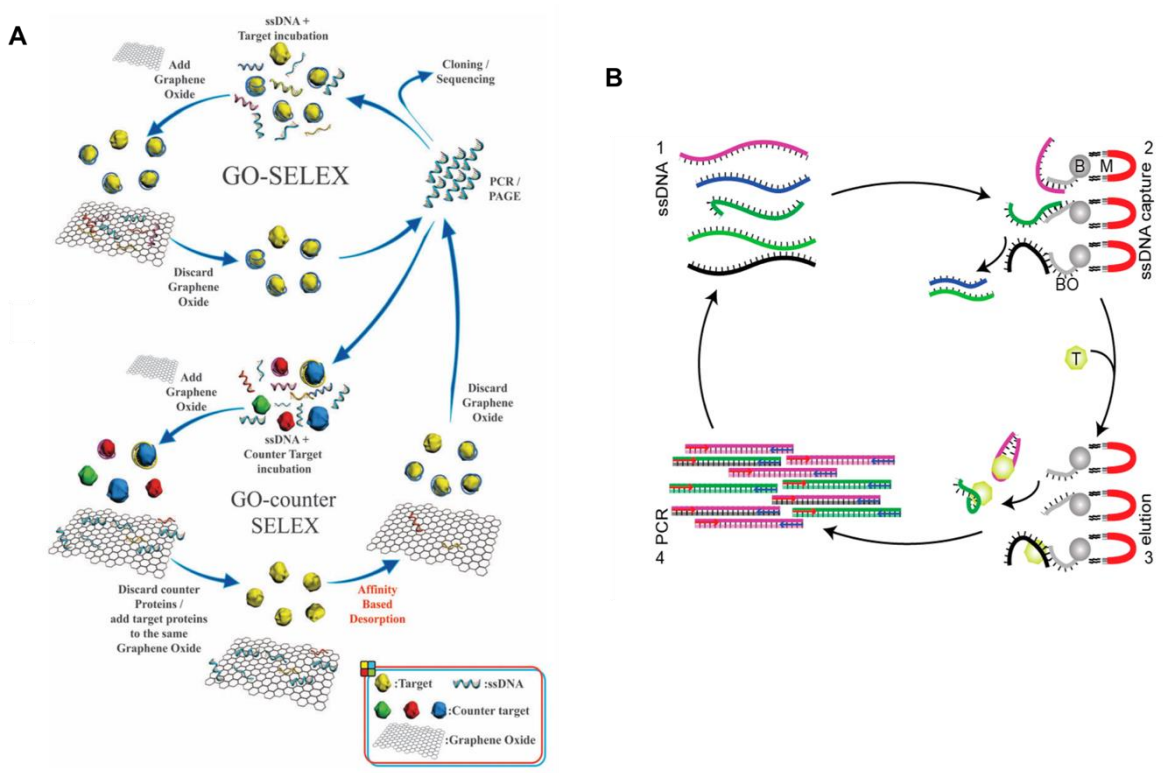

Figure 3.5. (A) Scheme of graphene oxide-based SELEX (GO-SELEX), Reproduced with permission from Royal Society of Chemistry [66]. **(B)** Scheme of capture SELEX. Reproduced with permission from American Chemical Society [69].

In case of GO-SELEX, the initial library containing ssDNA can be adsorbed on graphene oxide (GO) via π–π stacking. Addition of the target causes the release of nucleotide sequences which can interact with the target molecules. Here, the sequences are released because they possess higher affinity for the target molecules in comparison to binding to GO via π–π stacking [66]. In capture-SELEX, the initial ssDNA library is immobilised onto magnetic beads. Here, the ssDNA library is designed to hybridize to a complementary 5′-biotinylated DNA on the streptavidin-coated magnetic beads. Addition of the target causes the release of target-binding ssDNA from the complementary sequences due to their structure-switching. Therefore, target-unbound sequences remained on the beads and can be removed easily [69].

Table 3.2. Aptamers selected with GO-SELEX and Capture SELEX

Method of selection	Target moelcule	Ref.
GO-SELEX	T-2 toxin	[70]
	Patulin	[71]
	Tebuconazole	[67]
	Inabenfide	[67]
	Mefenacet	[67]
	Okdaic acid	[72]
	Gonyautoxin 1 and 4	[73]
	Benzylpenicillin	[74]
	Iprobenfos	[75]
	Edifenphos	[75]
	Ractopamine	[76]
Capture SELEX	Estradiol	[77]
	Organophosphorus pesticides	[78]
	Malachite Green	[79]
	Cd^{2+}	[80]
	Crystal violet	[81]
	Clenbuterol	[82]
	Melamine	[83]
	Tobramycin	[69]
	Acetamiprid	[84]

	ATP	[68]
	GTP	[68]
	Geniposide	[85]
	Aminoglycoside Antibiotics	[86]
	Bromocresol Purple	[87]

3.2.2. Characterization of aptamers

Major technological advances for *in vitro* aptamer selection have improved our ability to generate thousands of aptamer candidates. However, this comes with a challenge of high-throughput analysis to characterize and validate the developed aptamers for different applications [2]. Currently, aptamers are characterised with standard methods such as surface plasmon resonance (SPR) [88], isothermal calorimetry (ITC) [89], and capillary electrophoresis (CE) [90]. A new method was introduced in 2006 by Stephan Duhr et al. called Microscale thermophoresis (MST), which is capable of analyzing the interaction of binding ligands in solution [91].

MST utilises thermophoresis effect to determine equilibrium constants, such as dissociation constant (K_d value), associated with binding events between ligand and the target molecule. It measures the mobility of a fluorescently labeled molecule in a temperature field (Thermophoresis) and the sensitivity of fluorescence yield on temperature. This method is sufficiently sensitive to measure the interactions of small molecules to proteins. Importantly, it is easy to check the binding events in solution, allowing high flexibility in assay design and checking multiple cofactors that can influence the assay outcome [92].

3.2.3. Analytical applications of aptamers for the detection of small molecules

An important concern for an assay development is the transducer platform for signal production. For the detection of small molecules, mass-dependent detection platforms, such as SPR, quartz crystal microbalance (QCM), and cantilevers, are not suitable because it is difficult to get adequate signals based on the small mass changes induced by binding of small molecules using these platforms [11]. A sandwich assay format which is a widely used method in immunoassay is also not suitable for the detection of small molecules because these molecules are normally fit in the "cages" of the first aptamer, leaving minute space for the interaction with second aptamer [11, 32].

To address the challenges associated with signal generation, there is need to develop non-traditional assays to detect small molecules using aptamers. When aptamers interacts with their target molecules, they undergo significant structural changes, known as target-induced structure switching (TISS) (Figure 3.6.A) [10, 26]. These changes are enough to produce detectable signal [26]. Another assay-format, specific for aptamers is based on target-induced dissociation (TID) of complementary oligonucleotides. In case of TID, aptamers ability to hybridize with complementary sequences are used. Here, the helix structure formed between aptamer and its complementary sequence can be easily dissociated by competitive binding of the aptamer with its target (Figure 3.6.B) [10, 27, 28].

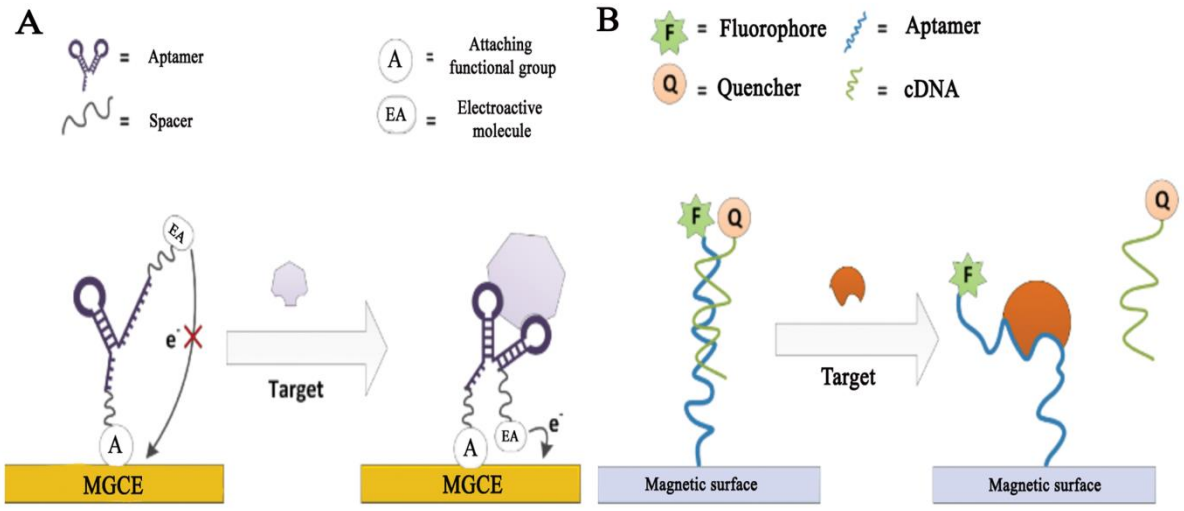

Figure 3.6. (A) Target-induced structure switching (TISS) type of assay. Here, the interaction between an aptamer and a target molecule leads to change in the conformation of the aptamer. The conformational changes can be utilised for signal generation, e.g., by using an electroactive molecule (EA) fused to the aptamer; **(B)** Target-induced dissociation (TID) type of assay. Here, the aptamer is hybridized with a complementary oligonucleotide (cDNA). The interaction between a target molecule and an aptamer leads to release of the cDNA sequence from the aptamer. The release of the cDNA can provide different types of signals in different assay formats, in the given example FRET-is used for signal generation. Adapted from [10] with permission. Copyright 2014, De Gruyter.

3.2.3.1 Aptamer-based assays using PCR for signal enhancement

Nucleotide nature of aptamers provides the advantage that they can be easily detected using PCR with high sensitivity [25]. This property of aptamer has been utilised in different types of applications such as loop-mediated isothermal amplification (LAMP) [93], rolling circle amplification [94], and isothermal signal amplification [95], proximity ligation assays [96], and nuclease protection assays [97].

3.3. Magnetic bead-based applications of aptamers

This section comprises the review article published as "Modh H, Scheper T, Walter JG. Aptamer-modified magnetic beads in biosensing. Sensors. 2018 Mar 30;18(4):1041." The article was reproduced with the permission of MDPI (publisher).

3.3.1. Summary

William Fullarton described the separation of iron material with a magnet in a patent in 1792, which paved the way for applications of magnetic fields in separation techniques [98]. Initially, magnetic properties of sediments were used for separation. Later, the magnetic beads synthesis process was improved and, in 1990s, Safariková et al. introduced coated magnetic beads for the extraction of organic compounds for the first time [99, 100]. Nowadays, novel analytical techniques and improvised-traditional methods have begun to incorporate micro- or nano-sized magnetic beads (MBs) [101]. The specific properties of MBs, such as colloidal stability of magnetic nanoparticles, homogenous size distribution, high and uniform magnetite content, a fast response to applied magnetic field, and presence of surface functional groups are essential for their analytical applications. Particularly, the superparamagnetic properties of MBs permit the easy isolation of analytes from complex matrices, such as biological and environmental samples, by attaching specific ligands on the surface of MBs and separating the MBs-bound analytes with the aid of an external magnetic field [98].

Currently, aptamers are emerging as favourable binding ligands due to number of advantages. Most importantly, the chemical synthesis of aptamers enables straightforward and controlled chemical modification with linker molecules and dyes [37]. Moreover, aptamers facilitate novel sensing strategies based on their oligonucleotide nature that cannot be realized with conventional protein-based ligands [10, 26-28]. Due to these benefits, the combination of aptamers and MBs was already used in various analytical applications which are summarized in the following review article.

Review article – Aptamer-Modified Magnetic Beads in Biosensing

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3.3.2. Abstract

Magnetic beads (MBs) are versatile tools for the purification, detection, and quantitative analysis of analytes from complex matrices. The superparamagnetic property of magnetic beads qualifies them for various analytical applications. To provide specificity, MBs can be decorated with ligands like aptamers, antibodies and peptides. In this context, aptamers are emerging as particular promising ligands due to a number of advantages. Most importantly, the chemical synthesis of aptamers enables straightforward and controlled chemical modification with linker molecules and dyes. Moreover, aptamers facilitate novel sensing strategies based on their oligonucleotide nature that cannot be realized with conventional peptide-based ligands. Due to these benefits, the combination of aptamers and MBs was already used in various analytical applications which are summarized in this article.

Keywords: aptamer; magnetic beads; analytical applications; electrochemical assays; optical assays; point-of-care-testing

3.3.3. Introduction

Aptamers are synthetic single-stranded (ss) DNA (deoxyribonucleic acid) or RNA (ribonucleic acid) molecules, which specifically bind to their target molecules with high affinity. They are selected by an iterative in vitro process termed systematic evolution of ligands by exponential enrichment (SELEX). In recent years, aptamers have been developed against a broad range of target molecules, including metal ions, small molecules, peptides, proteins, and even complex targets such as whole cells. Due to various advantages of aptamers, such as their animal free and cost effective production, high temperature stability, chemical stability, target versatility, and high affinity and selectivity for their targets, aptamers are appealing alternatives to antibodies (AB) for use in analytical applications [1].

The specific advantages offered by aptamers include the easy modification with functional groups resulting in the possibility to control the orientation of the aptamer after

immobilization. This controlled orientation facilitates high activity of immobilized aptamers, which is beneficial in their analytical applications [2]. In addition, aptamers can undergo considerable structural changes while interacting with target molecules. These changes have been extensively studied and exploited for the development of novel assays including target-induced structural switching (TISS) and target-induced dissociation (TID) of complementary oligonucleotides (Figure 1). These possibilities are specific for aptamers and allow for the design of sensing strategies even in cases, where conventional strategies, such as sandwich assays are not applicable. Moreover, aptamers can be regenerated and aptamer-modified sensors can be reused [3,4].

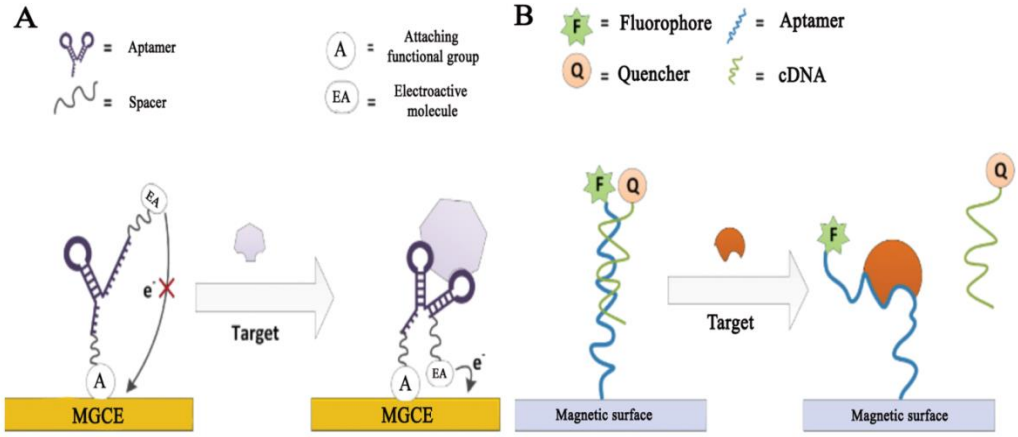

Figure 1. (**A**) Target-induced structure switching (TISS) type of assay. Here, the interaction between an aptamer and a target molecule leads to change in the conformation of the aptamer. The conformational changes can be exploited for signal generation, e.g., by using an electroactive molecule (EA) fused to the aptamer. In the figure, MGCE is a magnetic glass carbon electrode; (**B**) Target-induced dissociation (TID) type of assay. Here, the aptamer is hybridized with a complementary oligonucleotide (cDNA). The interaction between a target molecule and an aptamer leads to release of the cDNA sequence from the aptamer. The release of the cDNA can provide different types of signals in different assay formats, in the given example FRET-is used for signal generation. Adapted from [3] with permission. Copyright 2014, De Gruyter.

Recently, evolving analytical techniques and improved use of established methods have begun to incorporate micro- or nano-sized magnetic beads (MBs) [5]. The specific properties of MBs, such as colloidal stability of magnetic nanoparticles, homogenous size distribution, high and uniform magnetite content, a fast response to applied magnetic field, and presence of surface functional groups are essential for their analytical applications. The superparamagnetic properties of MBs permit the easy isolation of analytes from complex matrices, such as biological and environmental samples, by attaching specific ligands on the surface of MBs and separating the MBs-bound analytes with the aid of an external magnetic field. In order to provide different specificities, MBs can be functionalized with various reactive groups, such as

amines, carboxyls, epoxyls, and tosyls, which can be used to immobilize high affinity ligands such as aptamers, proteins, antibodies, etc. according to required applications [6]. Suspended modified MBs with immobilized ligands can be highly recommended for the detection of analytes in complex matrices and the isolation of targets from larger volumes [7]. In some applications, the magnetic properties of the MBs, such as magnetic-relaxation switch, have been used for signal generation [8,9]. Lately, magnetic separation processes have been introduced in biotechnology for the purification of proteins [10–16], protein digestion [17–19], separation of cells [20,21], and analytical applications [18,19,22–24].

The combination of MBs with aptamers opens up new possibilities in a number of applications, such as sample preparation, wastewater treatment, water purification, disease therapy, disease diagnosis including magnetic resonance imaging, cell labelling and imaging, and biosensors. Within this review article, first a brief introduction of magnetic beads and their applications will be given in Section 2. In Section 3, the use of aptamer-modified MBs in biosensing will be described and recent examples will be highlighted.

3.3.4. Magnetic Beads and Their Biological Applications

Within this section magnetic beads will first be briefly introduced together with potential strategies to modify their surfaces. Consequently, the use of magnetic beads in some of the important biological applications such as separating biomolecules from complex matrices will be described.

3.3.4.1. Magnetic Beads and Their Modification

In 1792, William Fullarton described the separation of iron material with a magnet in a patent, which paved the way for applications of magnetic fields in separation techniques [6]. Initially, magnetic properties of sediments were used for separation. In 1852, a company from New York separated magnetite from apatite and later on, magnetite was separated from iron from brass fillings, turnings, metallic iron from furnace products and plain gauge etc. Gradually, magnetic separation evolved into complex and diverse commercial applications. In 1950, introduction of high gradient magnetic separation (HGMS) systems allowed faster and broad magnetic separation applications [25]. Towler et al. [26] reported the use of micron sized magnetite particles with adsorbent, manganese dioxide, on the surface to recover radium, lead and polonium from seawater samples. Remarkably, Safariková et al. introduced silanized magnetite particles (blue magnetite [27]) in magnetic solid-phase extraction (MSPE) for the first time to preconcentrate organic compounds [28] prior to analysis. Nowadays, magnetic separations have

been widely used for protein purification [10–16], separation and purification of cells [20,21], and analytical applications [18,19,22–24].

In recent applications, MBs are largely composed of a magnetic core, a surface coating, and specific binding ligands at the surface (Figure 2). Generally, magnetic cores can be composed from various materials, which exhibit magnetic properties. Largely, they consist of either pure metals (e.g., Co, Fe, and Ni) or their oxides. In addition, transition-metal-doped oxides and metal alloys, including $CoPt_3$, FeCo, and FePt, are also good candidates. Among these magnetic materials, particularly iron oxides such as magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) are considered to be the most attractive candidates for biological applications, owing to their strong magnetic property and biocompatibility [6]. U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved the use of iron oxide MBs as magnetic resonance imaging (MRI) contrast agents [29]. Several approaches are available to synthesize the iron oxides. One widely used approach is co-precipitation from iron (III) chloride and iron (II) chloride solutions in the presence of aqueous ammonia solution [20,30,31]. Other methods are available including the extraction of bacterial magnetic particles (BMPs) from the flagella of magnetic bacteria from marine sediments [32,33].

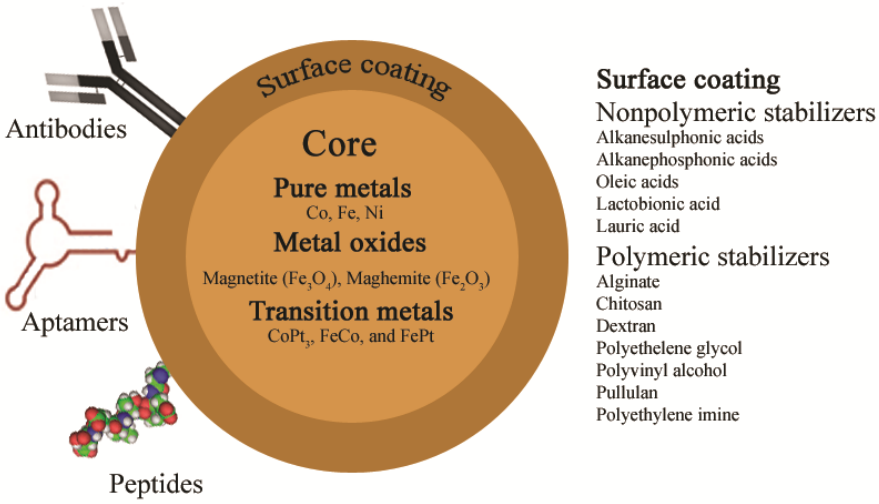

Figure 2. Composition of magnetic beads (MBs) used in analytical applications.

Finely divided iron is highly reactive toward oxidizing agents in the presence of water or humid air. Thus, surface coating of magnetic particles is required to obtain physically and chemically stable systems. Such stabilization can be achieved by surface coating of the magnetic particles in numerous ways (Figure 2). Surface coating can be performed by using stabilising surface coating material, encapsulation into polymeric shells and into liposomes [34].

Surface coating is primarily necessary to stabilize the newly formed surface of the particles and to prevent aggregation of the particles. Surface stabilization is generally achieved using nonpolymeric stabilizers based on organic monomers such as alkanesulphonic and alkanephosphonic acids, or phosphonates; oleic acid, lactobionic acid, lauric acid, or polymeric stabilizers, i.e., alginate, chitosan, dextran, polyethylene glycol, polyvinyl alcohol, pullulan, or polyethylene imine (Figure 2) [6]. Encapsulation into polymeric shells improves the water dispersibility and chemical and physical stability of the magnetic particles. In addition, the polymeric shell can provide a basis for conjugation of the magnetic particles to the targeting ligands by providing functional groups such as amine or carboxyl groups on the surface. Several ligands are available for the analytical purpose; the most popular among them are antibodies (ABs), aptamers, and peptides. The commonly used approach to attach ABs to MBs is the coupling of amine groups of the AB to carboxylated MBs via ethyl (dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) chemistry, but this can lead to random orientation of AB on the MBs. Different strategies have been attempted to avoid random orientation. For example, protein A can be used in order to immobilize the AB on the MB in a controlled orientation [35].

Currently, aptamers are also becoming popular for analytical applications. Several strategies are used for immobilising aptamers on the MBs. One of the popular strategies is to introduce amine groups on one terminus of the aptamer to allow immobilization to carboxylated MBs via EDC/NHS coupling. In contrast to AB immobilization, this results in highly oriented immobilization of aptamers, since only the terminal amine group can participate in coupling procedure. It is also possible to use streptavidin coated MBs and biotin-labelled aptamer for the attachment. Most important advantage here is the orientation of the aptamer, which can be easily controlled, as aptamers are chemically synthesized. During chemical synthesis, various modifications can be attached at defined positions within the aptamer sequence.

3.3.4.2. Separation of Biomolecules Using Modified MBs

The separation of biomolecules such as whole cells, proteins and peptides, and mRNA from complex matrices is very challenging. In this context, MBs modified with specific ligands allow to isolate the target molecule using strong magnets. This MB-based sample pre-treatment also allows to increase the concentration of target molecules in case of low concentration of the target molecules. In addition, time-consuming sample pre-treatment procedures like centrifugation, filtration and solid-phase extraction can be avoided [10]. In the following

paragraphs, some exemplary applications of MBs to separate the biomolecules will be briefly discussed.

Cell Separations

MB-based separation of cells from complex mixtures has become a popular tool and a valuable alternative to fluorescence activated cell sorting. In this process, specific aptamers or antibodies are anchored on the surface of magnetic particles. As example, Herr et al. immobilised an aptamer against leukemia cells on MBs. In this work, it was possible to specifically recognize the cells from complex mixtures including whole blood samples [36]. In a similar work by Zamay et al., MBs modified with aptamer against lung adenocarcinoma cells were used to separate circulating tumor cells (CTC) from human blood [37]. Interestingly, the applications of aptamer-modified MBs have been also integrated with microfluidic device to isolate cancer cell subpopulations [38]. Magnetic cell sorting (MACS) is used with antibodies since a long time [39]. The US FDA has also approved the Cellsearch® system as in vitro diagnostic system for the detection of CTC in the clinic [40].

mRNA Isolation

The isolation of mRNA using MBs is based on A-T pairing. Short sequence of dT (normally $dT_{(25)}$) can be covalently attached to MBs, which will hybridise to dA-tail of mRNA and the isolation of mRNA can be possible within 15 min [41]. This technique eliminates the cumbersome steps used in traditional methods of mRNA isolation such as use of centrifugation and membrane-based spin columns. Importantly, MB-based isolation of mRNA fulfils the demand of automated systems and microfluidic devices, thereby promoting fast processing and high sample throughput [42]. The possibility of high throughput analysis has facilitated the identification of genetic aberrations in cancer cells [43], understanding of biochemical pathways [44] and phylogenetic studies [45].

Protein and Peptide Enrichment

Traditionally, proteins are purified using expensive liquid chromatography systems, centrifuges, filters and other equipment. In addition, this purification process requires several steps and there is significant loss of protein/peptide at each step of purification. Purification using MBs reduces the amount of handling step and all the step can be done in a single test tube, which results in higher efficiency and reduced risk of contamination [46]. In some cases where intracellular proteins are targeted, it is even possible to combine the disruption of cells and separation of the protein form the complex mixture and thus shorten the total purification

time. For example, Ni^{2+} or Co^{2+} coated MBs can be used to easily purify His-tagged proteins from cells [6]. Alternatively, aptamer-modified MBs can be used for the isolation of His-tagged proteins [47] or other proteins [48].

These methods can be used to isolate and enrich proteins prior to analysis. For example, immunomagnetic assays rely on MBs modified with antibodies (AB). Enzyme-linked immunosorbent assay (ELISA) is a gold-standard method for the detection of the protein in complex mixture. In immunomagnetic assays, the capturing antibody is immobilized on the MBs, which can reduce the incubation time and efficiency of the assay, as MBs remain suspended throughout the procedure [49]. Morozov et al. have developed an immunomagnetic assay for the detection of streptavidin in a micorofluidic device. This has been highly successful in reducing the assay time (three minutes) and better sensitivity (2×10^{-17} M) of the assay [50]. While Section 2 provided a brief overview on the general biological applications of magnetic beads, the next section will focus on the analytical applications of aptamer-modified magnetic beads.

3.3.5. Aptamer-Modified Magnetic Beads in Analytical Applications

In aptamer-modified MB-based assays, aptamers are used as binding ligands and MBs are mostly used for the separation of the analyte from complex matrices. MBs have also been coupled with antibodies, but aptamer-based assays offer prominent advantages such as high stability, broad dynamic range, prolonged shelf life, and low cross reactivity [51]. Moreover, aptamers are synthesized chemically, which facilitates straightforward and highly controlled modification with functional groups and different labels [52]. Moreover, the use of aptamers facilitates new assay designs that cannot be realized by using ABs. Aptamers can undergo significant conformational change upon binding to the target molecule. This can be exploited in target-induced structure switching (TISS)-based assays. Another assay format specific for aptamers is based on target-induced dissociation (TID) of complementary oligonucleotides. In case of TID, aptamers ability to hybridize with complementary sequences are used. Here, the helix structure formed between aptamer and its complementary sequence can be easily dissociated by competitive binding of the aptamer with its target (Figure 1). TISS as well as TID can be used for signal generation in various sensing strategies and are especially useful in cases were conventional formats such as sandwich assays are not applicable [3,53,54].

As shown in Table 1, a growing number of research groups are already using aptamer-modified MBs in analytical applications using various sensor designs. In the following sections, the

applications have been broadly divided in the electrochemical, optical, piezoelectric and PCR-based assays.

Table 1. Examples of coupling magnetic beads in aptamer-based analytical applications.

Method	Analytes	Detection Limit	Reference
Electrochemical			
Voltammetric			
Differential pulse voltammetry (DPV)	Human activated protein C	2.35 μg mL^{-1}	[55]
DPV	Thrombin	5.5 fM	[56]
DPV	Thrombin	5 nM	[57]
DPV	Human liver hepatocellular carcinoma cells (HepG2)	15 cells mL^{-1}	[58]
DPV	Platelet derived growth factor BB (PDGF BB)	0.22 fM	[59]
DPV	Adenosine	0.05 nM	[60]
DPV	Hg^{2+}	0.33 nM	[61]
Squarewave voltammetry (SWV)	Tumor necrosis factor-alpha (TNF-α)	10 pg mL^{-1}	[62]
SWV	Ochratoxin A	0.07 pg mL^{-1}	[63]
Potentiometric			
Potentiometric carbon-nanotube aptasensor	Variable surface glycoprotein from African Trypanosomes	10 pM	[64]
Direct Potential Measurement	*Listeria monocytogenes*	10 cfu mL^{-1}	[65]
Chronopotentiometry	*Vibrio alginolyticus*	10 cfu mL^{-1}	[66]
Impedimetric			
Electrochemical impedance spectroscopy	*Salmonella*	25 cfu mL^{-1}	[67]
Impedimetric microfluidic analysis	Protein Cry1Ab	0.015 nM	[68]
Microfluidic impedance device	Thrombin	0.01 nM	[69]
Electrogenerated Chemiluminescence			
Electrochemiluminescence resonance energy transfer system	β-amyloid	4.2×10^{-6} ng mL^{-1}	[70]
Ratiometric electrochemiluminescence	Cancer cells	150 cells mL^{-1}	[71]
Optical			
Fluorescence			
Signal-on fluorescent aptasensor	Ochratoxin A	20 pg mL^{-1}	[72]
Aptamer-conjugated upconversion nanoprobes assisted by magnetic separation	Circulating tumour cells	20 cells mL^{-1}	[73]
Enzyme-linked aptamer assay	Oxytetracycline	0.88 ng mL^{-1}	[74]
Colorimetric			
Colorimetric assay (Methylene Blue-based)	Hg(II)	0.7 nM	[75]
Chemiluminescence			
Chemiluminescent	Hepatitis B Virus	0.1 ng mL^{-1}	[76]
Chemiluminescence (integrated microfluidic system)	Glycated haemoglobin	0.65 g dL^{-1} for HbA1c and 8.8 g dL^{-1} for Hb	[77]
Surface enhanced Raman scattering			
Molecular embedded SERS aptasensor	Aflatoxin B1	0.0036 ng mL^{-1}	[78]
Universal SERS aptasensor	Aflatoxin B1	0.54 pg mL^{-1}	[79]
Induced Target-Bridged Strategy	platelet derived growth factor BB	3.2 pg mL^{-1}	[80]

Piezoelectric			
Quartz crystal microbalance sensor	*Salmonella enterica*	100 cfu mL^{-1}	[81]
Magnet-quartz crystal microbalance system	Acute leukemia cells	8×10^3 cells mL^{-1}	[82]
PCR-based assays			
Apta-qPCR	ATP	17 nM	[54]
Apta-qPCR	Ochratoxin A	0.009 ng mL^{-1}	[53]
Rolling circle amplification	Cocaine	0.48 nM	[83]
Micromagnetic aptamer PCR	PDGF-BB	62 fM	[84]
Real-time PCR	*Escherichia coli*	100 cfu mL^{-1}	[85]
Magnetic relaxation			
Magnetic nanosensors	CCRF-CEM cell	40 cells mL^{-1}	[8]
Magnetic relaxation switch	*Pseudomonas aeruginosa*	50 cfu mL^{-1}	[9]

3.3.5.1. Electrochemical

Since the first use of electrochemical (EC) assay for the detection of glucose by Clark and Lyons in 1963 [86], the applications have evolved in different kind of assays including ABs and aptamer-based assays. EC assays are generally rapid, highly sensitive, cost-effective and easy to miniaturize, which is highly attractive for the development of modern bioassays. In aptamer-based electrochemical assays, the change in electrochemical signals (current, voltage, and impedance) due to interaction of analytes and aptamers is measured [87].

EC assays can be broadly classified as amperometric, potentiometric, voltammetric, impedimetric, and electrogenerated chemiluminescence (ECL) assays, according to their working principles. In Table 1, recent work on aptamer-modified MBs in different electrochemical assays is summarized.

Voltammetric Assays

In voltammetric assays, a specific potential is applied to a working electrode in comparison to a reference electrode. The electrochemical reduction or oxidation at the surface of the working electrode results in the generation of a current. Here, amperometric assays are included as a subclass of voltammetric assays as, in amperometric assays also, a constant potential is applied on the working and reference electrode, and the change in current is measured over a period of time. In case of voltammetric assays, a potential range is applied and the changes in both, current and potential are observed. In both assays, the observed change in current is proportional to the concentration of the analyte. Different voltammetric modes are available such as cyclic voltammetry (CV), differential pulse voltammetry (DPV), squarewave voltammetry (SWV), and alternating current voltammetry (ACV) [88]. CV is majorly used to evaluate the electrode surface including purity, stability, and reproducibility of the electrode and to examine

the aptamer immobilization on the electrode surface since it allows to check the redox behaviour over a wide potential range [58,88,89].

Differential Pulse Voltammetry (DPV)

Due to its sensitivity and high selectivity, DPV is preferred in analytical applications. In DPV, different electroactive labels are used to generate the signal including small molecules and enzymes, such as horseradish peroxidase (HRP), alkaline phosphatise (AP), and glucose oxidase (GOD), etc. Among these enzyme labels, HRP is one of the most commonly used. For example, Zhao et al. [56] developed a highly sensitive and selective assay for the detection of thrombin (Figure 3A). In this assay, the capture probe was prepared to capture thrombin from the sample solution by immobilizing a thrombin aptamer-1 on MB-AuNP. The detection probe was prepared from another thrombin aptamer-2, horseradish peroxidase (HRP), thiolated chitosan (CS) nanoparticle and gold nanoparticle (CS-AuNP-HRP-Apt2). Presence of thrombin resulted in formation of the sandwich structure of MB-AuNP-Apt1/thrombin/Apt2-HRP-AuNP-CS. The sandwich structures were captured on the surface of a screen printed carbon electrode (SPCE) by a magnet located at the edge of SPCE. Due to the presence of HRP within the sandwich structure, the oxidation of hydroquinone (HQ) with H_2O_2 was dramatically accelerated. The observed electrochemical signal was proportional to the concentration of thrombin in the samples. A similar assay was also developed by Sun et al. [58] for the detection of human liver hepatocellular carcinoma cells (HepG2).

Centi et al. [57] have developed an electrochemical sandwich assay coupled to magnetic beads for the detection of thrombin in plasma. In this work, a microfluidic device was developed for the detection. The electrodes were screen-printed on a magnetic bar. Two different aptamers against thrombin were used where the first aptamer sequence was immobilised on MBs, to capture thrombin from the samples, using streptavidin-biotin interactions. The second aptamer was linked to alkaline phosphatase. The presence of thrombin in the sample resulted in current generation at the electrode, as more product was formed in the presence of alkaline phosphatase.

Zheng et al. [59] have developed an assay based on aptamer-conjugated to methylene blue for the detection of human platelet-derived growth factor BB (PDGF-BB). In this work, boronic acid modified SiO_2@Fe_3O_4@PDA@AuNP composite was used to capture PDGF-BB from the sample. An aptamer against PDGF was linked to SiO_2-methylene blue sphere, where methylene blue was used for its high electron-transfer efficiency. Due to the magnetic property of the electrode, the

SiO_2@Fe_3O_4@PDA@AuNP/PDGF/aptamer-SiO_2-methylene blue could be easily enriched on the electrode surface. Later on, the electrochemical responses could be detected to quantify PDGF.

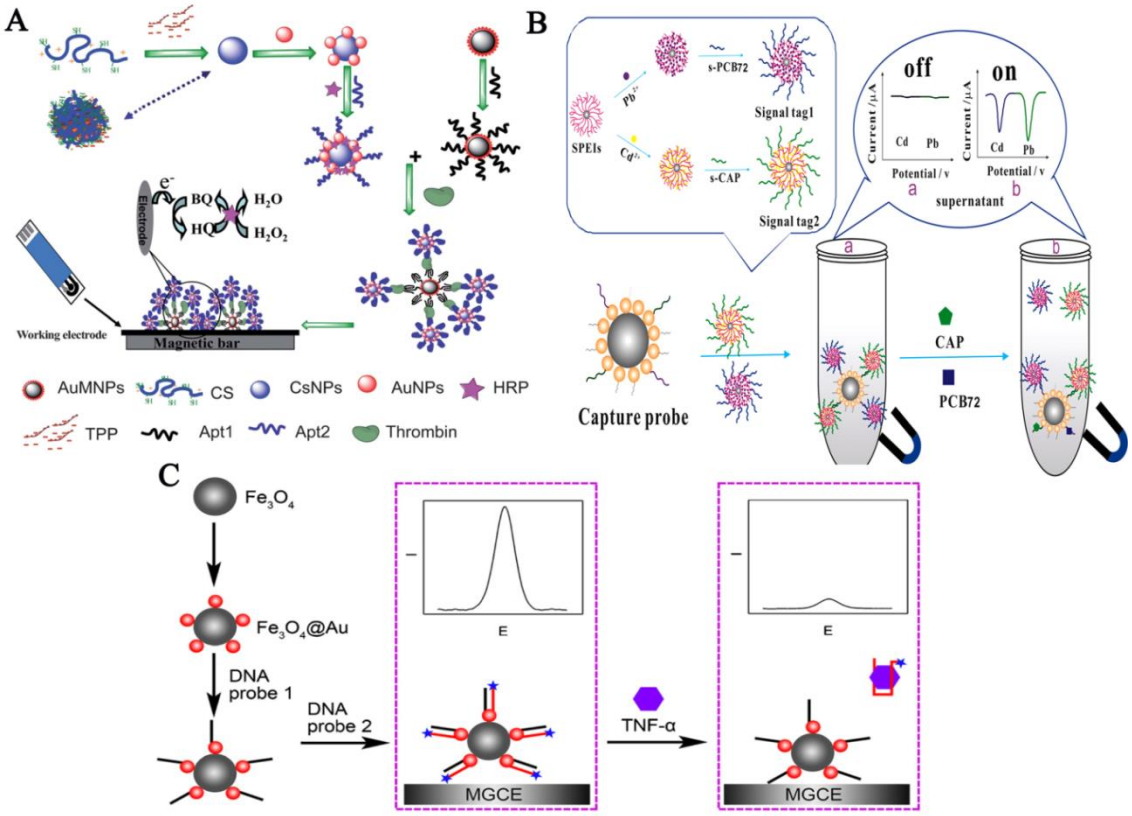

Figure 3. Utilization of MBs in aptamer-based electrochemical assays. (**A**) Using an electric signal mediator. Here, the electroactive molecules (HRP) were brought close to the electrode using aptamer-modified MBs. Reproduced with permission from [56]. Copyright 2012, Royal Society of Chemistry; (**B**) Signal-on type of electrochemical assay. The interactions between aptamers and the target molecules (Chloramphenicol and PCB 72) lead to generation of electrochemical signal. Reproduced with permission [90]. Copyright 2015, Elsevier; (**C**) Signal off type of electrochemical assay. In this type of assay, the interaction between the aptamer and the target molecule leads to reduction in electric signal. Reproduced with permission from [62]. Copyright 2017, Springer.

While in the previous examples, proteins were detected with aptamer-modified MBs, also small molecules are suitable targets, which can be detected e.g., by using the aptamer-specific TISS and TID-based assays. In this context, a TID-based label-free assay was developed by Yang et al. [60] for the detection of adenosine using thionine (Th) for generation of the electrochemical signal. In this work, adenosine aptamer was immobilised on MBs and an oligonucleotide complementary to the target-binding site of the aptamer (abbreviated as cDNA) was hybridized to the immobilized aptamer. Addition of adenosine resulted in the formation of aptamer-adenosine complex and the release of cDNA from the aptamer due to TID. The released cDNA was captured on the sensing electrode through DNA hybridization. As the cDNA is modified with thiol groups at the 5′ termini, AuNP can attach to the cDNA via the formation of S-Au

bonding. Subsequently, the electroactive molecules, thionine, are adsorbed on the surfaces of the AuNPs and result in signal generation.

A TISS-based assay in which the structural changes of the aptamer were exploited for signal generation was developed by Wu et al. [61] for the detection of Hg^{2+} using streptavidin modified magnetic beads (Fe_3O_4–SA) and thionine, as electron mediator. In this work, Streptavidin-modified MBs (MB-SA) were immobilized onto the glassy carbon electrode (GCE) and provided magnetic character to the electrode. Then biotin-labelled aptamer against Hg^{2+} was immobilized to the electrode via SA-biotin interaction. Addition of Hg^{2+} resulted in a stable folded structure of thymine (T)-Hg^{2+}-T where Th can easily intercalate. The detection of Hg^{2+} was achieved by recording the DPV signal of Th.

Squarewave Voltammetry (SWV)

SWV is the popular pulse technique and widely considered for automatous and kinetic studies complementary to cyclic voltammetry. Here, TID-based assays are very popular and the interaction of the aptamer with the target molecule can result in electrical signal gain (signal-on) or signal suppression (signal-off).

Yan et al. [90] have developed an assay for the simultaneous detection of two different molecules, chloramphenicol and polychlorinated biphenyls-72 using a signal-on mechanism (Figure 3B). In this work, aptamers were immobilised on MBs and hybridised with a cDNA sequences attached to CdS or PbS QDs as electrochemical signal tracers. Binding of chloramphenicol caused the release of CdS QDs and binding of polychlorinated biphenyls-72 caused the release of PbS QDs. The released CdS and PbS QDs were simultaneously detected through the square wave voltammetry (SWV), which can switch the signals of the biosensor to "on" state. Hao et al. [63] have also developed an assay based on signal-on approach for the detection of ochratoxin A (OTA). In this work, addition of OTA caused the release of CdTe QDs modified with cDNA, from the MB-modified aptamers. After magnetic separation of aptamer-modified MBs, CdTe QDs remaining in the supernatant were dissolved by HNO_3 and the concentration of Cd ions, which was directly proportional to the concentration of OTA, was detected by SWV.

Miao et al. [62] have developed a signal-off assay for the detection of TNF-α (Figure 3C). In this simple assay, methylene blue-tagged aptamer was immobilised on magnetic glassy carbon electrode (MGCE) using a cDNA. Addition of the target molecule resulted in release of methylene blue-tagged aptamer, resulting in decrease in electrochemical signal by SWV.

Potentiometric Assays

In potentiometric sensors, the change in electric potential between two electrodes is detected by a field-effect transistor (FET) [91]. Here, the indicator electrode reports change in electric potential according to analyte concentration and the reference electrode provides constant electric potential.

Recently, Zhao et al. [66] have developed a potentiometric sensor for the detection of *Vibrio alginolyticus*, which is an opportunistic marine pathogen and can cause otitis, wound infection, and chronic diarrhoea in mammals. In this work, a cDNA sequences were immobilized on the surface of the magnetic beads using streptavidin-biotin interaction. The aptamer and H1/H2 (two different oligonucleotides) hybridize successively with the cDNA to form the DNA structure-modified magnetic beads. The resulted DNA structure can interact with protamine (polycation) due to electrostatic interactions, which can be detected by the polycation-sensitive electrode. When a sample containing *Vibrio alginolyticus* was added, the aptamers interact with the target due to its high affinity with the target. Consequently, the DNA structure disassembled and a reduction in potential was observed which was proportional the target concentration. A similar assay was also used for the detection of small molecules such as bisphenol A [92].

Impedimetric Assays (EIS)

In impedimetric assays, electrochemical impedance spectroscopy (EIS) is popular due to its high sensitivity. EIS involves the analysis of the resistive and capacitive properties, which are based on the perturbation of a system at equilibrium by a small amplitude of excitation signal. EIS allows rapid and accurate detection of the small changes along the electrode by a transducer. The signal is enhanced by an amplifier, which makes EIS appealing in analytical applications. In addition, EIS is a simple technique and the detection in EIS does not require attachment of capture molecules to the electrodes and thus allows label-free detection.

Wang et al. [69] have developed a microfluidic analysis system assay for the detection of thrombin using aptamer-modified magnetic separation. In this work, thrombin aptamer-modified MBs were used to capture and separate the target protein from serum. Later on, the bound complex was injected into the microfluidic flow cell for impedance measurement. Similar analysis system was also developed by Jin et al. [68] for the detection of Cry1Ab protein to detect genetically modified crops. Another interesting impedimetric assay based on TID mechanism was developed by Lee et al. [93] for the detection of prostate-specific antigen

(PSA). Combining PSA aptamer-modified magnetic nanoparticles with rolling circle amplification (RCA) has provided a better sensitivity of 0.74 pg mL^{-1} PSA in human serum.

Electrogenerated Chemiluminescence

Electrogenerated chemiluminescence (ECL), also called electrochemiluminescence, refers to the emission of light via electron transfer reactions from electrochemically generated reagents. ECL combines the sensitivity and wide dynamic range from chemiluminescence (CL) with the advantages offered by electrochemical methods, such as simplicity, stability, and facility to be miniaturized [94,95]. Lately, ECL has been widely accepted in different analytical applications including fundamental studies to detecting trace amount of target molecules. Among different luminophores, use of luminol, quantum dots (QDs) and ruthenium(II) complexes, have been widely employed in ECL assays [96].

Ke et al. [70] have developed an assay for the detection of β-amyloid (Aβ) using a sandwich-type ECL sensing platform. In this work, $Ru(bpy)_3^{2+}$ was used as ECL donor and gold nanorods (GNRs) were used as ECL acceptor. Here, resonance energy transfer (RET) donor nanohybrids were prepared with mesoporous carbon nanospheres (MCNs)@nafion/$Ru(bpy)_3^{2+}$/Aβ antibody. After incubation with target Aβ protein and GNRs-attached aptamer, prominent decrease in ECL signal was observed due to the quenching effect between $Ru(bpy)_3^{2+}$ and GNRs. This innovative approach performed well with sensitivity of 4.2 fg mL^{-1} in real Alzheimer's patient cerebrospinal fluid samples.

In a TID-based approach, Wang et al. [71] have developed a novel ECL sensing system for the detection of HL-60 cancer cells. Here, Ag-polyamidoamine (PAMAM) was prepared and functionalized with cDNA and bio-bar-code DNA (bbcDNA). The prepared composite was hybridized with the aptamer-modified MBs. Addition of HL-60 cancer cells resulted in the release of cDNA-Ag-PAMAM composite in the supernatant. For the detection, an oligonucleotide complementary to cDNA was immobilised on the electrode surface resulting in hybridization of released cDNA-Ag-PAMAM.

3.3.5.2. Optical

Optical assays have been widely used due to their specific advantages such as high sensitivity, quick response, high signal-to-noise ratio, reduced cost of manufacture, and relatively simple operation. Aptamers are preferred ligands in optical assays due to flexibility in modification with various fluorophores and other labels. As already described for electrochemical assays, application of aptamer-modified MBs can be highly advantageous due to easy separation of the

target molecule from the complex matrices, which offers high signal-to-noise ratio during measurement. The assays can be classified based on the detection principle including fluorescence, colorimetry, chemiluminescence, surface plasmon resonance (SPR), and Raman scattering.

Fluorescence Based Assays

Aptamer-based fluorescence assays can be mainly divided into labelled and label-free assays. Easy modification of aptamers with fluorophores and quenchers during chemical synthesis facilitates the design of various assays.

Luo et al. [97] developed a nicking enzyme assisted signal amplification (NEASA)-based assay relying on TID mechanism (Figure 4A). In their work, ampicillin aptamer was immobilised on MBs and attached to a complementary sequence (cDNA) through Watson-Crick base pairing. Addition of ampicillin resulted in release of cDNA which can bind to Taqman probe having fluorescent and quencher probes at opposite end. The nicking enzyme cleaved the Taqman probe only when it was bound to cDNA. The decrese in fluorescence signal was proportional to the concentration of ampicillin.

Upconversion nanoparticles (UCNPs), nanocrystals containing lanthanide ions, emerged as an important fluorophor, as they lack autofluorescence and their use result in high signal-to-noise ratio. In addition, the optical properties of UCNP can be tuned with different lanthanide dopants such as Er^{3+}, Tm^{3+}, and Ho^{3+} [98,99]. Fang et al. [73] developed an assay for the detection of circulating tumor cells (CTC) using UCNPs. In their work, UCNPs were modified with the aptamer and biotinylated-PEG. Aptamer was used to recognize CTC and biotinylated-PEG was used to attach UCNP to the MBs. Here, whole blood samples were mixed with the modified UCNPs and it was possible to detect as low as 10 cells into 0.5 mL of whole blood samples. Efforts have been also made to further increase the sensitivity of fluorescence-based assays. In this context, Wang et al. [100] used RuBpy-doped silica nanoparticles (RSiNPs), which are highly photostable and provide significant enhancement in fluorescent signal when compared with single RuBpy dye molecules. In this work, aptamer-modified MBs were used to separate thrombin from human serum.

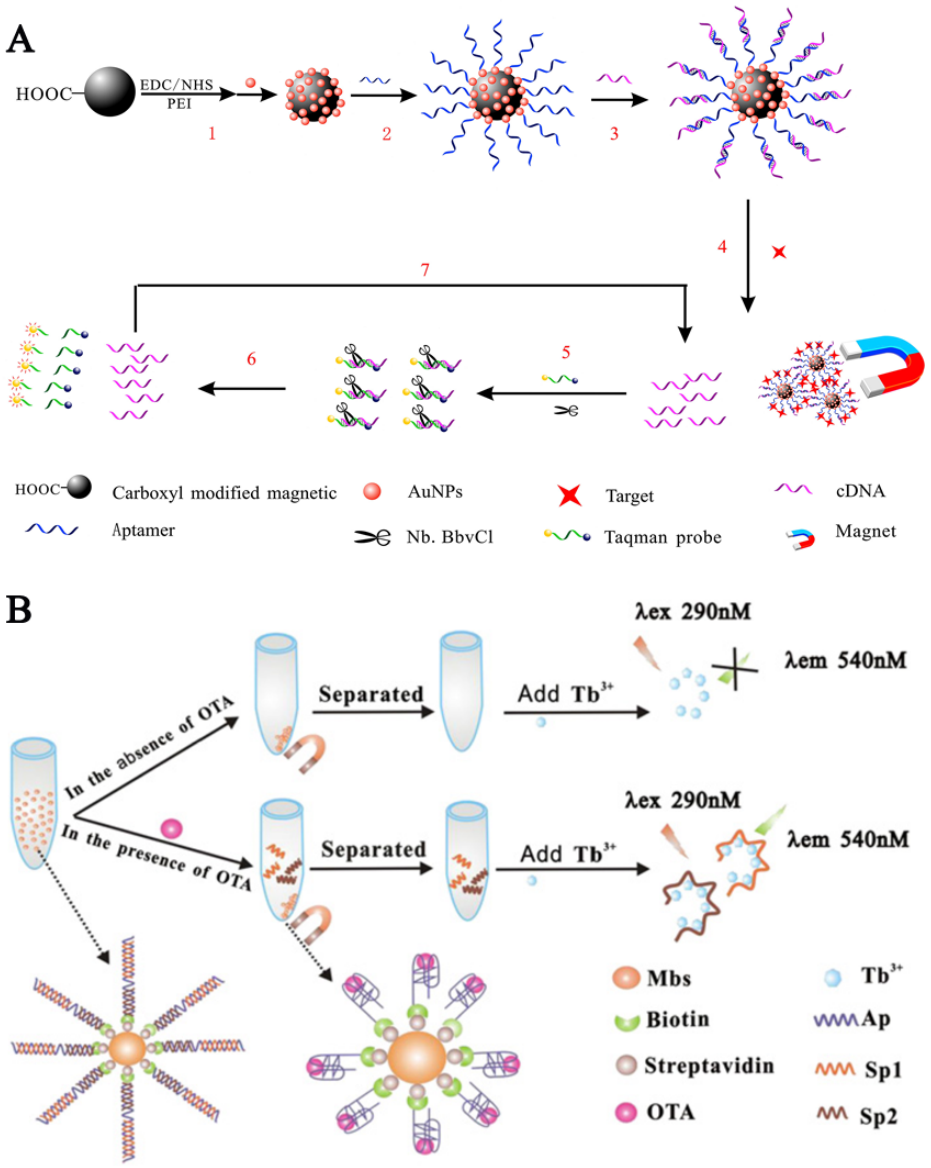

Figure 4. Fluorescence-based assays. (**A**) Combining fluorophores and quencher molecules. Here the interaction between the aptamer and the target molecule leads to the release of quencher molecule and the increase of fluorescence signal. Reproduced with permission from [97]. Copyright 2017, Elsevier; (**B**) Label-free assay. Being oligonucleotides, aptamers can specifically interact with dyes specific for ssDNA or dsDNA. In this example, Tb^{3+} was used which interacts specifically with ssDNA (cDNA), which was released due to TID from ochratoxin A (OTA) aptamer. Reproduced with permission from [72]. Copyright 2013, Elsevier.

Aptamer-modified MBs have been also used in enzyme-linked aptamer sandwich assays. John Bruno et al. [101] used aptamer-modified MBs to capture *Campylobacter jejuni* from the samples. Later on, a second aptamer modified with QDs was introduced. The bound complex was brought on the photo detector using external magnet. The whole detection procedure could be finished in 15 min and resulted in high sensitivity. Similar detection principle was also used by Hao et al. for the detection of thrombin [102].

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To reduce the labelling cost and reducing the effect of labelling on aptamer conformation, label free assays have attracted big attention. Being DNA sequences, aptamers can also bind to DNA binding chemicals, such as crystal violet [103], SYBR Green I (SGI) [104,105], 4',6-diamidino-2-phenylindol (DAPI) [106], malachite green [107], OliGreen [108] and terbium (III) (Tb^{3+}). Zhang et al. [72] developed a label-free fluorescent aptasensor based on the Tb^{3+}, structure-switching of anti-OTA aptamer and MBs for the detection of ochratoxin A in wheat (Figure 4B).

Colorimetric Assay

Colorimetric assays are widely used in analytical application due to simplicity of the measurement. Among aptamer-based colorimetric assays, AuNP-based assays are widely used. MBs-based separation in these assays provide better signal-to-noise ratio and sensitivity.

For example, Liang et al. [109] reported an aptamer-protein interaction-induced aggregation assay for the detection of thrombin. In their work, two different aptamers against human α-thrombin were immobilised on nanoroses (MB-AuNP core-shell structure in a flowerlike shape). Addition of human α-thrombin in solution resulted in aggregation of nanoroses and thus in a characteristic change in UV-Vis absorption spectra of the colloid. An interesting assay was also developed by Wang et al. [75] for the detection of Hg(II) based on hybridization chain reaction (HCR). In this work, aptamer against Hg(II) were immobilized on the MB-AuNP. HCR process is inhibited in the presence of Hg(II) enabling less methylene blue to intercalate into the dsDNA structure.

The use of peroxidase-like activity of magnetic nanoparticles is exploited in different colorimetric assays. Kim et al. [110] reported an assay for the detection of metal ions. Here, the aptamers were adsorbed on the positively charged surface of MBs, which reduces the catalytic activity of MBs. Addition of the target molecules released the adsorbed aptamers from MBs surface and MBs recover the peroxidase-like activity. Similar assays were also developed for the detection of ochratoxin A in cereal samples [111] and thrombin in blood plasma [112].

Chemiluminescence Assays

In chemiluminescence (CL) assays, the light emitted during a chemical reaction is detected. The unique characteristics of CL include high sensitivity, wide dynamic ranges and simple instrumentation. In case of CL, an excitation light-source is not required which is highly cost effective.

A label-free CL detection of adenosine in human serum was realized by Yan et al. [113]. In their work, 3,4,5-trimethoxyl-phenylglyoxal (TMPG) was used as the signalling molecule for CL, as TMPG can intercalate with guanine (G) nucleobases. Firstly, the cDNA was immobilized on the surface of MBs and hybridized with a G-rich adenosine aptamer. Addition of adenosine containing sample caused the release of the aptamer from MBs modified with cDNA and a decrease in CL signal was observed, which was proportional to adenosine concentration.

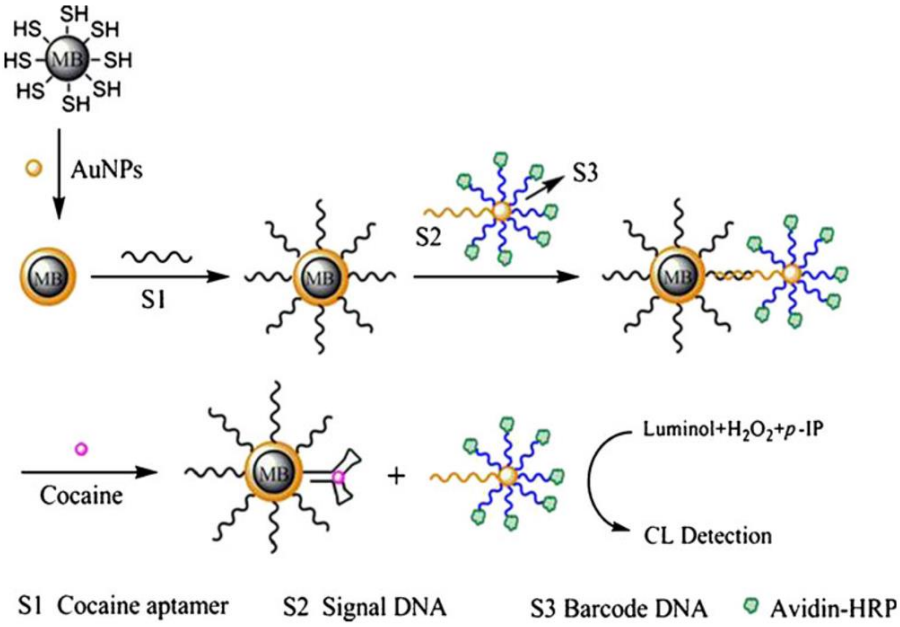

Figure 5. Chemiluminescence assay. This assay is based on TID. Here, the interaction between the aptamer and the target molecule (cocaine) caused the release of cDNA attached to HRP-modified AuNPs. Released HRP generated chemiluminescence signal which was proportional to cocaine concentration. Reproduced with permission from [114]. Copyright 2011, Springer.

In contrast, HRP-based catalysis can be widely used for the generation of CL signal. As an example for CL assays using HRP, Li et al. [114] immobilized aptamers directed against cocaine on the surface of AuNP-functionalized MBs (MB-AuNP). Therefore, aptamers were hybridized with the cDNA immobilized on the double-functional AuNP modified with HRP (HRP-AuNP). When cocaine was introduced, a dissociation of the aptamer was achieved due to binding of the aptamer to cocaine. Consequently, HRP-AuNP were eluted from the MB-AuNP due to

target-induced dissociation (TID). The recorded CL signals were proportional to the concentration of cocaine (Figure 5).

Surface-Enhanced Raman Scattering-Based Assays

Surface-enhanced Raman scattering (SERS) relies on the principle that the Raman scattering intensity of molecules will be greatly improved after their adsorbtion onto the metal surface.

Quansheng et al. [78] developed a SERS assay for ultrasensitive detection of aflatoxin B1 (AFB1) detection in peanut oil (Figure 6). In this study, AFB1 aptamers were immobilised on the MBs and cDNA was immobilised on gold nanorods (cDNA-AuNRs). Presence of AFB1 resulted in the release of cDNA-AuNRs and decrease in SERS signal was observed. In an interesting work, aptamer-conjugated magnetic beads were used for the separation of circulating tumor cells from whole blood samples and the tumor cells were detected using surface-enhanced Raman scattering imaging [115].

Yoon et al. [116] developed SERS-based magnetic aptasensors for the detection of thrombin in serum samples. In this work, two different aptamers against thrombin were used for the detection. One aptamer was immobilised on MBs and second aptamer was immobilised on AuNP-coated with Raman reporter molecules, X-rhodamine-5-(and -6)-isothiocyanate (XRITC). Addition of thrombin resulted in the formation of sandwich aptamer complexes and an increase of SERS signal according to thrombin concentration in the sample was observed.

Piezoelectric Assays

Since the discovery of piezoelectric effect in 1880 by the Curie brothers, the piezoelectric effect has been very popular in analytical applications. Lately, the progress made in the fields of microelectronics and microfluidics further promotes the development of label-free piezoelectric assays [117]. Particularly, quartz-crystal microbalance (QCM)-based assays have become popular in analytical applications of aptamers. Utilizing MBs in these assays is highly advantageous due to their inherent piezoelectric properties, and potential to concentrate the analyte molecules at the QCM surface [118].

Ozalp et al. [81] developed a QCM biosensor for the detection of *Salmonella* cells in food samples. Here, aptamer-modified MBs were, firstly, used to capture the target pathogens from the food samples. The magnetically separated pathogens were detected by QCM sensor and 100 cfu mL^{-1} *Salmonella* cells could be detected in milk samples. In a similar assay, Pan et al. [82] detected leukemia cells in complex matrices. In this work, aptamer-modified MBs were used to

capture leukemia cells from the biological sample and to approximate them to the QCM sensor using an external magnet.

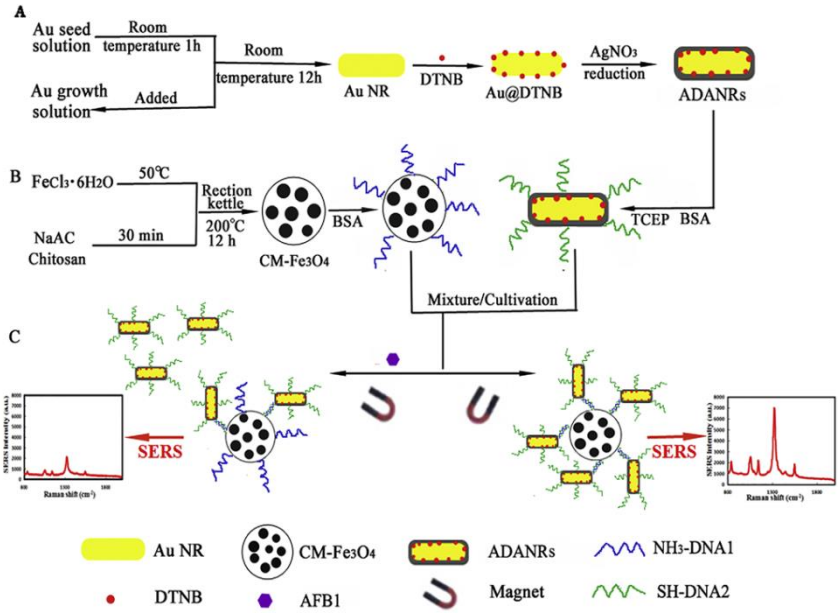

Figure 6. Surface-enhanced Raman scattering-based assays. (**A**) Immobilization of aflatoxin B1 (AFB1) aptamer on gold nanorods (AuNRs). (**B**) Immobilization of cDNA on chitosan-modified MBs. (**C**) Schematic representation of AFB1 measurement. Here, the binding of AFB1 induced the release of cDNA and, in turn, AuNRs from the MBs and a decrease in SERS signal was observed. Reproduced with permission from [78]. Copyright 2018, Elsevier.

An interesting approach was used by Song et al. [119] for the detection of ATP via DNAzyme-activated and aptamer-based target-triggered circular amplification. In this work, AuNPs were used for mass amplification and captured on the modified gold electrode. The amplification scheme involved circular nucleic acid strand-displacement polymerization, aptamer binding strategy and DNAzyme signal amplification. Presence of ATP resulted in a two-cycle amplification process, triggered by the aptamer recognition of a target molecule.

PCR-Based Assays

Being oligonucleotide sequences, aptamers can be easily amplified and quantified using qPCR with high sensitivity and reproducibility. Recently, different assays based on this property of aptamers have been developed including Apta-qPCR, micromagnetic aptamer PCR (MAP), and assays involving PCR-based amplification strategies as loop-mediated isothermal amplification (LAMP), rolling circle amplification, and isothermal signal amplification, proximity ligation assays, and nuclease protection assays. In these assays, MBs provides opportunity to separate target-bound and unbound aptamers, which is very important to get minimum background and high signal-to-noise ratios and in turn high sensitivity and reproducibility.

Our group [53] developed an Apta-qPCR assay for the detection of ochratoxin A in beer samples (Figure 7). In this work, ochratoxin A aptamer was hybridized to a corresponding cDNA, which was immobilized on MBs. Addition of the target molecules caused TID of aptamer from the MBs and the released aptamers were quantified using qPCR. This assay was able to detect 0.009 ng mL^{-1} OTA in beer samples. Similar assay was also used for the quantification of ATP present in HeLa cell lysate with the sensitivity of 17 nM ATP [54].

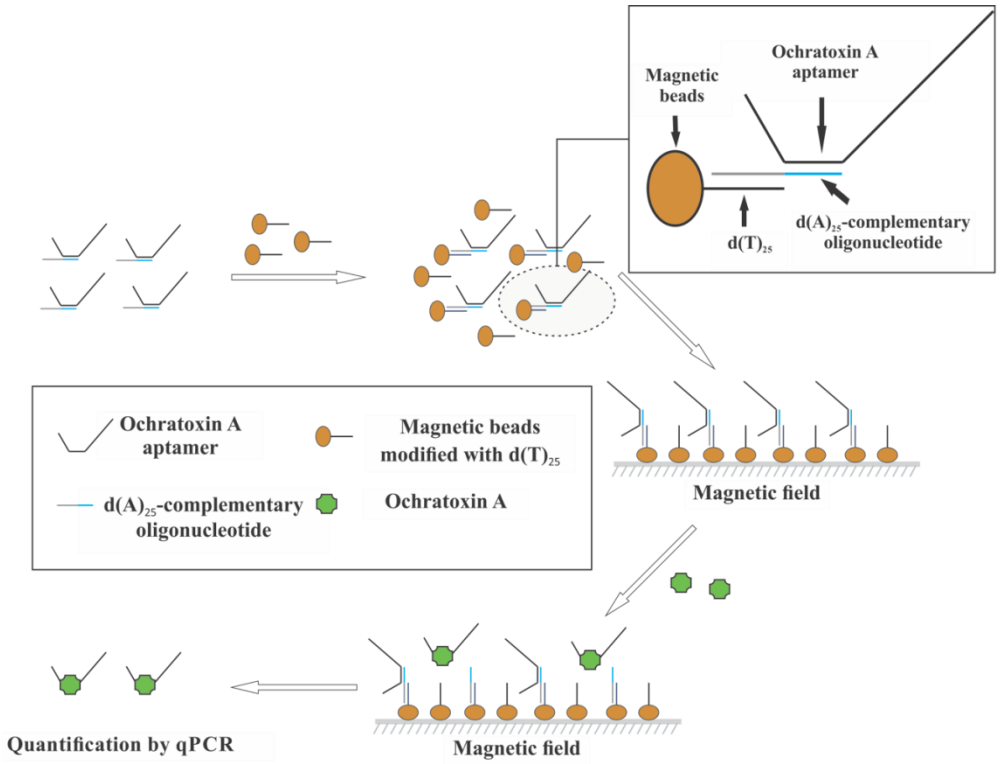

Figure 7. Apta-qPCR. This assay is based on TID, where the interaction of the target molecules (ochratoxin A) caused the release of aptamer from the cDNA-modified MBs. The released aptamers were quantified using qPCR. Reproduced with permission from [53]. Copyright 2017, Wiley.

Csordas et al. [84] developed an interesting concept for the detection of PDGF-BB using combination of antibody and aptamer in MAP. In this work, high-gradient magnetic field sample preparation was integrated within a microfluidic device with aptamer-based real-time PCR readout. Antibody-modified MBs were used for capturing PDGF-BB and an aptamer against PDGF-BB, which was modified with flanking PCR primer sequences, was added after washing. The bound aptamers were quantified using qPCR.

Ozalp et al. [85] developed a qPCR-based assay where aptamer-modified MBs were used to preconcentrate the *Escherichia coli* or *Salmonella typhimurium*. Later on, bacterial genomes were extracted which was quantified using qPCR. A similar assay was also developed by Feng et al. [120] for the detection of *Listeria monocytogenes* using a LAMP assay.

3.3.6. Conclusions and Future Trends

Aptamers are attractive bioreceptors in analytical applications due to their small size, animal free- and cost-effective production, high stability (especially DNA aptamers), target versatility, high binding affinity and selectivity for their target molecules. In addition, several properties of aptamers including ease of chemical modification, measurable structural changes induced upon interaction of the aptamer with the target molecule, and the potential to amplify aptamers via PCR are advantageous in comparison to other binding ligands such as ABs. Due to these significant advantages of aptamers, they have been widely used for the detection of different analytes ranging from metal ions, small molecules, proteins to whole cells in diverse assay formats. In this review article, the focus was put on the magnetic bead-based analytical applications of aptamers. Utilization of MBs in aptamer-based applications allows to rapidly detect the analyte in the complex matrices with high signal-to-noise ratio. Recent developments in the synthesis of MBs resulted in MBs with better homogenous size distribution, high and uniform magnetite content, and a fast response to applied magnetic field, as well as high colloidal stability of magnetic nanoparticles.

In this review, different assay formats have been discussed where MBs were coupled with aptamers for the analytical applications. In many of the applications, MBs help to separate the target molecule from complex matrices. In some applications the aptamer-modified beads are also enriched directly on the sensor surface, thereby representing a surface modification used to immobilize the target. In few applications, magnetic properties of the MBs, such as magnetic-relaxation switch, have been used for signal generation.

Application of aptamer in analytical techniques is still in development phase, as many commercial applications use ABs. Slowly but steadily, aptamers are developed against a range of molecules. For some of them the development of other ligands, such as antibodies, is not easy, e.g., due to low immunogenicity or high toxicity. Moreover, aptamers seem to be especially advantageous for the detection of small molecules. In this context, TISS and TID mechanism provide the possibility to design assays that can detect small molecules, while other strategies like sandwich assays are not suitable for detection of small molecules. These advantages of aptamers and their combination with those of MBs, can further boost the development of new analytical procedures. The use of MBs in these assays can result in rapid detection of the target molecules even within complex matrices with no need for time-consuming sample pre-treatment procedures. Taking together the strengths of aptamers and

MBs can therefore be especially advantageous in the development of POCT, where complex samples have to be analyzed within minutes.

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4. Experimental Investigations

The purpose of this study is to a develop novel a aptamer-based assay, Apta-qPCR, for the detection of small molecules. For this purpose, the Apta-qPCR assay was optimised for the detection of small molecules of different importance, including ATP, ochratoxin A and oxytetracycline. In addition, a rapid assay was also developed that can detect ATP and ochratoxin A within 15 minutes. The experimental part of this thesis consists of three chapters. In chapter 1, ATP was used as a model molecule for the development of Apta-qPCR. In chapter 2, the Apta-qPCR was transferred to the detection of a food toxin, ochratoxin A. Chapter 3 consists of unpublished work, where the development of Apta-qPCR for the detection of oxytetracycline and development of a rapid colorimetric assay for the detection of ATP and ochratoxin A were performed.

Chapter 1

Detection of ATP using Apta-qPCR

4.1. Detection of ATP using Apta-qPCR

The result of this chapter were published as "Modh H, Witt M, Urmann K, Lavrentieva A, Segal E, Scheper T, Walter JG. Aptamer-based detection of adenosine triphosphate via qPCR. Talanta. 2017 Sep 1;172:199-205." The article was reproduced with the permission of Elsevier (publisher).

4.1.1. Summary

Aptamers are highly advantageous for the detection of small molecules because of their structural flexibility. It is possible to develop assays based on TID and TISS, exploiting the structure flexibility of aptamers. In TID-based assay, first the aptamer is bound to its respective complementary sequence. Addition of the aptamer´s target causes the release of aptamers from the complementary sequence as aptamers have higher binding affinity toward its target molecule.

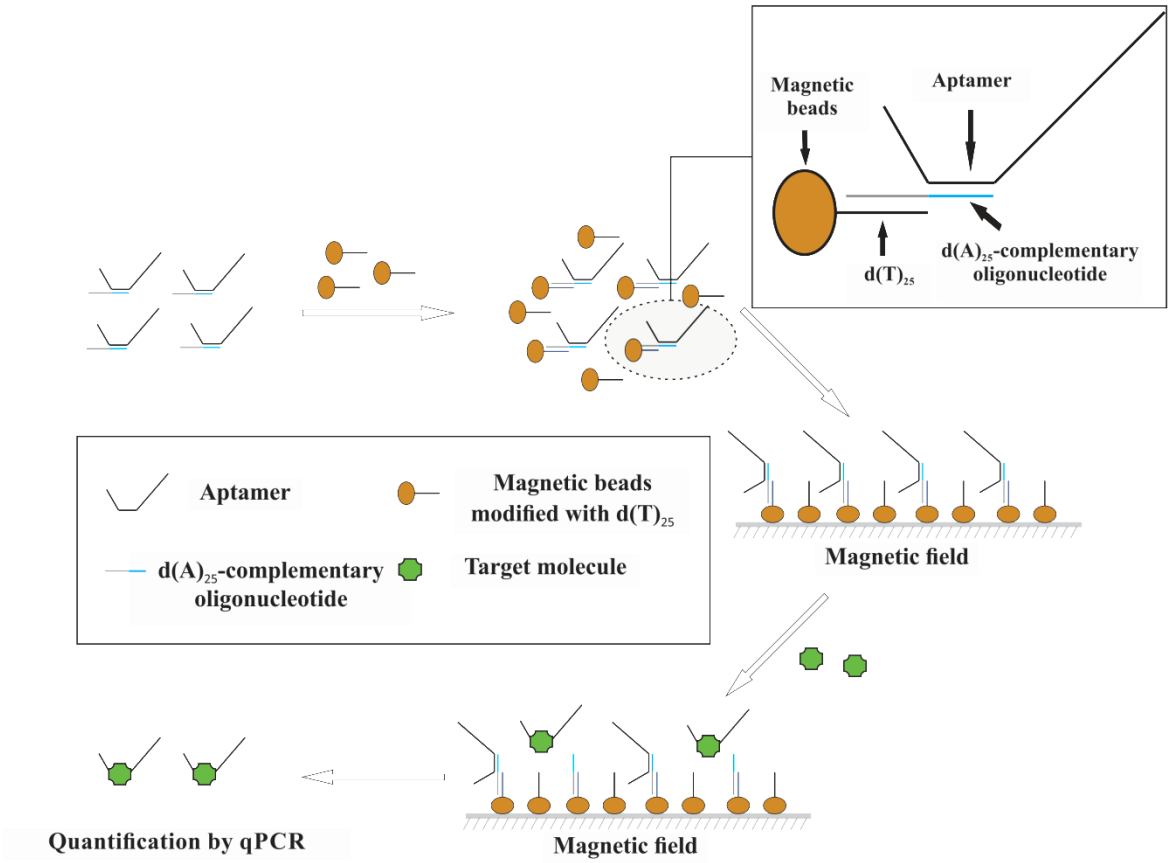

Figure 4.1. Scheme of detection of small molecules using Apta-qPCR.

In this work, a novel TID-based assay was developed in form of Apta-qPCR. The principle of Apta-qPCR assay is depicted in Figure 4.1. Briefly, aptamers are first immobilised on magnetic beads using the complementary sequences. Here, the complementary sequence is designed in a

way that it binds to the part of aptamer which is essential for the interaction with the target molecule. When a sample containing target is introduced to the aptamer/complementary sequence/magnetic beads complex (apta-beads complex), the aptamer gets released into the supernatant from the apta-beads complex. The released target-bound aptamer can be easily separated using a magnetic stand and quantified using qPCR. Here, qPCR-based detection of aptamers provides high sensitivity of the assay as it is possible to amplify and quantify a very minute amount of aptamer. In addition, it is also possible to detect a broad range of aptamer concentration with qPCR, which provides a broad linear detection range to Apta-qPCR assay.

In this work, ATP molecule was used as a model molecule for Apta-qPCR because the aptamer against ATP is well characterized and the complementary sequences required to develop TID-based assays are already available. In addition, ATP detection is very important in biochemical studies and clinical diagnosis. As expected, the assay resulted in high sensitivity with detection limit of 17 nM ATP in HeLa cell lysate. It was also possible to get broad linear range of detection from 50 nM to 5 mM, which reduces the requirement of dilutions where potential manual errors can arise [28].

Article - Aptamer-based detection of adenosine triphosphate via qPCR

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4.1.2. Abstract

Sensitive and specific detection and quantification of small molecules often remain challenging. We developed a novel magnetic bead-based aptamer-assisted real-time PCR (Apta-qPCR) assay to provide a versatile platform for quantification of small molecules. The assay has been realized for the detection of ATP as a model system. The assay relies on a combination of qPCR with the target-induced dissociation (TID) of ATP aptamer from an oligonucleotide, complementary to the ATP binding site of the aptamer. The complementary oligonucleotide was immobilized on deoxythymidine (dT)-modified magnetic beads (dT-beads) and hybridized with the aptamer. The presence of ATP resulted in dissociation of the aptamer from the dT-beads and the dissociated aptamer was quantified using qPCR. The Apta-qPCR assay was able to detect 17 nM ATP with a broad dynamic range from 50 nM to 5 mM. The assay is label-free, and real-time PCR-based detection of aptamer facilitates high sensitivity. The presented method is highly versatile and can be applied to various aptamer-target pairs to allow detection of a broad range of target analytes.

4.1.3. Introduction

Nucleic acid aptamers have come up with broad applicability in various fields ranging from detection of environmental pollutants to measurement of carcinogens and drug levels in blood [1]. They are the congeners of antibodies in terms of their binding properties to corresponding

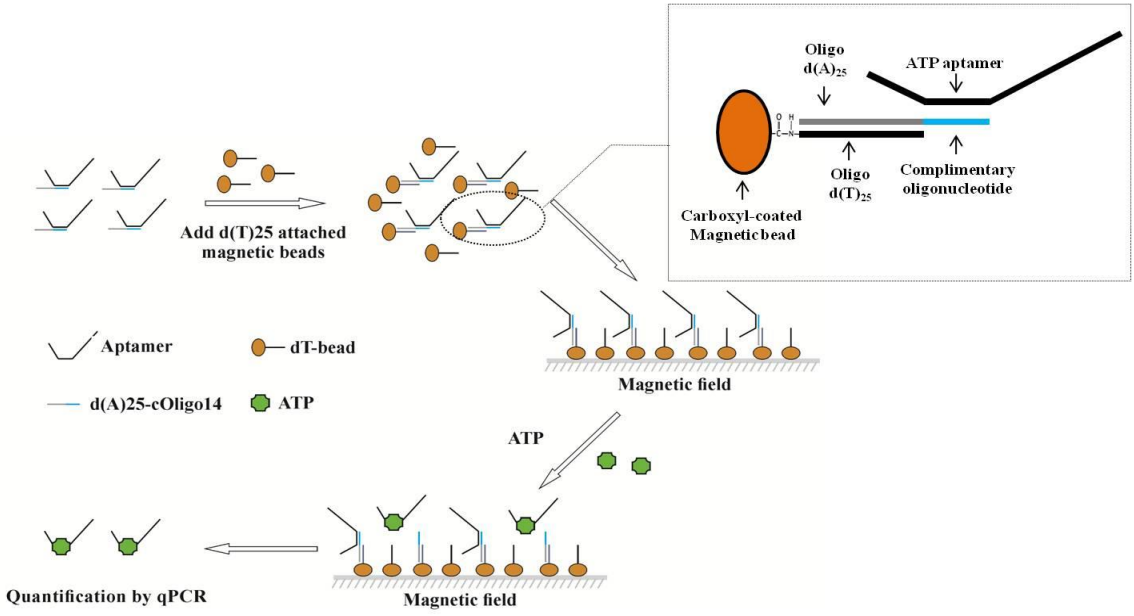
target molecules. While antibodies bind to the epitopes of the antigen, aptamers bind to the so-called aptatopes of respective target molecules [2]. Aptamers have been developed and studied as capture probes for many targets ranging from whole cells [3-5], proteins [6, 7], or small molecules like antibiotics [8, 9], dyes [10], metal ions [11], pesticides [12], toxins [13] and others. They can be easily modified and conjugated to solid matrices for affinity separation [7] or to transducer surfaces [8, 14, 15] to exploit their interaction with the target molecule for precise detection and quantification.

Small molecules are routinely quantified by hyphenated gas chromatographic (GC) techniques and high-performance liquid chromatography (HPLC) with UV and/or fluorescence detection [16, 17]. These techniques offer high sensitivity but require additional steps of extraction and clean-up processes which are time-consuming and costly. Simple and label-free methods, e.g. for the detection of small molecules (e.g., metal ions) [18], are scarce and limited to specific target analytes. Alternatively, small molecules can be detected using aptamers due to the conformational changes in aptamers during their binding to the target molecule. These conformational changes can be transduced to colorimetric [19, 20], fluorescence [21], mass [22], surface plasmon resonance [8], or electrochemical signals [23, 24]. Target-induced dissociation (TID) of oligonucleotides complementary to the target-binding site of the aptamer is another mechanism that can be exploited to convert the binding of a small molecule target into a detectable signal.

In TID, binding of the target induces dissociation of the complementary sequence from the aptamer [25]. Aptamers come with the advantage of consisting of an oligonucleotide sequence; thus they allow the straightforward design of complementary oligonucleotides for competitive assays and easy amplification via PCR to further enhance the sensitivity of the assay [26]. In the early 1990s, Cantor's group was the first to utilize the amplification potential of DNA in so-called immuno-PCR, where specific antibodies were labeled with double-stranded DNA, and the latter served as a template for qPCR following the formation of an immunocomplex [27]. The Apta-PCR represents a further advancement and simplification of immuno-PCR, where an aptamer molecule replaces the cumbersome antibody-DNA complex. Recently, the aptamer specificity has been combined with qPCR sensitivity in various approaches for the ultrasensitive detection of proteins, including a proximity ligation assay [28, 29], nuclease protection assay [30], capillary electrophoresis [31], and the use of target-modified magnetic microparticles [32, 33]. Up to now the combination of aptamers and qPCR was solely applied to the detection of

proteins, while the detection of small molecules has not been realized. To overcome this limitation, we used an aptamer directed against ATP as a model system to demonstrate the applicability of Apta-PCR for small molecule detection for the first time.

ATP is acknowledged as the mediator of energy exchanges that occur in all living cells and plays a critical role in the regulation of cellular metabolism and biochemical pathways in cell physiology [34]. The highly sensitive and selective detection of ATP is essential for biochemical studies as well as in clinical diagnosis [35, 36]. The DNA aptamer against ATP was originally created by Huizenga and Szostak through *in vitro* selection [37]. The aptamer was reported to bind ATP and adenosine monophosphate (AMP) [38], while no binding of cytidine triphosphate (CTP), guanosine triphosphate (GTP), and uridine triphosphate (UTP) was observed [39]. NMR studies revealed that the aptamer (G5 – A10, and G18 – A23) binds two ATP molecules (Figure S-1) [40].



Scheme 1. Schematic representation of ATP detection by magnetic bead-based aptamer-assisted qPCR (Apta-qPCR) assay.

So far, the combination of qPCR with aptamers has been limited to the detection of large biomolecules [41-45]. This study is the first to report on the application of Apta-qPCR for the detection of small molecules. We have recently used TID for the development of an aptamer microarray for sensitive detection of ethanolamine [25]. In this study, the TID strategy was applied to the ATP-binding aptamer (figure S-2). The complementary sequence was chosen to hybridize to the part of ATP aptamer which is directly involved in binding of the target (Table 1) [40]. In the absence of ATP, the ATP aptamer hybridizes with the complementary sequence,

which is immobilized on dT-modified beads (dT-beads). In the presence of ATP, the ATP aptamer is released from the dT-beads and quantified by qPCR. The Apta-qPCR assay is illustrated in Scheme 1. The sensitivity of the optimized Apta-qPCR assay for ATP was found to be 17 nM in cell lysates obtained from HeLa cells, and the dynamic range was 50 nM to 5 mM. The approach combines the specificity of aptamers with the sensitivity of qPCR, and by transfer of the method to other aptamers, detection and quantification of a wide range of target analytes could be feasible.

Table 1. List of sequences used in the work

Name of the sequence	Sequence*
ATP PCR Aptamer	5´GGAACACTATCCGA*CTGGCACC*A*CCTGGGGGAGTATT*GCGGAGGAAGGTCCTTGGGCATGTCTAGCGATCC3′
dA-cOligo11	3´A_{25}-TT*TGGACCCCCTC*-5´
dA-cOligo14	3´A_{25}-TT*TGGACCCCCTCATA*-5´
dA-cOligo17	3´A_{25}-TT*TGGTGGACCCCCTCATA*-5´
dA-cOligo20	3´A_{25}-TT*CGTGGTGGACCCCCTCATAA*-5´
dA-cOligo25	3´A_{25}-TT*GACCGTGGTGGACCCCCTCATAA*-5´
Random sequence	5′- NH_2-C_{12}-TGGACCCCCTC-3′
Forward primer	5′-GGAACACTATCCGACTGGCACC-3′
Reverse primer	5′-GGAACCCGTACAGATCGCTAGG-3′
dT	5′-NH_2-C_6-TTTTTTTTTTTTTTTTTTTTTTTTT-3′
Cy5-labeled ATP PCR aptamer	5´-Cy5-GGAACACTATCCGA*CTGGCACC*A*CCTGGGGGAGTATT*GCGGAGGAAGGTCCTTGGGCATGTCTAGCGATCC-3′

*Underlined bases correspond to primer binding regions. Italics bases indicate complementary sequences within the aptamer and oligonucleotides. dA means $d(A)_{25}$ and dT means $d(T)_{25}$. 2 T nucleotides were used as spacer between $d(A)_{25}$ region and complementary sequence. Justification of the sequences used in the assay is given in supplementary information (Figure S-2).

4.1.4. Experimental section

4.1.4.1. Chemicals and materials

All DNA oligonucleotides including 5'-amino modified DNA were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The sequences utilized in the study are shown in Table 1. The concentrations of oligonucleotides were determined using NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) with the corresponding extinction coefficients. Adenosine triphosphate (ATP), cytidine triphosphate (CTP), guanosine triphosphate (GTP), thymidine triphosphate (TTP), and Dynabeads® MyOne™ Carboxylic acid were all purchased from Life Technologies GmbH (Darmstadt, Germany). 2-(*N*-morpholino) ethane sulfonic acid (MES) was purchased from AppliChem GmbH (Darmstadt, Germany). Adenosine monophosphate (AMP), and 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC) were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). SYBR Green Real-Time PCR Master Mix was purchased from Promega GmbH (Mannheim, Germany). HeLa cells were purchased from German

Collection of Microorganisms and Cell Cultures (DSMZ) GmbH (Braunschweig, Germany). All chemicals were of analytical grade. All stock solutions and buffers were prepared with deionized water (arium 611, Sartorius AG, Göttingen, DE).

4.1.4.2. Preparation of dT-beads

Magnetic beads (7-12x10^9 beads mL^{-1}, Dynabeads® MyOne™ Carboxylic Acid, Invitrogen) of about 1.05 µm diameter with carboxyl groups on the surface were used. 100 µL suspension of magnetic beads (10 mg beads mL^{-1}) was dispensed to a micro-tube and washed three times with 500 µL MES buffer (25 mM MES, pH 4.5). The carboxyl groups on the magnetic beads were activated using 500 µL 50 mM EDC in MES buffer for 30 min. 100 µL 5'-amino modified dT (0.5 µM, 1 µM, 1.5 µM, 2.5 µM, 5 µM, 10 µM, and 20 µM) in MES buffer was added to the micro-tube after removing unreacted EDC. Immobilization was carried out by incubation of dT and magnetic beads at room temperature for 2 h with slow tilt rotation to prevent sedimentation of the magnetic beads. Magnetic beads were washed three times with 200 µL MES buffer to remove non-immobilized dT. In order to quench the non-reacted activated carboxylic acid groups on the magnetic beads, the magnetic beads were incubated with 200 µL 50 mM Tris pH 7.5 for 15 min. The dT-beads were washed twice with 200 µL of the aptamer selection buffer (20 mM Tris-HCl buffer containing 300 mM NaCl, 5 mM $MgCl_2$, pH 8.3) and then stored in 200 µL selection buffer at 4 °C. In order to confirm the immobilization of dT on the magnetic beads, all washing fractions were collected, and dT was quantified using NanoDrop ND 1000.

4.1.4.3. Hybridization of aptamer to dT-beads using complementary sequences

6.25 µL 100 nM aptamer was mixed with 6.25 µL 100 nM complementary oligonucleotides (dA-cOligo) (1:1; aptamer:dA-cOligos) in 6.25 µL selection buffer in a micro-tube. Different lengths of dA-cOligos (11, 14, 17, 20, and 25 nt) were investigated to improve the efficiency of TID mechanism. During incubation, the solution was heated up to 95 °C for 5 min and cooled to room temperature for 20 min. 6.25 µL dT-beads suspension (labeled with 2.72 pmol, 6.61 pmol, 10.23 pmol, and 16.96 pmol dT) was added to the reaction mixture at room temperature and incubated for 20 min with slow tilt rotation. The micro-tube was then placed under magnetic field using DynaMag™-2 Magnet for 5 min. The unbound aptamers, remaining in the supernatant, were removed by washing with selection buffer five times (20 min of incubation each time) and were quantified using qPCR. Magnetic beads without immobilized sequence, magnetic beads immobilized with a random sequence, and the reaction mixture without addition of dA-cOligo were used as negative controls.

4.1.4.4. qPCR detection of aptamer

1 µL aliquot of aptamer-containing solution (standards or sample fractions) was mixed with 12.5 µL of GoTaq® qPCR Master Mix (2X), 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer, and 10.5 µL of nuclease-free water to make a total volume of 25 µL. The final PCR mixture contained 200 nM forward primer, 200 nM reverse primer, and 1µL aliquot of aptamer in 1× GoTaq® qPCR Master Mix. qPCR was carried out in a 96-well PCR plate (Sarstedt) covered with strip caps. A melting curve analysis was performed from 55 °C to 85 °C to detect potential nonspecific product. The thermal cycling regime was as follows: initial denaturation for 2 min at 95 °C, cycling for 30 s at 95 °C, 15 s at 55 °C and 15 s at 72 °C, repeated 40 times on the Bio-Rad iCycler real-time PCR machine.

4.1.4.5. Detection of ATP using Apta-qPCR

6.25 µL 100 nM aptamer was mixed with 6.25 µL 100 nM 14 nt complementary sequence (dA-cOligo14) in 6.25 µL selection buffer in a micro-tube. During incubation, the solution was heated up to 95°C for 5 min and then cooled to room temperature for 20 min. 6.25 µL dT-beads suspension (labeled with 6.61 pmoldT) was added to the reaction mixture at room temperature and incubated for 20 min. The micro-tube was placed under magnetic field using DynaMag™-2 magnet for 5 min. The unbound aptamers were removed by washing twice with selection buffer (20 min each). After washing, 25 µL of selection buffer containing different concentrations of ATP were added to the micro-tube and incubated for 20 min. The aptamer released by TID was collected in the supernatant after magnetic separation of the beads and quantified using qPCR.

4.1.4.6. Microscale thermophoresis (MST) experiments

Binding studies were performed using microscale thermophoresis to characterise the binding between ATP aptamer and ATP in comparison to ATP aptamer and cOligo14. ATP aptamer was labeled with a Cy5-dye at the 5'-terminus. The concentration of fluorescently labeled aptamer was kept constant at 30 nM in selection buffer in all MST experiments. To check the binding of ATP, the concentration of ATP was varied by serial dilution (50 µM to 1.52 nM). In case of cOligo14, the concentration of cOligo14 was varied from 25 µM to 1.52 nM. Before incubation, the aptamer was heated to 95 °C for 5 min and cooled to room temperature for 20 min prior to addition of the ligand. After mixing aptamer and the respective ligand, solutions were incubated for 20 min at room temperature. The measurements were carried out using Nanotemper monolith NT.115 (Nanotemper Technology GmbH, Munich, Germany) at 40% MST power at room temperature. For data analysis, NT.Analysis software (Nanotemper Technology

GmbH, Munich, Germany) was used. The curves were fitted using the K_d model of theNT.Analysis Software and the graphs were drawn afterwards with Origin 7G (OriginLab Corporation, Northampton,MA, USA).

4.1.4.7. ATP detection in biological samples using Apta-qPCR and standard ATP assay

HeLa cells were grown in DMEM medium supplemented with 10% FCS, 1% penicillin and 1% streptomycin in a culture flask. Cells were routinely cultured in a humidified 5% CO_2 incubator at 37°C to approximately 70% confluency. The cells were harvested by trypsin treatment for 2 min at 37°C and washed twice by centrifugation (5740 g, 5 min) in selection buffer and then stored in the same buffer at 4°C. Cell lysis (approximately 100,000 cells mL^{-1}) was performed by ultrasonic cell crusher (T 10 basic ULTRA-TURRAX®, IKA, Staufen, Germany) for 30 min on ice, and the lysed cells were ready for ATP assays. The Apta-qPCR ATP detection protocol was the same as stated above. For comparison, a commercial assay (CellTiter-Glo® Assay, Promega) for ATP quantification was used according to the instructions supplied by the manufacturer. We have observed a lack of ATP in cell lysates stored for 24 h. Different concentrations of ATP were spiked to the cell lysate stored for 24 h to obtain biological samples with defined ATP concentrations and quantification of ATP in cell lysates was compared using both assays.

4.1.5. Results and discussion

4.1.5.1 Principle of Apta-qPCR assay

The principle of Apta-qPCR assay is depicted in scheme 1. The envisaged assay relies on target-induced dissociation of ATP aptamer from a complementary oligonucleotide. The complementary oligonucleotide was designed to bind to the target-binding portion of the aptamer and additionally contains a dA tail to ensure hybridization with dT immobilized on magnetic beads. Addition of ATP results in dissociation of the aptamer from the complementary oligonucleotide, and thus release of the aptamer from the magnetic beads. The magnetic beads were separated with a magnetic stand and the dissociated aptamer present in the supernatant was quantified by qPCR.

In the following sections, the development and optimization of the Apta-qPCR are described, and finally the assay ischaracterized concerning sensitivity, specificity, and applicability to complex biological samples.

4.1.5.2. Preparation of dT-beads

In the developed assay, dT was immobilized on the magnetic beads to capture free aptamers from the solution. One-step EDC-coupling was used for immobilization. Seven different

concentrations of dT (50 pmole mg^{-1}, 100 pmole mg^{-1}, 150 pmole mg^{-1}, 250 pmole mg^{-1}, 500 pmole mg^{-1}, 1000 pmole mg^{-1}, 2000 pmole mg^{-1} magnetic beads) were used for the immobilization. Using higher concentrations of dT resulted in higher immobilization densities, as depicted in Figure 1. Yet, a decrease in the conjugation efficiency was observed at higher dT concentrations (Figure 1). Non-specific adsorption of DNA probes on the magnetic beads needs to be prevented as this can reduce the hybridization efficiency of dT [46]. From above mentioned different concentrations of dT; magnetic beads modified with 100 pmole mg^{-1} dT (87.1 pmole mg^{-1} immobilized), 250 pmole mg^{-1} dT (211.5 pmole mg^{-1} immobilized), 500 pmole mg^{-1} dT (327.4 pmole mg^{-1} immobilized), and 1000 pmole mg^{-1} dT (542.6 pmole mg^{-1} immobilized) were used for the optimization of aptamer release in later experiments.

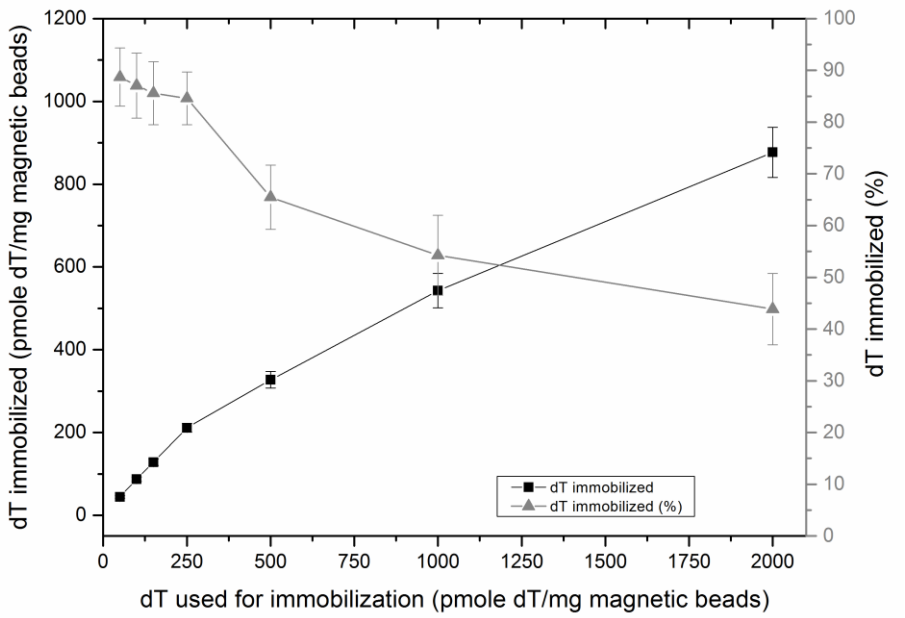


Fig. 1. Immobilization of amino-modified dT on carboxyl magnetic beads using EDC-coupling. Y-axis 1 shows amount of immobilized dT in respect to amount of dT used for immobilization; Y-axis 2 shows percentage of immobilized dT in respect to amount of dT used for immobilization.

4.1.5.3. Optimization of aptamer release using dT-beads

During the Apta-qPCR assay, the binding of the aptamer to dT-beads occurs through an indirect hybridization process mediated by a complementary oligonucleotide (Scheme 1, Figure S-2). The complementary oligonucleotides were designed to bind the portion of the ATP aptamer where two ATP molecules can bind [40]. $d(A)_{25}$ (dA) was added to 3'-terminus of complementary oligonucleotide to immobilize complementary oligonucleotides on dT-beads, and there are two

thymidine (T) nucleotides as a small spacer between dA and complementary sequence (Table 1, Figure S-2).

Here, two subsequent hybridization processes (hybridization between an aptamer and a dA-cOligo and hybridization between a dA-cOligo and the dT-beads) needed to be optimized for the assay. Different densities of dT on magnetic beads were incubated with 1:1 ratio of the aptamer to the dA-cOligo to get an optimal ratio of aptamer:dA-cOligo:dT for hybridization and TID of the aptamer from the complementary oligonucleotide in the presence of ATP. Here, the effects of the length of the complementary oligonucleotides (11, 14, 17, 20, and 25 nt) on the hybridization were studied, and the results are presented in Figure 2.

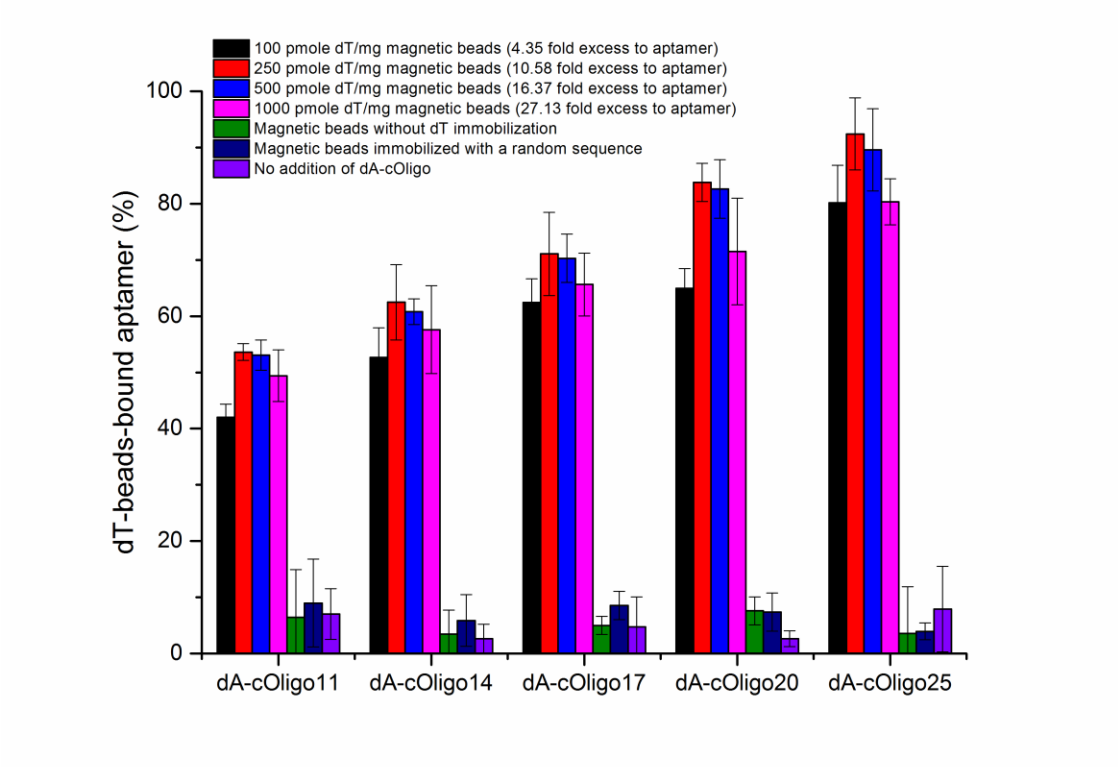


Fig. 2. Optimization of aptamer-hybridization using dT-beads. Aptamer and dA-cOligo were used in 1:1 ratio. dT, immobilized on the magnetic beads, was used in different ratios compared to aptamer. Beads with no immobilized dT (-dT), magnetic beads immobilized with a random sequence and the reaction mixture without addition of dA-cOligo (-dA-cOligo) were used as negative controls.

The higher density of dT resulted in fewer hybridization events. The maximum hybridization events were observed at the ratio of 1:1:10.58 (aptamer:dA-cOligo:dT). This observation can be ascribed to electrostatic repulsion caused by the dense dT decoration on the magnetic beads resulting in reduced interaction of the dA-tail of dA-cOligo with dT modified beads [47]. In addition, the effect of the length of the complementary sequence and the ratio of aptamer:dA-cOligo:dT were studied conjointly. More hybridization of aptamer was observed in the case of

dA-cOligo with longer complementary sequences. In the case of dA-cOligo25, 92.39% of aptamer can hybridize with dT-beads (Ratio of aptamer:dA-cOligo:dT = 1:1:10.58) (Figure 2).

4.1.5.4. Detection of ATP using Apta-qPCR

Here, dT was used as a spacer between the magnetic beads surface and aptamer-binding sites. Earlier the complementary oligonucleotide was directly immobilized on the magnetic beads for Apta-qPCR assay, and it was possible to hybridize aptamers to the oligonucleotide, but ATP measurement was not reproducible (data not shown here). This can be explained by steric hindrance due to the close proximity of magnetic bead surface. When ATP binds to the aptamer, the hybridization between the aptamer and the dA-cOligo is disturbed. This, in turn, causes the release of the aptamer from the dT-beads. Since the separation of aptamer and complementary sequences due to ATP binding also depends on the length of the complementary sequence, five complementary oligonucleotides of different length (11, 14, 17, 20, and 25 nt) were used in this assay. The complementary region was chosen based on published ATP binding sites [37, 40, 41, 43]. ATP detection was found to be optimal with 14 nt complementary sequence. When longer complementary oligonucleotides were used, the TID induced by ATP addition was significantly lower (Figure S-3). Also, for the 11 nt complementary sequence, hybridization was less efficient in comparison to 14 nt complementary sequence (Figure 2). We found that at a molar ratio of 1:1:10.58 aptamer:dA-cOligo:dT, 62% aptamer were bound to dT-beads which allowed optimal detection of ATP.

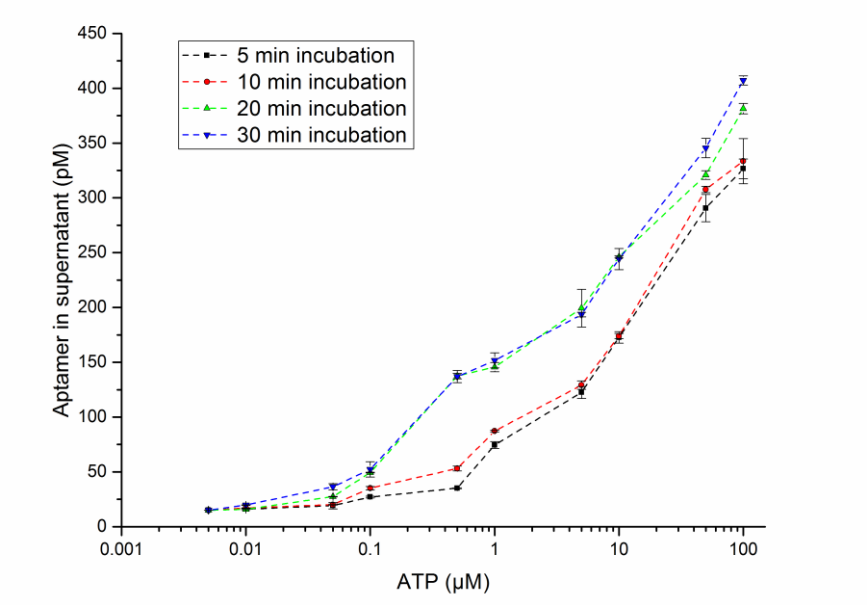


Fig. 3. Effect of incubation time. Both incubation steps in the assay (incubation of aptamer with dA-cOligo and incubation of dT-beads with the aptamer/dA-coligo complex) were optimized for ATP detection.

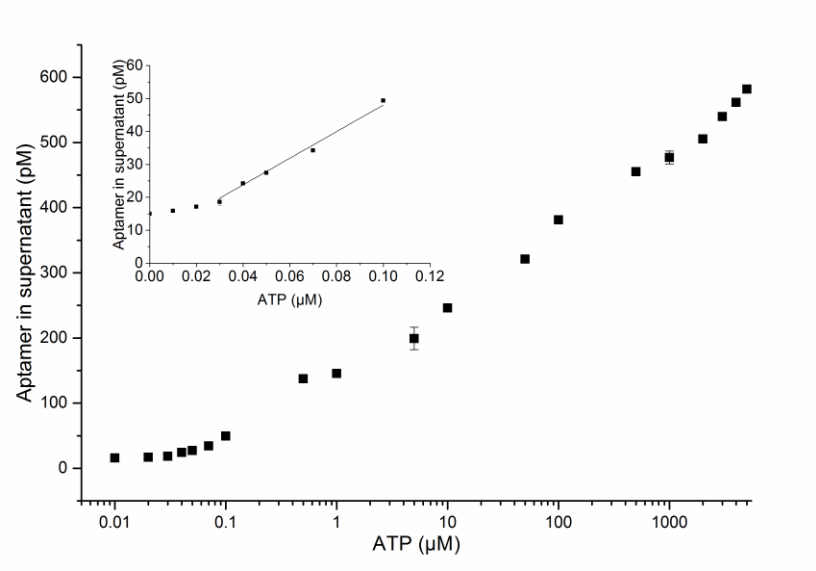


Fig. 4. ATP measurement using Apta-qPCR assay. The dissociation of aptamer was induced with different concentrations of ATP. Inset: The LOD for the Apta-qPCR assay was found to be 13 nM (Equation: y=7.63 + 403.94x; R^2 value: 0.993). The error bars represent the standard deviations based on three independent measurements.

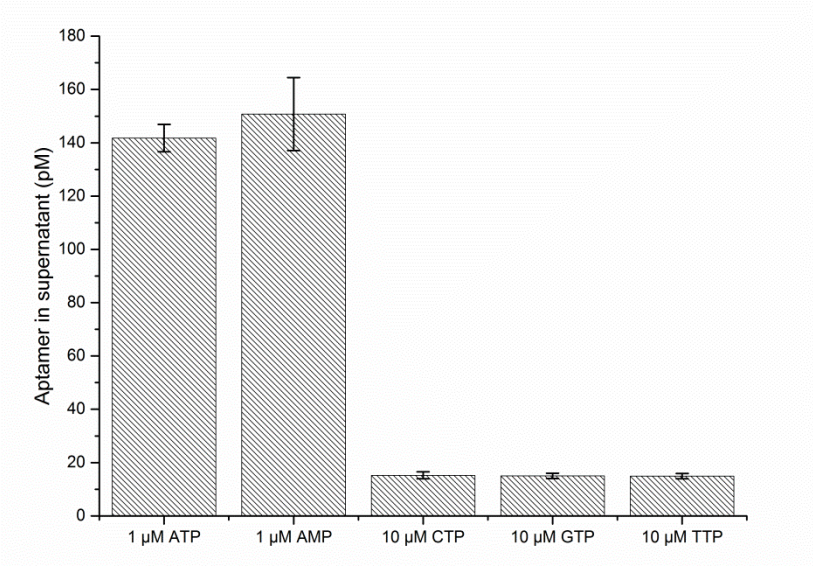


Fig. 5. The specificity of Apta-qPCR assay. The dissociation of aptamer from magnetic beads was observed for ATP and AMP (1 μM); CTP, GTP, and TTP (each 10 μM, 10-fold of ATP). The error bars represent the standard deviations based on three independent measurements.

To evaluate the stability of the aptamer:dA-cOligo:dT-beads complex, the complex was washed five times with the selection buffer, and the presence of aptamer in each washing step was checked with qPCR. It was found that the first washing step is the most crucial. In the subsequent washing steps, only minor amounts of aptamer were measured, demonstrating stable binding of the aptamer:dA-cOligo:dT-beads complex (Figure S-4A, B, and C). For

quantification of ATP using the assay, ATP was added after the second washing step. To accelerate the detection of ATP, the effect of incubation time on both incubation steps (incubation of aptamer with dA-cOligo and incubation of dT-beads with the aptamer/dA-cOligo complex) was investigated. The data is presented in Figure 3, revealing theincubation time of 20 min is optimal. Although the incubation time can be reduced to 5 min, this affects the sensitivity of ATP measurement. For example, concentrations lower than 500 nM ATP could not be detected with incubation times shorter than 20 min. This observation can be attributed to the initial competitive interactions between ATP and dA-cOligo to bind with the aptamer until a 'pseudo-equilibrium' is attained.

Figure 4 reveals that the release of aptamer increases with higher ATP concentrations from 50 nM to 5 mM. The correlation coefficient was found to be 0.993 between the removal of the aptamer from the dT-beads and logarithm of the ATP concentrations over the range of 50 nM to 5 mM (Figure 4). The saturation for the ATP measurement was reached above 5 mM (Figure S-5).

The limit of detection (LOD) for ATP measurement was found to be 13 nM based on 3σ/slope, where σ is the standard deviation of the blanksamplesof three Apta-qPCR assays (Figure 4) [48]. The specificity of the assay was examined by comparison of its response on 1 µM ATP and AMP and 10 µM of other related small molecules including CTP, GTP, and TTP. As expected due to the known cross-reactivity of the used aptamer to AMP, ATP and AMP resulted in a release of the aptamer [38]. The release of the aptamer from the dT-beads in the presence of other small molecules (CTP, GTP, and TTP) was much lower (Figure 5).

4.1.5.5. Binding studies of aptamer to ATP and cOligo14

To further investigate the ATP-induced dissociation of cOligo14 from the aptamer, binding studies of ATP and cOligo14 to the aptamer were performed using microscale thermophoresis (MST). As can be seen in Figure S-6, the binding constant of cOligo14 (90 nM) to the aptamer was lower than the binding constant of ATP (1.2 µM). Interestingly, the binding of ATP to aptamer resulted in strong change in signal (signal amplitude of 70) in comparison to the binding of cOligo14 (signal amplitude of 50). This observation suggests large structural changes in aptamer upon binding of ATP. Despite the rather poor affinity of ATP to the aptamer, ATP can replace cOligo14 from the aptamer [40, 43]. Here, we suspect a three-way equilibrium between ATP, cOligo14 and ATP aptamer for ATP-induced dissociation of cOligo14 from the aptamer.

4.1.5.6. ATP detection in biological samples

The applicability of the Apta-qPCR assay to quantify ATP in complex samples was investigated by detection of cellular ATP in HeLa cells. Comparative experiments were performed using commercially available CellTiter-Glo® Assay. The developed assay was able to detect ATP and AMP in freshly lysed cells (62 cells) (876 nM with Apta-qPCR assay and 902 nM with CellTiter-Glo® Assay). No ATP was found in cell lysates stored for 24 h after lysis. For the characterization of Apta-qPCR assay performance in the cell lysate, different concentrations of ATP were spiked to the cell lysates stored for 24 h. The correlation coefficient was found to be 0.964 within the range of 50 nM to 5 mM ATP and a LOD of 17 nM was achieved which is similar to that in selection buffer (Figure 4, 6 and S-7).

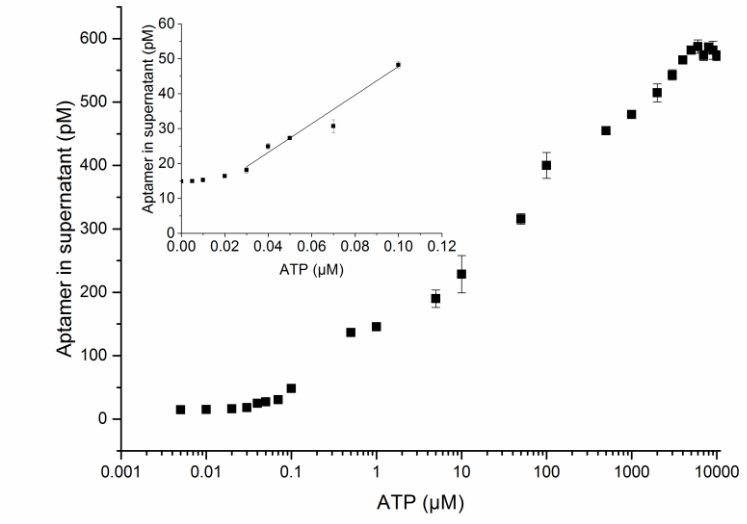


Fig. 6. ATP measurement in cell lysate using Apta-qPCR assay. Inset: The LOD for the Apta-qPCR assay was found to be 17 nM (Equation: y=6.73 + 410.70x; R^2 value: 0.964). The error bars represent the standard deviations based on three independent measurements.

Table 2. Recovery of ATP in cell lysate

	Added	Apta-qPCR assay		CellTiter-Glo® Assay	
Sample	ATP (µM)	Concentration (µM)	Recovery (%)	Concentration (µM)	Recovery (%)
1	0.1	0.097	97	0.005	5
2	0.5	0.477	95	0.527	105
3	1	1.042	104	1.062	106
4	2.5	2.611	104	2.565	103
5	5	5.398	108	5.044	101
6	7.5	7.890	105	7.492	100
7	10	10.757	108	9.977	100

The measurement using the developed assay was compared with CellTiter-Glo® Assay by the addition of previously known concentrations of ATP to the cell lysates stored for 24 h. The measurements were repeated three times and the recovery with both assays was 95–108% of ATP in the range of 0.5 µM to 10 µM. The CellTiter-Glo® Assay was not able to detect 0.1 µM ATP whereas Apta-qPCR assay was able to detect 0.1 µM ATP (Table 2). This demonstrates the applicability of the developed assay for ATP detection in complex samples and indicates its superiority especially for low concentrations of ATP. Nonetheless, while in the present study cell lysate was free of AMP (due to storage for 24 h) [39], fresh lysates will contain ATP and AMP. In this case the aptamers cross-reactivity towards AMP will hinder selective detection of ATP. Nonetheless, the Apta-qPCR allows for sensitive detection of ATP in the absence of AMP. Moreover, since ATP aptamer was used as a model system, this study provides a way to develop Apta-qPCR assays to quantify other small molecules by using corresponding aptamers. A detailed comparison of the Apta-qPCR with other recent aptamer-based methods for the detection of ATP is given in Table S-1.

4.1.6. Conclusions

In this work, the capability of an aptamer to bind specifically to its target has been combined with its potential for PCR-based amplification for the detection of a small molecule. The design of the Apta-qPCR assay is based on target-induced dissociation of a complementary oligonucleotide from the aptamer. Therefore, the assay avoids the need of labeling and covalent immobilization of the aptamer. dT-modified magnetic beads were used to separate ATP-bound aptamer from unbound aptamers and, after separation; the ATP-bound aptamers were quantified using qPCR. The sensitivity of this method was found to be 13 nM ATP with a wide dynamic range from 50 nM to 5 mM. Additionally, the assay requires small sample volumes (6.25 µL) and 2.5 h for the detection of ATP. Although the used aptamer was able to detect ATP and AMP, the specificity of the assay was confirmed using similar molecules (CTP, GTP, and TTP). The successful detection of ATP in HeLa cell lysate with LOD of 17 nM demonstrates the potential of Apta-qPCR for quantification of target molecules in biological samples. Additionally, the Apta-qPCR was compared with CellTiter-Glo® assay for the detection of ATP in cell lysate using defined ATP concentrations. Both assays were able to recover 95-108% of ATP in the range of 0.5 µM to 10 µM. The Apta-qPCR shown here can be easily extended for detection of other molecules as long as a specific aptamer is available.

4.1.8. Acknowledgements

The German Research Foundation (DFG - SCHE 279/32-1) supported parts of this work. German Academic Exchange Service (DAAD) is acknowledged for the financial support to Harshvardhan Modh. We thank Didem Ag Seleci (Institute of Technical Chemistry, Leibniz University of Hannover) for providing HeLa cells.

4.1.9. Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at...

4.1.10. References

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4.1.11. Supplementary Information – Aptamer-based detection of adenosine triphosphate via qPCR

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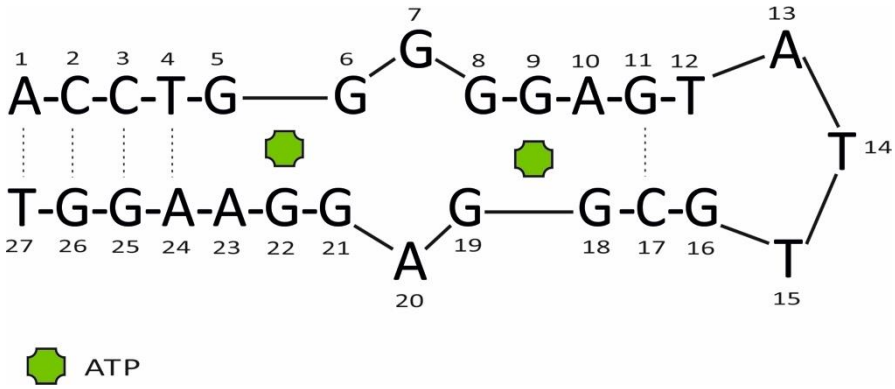

Figure S-1: ATP binding to ATP aptamer (adapted from Huizenga et al.[1])

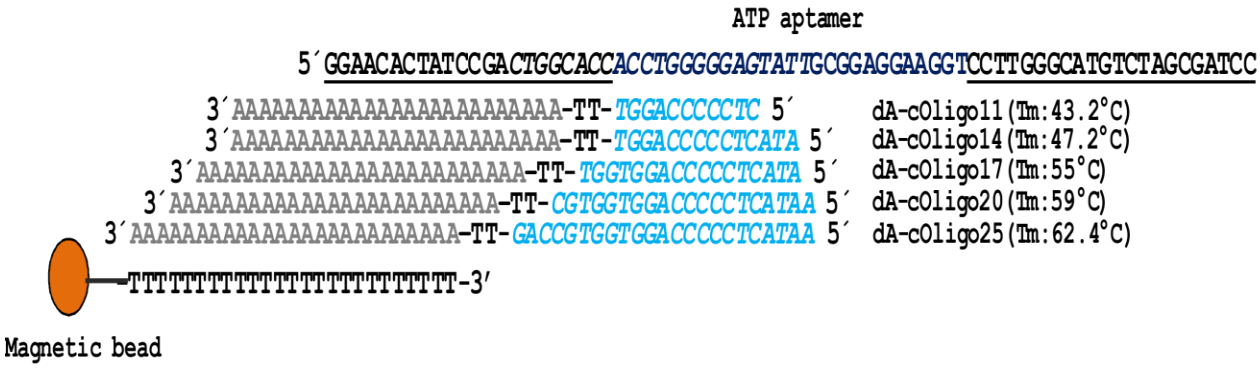

Figure S-2: Justification of the sequences used in the Apta-qPCR assay. Different lengths of complementary sequences were used to optimize the target-induced dissociation of ATP aptamer. The bases in blue are complementary to the ATP aptamer. 2 T nucleotides were used as a small-spacer between $d(A)_{25}$ and complementary sequence. Here, dT-beads were used in order to avoid the interference of magnetic beads with ATP-induced dissociation of complementary sequence. Melting temperature analysis suggested the ATP-induced dissociation is better with short complementary sequences.

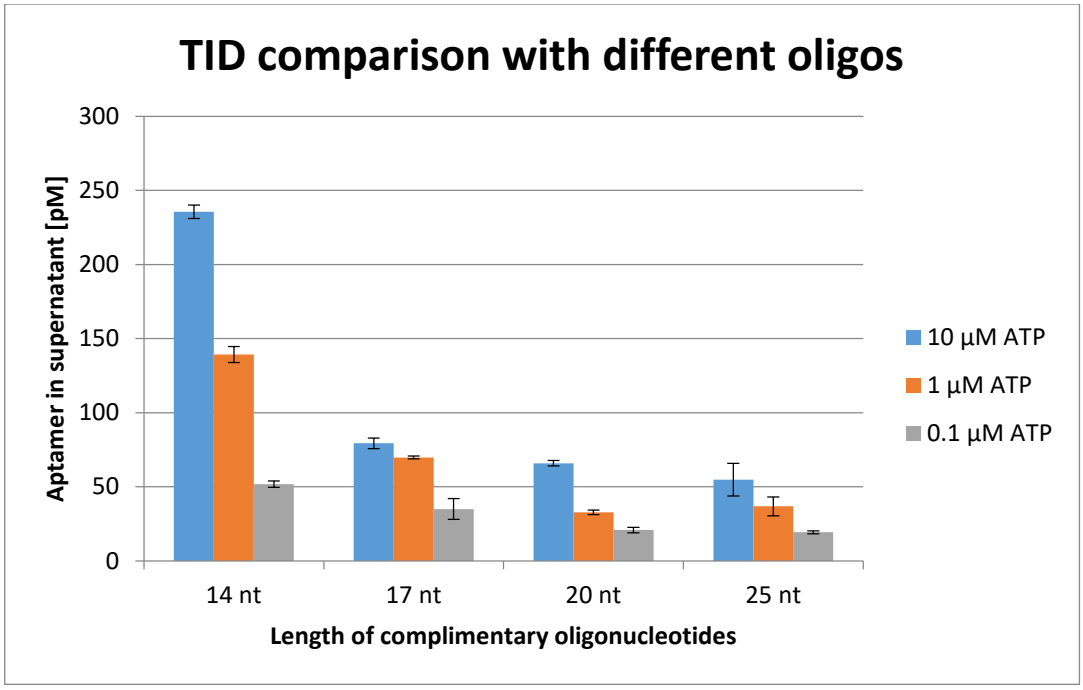

Figure S-3: Comparison of target-induced dissociation (TID) due to ATP addition with different lengths of complementary sequences. In case of 11 nt long complementary sequence, aptamer hybridization efficiency with dT-beads was too low (~50%). This hybridization efficiency will not allow detection of low ATP concentration.

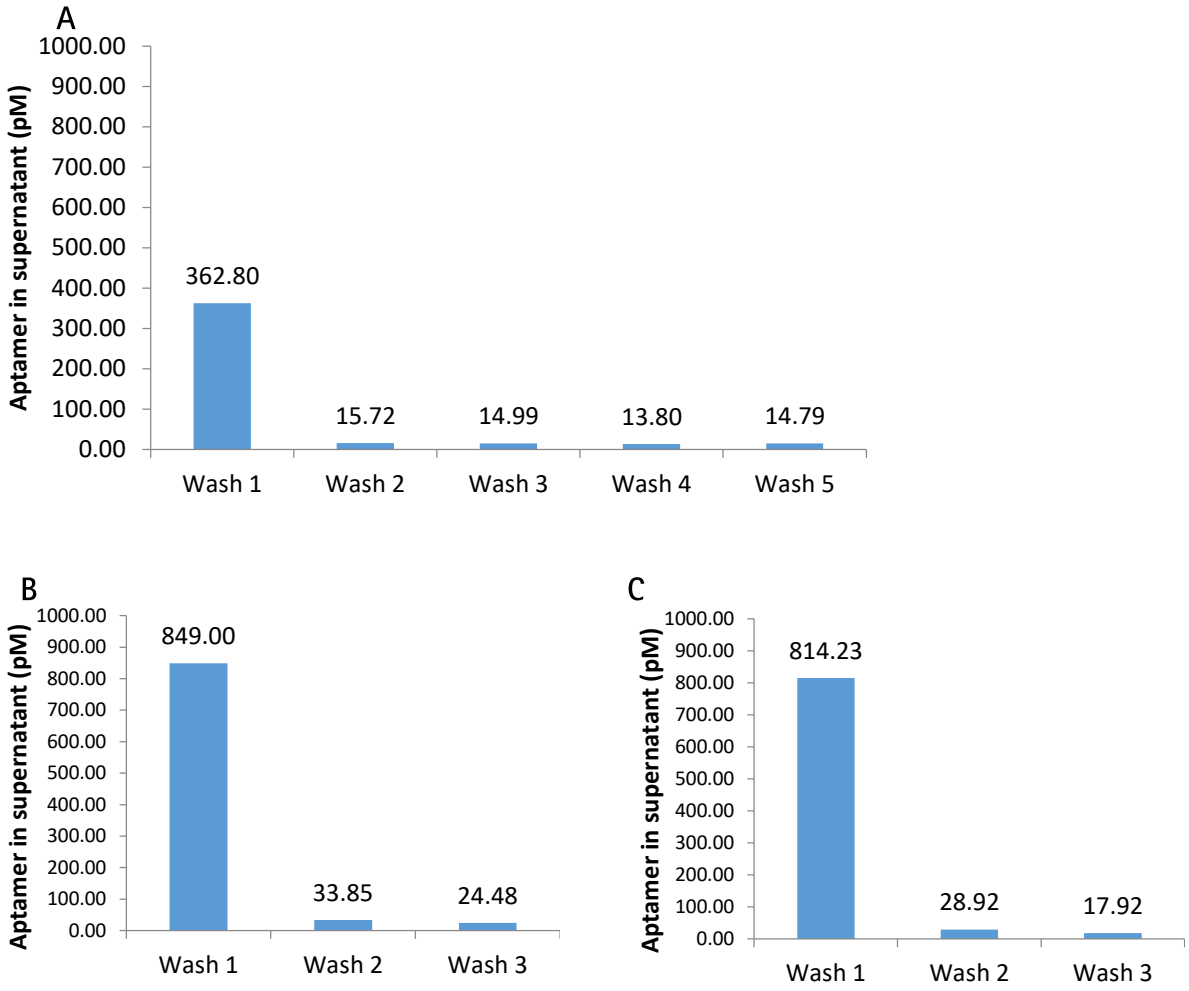

Figure S-4: Removal of aptamer in the washing steps using selection buffer. Here, 1000 pM of aptamer was used as initial concentration. (A) Aptamer:dA-cOligo14:dT-beads complex was washed with selection buffer 5 times (ratio: 1:1:10.58). Presence of aptamer in each supernatant was checked by qPCR. (B) Non-specific binding of aptamer to magnetic beads was checked. Magnetic beads (without dT immobilization) were incubated with aptamer/dA-cOligo14 complex. The washing solutions using S-buffer were checked for the presence of aptamer. (C) Non-specific binding of aptamer to dT-beads was checked. dT-beads were incubated with aptamer (without dA-cOligo). The washing solutions using S-buffer were checked for the presence of aptamer.

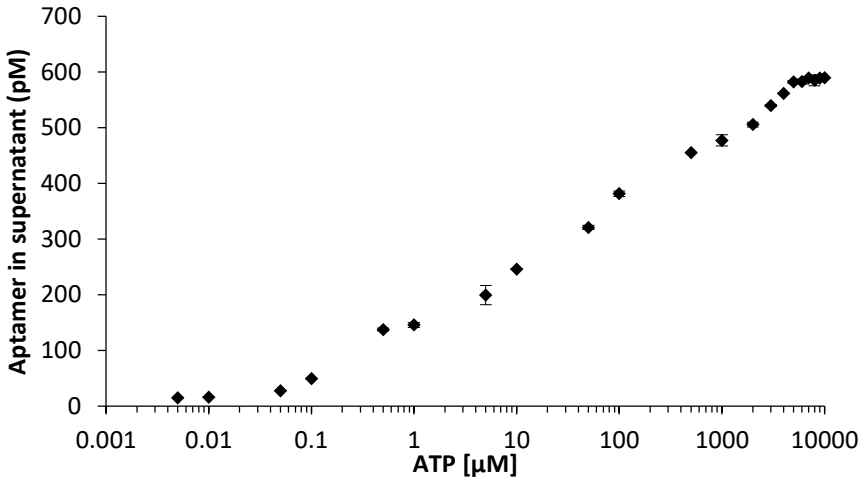

Figure S-5: ATP measurement by magnetic bead-based Apta-qPCR assay. The dissociation of aptamer was induced with different concentrations of ATP. The error bars represent the standard deviation of three independent measurements. Apta-qPCR was able to detect ATP with a wide dynamic range from 50 nM to 5 mM.

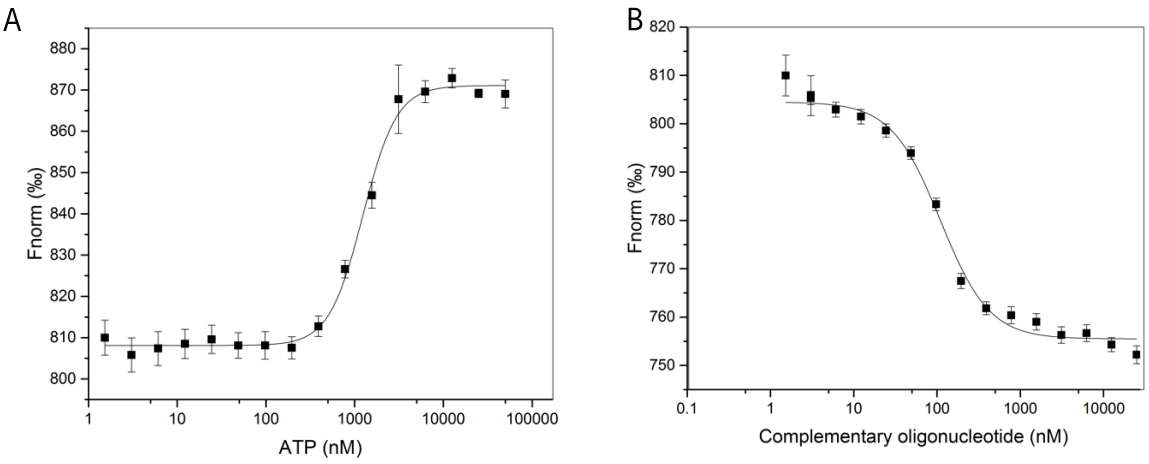

Figure S-6: Binding studies of aptamer to (A) ATP and (B) complementary oligonucleotides (cOligo14) using microscale thermophoresis (MST).

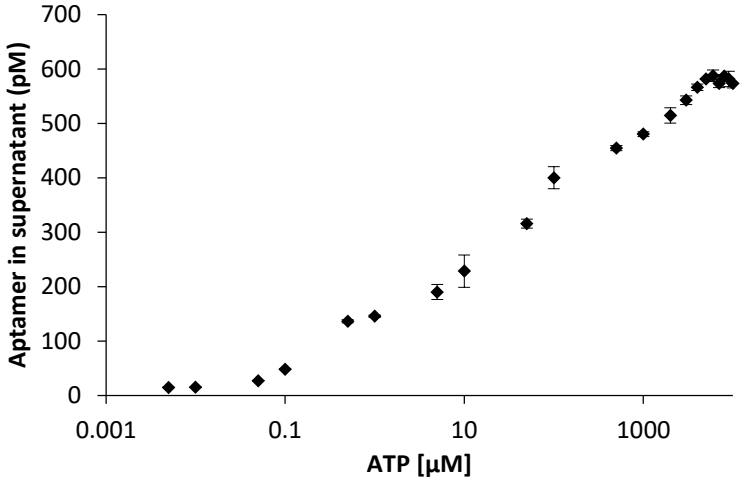

Figure S-7: ATP measurement in cell lysate by magnetic bead-based Apta-qPCR assay. The error bars represent the standard deviation of three independent measurements. Apta-qPCR was able to detect ATP with a wide dynamic range from 50 nM to 5 mM.

Table S-3: Comparison of recent methods used for ATP detection

Detection method	Signal-generation mode	Linear range	LOD	Ref.
Turn-off fluorescence-based aptasensor	Aptamer-triggered dsDNA concatamers	0 - 5000 μM	6.1 μM	[2]
Turn-on fluorescence-based aptasensor	Aggregation-induces emission-based fluorescence probe	0.1–1.0 mM	24 μM	[3]
Fluorescence-based aptasensor	single-stranded DNA adsorption on graphene oxide	3–320 μM	450 nM	[4]
Fluorescence-based aptasensor	Nucleic acid stain PicoGreen	100 nM– 5 μM	14.2 nM	[5]
Fluorescence-based aptasensor	Nucleic acid stain SYBR Green I	100 nM – 10 mM	23 nM	[6]
Electrochemical aptasensor	Ferrocene-aptamer conjugate	0.5–500 nM	0.32 nM	[7]
Electrochemical aptasensor	Dual-signaling strategy using ferrocene and methylene blue	10 nM–100μM	2.1 nM	[8]
Electrochemical aptasensor	Toehold-mediated strand displacement reaction	5 nM- 10 μM	5 nM	[9]
Isothermal amplified detection	DNA molecular gate	50 nM – 1 μM	10 nM	[10]
Chemiluminescence resonance energy transfer	Structure-switching	1 - 10 μM	100 nM	[11]
Fluorescence-based aptasensor	Apta-qPCR	50 nM – 5 mM	13 nM	*Our method**

*Apta-qPCR was able to detect ATP with the sensitivity of 13 nM which is comparable to available methods. With Apta-qPCR, width of dynamic range has been broadened.

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Chapter 2

Detection of Ochratoxin A using Apta-qPCR

4.2. Detection of ochratoxin A using Apta-qPCR

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4.2.1. Summary

In the previous work, ATP was used as a model molecule for the development and optimization of Apta-qPCR. To extend the applications of Apta-qPCR, it was transferred here to detect a food toxin, ochratoxin A (OTA).

OTA is most-abundant mycotoxin and can be present in a variety of crops, including final food products such as grain, pork products, coffee, wine and beer. OTA is highly nephrotoxic and is suspected to be the main etiological agent responsible for human Balkan endemic nephropathy and associated urinary tract tumors. According to International Agency for Research on Cancer (IARC), OTA is a "possible carcinogen to humans" (group 2B). Considering these adverse effects, regulation of OTA content in food products is regulated by international and governmental agencies. The European Commission Regulation (EC) has established a maximum level of OTA at 5 mg/kg for raw cereal grains, 3 mg/kg for all cereal-derived products, and 10 mg/kg for soluble coffee. In addition, OTA in grape juices, wines should be less than 2 ng/mL. Though the concentration limits are high, OTA is highly stable in different conditions and cannot be metabolised in animal or human body. It gets accumulated over a longer exposure in body. It is very important to develop highly sensitive and rapid detection platform for the detection of OTA.

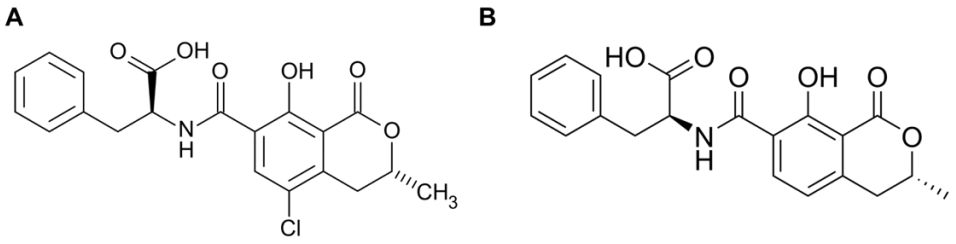

Figure 4.2. Chemical structure of **(A)** ochratoxin A and **(B)** ochratoxin B

Aptamers against OTA were developed by Cruz Aguado *et al.* in 2008. The aptamer used in this work is highly sensitive and specific for the detection of OTA. The K_d value of the aptamer was checked using MST experiments and it was found to be 13 ng/mL (32 nM) in aptamer selection buffer and 98.4 ng/mL (242.2 nM) in OTA-spiked Herrenhäuser beer samples. The selectivity of

the aptamer was confirmed using ochratoxin B which is different from ochratoxin A at a single site (Figure 4.2.).

In this work, Apta-qPCR was used to detect OTA in beer. This assay is highly sensitive with the detection limit of 0.009 ng/mL (0.022 nM) and, also provides broad linear range of detection from 0.039–1000 ng/mL (0.1-2500 nM). The total assay procedure requires 2.5 h to get the results. Additionally, the assay needs low sample volumes (6.25 µL) and the detection of OTA in one sample costs approximately €0.70 based on the costs of chemicals and biomolecules used in the assay [27].

Research Article

Article - Detection of Ochratoxin A by aptamer-assisted real-time PCR-based assay (Apta-qPCR)

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Keywords: Apta-qPCR, Aptamer, Food toxin, Ochratoxin A, Target-induced dissociation

Abbreviations: Apta-qPCR, aptamer-assisted real-time PCR-based assay; **OTA,** Ochratoxin A; **dA-cOligo,** $d(A)_{25}$-complementary oligonucleotide; **dT,** $d(T)_{25}$; **dT beads,** dT-modified magnetic microparticles; **Apta-beads complex,** Aptamer:dA-cOligo:dT beads

4.2.2. Practical application

Ochratoxin A is a prominent mycotoxin, ubiquitously present in a variety of crops. There is an urgent need to develop a robust, rapid and highly sensitive method for Ochratoxin detection. Aptamers developed against Ochratoxin A have been successfully used in various applications. In addition, recent applications of various aptamers show the potential to substitute antibodies due to its reduced production cost and high stability. In this study, an aptamer-assisted real-time PCR based assay (Apta-qPCR) is realized for the detection of Ochratoxin A following target-induced dissociation from complementary oligonucleotides. Apta-qPCR assay features sensitive, robust, and selective detection of Ochratoxin A and has potential to be used in complex samples. Moreover, the described assay can be easily transferred to the detection of other small molecules with corresponding aptamers.

4.2.3. Abstract

Detection of food toxins with high sensitivity is very important and challenging. Ochratoxin A (OTA) is frequently present as food contaminant in contaminated grains and grain derivatives like bread and beer. In this work, a target-induced dissociation (TID)-based aptamer-assisted real-time PCR based assay (apta-qPCR) is developed that features effective detection of OTA. Apta-qPCR effectively combines the capabilities of aptamer to be amplified, being a nucleotide sequence, with its specific interaction with the corresponding target molecule. Compared to commonly used fluorescence-based and colorimetric methods, the sensitivity of qPCR to detect a nucleotide sequence (aptamer) has ameliorated the sensitivity of the aptamer-based detection of OTA. Here, the OTA aptamer was immobilized on the magnetic beads coated with $dT_{(25)}$ (dT beads). A sequence complementary to the OTA-binding portion of the aptamer was used as a linker between dT beads and the aptamer sequence. When OTA was added, the aptamer was released from the dT beads due to target-induced dissociation (TID). The resulting assay was able to detect 0.009 ng/mL OTA with a wide dynamic range of 0.039 to 1000 ng/mL. Apta-qPCR can be easily transferred to other small molecules for highly sensitive detection using corresponding aptamers.

4.2.4. Introduction

Food safety for humans and animals is a global health objective, and foodborne diseases caused by the consumption of contaminated food represents a major health risk [1]. Mycotoxins are biologically active fungal secondary metabolites often found in a variety of crops, including commodities largely consumed by humans and animals [2, 3]. Ochratoxin A (OTA) is a ubiquitous mycotoxin produced by Aspergillus and Penicillium genera, in particular by *Aspergillus ochraceus* and *Penicillium viridicatum*. OTA is highly nephrotoxic and is suspected to be the main etiological agent responsible for human Balkan endemic nephropathy (BEN) and associated urinary tract tumors [4, 5]. According to International Agency for Research on Cancer (IARC), OTA is a 'possible carcinogen to humans' (group 2B) [6]. Considering these adverse effects, regulation of OTA content in food products is regulated by International and governmental agencies. The European Commission Regulation (EC) has established a maximum level of OTA at 5 mg/kg for raw cereal grains, 3 mg/kg for all cereal-derived products, and 10 mg/kg for soluble coffee. In addition, OTA in grape juices, wines (red, white and rose) should be less than 2 ng/mL [7, 8].

The design of highly sensitive, more convenient, rapid and robust assays for the detection of OTA in food products and serum are in great demand. Standardized methods for OTA detection include chromatographic methods where thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), or gas chromatography (GC) coupled to fluorescence detection [9, 10]. During the last decade, various assays including enzyme-linked immunosorbent assay (ELISA), lateral flow immunoassay, flow-through immunoassay, surface plasmon resonance (SPR) assays, and electrochemical immunosensors have been developed for the detection of OTA in order to improve the detection [11]. However, antibody-based detection systems are hindered by the time required for antibody preparation, the effect of modification on antibodies, solvent effects and the thermal instability of antibodies [12]. Multiple strategies have been used to overcome the drawbacks of antibody-based detection systems including molecular imprinted polymers (MIPs) [13], aptamers [14, 15] and phage display libraries [16]. Among these, DNA aptamers have been widely and successfully used for the detection of small molecules including OTA [17].

DNA aptamers are single-stranded oligonucleotides selected *in vitro* from combinatorial oligonucleotide libraries by the systematic evolution of ligands by the exponential enrichment (SELEX) process. Aptamers are highly specific to the target molecule and high-purity aptamers can be synthesized chemically at low cost. Aptamers are thermostable and can be modified chemically to enhance stability, and to enable detection. Due to these advantages, aptamers have been used in various applications including biosensor development [18], purification using affinity separation [19, 20], and medical applications including diagnostics [21] as well as drug delivery approaches [22]. Particularly in diagnostic applications, aptamers have been used in various detection techniques including photometric, electrochemical, mass, and surface plasmon resonance [23].

Aptamers against OTA (OTA aptamer) were selected by Cruz Aguado *et al.* in 2008. The specificity of the OTA aptamer was confirmed using warfarin, a structure analogue of OTA [24]. Considering the importance of OTA detection, various assays have been recently developed to detect OTA using aptamer. These assays mainly involve fluorescence [25, 26], electrochemical [27], gold nanoparticle-based colorimetric assays [28], surface plasmon resonance [29], filtration [30], and chemiluminescence [11, 31]. In these assays, the measurement is limited to the aptamer-target binding and the changes due to the interactions in a 1:1 ratio. Being an oligonucleotide, the aptamer can be easily amplified and quantified using qPCR. Recently,

various assays including rolling circle amplification [32], isothermal signal amplification [33], split aptamer assay [34], proximity ligation assay [35], nuclease protection assay [36] and the use of modified magnetic microparticles [37] have utilized amplification-based aptasensing strategies. The strategy to amplify offers a major advantage because the release of a single sequence can be monitored with high sensitivity in a robust way using qPCR.

In this work, dT-modified magnetic microparticles (dT beads) were used for aptamer-assisted qPCR based assay (Apta-qPCR). Aptamers were immobilized on the dT beads using a sequence complementary to the target-binding portion of the aptamer. Addition of OTA resulted in the release of aptamer from the dT-beads and the released aptamers were amplified and quantified using qPCR. The assay resulted in 0.009 ng/mL sensitivity for OTA detection. Additionally, the assay is able to detect a broad range of OTA concentration from 0.039 to 1000 ng/mL.

4.2.5. Materials and Methods

4.2.5.1. Chemicals and materials

The DNA sequences used in this work were all synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) (Table 1). The oligonucleotide concentrations were determined with NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) using the extinction coefficients of the respective oligonucleotide. OTA was purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). The magnetic beads (Dynabeads® MyOne™ Carboxylic acid) were purchased from Life Technologies GmbH (Darmstadt, Germany). 2-(*N*-morpholino) ethane sulfonic acid (MES) was purchased from AppliChem GmbH (Darmstadt, Germany). 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) was purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). SYBR Green Real-Time PCR Master Mix was purchased from Promega GmbH (Mannheim, Germany). Herrenhäuser premium pilsener beer was used as a complex sample matrix. All chemicals were of analytical grade. All stock solutions and buffers were prepared with deionized water (arium 611, Sartorius AG, Göttingen, DE).

Table 1. List of oligonucleotides used in this work

Name of the sequence	Sequence (5' to 3')*
Ochratoxin A Aptamer	TGGTGGCTGTAGGTCAGCATCTGATCGGGTGTGGGT GGCGTAAAGGGAG*CATCGGACA*ACG
dA-Complementary sequence (dA-cOligo)	AAAAAAAAAAAAAAAAAAAAAAAAA-TA-*TGT CCG ATG* C
Random sequence	NH_2-C_{12}-TGGACCCCCTC
Forward primer	TGGTGGCTGTAGGTCA
Reverse primer	CGTTGTCCGATGCTC
dT	TTTTTTTTTTTTTTTTTTTTTTTTT-C_6-dT-NH_2

*Underlined bases correspond to primer binding regions. Italics bases indicate complementary sequences within the aptamer and oligonucleotides. In dA-cOligo, TA was used as a spacer between $d(A)_{25}$ region and complementary sequence.

4.2.5.2. Preparation of dT-beads

Here, 3'-amino modified dT was conjugated to the magnetic beads (7-12x10^9 beads/mL, Dynabeads® MyOne™ Carboxylic acid, Invitrogen) of about 1.05 µm diameter with carboxyl groups on the surface. The conventional EDC-coupling reaction was used to form amide bond between the carboxyl group on the magnetic beads and a primary amine group at the 3'-end of dT. In brief, 100 µL suspension of magnetic beads (10 mg/mL) was dispensed to a micro-tube and washed with 500 µL MES buffer (25 mM MES, pH 4.5) three times. The carboxyl groups on the magnetic beads were activated using 500 µL 50 mM EDC in MES buffer for 30 min. 100 µL 3'-amino modified dT in MES buffer was added to the micro-tube after removing unreacted EDC. Immobilization was carried out by incubation of dT and magnetic beads at room temperature for 2 h with slow tilt rotation to prevent sedimentation of the magnetic beads. Magnetic beads were washed three times with 200 µL MES buffer to remove non-immobilized dT. In order to quench the non-reacted carboxylic acid groups on the magnetic beads, the magnetic beads were incubated with 200 µL 50 mM Tris, pH 7.5 for 15 min. The dT-beads were washed with 200 µL of the aptamer selection buffer (20 mM Tris-HCl buffer containing 150 mM NaCl, 5 mM $MgCl_2$, pH 8.2) twice and then stored in 200 µL selection buffer at 4 °C. In

order to confirm the immobilization of dT on the magnetic beads, all washing fractions were collected and dT was quantified using NanoDrop ND 1000.

4.4.5.3. Hybridization of aptamer to dT-beads using a complementary sequence

6.25 µL 100 nM aptamer was mixed with 6.25 µL 100 nM complementary oligonucleotides (dA-cOligo) in a micro-tube containing 6.25 µL selection buffer. The complementary oligonucleotide was adapted from J. Chen et al. [38]. During the incubation, the mixture was heated up to 90°C for 5 min and cooled to room temperature for 20 min to ensure proper hybridization. 6.25 µL dT-beads suspension was added to the reaction mixture at room temperature and incubated for 20 min with slow tilt rotation. The micro-tube was then placed under magnetic field using DynaMag™-2 Magnet for 5 min. The unbound aptamers, remaining in the supernatant, were removed. The complex was washed ten times (20 min of incubation each time) with 25 µL selection buffer and the presence of aptamer in each washing fraction was checked using qPCR. Magnetic beads without immobilization, magnetic beads immobilized with a random sequence, and the reaction mixture without addition of dA-cOligo were used as negative controls.

4.2.5.4. qPCR-based detection of released aptamer

1 µL aliquot of aptamer-containing solution was mixed with 12.5 µL of GoTaq® qPCR Master mix (2X), 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer, and 10.5 µL of nuclease-free water to make a total volume of 25 µL. The final PCR mixture contained 200 nM forward primer, 200 nM reverse primer, and 1 µL aliquot of aptamer in 1× GoTaq® qPCR Master mix. qPCR was carried out in a 96-well PCR plate (Sarstedt) covered with strip caps. A melting curve analysis was performed from 55°C to 85°C to detect potential nonspecific products. The thermal cycling regime was as follows: initial denaturation for 2 min at 95°C, cycling for 30 s at 95°C, 15 s at 46°C and 15 s at 72°C, repeated 40 times on the Bio-Rad iCycler real-time PCR machine.

4.2.5.5. Detection of OTA using Apta-qPCR

6.25 µL 100 nM aptamer was mixed with 6.25 µL 100 nM dA-cOligo in a micro-tube containing 6.25 µL selection buffer. During the incubation, the mixture was heated up to 90°C for 5 min and then cooled to room temperature for 20 min to ensure hybridization. 6.25 µL dT-beads, with 7.5 pmol dT immobilized on the beads surface, were added to the reaction mixture at room temperature for 20 min. The micro-tube was placed under magnetic field using DynaMag™-2 magnet for 5 min. The unbound aptamers were removed by washing with selection buffer 2 times (20 min each). After washing, 25 µL of selection buffer containing different

concentrations of OTA were added to the micro-tube and incubated for 20 min. The aptamer released by target-induced dissociation was collected in the supernatant and quantified using qPCR. To test the Apta-qPCR assay in complex samples, different OTA concentrations were spiked in Herrenhäuser premium pilsener beer purchased from a local market in Hannover, Germany. The limit-of-detection (LOD) was calculated by 3σ/slope method, where σ is the standard deviation of the blank samples of three Apta-qPCR assays.

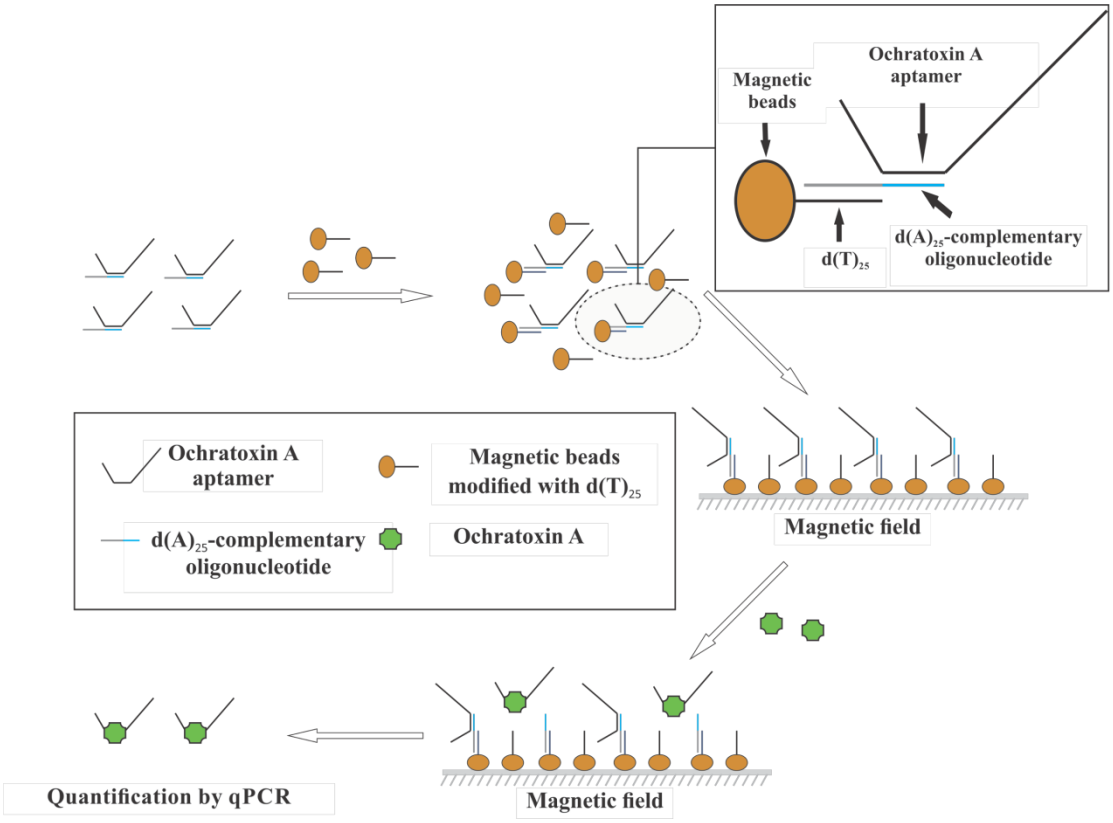

Figure 1. Schematic illustration of Apta-qPCR for Ochratoxin A detection. Binding of OTA results in dissociation of the aptamer from the dT beads. The dissociated aptamer was separated from the dT beads using a magnetic stand and was quantified using real-time PCR.

4.2.6. Results and Discussion

4.2.6.1. Principle of the Apta-qPCR assay

The principle of Apta-qPCR assay is depicted in Figure 1. The envisaged assay relies on target-induced dissociation of OTA aptamer from a complementary oligonucleotide. The complementary oligonucleotide was designed to bind to the target-binding portion of the aptamer and additionally contains a dA tail to ensure hybridization with dT immobilized on the magnetic beads. Addition of OTA results in dissociation of the aptamer from the complementary oligonucleotide, and thus release of the aptamer from the magnetic beads. The magnetic beads

were separated with a magnetic stand and the dissociated aptamer present in the supernatant was quantified by qPCR.

4.2.6.2. Preparation of dT-beads

In the developed assay, dT was immobilized on the magnetic beads in order to immobilize the aptamers on the magnetic beads. One-step EDC-coupling was used for immobilization.

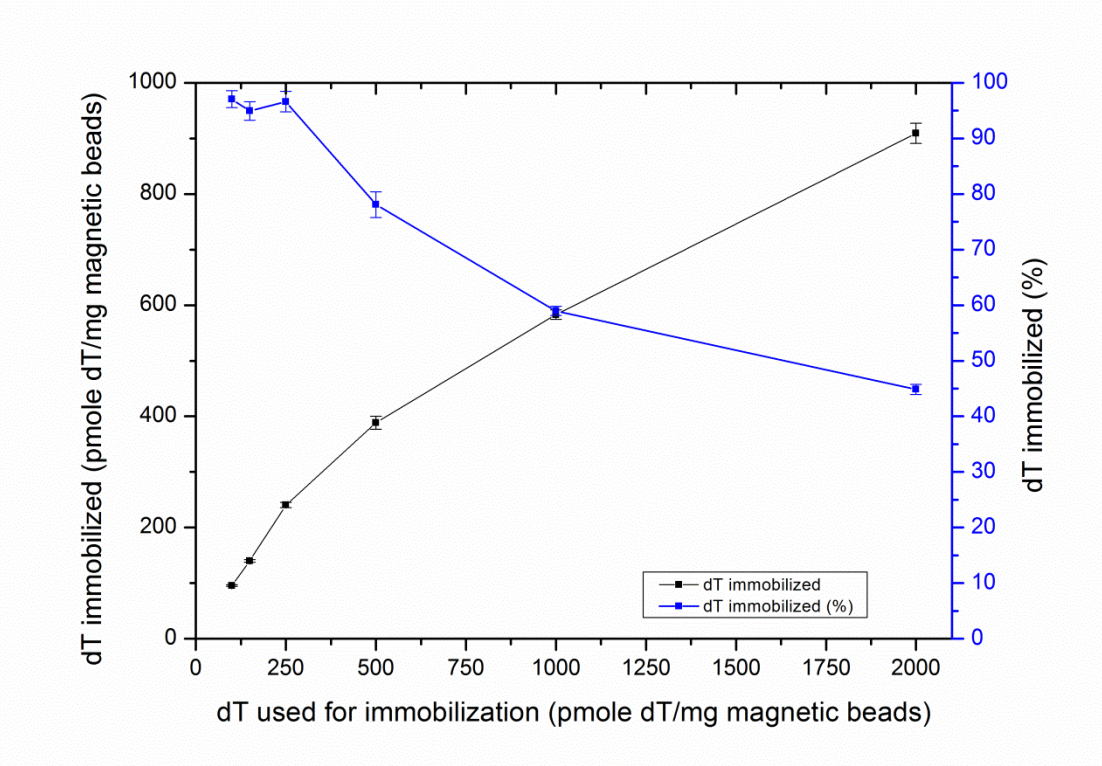

Figure 2. Immobilization of amino-modified dT on carboxyl magnetic beads using EDC-coupling. Y-axis, in black, shows the amount of immobilized dT; Y-axis, in blue, shows percentage of immobilized dT in respect to amount of dT used for immobilization.

Six different amounts of dT/mg magnetic beads (100 pmol mg^{-1}, 150 pmol mg^{-1}, 250 pmol mg^{-1}, 500 pmol mg^{-1}, 1000 pmol mg^{-1}, 2000 pmol mg^{-1} magnetic beads) were used to optimize the Apta-qPCR assay as the density of dT on the magnetic beads can affect the immobilization of aptamer on the magnetic beads. Non-reacted activated carboxyl groups were blocked with 50 mM Tris buffer and beads were then washed with selection buffer 2 times. Using higher concentrations of oligo dT resulted in higher immobilization densities. Yet, a decrease in the immobilization efficiency was observed at high dT concentrations (Figure 2).

4.2.6.3. Optimization of aptamer dissociation from dT-beads

As shown in Figure 1, the aptamer is in contact with the magnetic beads with the help of a complementary oligonucleotide. The complementary oligonucleotide was adapted from J. Chen

et al. as the described 10 nucleotide-long complementary oligonucleotide provided a stable duplex with the aptamer and the highest target-induced dissociation in response to OTA [38]. On 5′-terminus, the complementary oligonucleotide was extended with $d(A)_{25}$ (dA) and two nucleotides (T and A, from 5′ to 3′) as an additional spacer to get efficient hybridization between the OTA aptamer and the complementary oligonucleotide [37].

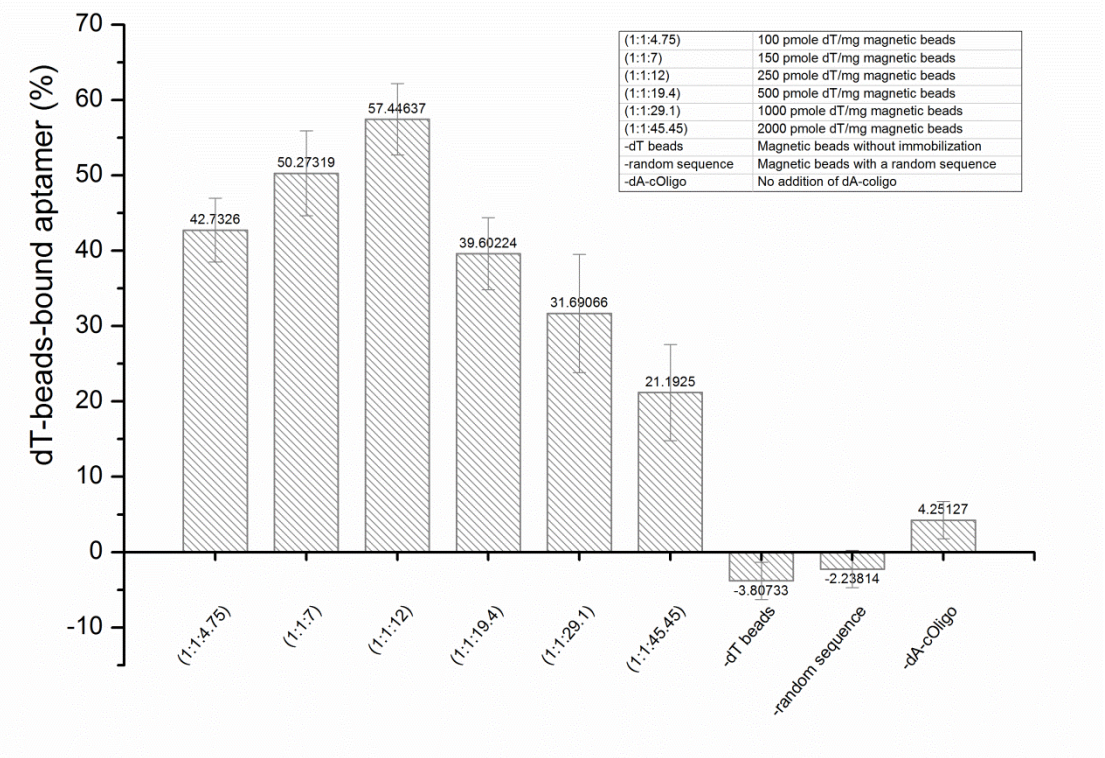

Figure 3. Characterization of apta-beads complex. dT beads with different dT oligo densities on the magnetic beads were incubated with 1:1 ratio of aptamer to dA-cOligo. Maximum hybridization was observed with 1:1:12 of aptamer:dA-cOligo:dT.

To optimize the hybridization between dA-cOligo and dT-beads, dT beads with different dT oligo densities on magnetic beads were incubated with 1:1 ratio of aptamer to dA-cOligo. The higher density of dT resulted in reduced hybridization of aptamer (Figure 3). This can be ascribed to electrostatic repulsion as a result of higher density of dT decoration on the magnetic beads. This repulsion interferes with the interaction of the dA-tail of dA-cOligo with dT modified beads. Optimal hybridization was observed using a 1:1:12 ratio of Aptamer:dA-cOligo:dT (immobilized on the magnetic beads).

The stability of aptamer/dA-cOligo/dT-beads complex (hereinafter, aptamer/dA-cOligo/dT-beads is mentioned as 'apta-beads complex') was investigated by ten subsequent washing steps. The washing steps were analyzed for the presence of aptamer. Release of aptamer was only observed in the first two washing steps (Figure 4). In 10 washing steps, 35% aptamer-release

was observed in first washing step and 7% aptamer-release was observed in second washing step. In further washing steps, no release of the aptamer from the apta-beads complex was detected.

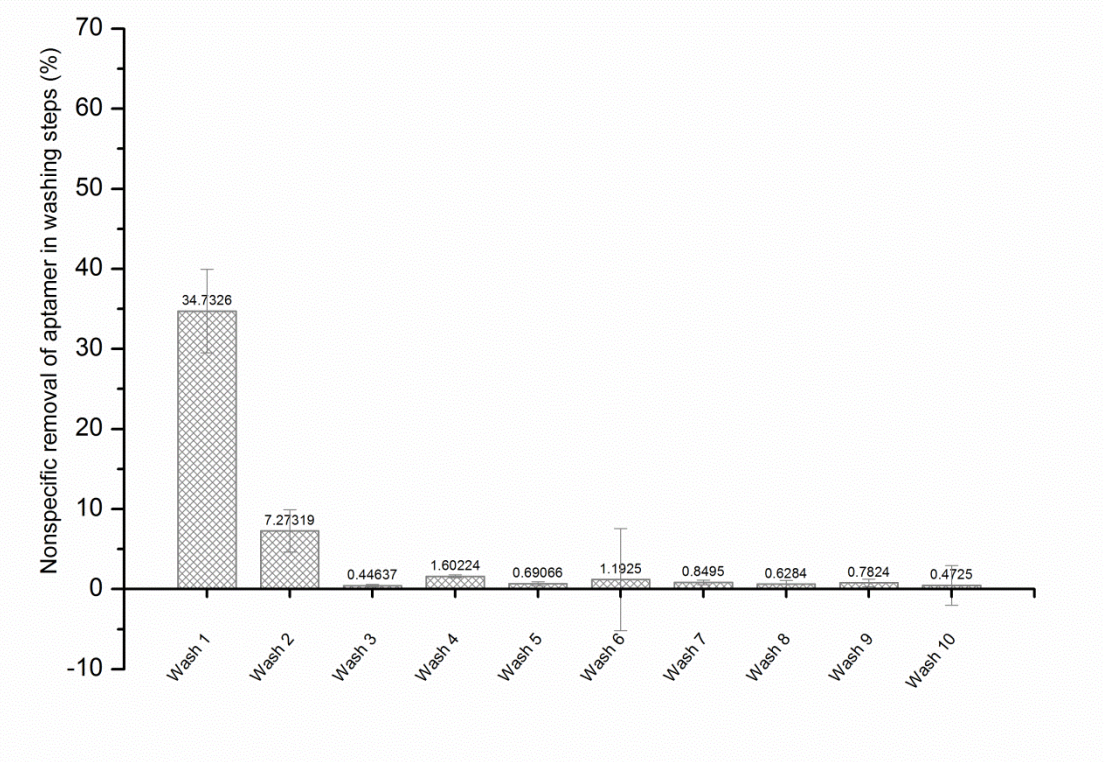

Figure 4. Release of aptamer in the washing steps using selection buffer. Here, 1 nM aptamer was immobilized in apta-beads complex. Release of aptamer was only observed in first two washing steps. The apta-beads complex remained stable after wash 2.

4.2.6.4. Detection of OTA using Apta-qPCR

The scheme of Apta-qPCR assay is shown in Figure 1. When an OTA containing sample is added to the apta-beads complex, the OTA aptamer binds to OTA and is released from the complex. The magnetic beads were separated from the OTA-bound aptamers using a magnetic stand. The supernatant containing OTA-bound aptamers were subjected to qPCR.

As discussed in section 3.3, the complementary oligonucleotide was adapted from a previous work and the apta-beads complex was stable after second washing step. For detection of OTA, the OTA samples were added to the apta-beads complex after second washing step. To further optimize the assay, the effect of incubation time was also evaluated. As can be seen in Figure 5, the optimum time for incubation was found to be 20 min for OTA-induced dissociation of aptamer. Although the incubation time of 5 min and 15 min is capable of detecting 1.25 ng/mL and 0.625 ng/mL of OTA respectively but it compromised the LOD of the assay. Conclusively, OTA-induced dissociation at lower concentration requires 20 min of incubation time.

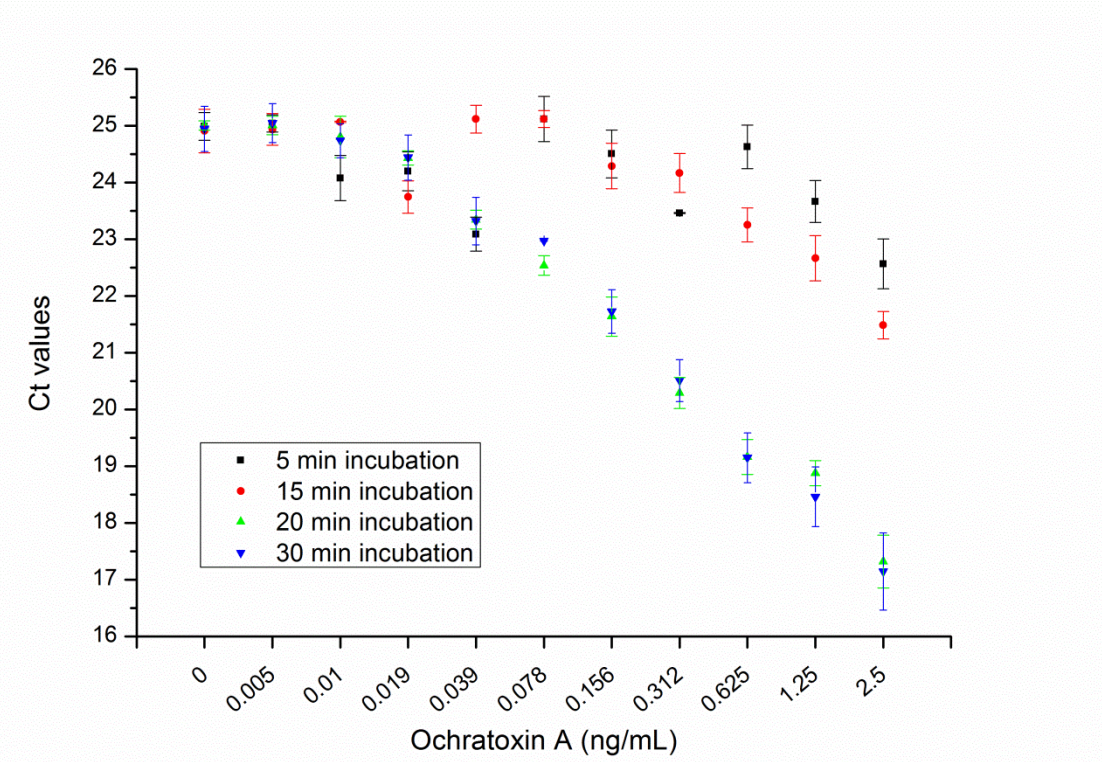

Figure 5. Incubation time investigation of OTA-induced dissociation of aptamer from apta-beads complex. The optimum time for OTA-induced dissociation was found to be 20 min.

Table 2. Comparison of recent aptamer-based assays for the detection of Ochratoxin A

Method	LOD	Detection range	Ref
Chemiluminescence resonance energy transfer (CRET) aptasensor	0.22 ng/mL	0.1 to 100 ng/mL	[11]
Luminescence resonance energy transfer-based aptasensor	0.027 ng/mL	0.05 to 100 ng/mL	[31]
Single-walled carbon nanohorn aptasensor	7.36 ng/mL	8.56 to 2140 ng/mL	[39]
Monolithically integrated optoelectronic aptasensor	2 ng/mL	4 to 100 ng/mL	[27]
Picogreen dye-based fluroscence aptasensor	0.058 ng/mL	0.128 to 4 ng/mL	[25]
Fluorescence-based nano-graphite sensing	8 ng/mL	8.5 to 171	[26]

		ng/mL	
Dot immunogold filtration assay	10 ng/mL	20 to 1000 ng/mL	[30]
Surface plasmon resonance	0.01 ng/mL	0.05 to 10 ng/mL	[29]
Impedimetric immunosensor	0.01 ng/mL	0.05 to 5 ng/mL	[40]
Electrochemical immunosensor	1 ng/mL	2.5 to 100 ng/mL	[41]
Apta-qPCR assay	0.009 ng/mL	0.039 to 1000 ng/mL	

The LOD for OTA detection was found to be 0.009 ng/mL based on 3σ/slope, where σ is the standard deviation of the blank samples of three Apta-qPCR assays (Inset, Figure 6). One of the notable advantages of Apta-qPCR includes the broad range of analyte concentration detection ranging from 0.039 to 1000 ng/mL, which reduced the need of dilution steps (Figure 6). Interestingly, OTA aptamer was reported to exhibit a K_d value of 144.57 ng mL^{-1} and a dynamic range of 8.07 to 2000 ng mL^{-1} using equilibrium dialysis [24]. In addition, different dynamic range of OTA detection has been reported with different assay formats using the OTA aptamer (Table 2). Apta-qPCR assay resulted in higher sensitivity by using qPCR to detect the oligonucleotides. In Apta-qPCR assay, the signal (here, the concentration of dissociated aptamer) is amplified with the help of qPCR, which is not the case in traditional fluorescence, plasmonic, colorimetric, and electrochemical assays, where the measurement is limited to the aptamer-target binding and the changes due to the interactions in 1:1 ratio. To investigate the specificity of the assay, Ct value obtained for 10 ng/mL OTA was compared with 100 ng/mL Ochratoxin B (OTB). The Ct value obtained for 100 ng/mL OTB was found to be 23.3 and the Ct values with 10 ng/mL OTA was found to be 15.18. This observation clearly indicates the specificity of Apta-qPCR assay for the detection of OTA.

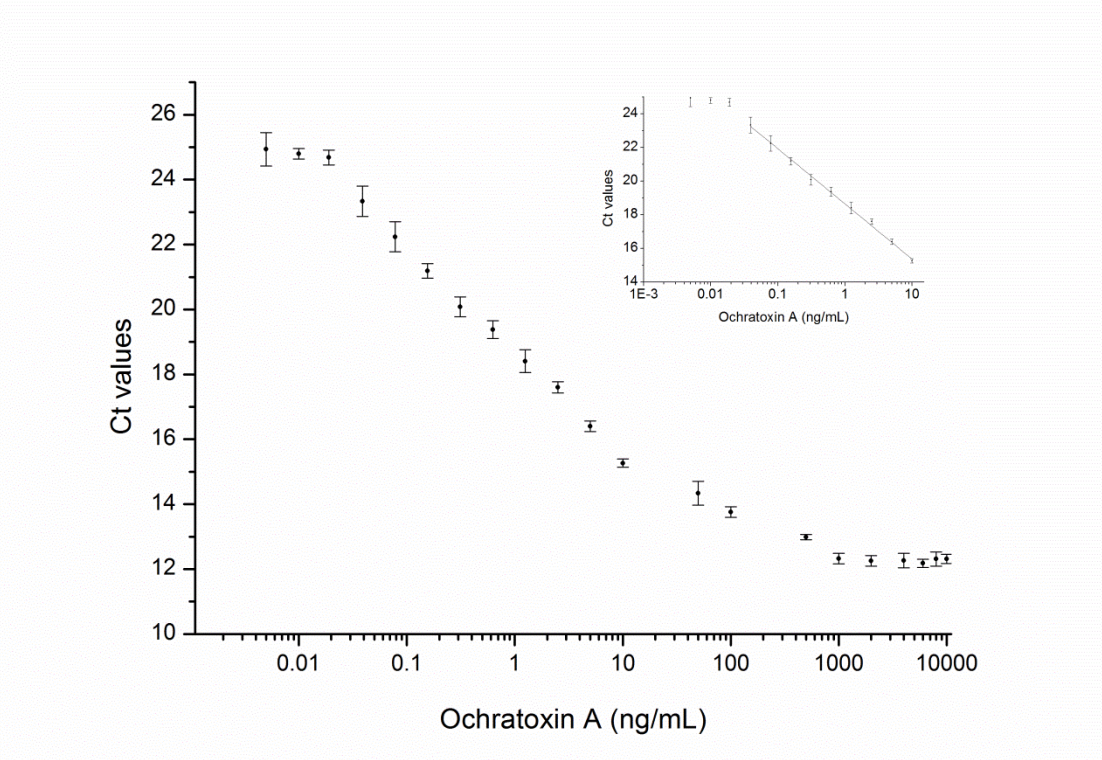

Figure 6. The aptamer-dissociation from apta-beads complex in response to OTA addition. The decrease in Ct in response to higher OTA concentrations reflects to the dissociation of aptamer from apta-beads complex into the supernatant. The assay was able to detect OTA with the linear relationship of Ct values from 0.039 to 1000 ng/mL, R^2 = 0.95. The limit-of-detection (LOD) of the Apta-qPCR was found to be 0.009 ng/mL OTA as shown in Inset figure.

The Apta-qPCR was further investigated for the applicability to quantify OTA in complex beer samples. Different concentrations of OTA were spiked in beer and the assay was able to detect 0.078 ng/mL OTA in beer (Inset, Figure 7). The reason behind the higher LOD in beer samples can be explained from microscale thermophoresis (MST) analysis of aptamer interaction with OTA [20]. In brief, the presence of beer in the selection buffer affected the K_d value of the aptamer for OTA binding. As mentioned in the previous work of our group, the presence 25% beer in the buffer changed the K_d value of from 13 ng/mL (K_d value in selection buffer) to 98.4 ng/mL. The assay demonstrated the broad range of OTA concentrations detection from 0.1 to 1000 ng/mL in complex beer samples (Figure 7). A comparison of the developed Apta-qPCR assay with other recent aptamer-based assays for the detection of OTA is shown in Table 2. The detection performances have been improved in terms of sensitivity and the broad range of detection in comparison to previously reported assays using aptamers/antibodies. Our present Apta-qPCR assay relies on the target-induced dissociation where labeling and the covalent immobilization of the aptamer are not required.

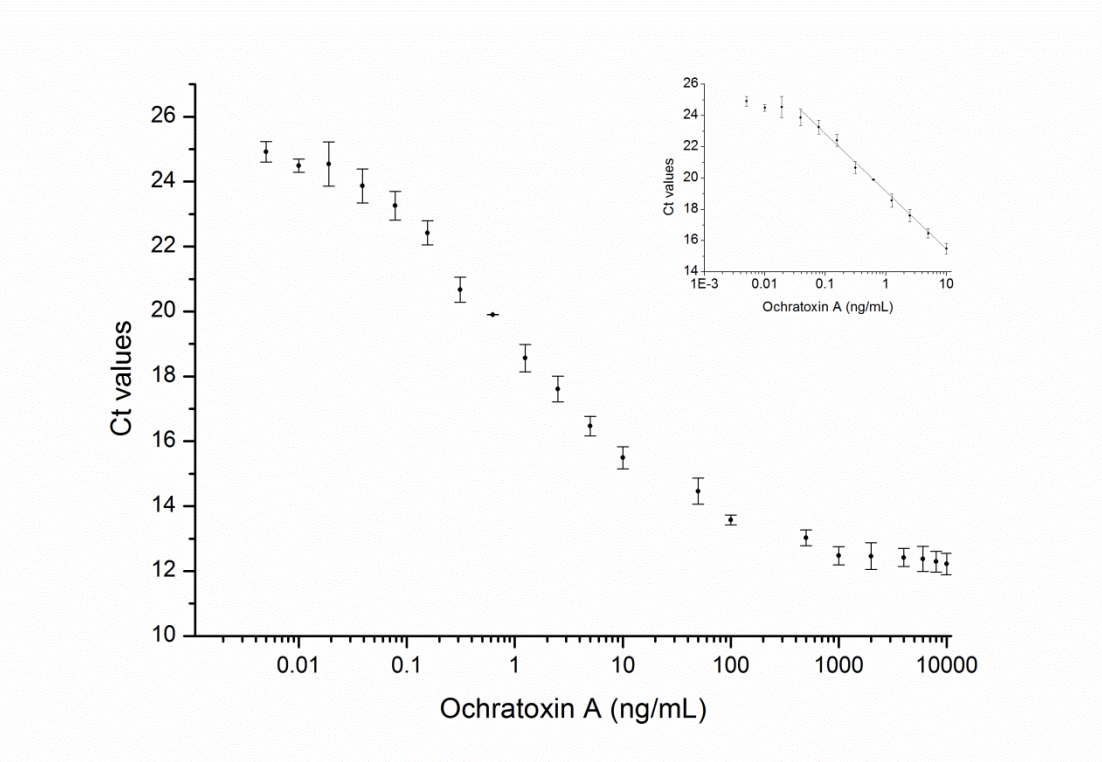

Figure 7. The aptamer-dissociation from apta-beads complex in response to spiked OTA in beer. The decrease in Ct in response to higher OTA concentrations reflects to the dissociation of aptamer from apta-beads complex into the supernatant. The assay was able to detect OTA with the linear relationship of Ct values from 0.1 to 1000 ng/mL, R^2 = 0.95. The limit-of-detection (LOD) of the Apta-qPCR was found to be 0.078 ng/mL OTA as shown in Inset figure.

4.2.7. Concluding remarks

In this work, the Apta-qPCR assay has been used for the quantification of OTA for the first time. The Apta-qPCR assay is based on target-induced dissociation (TID) of a complementary oligonucleotide from the aptamer. Here, the presence of OTA results in dissociation of the complementary oligonucleotide from the aptamer and, in turn, release of the aptamer from the dT-modified beads. OTA-bound aptamers were easily separated from the dT beads using a magnetic stand and quantified in the supernatant using qPCR. As discussed previously, aptamers offer an important advantage as they can be easily amplified, which allows the quantification of a minute amount of dissociated aptamer by qPCR. The assay resulted in a LOD of 0.009 ng/mL for OTA. The advantage of using qPCR for the assay gave broad range of OTA detection from 0.039 to 1000 ng/mL using Apta-qPCR assay, which reduces the requirement of dilution steps. In this work, the assay has been also optimized to detect OTA in complex samples. The advantages of the assay also include no requisite of labeling and covalent immobilization of the aptamer. Additionally, the assay needs low sample volumes (6.25 µL) and

the detection of OTA in one sample costs approximately € 0.70 based on the costs of chemicals and biomolecules used in the assay. With an assay time of 2.5 h, the assay is not rapid in comparison to many other reported assays, but Apta-qPCR provides high sensitivity with a broad range of detection in a robust way. An alternative way to quantify oligonucleotides in comparison to qPCR can reduce the detection time. The Apta-qPCR shown here can be easily extended for detecting other molecules as long as a specific aptamer is available.

4.2.8. Acknowledgments

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Conflict of interests: The authors have declared no conflicts of interest.

4.2.9. References

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Chapter 3

Unpublished work

Summary of unpublished work

Antibiotics are natural compounds of low molecular weight that can be produced by fungi or bacteria, possessing antibacterial activity to kill or inhibit the growth of many microorganisms. Antibiotics are widely used in animal husbandry mainly to prevent bacterial infections and to increase growth rate. If these antibiotics are improperly administrated, however, their residual or degradation products may be present in the final products, which cause harmful effects on consumers. In particular, a trace amount of antibiotic compounds in milk can foster the development of antibiotic-resistant bacteria [102]. To address this issue, many countries have set definitive maximum residue limits in food products, for which suitable analytical methods need to be developed to control the concentration of these compounds below the maximum residue limit level [103].

Oxytetracycline (OTC) has been approved for use in a variety of food producing animals including cattle, sheep, goats, poultry, and fish, as an additive in feed or drinking water to maintain optimal health. For this reason, a lot of efforts have been made to develop highly sensitive and specific assays for the detection of antibiotic content in food products [19, 104, 105]. As traditional detection methods for antibiotics are time-consuming [106], novel assays have been developed but they usually lack sensitivity and specificity [26, 107].

Here, the application of Apta-qPCR was extended to the detection of an antibiotic, oxytetracycline. The assay resulted in the detection limit of 0.025 ng/mL (0.054 nM) with a linear range of detection from 0.078 to 1000 ng/mL (0.17 to 2172 nM). In the next section, the optimization of Apta-qPCR for the detection of oxytetracycline is described.

In addition to developing Apta-qPCR (which provides result in 2.5 h), a rapid assay was also developed, which can provide results within 15 minutes. This assay could enable on-site detection of small molecules as it can be easily integrated in microfluidic devices or smartphone-based sensors. The development of this colorimetric assay is also described in the following section.

4.3. Optimization of Apta-qPCR for the detection of oxytetracycline

4.3.1. Introduction

Tetracyclines are broad-spectrum antibiotics widely used in human therapy (wide range of infections), animal husbandry and in aquaculture [31]. Oxytetracycline (OTC) is one of the most frequently used molecules from this class [26]. OTC works by inhibiting the translation process in bacteria. It binds to the 30S ribosomal subunit and prevents the amino-acyl tRNA from binding to the A site of the ribosome. The binding is reversible in nature. Due to lipophilic property of OTC, it can easily pass through the cell membrane or passively diffuses through porin channels in the bacterial membrane [108].

OTC is widely used for the treatment of infectious diseases in animals. A low dose of OTC is enough for its effectivity and then it gets cleared from the body after a short time of residence. Only a fraction of the ingested antibiotics is metabolized, hence a large proportion of the antibiotics is either accumulated in tissues or excreted and released into the environment via manure and sludge used as fertilizer on the field or in aquaculture. This is a big issue because this can result in the development of bacterial strains which are resistant to the OTC treatment. Thus it is highly important to check animal products such as milk, meat, eggs and seafood for the presence of antibiotics [102].

Normally, microbiological assays are used in combination with confirmatory methods such as LC-MS to detect antibiotics in food products. These methods are not feasible for the rapid detection of antibiotics and it is also possible to get false positive results in case of closely related molecules [106]. Aptamer-based assays offer a big advantage here because they allow to develop rapid assays and exhibit high specificity towards the target molecules [102].

In this work, Apta-qPCR assay was used to detect OTC. This assay is highly sensitive with LOD of 0.01 ng/mL of OTC in binding buffer. In addition, this assay allows to detect a broad range of OTC from 0.04–1000 ng/mL reducing the requirement of dilution steps. The whole detection process can be finished in 2.5 h.

4.3.2. Experimental section

4.3.2.1. Chemicals and materials

The DNA sequences used in this work were all ordered from Integrated DNA Technologies, Inc. (Coralville, IA) (Table 1). The oligonucleotide concentrations were determined with NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) using the extinction coefficients of the respective oligonucleotide. OTC was purchased from Sigma-Aldrich Chemie

GmbH (Munich, Germany). The magnetic beads (Dynabeads® MyOne™ Carboxylic acid) were purchased from Life Technologies GmbH (Darmstadt, Germany). 2-(*N*-morpholino) ethane sulfonic acid (MES) was purchased from AppliChem GmbH (Darmstadt, Germany). 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) was purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). SYBR Green Real-Time PCR Master Mix was purchased from Promega GmbH (Mannheim, Germany). All chemicals were of analytical grade. All stock solutions and buffers were prepared with deionized water (arium 611, Sartorius AG, Göttingen, DE).

Table 4.1. List of oligonucleotides used in this work

Name of the sequence	Sequence (5' to 3')*
Oxytetracycline Aptamer [19, 109]	CGT ACG GAA TTC GCT AGC ACG TTG ACG CTG GTG CCC GGT TGT GGT GCG AG*T GTT GTG TGG ATC* CGA GCT CCA CGT G
dA-Complementary sequence (dA-cOligo)	AAAAAAAAAAAAAAAAAAAAAAAA *GAT CCA CAC AAC A*
Random sequence	NH_2-C_{12}-TGGACCCCCTC
Forward primer	CGT ACG GAA TTC GCT AGC
Reverse primer	CAC GTG GAG CTC GGA TCC
dT	TTTTTTTTTTTTTTTTTTTTTTTTT-C_6-dT-NH_2

*Underlined bases correspond to primer binding regions. Italics bases indicate complementary sequences within the aptamer and oligonucleotides.

4.3.2.2. Preparation of dT-beads

Here, 3'-amino modified dT was conjugated to the magnetic beads (7-12x10^9 beads/mL, Dynabeads® MyOne™ Carboxylic acid, Invitrogen) with carboxyl groups on the surface (1.05 µm diameter). The conventional EDC-coupling reaction was used to couple dT sequences on MBs. In brief, 100 µL suspension of magnetic beads (10 mg/mL) was dispensed to a micro-tube and washed with 500 µL MES buffer (25 mM MES, pH 4.5) three times. The carboxyl groups on the magnetic beads were activated using 500 µL 50 mM EDC in MES buffer for 30 min. 100 µL 3'-amino modified dT in MES buffer was added to the micro-tube after removing unreacted EDC. Immobilization was carried out by incubation of dT and magnetic beads at room temperature for 2 h with slow tilt rotation to prevent sedimentation of the magnetic beads. Magnetic beads

were washed three times with 200 µL MES buffer to remove non-immobilized dT. In order to quench the non-reacted carboxylic acid groups on the magnetic beads, the magnetic beads were incubated with 200 µL 50 mM Tris, pH 7.5 for 15 min. The dT-beads were washed with 200 µL of the OTC aptamer selection buffer [19] (20 mM Tris-HCl buffer containing 100 mM NaCl, 2 mM $MgCl_2$, 5 mM KCl, 1 mM $CaCl_2$, 0.02 Tween 20, pH 7.6) twice and then stored in 200 µL selection buffer at 4 °C. In order to confirm the immobilization of dT on the magnetic beads, all washing fractions were collected and dT was quantified using NanoDrop ND 1000.

4.3.2.3. Hybridization of aptamer to dT-beads using a complementary sequence

6.25 µL 100 nM aptamer was mixed with 6.25 µL 100 nM complementary oligonucleotides (dA-cOligo) in a micro-tube containing 6.25 µL selection buffer. The complementary oligonucleotide was adapted from C. Fang et al. [110]. During the incubation, the mixture was heated up to 90°C for 5 min and cooled to room temperature for 20 min to ensure proper hybridization. 6.25 µL dT-beads suspension was added to the reaction mixture at room temperature and incubated for 20 min with slow tilt rotation. The micro-tube was then placed under magnetic field using DynaMag™-2 Magnet for 5 min. The unbound aptamers, remaining in the supernatant, were removed. The complex was washed ten times (20 min of incubation each time) with 25 µL selection buffer and the presence of aptamer in each washing fraction was checked using qPCR. Magnetic beads without immobilization, magnetic beads immobilized with a random sequence, and the reaction mixture without addition of dA-cOligo were used as negative controls.

4.3.2.4. qPCR-based detection of released aptamer

1 µL aliquot of aptamer-containing solution was mixed with 12.5 µL of GoTaq® qPCR Master mix (2X), 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer, and 10.5 µL of nuclease-free water to make a total volume of 25 µL. The final PCR mixture contained 200 nM forward primer, 200 nM reverse primer, and 1 µL aliquot of aptamer in 1× GoTaq® qPCR Master mix. qPCR was carried out in a 96-well PCR plate (Sarstedt) covered with strip caps. A melting curve analysis was performed from 55°C to 85°C to detect potential nonspecific products. The thermal cycling regime was as follows: initial denaturation for 2 min at 95°C, cycling for 30 s at 95°C, 30 s at 48°C and 15 s at 72°C, repeated 40 times on the Bio-Rad iCycler real-time PCR machine.

4.3.2.5. Detection of OTC using Apta-qPCR

6.25 µL 100 nM aptamer was mixed with 6.25 µL 100 nM dA-cOligo in a micro-tube containing 6.25 µL selection buffer. During the incubation, the mixture was heated up to 90°C for 5 min

and then cooled to room temperature for 20 min to ensure hybridization. 6.25 µL dT-beads, with 7.5 pmol dT immobilized on the beads surface, were added to the reaction mixture at room temperature for 20 min. The micro-tube was placed under magnetic field using DynaMag™-2 magnet for 5 min. The unbound aptamers were removed by washing with selection buffer 2 times (20 min each). After washing, 25 µL of selection buffer containing different concentrations of OTC were added to the micro-tube and incubated for 20 min. The aptamer released by target-induced dissociation was collected in the supernatant and quantified using qPCR. The limit-of-detection (LOD) was calculated by 3σ/slope method, where σ is the standard deviation of the blank samples of three Apta-qPCR assays.

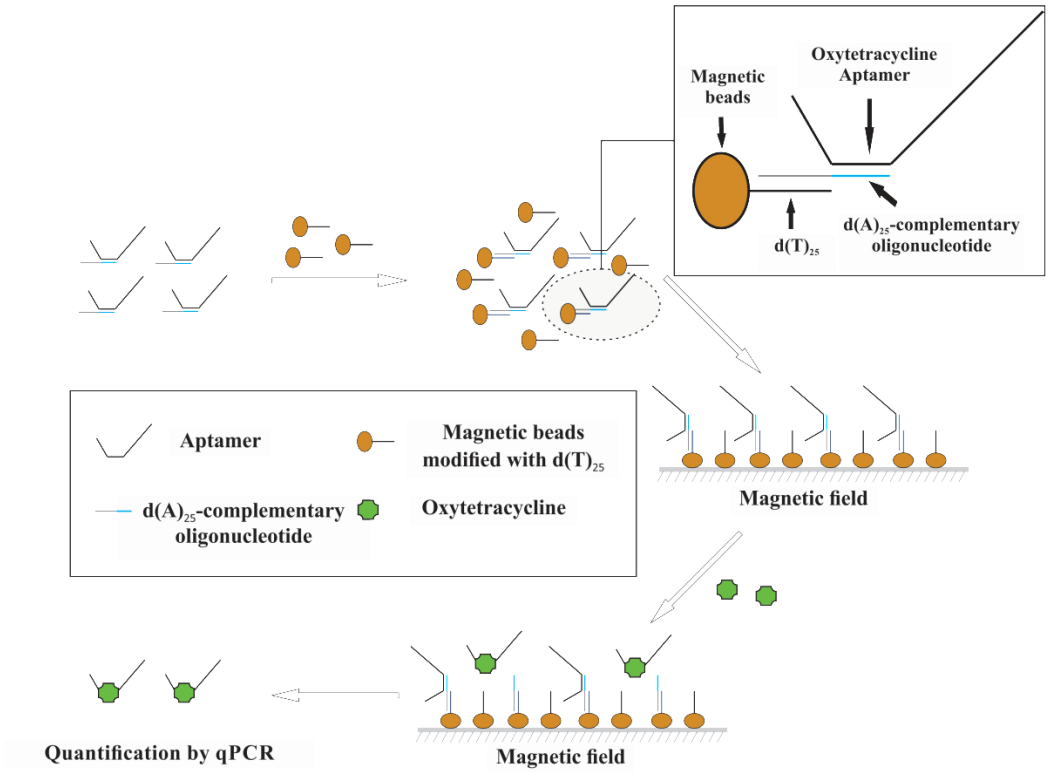

Figure 4.3. Scheme of Apta-qPCR for the detection of oxytetracycline

4.3.3. Results and discussion

The principle of Apta-qPCR assay is depicted in Figure 4.3. The envisaged assay relies on target-induced dissociation of OTC aptamer from a complementary oligonucleotide. The complementary oligonucleotide was designed to bind to the target-binding portion of the aptamer and additionally contains a dA tail to ensure hybridization with dT immobilized on the magnetic beads. Addition of OTC results in dissociation of the aptamer from the complementary oligonucleotide, and thus release of the aptamer from the magnetic beads. The magnetic beads

were separated with a magnetic stand and the dissociated aptamer present in the supernatant was quantified by qPCR.

4.3.3.1. Preparation of dT-beads

In the developed assay, dT was immobilized on the magnetic beads in order to immobilize the aptamers on the magnetic beads. One-step EDC-coupling was used for immobilization. Six different amounts of dT/mg magnetic beads (100 pmol mg^{-1}, 150 pmol mg^{-1}, 250 pmol mg^{-1}, 500 pmol mg^{-1}, 1000 pmol mg^{-1}, 2000 pmol mg^{-1} magnetic beads) were used to optimize the Apta-qPCR assay as the density of dT on the magnetic beads can affect the immobilization of aptamer on the magnetic beads. Non-reacted activated carboxyl groups were blocked with 50 mM Tris buffer and beads were then washed with selection buffer 2 times. Using higher concentrations of oligo dT resulted in higher immobilization densities. Yet, a decrease in the immobilization efficiency was observed at high dT concentrations (Figure 4.4.).

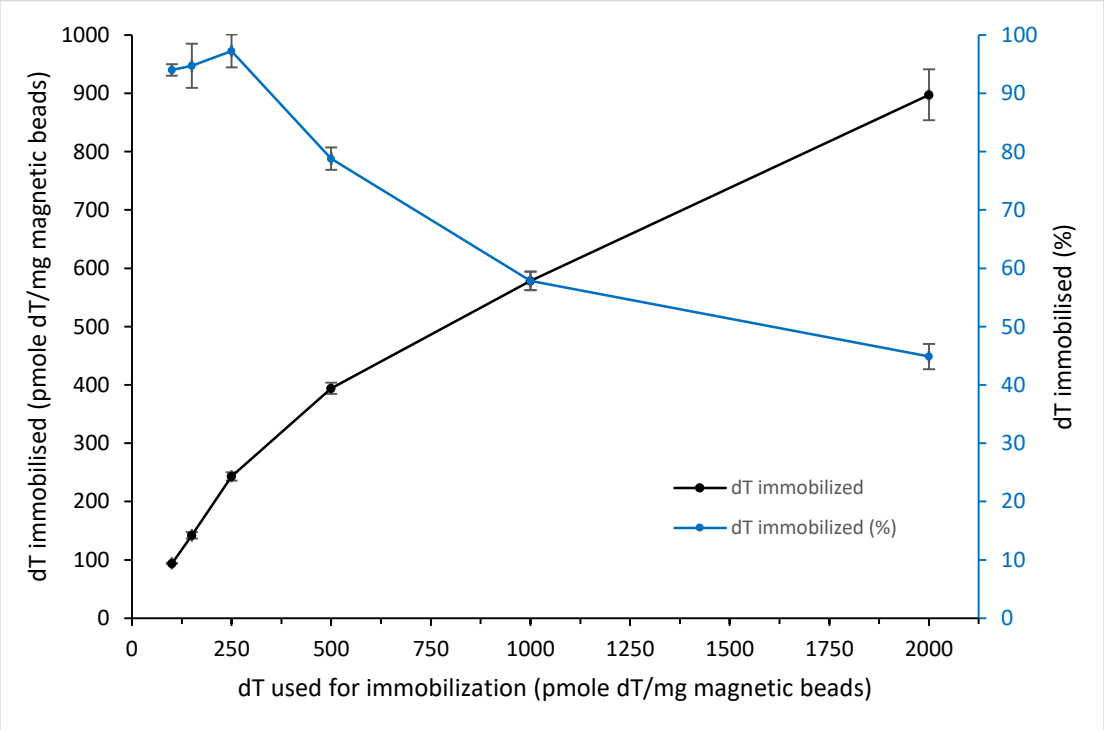

Figure 4.4. Immobilization of amino-modified dT on carboxyl magnetic beads using EDC-coupling. Y-axis, in black, shows the amount of immobilized dT; Y-axis, in blue, shows percentage of immobilized dT in respect to amount of dT used for immobilization.

4.3.3.2. Optimization of aptamer dissociation from dT-beads

As shown in Figure 4.3, the aptamer is immobilized on the magnetic beads with the help of a complementary oligonucleotide. The complementary oligonucleotide was adapted from C. Fang et al. [110] as the described 13 nucleotide long complementary oligonucleotide provided a

stable duplex with the aptamer and the highest target-induced dissociation in response to OTC [38].

To optimize the hybridization between dA-cOligo and dT-beads, dT beads with different dT oligo densities on magnetic beads were incubated with 1:1 ratio of aptamer to dA-cOligo. The higher density of dT resulted in reduced hybridization of aptamer (Figure 4.5.). This can be ascribed to electrostatic repulsion as a result of higher density of dT decoration on the magnetic beads. This repulsion interferes with the interaction of the dA-tail of dA-cOligo with dT modified beads. Optimal hybridization was observed using a 1:1:12 ratio of Aptamer:dA-cOligo:dT (immobilized on the magnetic beads).

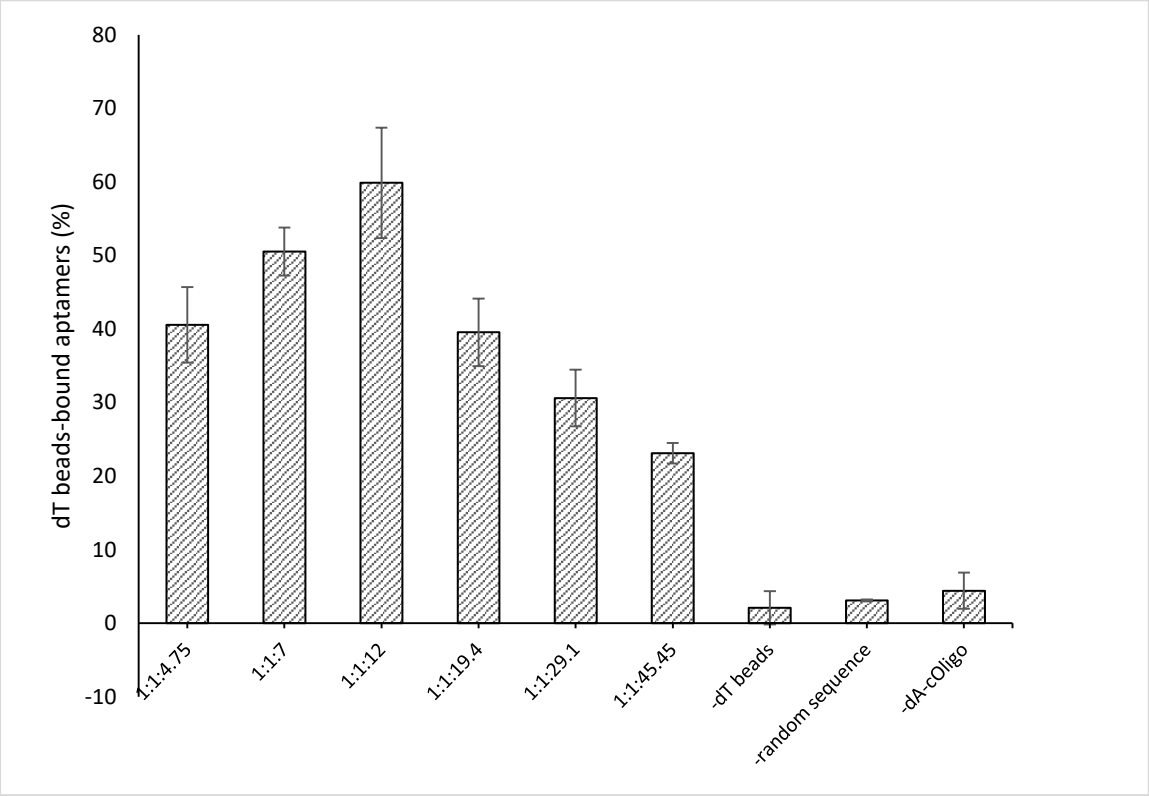

Figure 4.5. Characterization of apta-beads complex. dT beads with different dT oligo densities on the magnetic beads were incubated with 1:1 ratio of aptamer to dA-cOligo. Here, 1:1:4.75 = 100 pmole dT/mg magnetic beads; 1:1:7 = 150 pmole dT/mg magnetic beads; 1:1:12 = 250 pmole dT/mg magnetic beads; 1:1:19.4 = 500 pmole dT/mg magnetic beads; 1:1:29.1 = 1000 pmole dT/mg magnetic beads; 1:1:45.45 = 2000 pmole dT/mg magnetic beads; -dT beads = Magnetic beads without immobilisation; -random sequence = Magnetic beads with a random sequence; -dA-cOligo = No addition of dA-cOligo. Maximum hybridization was observed with 1:1:12 of aptamer:dA-cOligo:dT.

The stability of aptamer/dA-cOligo/dT-beads complex (hereinafter, aptamer/dA-cOligo/dT-beads is mentioned as 'apta-beads complex') was investigated by ten subsequent washing steps. The washing steps were analyzed for the presence of aptamer. Release of aptamer was only

observed in the first two washing steps (Figure 4.6.). In 10 washing steps, 33% aptamer-release was observed in first washing step and 8% aptamer-release was observed in second washing step. In further washing steps, no release of the aptamer from the apta-beads complex was detected.

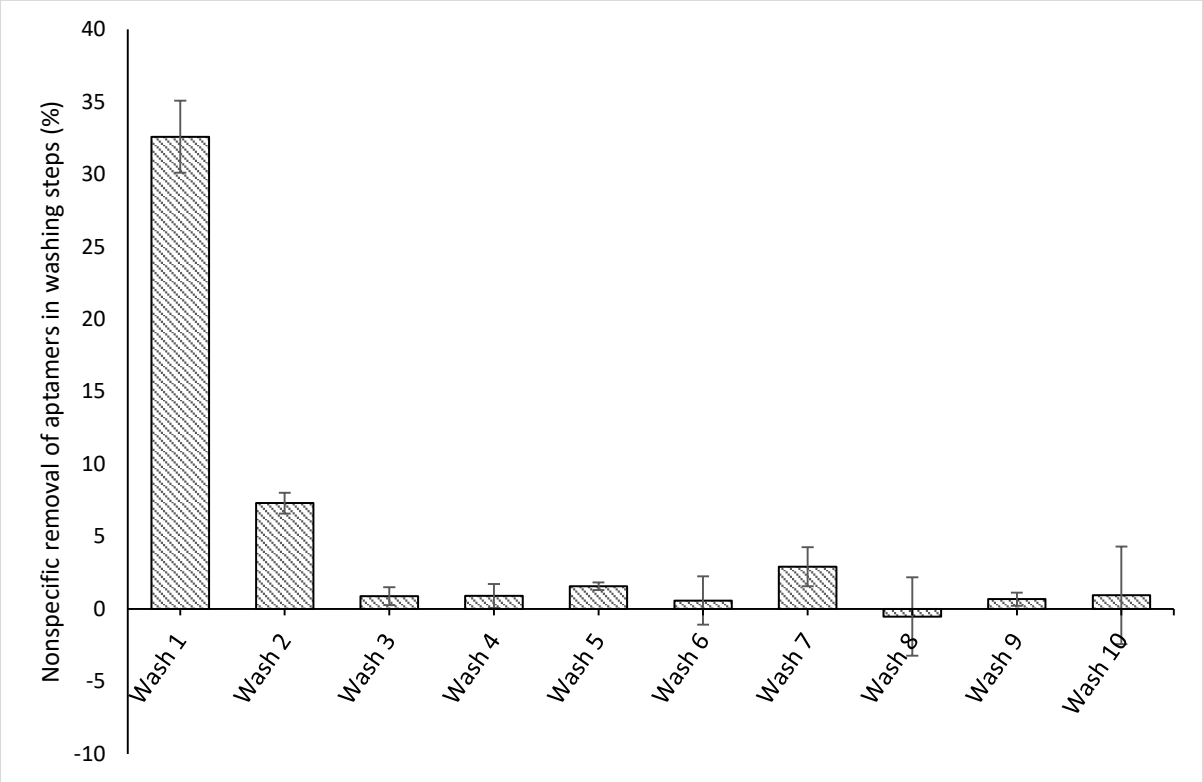

Figure 4.6. Release of aptamer in the washing steps using selection buffer. Here, 1 nM OTC aptamer was immobilized in apta-beads complex. Release of aptamer was only observed in first two washing steps. The apta-beads complex remained stable after wash 2.

4.3.3.3. Detection of OTC using Apta-qPCR

The scheme of Apta-qPCR assay is shown in Figure 4.3. When the sample containing OTC is added, the OTC aptamer binds to OTC and gets released from the apta-beads complex. In turn, the target-bound OTC aptamer can be separated easily from unbound aptamers using a magnetic stand, and collected as supernatant. Later, the concentration of aptamer in the supernatant was obtained using qPCR, which is proportional to the OTC concentration.

As discussed in section 3.2, the apta-beads complex was stable after second washing step. For detection of OTC, the sample containing OTC were added to the apta-beads complex after second washing step. The detection limit was found to be 0.025 ng/mL (0.054 nM) based on 3σ/slope, where σ is the standard deviation of the blank samples of three Apta-qPCR assays (Inset, Figure 4.7.). In case of OTC detection, it was possible to get broad range of detection,

from 0.078 to 1000 ng/mL (0.17 to 2172 nM) (Figure 4.7.), which reduces the requirements of dilution. To investigate the specificity of the assay, Ct value obtained for 10 ng/mL OTC was compared with 100 ng/mL Chloramphenicol. The Ct value obtained for 100 ng/mL Chloramphenicol was found to be 24.4 and the Ct values with 10 ng/mL OTC was found to be 16.24. This observation clearly indicates the specificity of Apta-qPCR assay for the detection of OTC.

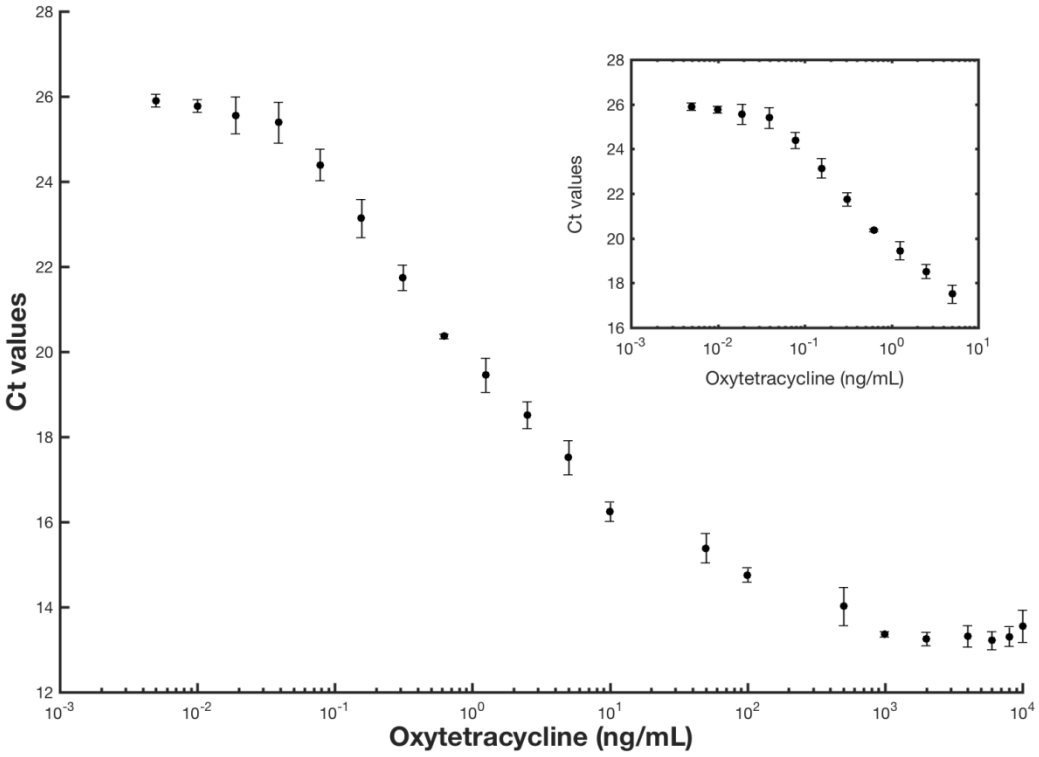

Figure 4.7. The aptamer-dissociation from apta-beads complex in response to OTC addition. The decrease in Ct in response to higher OTC concentrations reflects to the dissociation of aptamer from apta-beads complex into the supernatant. The assay was able to detect OTC with the linear relationship of Ct values from 0.078 to 1000 ng/mL, R^2 = 0.95. The limit-of-detection (LOD) of the Apta-qPCR was found to be 0.025 ng/mL OTC as shown in Inset figure.

4.3.4. Conclusions

In this work, the Apta-qPCR assay was used for the detection of oxytetracycline. The Apta-qPCR assay is based on target-induced dissociation (TID) of a complementary oligonucleotide from the aptamer. Here, the presence of OTC results in dissociation of the complementary oligonucleotide from the aptamer and, in turn, release of the aptamer from the dT-modified beads. OTC-bound aptamers were easily separated from the dT beads using a magnetic stand

and quantified in the supernatant using qPCR. The assay resulted in a LOD of 0.025 ng/mL (0.054 nM) for OTC with a broad range of detection from 0.078 to 1000 ng/mL (0.17 to 2172 nM).

4.4. Colorimetric assay for the detection ATP and Ochratoxin A

4.4.1. Introduction

The structural flexibility of aptamer allows us to develop assays which were not possible using traditional protein-based binding ligands. The possibility to develop assays which are based on target-induced dissociation (TID) and target-induced structure switching (TISS) is highly advantageous [10, 26-28]. In previous work, TID-based assay was developed in terms of Apta-qPCR [27, 28]. Here, an assay based on TISS was developed where there is no need to immobilise aptamer and even more importantly, there is no need to find a complementary oligonucleotide, reducing the requirement of finding target binding sites. Here, the developed colorimetric assay, for the detection of ATP and OTA, can provide results within 15 minutes. At the moment, the limitation of the assay includes the sensitivity of the assay but there is potential for further improvement.

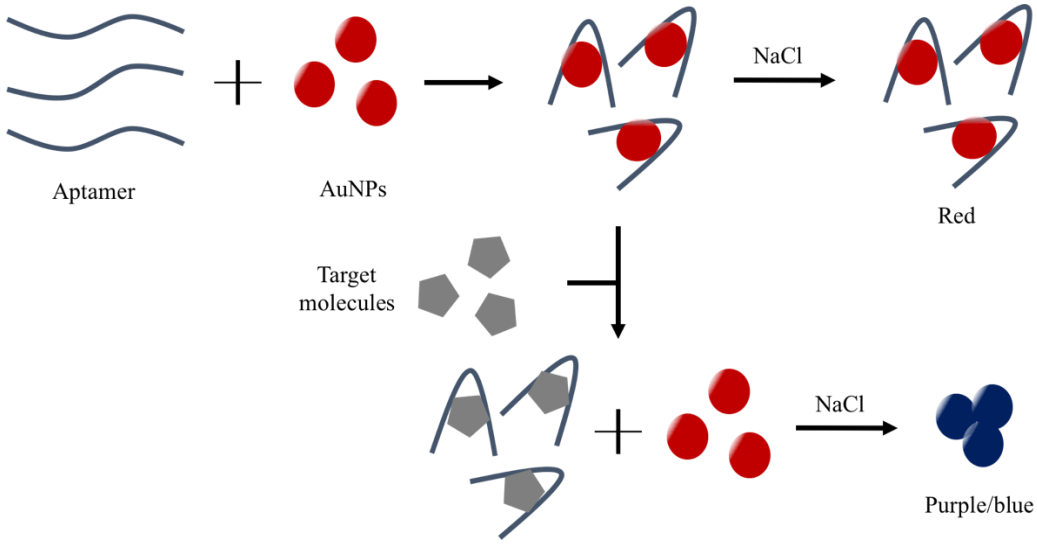

Figure 4.8. Scheme of aptamer-based colorimetric assay for the detection of ATP and ochratoxin A.

Recently, gold nanoparticles (AuNPs) have emerged as an important analytical tool for the development of colorimetric assays for the detection of various substances including DNA [111], metal ions [112], and proteins [113]. The advantages of using AuNPs in colorimetric assays include the simplicity of the assay, no need to use complicated and expensive analytical instruments, and importantly, high extinction coefficients (>1000 times larger than those of

organic dyes). The principle of using AuNPs as a colorimetric reporter relies on its unique SPR property. The homogenous AuNP solution is red whereas the aggregated AuNP solution is purple (or blue) [114]. This special SPR property of AuNP can be well explained by the Mie theory [115]. The aggregation of AuNP can be induced using high concentration of salt as example, high NaCl concentration. Interestingly, AuNP interacts non-specifically with ssDNA and binding of ssDNA protect AuNPs from salt (NaCl)-induced aggregation [26]. As aptamers are ssDNA, this property was used to develop the assays for the detection of ATP and OTA using aptamers. The scheme of the assay is shown in figure 4.8.

4.4.2. Experimental section

4.4.2.1. Chemicals and materials

The DNA sequences used in this work were all ordered from Integrated DNA Technologies, Inc. (Coralville, IA) (Table 1). The oligonucleotide concentrations were determined with NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) using the extinction coefficients of the respective oligonucleotide. ATP was purchased from Life Technologies GmbH (Darmstadt, Germany) and OTA was purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). All chemicals were of analytical grade. All stock solutions and buffers were prepared with deionized water (arium 611, Sartorius AG, Göttingen, DE).

Table 4.2. List of oligonucleotides used in this work

Name of the sequence	Sequence (5' to 3')
ATP Aptamer [17, 28]	GGA ACA CTA TCC GAC TGG CAC CAC CTG GGG GAG TAT TGC GGA GGA AGG TCC TTG GGC ATG TCT AGC GAT CC
Ochratoxin A aptamer [27, 45]	TGG TGG CTG TAG GTC AGC ATC TGA TCG GGT GTG GGT GGC GTA AAG GGA GCA TCG GAC AAC G

4.4.2.2. Synthesis of AuNPs

For the synthesis of AuNPs, the protocol from Liu et al. [116] was followed. Briefly, all the glasswares were cleaned using aqua-regia (3:1 concentrated HCl:HNO_3) solution for 15 min. Later, the glasswares were rinsed using copious amount of water. In the two-neck flask, 50 mL 1 mM gold chloride (HAuCl4) solution was added and heated until refluxing start. When the solution started to reflux, the stopper was removed and 10 mL 38.8 mM sodium citrate was added. The stopper was placed back. The color of the solution turned from pale yellow to dark

red in 1 min. Solution was cooled to room temperature. The size of the particle and the concentration of the AuNP solution was confirmed using UV absorption spectroscopy.

4.4.2.3. Colorimetric detection of ATP and OTA

A mixture of 37.5 µL of AuNPs (5.4 nM), 10 µL ATP (with different concentration), 27.5 µL aptamer (2.18 µM) was mixed and shaken mildly for 10 min at RT. Later, 75 µL 2X binding buffer (ATP binding buffer (1x): 20 mM Tris-HCl buffer containing 300 mM NaCl, 5 mM $MgCl_2$, pH 8.3; OTA binding buffer (1x): 20 mM Tris-HCl buffer containing 150 mM NaCl, 5 mM $MgCl_2$, pH 8.2) was mixed and shaken mildly for 5 min at RT. The absorbance of the final mixture was checked using UV absorption spectroscopy at 520 nm.

4.4.3. Results and discussion

4.4.3.1. AuNP characterization

The size of the AuNP was confirmed using UV absorption spectroscopy. Here, the absorbance of AuNPs synthesised using Liu et al. [116] protocol was compared with AuNPs (15 nm) from Sigma Aldrich. Both particles showed similar absorbance maximum at 520 nm (Figure 4.9.). In addition, the 15 nm size of the AuNPs was also confirmed using the ratio of the absorbance at 520 nm and 450 nm, which was 1.61 [117]. The concentration of AuNPs was also checked using UV absorption spectroscopy using 520 nm as absorption maximum. The concentration of AuNPs was found to be 5.4 nM.

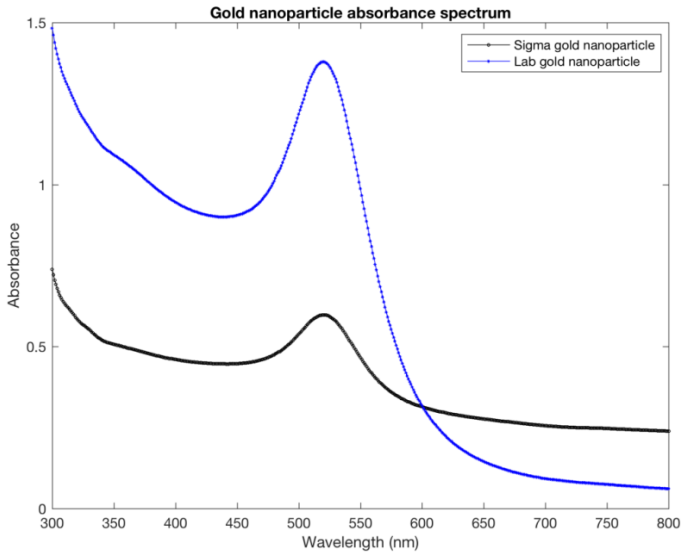

Figure 4.9. AuNPs size determination. Here, the AuNPs synthesized in the lab are compared with the AuNPs from Sigma Aldrich having particle size of 15 nm. Both particles have absorption maximum at 520 nm.

4.4.3.2. Colorimetric detection of ATP and OTA

As explained earlier, this assay is based on NaCl-induced aggregation of AuNPs. As both the aptamer selection buffers (For ATP and OTA) contains NaCl, addition of selection buffer was able to induce the aggregation of AuNPs. Aptamers, being ssDNA, non-specifically interact with AuNPs and they are able to protect AuNPs from NaCl-induced aggregation resulting in red colloids. In presence of the analytes, aptamers interact with the target molecules and are released from the AuNPs. In this case, the AuNPs are no more protected against NaCl-induced aggregation and the color of colloids turns to purple/blue. The color change could be seen with naked eye (Figure 4.10.A) and is proportional to the concentration of the analyte in the solution (Figure 4.10.B, for ATP detection). With this assay, it was possible to detect ATP from 0.066 µM to 666 µM in a linear range with detection limit of 0.05 µM. In case of OTA detection, the linear range for detection was from 0.05 to 50 nM with the detection limit of 0.04 nM (Figure 4.11.). At the moment, this is proof-of-concept and the measurements were performed in selection buffer. The sensitivity of the assay can be also improved by adjusting buffer composition according to assay requirements and adjusting incubation time.

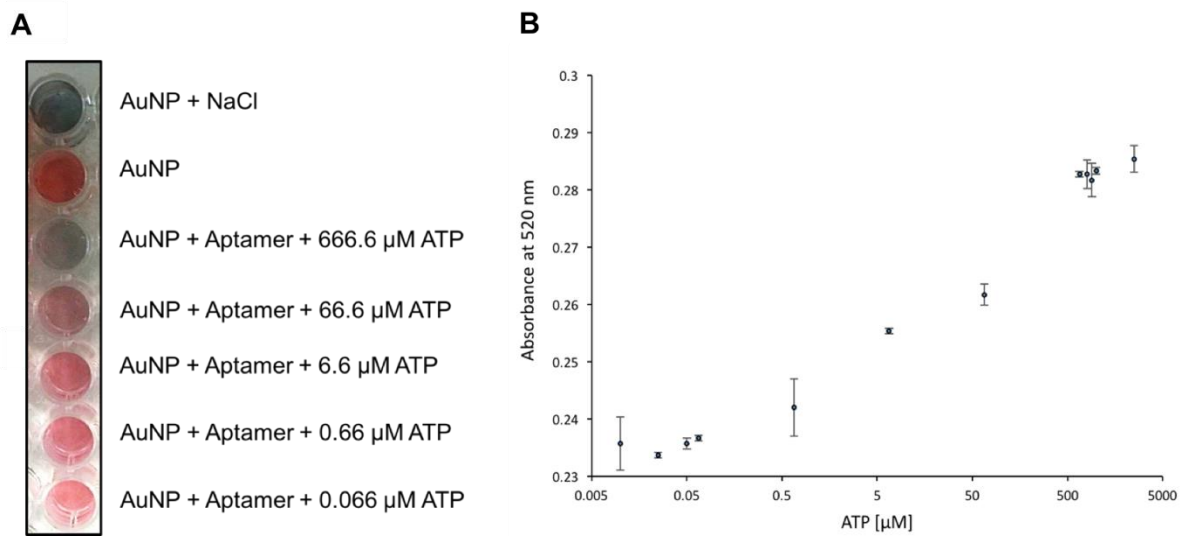

Figure 4.10. Colorimetric detection of ATP. **(A)** Naked-eye observation of ATP measurement. **(B)** Linear range for the detection of ATP was found to be from 0.066 µM to 666 µM.

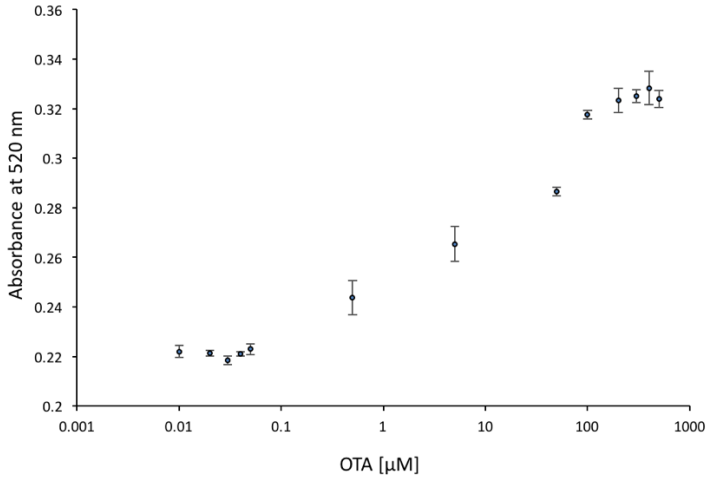

Figure 4.11. Colorimetric detection of OTA. Linear detection range for the detection of OTA was found to be 0.05 to 50 nM.

4.4.4. Conclusions

Colorimetric assays are popular for developing on-site detection sensors because of their easy operation steps and easy signal detection. In this work, a AuNP-based colorimetric assay was developed to detect small molecules, which can provide results within 15 minutes. As proof-of-concept, it was tested for the detection of ATP and OTA. It was possible to detect ATP from 0.066 µM to 666 µM in a linear range with detection limit of 0.05 µM, and OTA from 0.05 to 100 nM in a linear range with detection limit of 0.04 nM. The detection was performed in their respective selection buffer but there is a scope to optimise this assay in different matrices. Importantly, this assay can be easily extended to the detection of other analytes.

5. Conclusions and outlook

The objective of this project was to develop a rapid and sensitive assay for the detection of small molecules. Traditionally, small molecules are analysed by bench-top methods such as HPLC in combination with UV/fluorescence based detection or LC-MS. In last years, antibody and enzyme-based assays have also provided an alternative but usually it is difficult to develop these protein-based assays, especially against small molecules due their small size. In this work, a novel aptamer-based assay, Apta-qPCR, was developed and optimized for the detection of different small molecules including ATP, a food toxin (ochratoxin A), and an antibiotic (oxyteratracycline).

The main advantage of this assay is the high sensitivity and specificity, broad range of detection (reducing the dilution steps). The advantages offered by this assay originate from the integration of MB-based separation of target-bound and unbound aptamers (low signal-to-noise ratio) with qPCR based detection of aptamers (high sensitivity and broad range of detection). The potential of aptamers to detect the targets with high sensitivity is not fully realised in normal assays including SPR, colorimetric assays, electrochemical assays, filtration, and chemiluminescence-based assays. In these assays, the measurement is limited to aptamer-target binding and the changes due to these binding in 1:1 ratio. Nucleotide nature of aptamers provides an advantage that they can be easily detected using qPCR, where the aptamers can be amplified, and in turn the changes due to aptamer-target binding is significantly amplified.

Apta-qPCR assay

Detection principle

Apta-qPCR relies of target-induced dissociation (TID) of aptamers from a complementary oligonucleotide. Here, aptamers are first immobilised on magnetic beads using the complementary sequences. Here, the complementary sequence is designed in a way that it binds to the part of aptamer which is essential for the interaction with the target molecule. When a sample containing target is introduced to the aptamer/complementary sequence/magnetic beads complex (apta-beads complex), the aptamer gets released into the supernatant from the apta-beads complex. The released target-bound aptamer can be easily separated using a magnetic stand and quantified using qPCR.

Detection of ATP using Apta-qPCR

In this work, the specificity of the aptamers has been combined with the possibility of aptamers to be sensitively detected with PCR-based amplification to detect small molecules. ATP

molecule was used as a model molecule here for Apta-qPCR because the aptamer against ATP is well characterized and the complementary sequences required to develop TID-based assays are already available. In addition, ATP detection is very important in biochemical studies and clinical diagnosis.

The sensitivity of the Apta-qPCR assay was found to be 17 nM ATP in HeLa cell lysate with a broad dynamic range from 50 nM to 5 mM, confirming its applicability in biological samples. The assay requires small sample volumes (6.25 µL) and 2.5 h for the detection of ATP. The specificity of the assay was confirmed using similar molecules (CTP, GTP, and TTP). Additionally, the Apta-qPCR was compared with CellTiter-Glo® assay for the detection of ATP in cell lysate using defined ATP concentrations. Both assays were able to recover 95-108% of ATP in the range of 0.5 µM to 10 µM, but Apta-qPCR provided better performance at lower concentartion of ATP.

Detection of ochratoxin A using Apta-qPCR

OTA is most-abundant mycotoxin and can be present in a variety of crops, including final food products such as grain, pork products, coffee, wine and beer. OTA is highly nephrotoxic and is suspected to be the main etiological agent responsible for human Balkan endemic nephropathy and associated urinary tract tumors.

With Apta-qPCR assay, the limit of detection was found to be of 0.009 ng/mL (0.022 nM) and detection range was from 0.039–1000 ng/mL (0.1-2500 nM) OTA in beer samples. The assay needs low sample volumes (6.25 µL), 2.5 h of detection time and the detection of OTA in one sample costs approximately € 0.70 based on the costs of chemicals and biomolecules used in the assay.

Detection of oxytetracycline using Apta-qPCR

Oxytetracycline (OTC) is the most popular broad-spectrum antibiotic from tetracycline (TC) group of antibiotics. OTC is widely used in animal and aquaculture to prevent bacterial infections and increase their growth rate. Abuse of OTC especially, in farm animals can cause the accumulation of the antibiotic in food products including, meat, milk and eggs. Ultimately, this can cause a serious implication to human health in terms of development of antibiotic resistance.

Here, Apta-qPCR was also applied for the detection of antibiotics. The limit of detection was found to be of 0.025 ng/mL (0.054 nM) with linear detection range of 0.078 to 1000 ng/mL (0.17 to 2172 nM) OTC in selection buffer.

Here, Apta-qPCR was proven to be powerful tool for the detection of different small molecules. It offers high sensitivity and broad range of linear detection requiring low sample volume (6.25 µL) and 2.5 h for the detection process. To reduce the detection time, qPCR can be more optimised in terms of reducing the time requirement because qPCR consumed approximately 80% time of the total detection process.

Colorimetric assay

Detection principle

Here, the colorimetric assay was developed using AuNPs. The homogenous AuNP colloid is red whereas the aggregated AuNP colloid is purple (or blue). The aggregation of AuNP can be induced using high concentration of salt as example, high NaCl concentration. Interestingly, ssDNA can interact non-specifically with AuNPs, and protect AuNP solution against salt-induced aggregation. Here, aptamers were used as ssDNA that can interact with AuNPs. In absence of aptamer-target molecule, aptamers are bound to AuNPs and AuNPs are not aggregated in presence of NaCl (colloid remains red). Addition of the target causes the release of aptamers from AuNPs and, here, the AuNPs are no more protected against NaCl-induced aggregation (colloid turns purple/blue).

Detection of ATP and OTA

As proof-of-concept, the colorimetric assay was tested for the detection of ATP and OTA. It was possible to detect ATP from 0.066 µM to 666 µM in a linear range with detection limit of 0.05 µM, and OTA from 0.05 to 50 nM in a linear range with detection limit of 0.04 nM. The assay could be finished in 15 minutes. There are still opportunities to optimise the assay by modifying binding conditions, e.g, buffer composition, incubation time, that can improve the sensitivity of the assay and detection time. This detection protocol can also be easily integrated in smartphone-based sensors and microfluidic devices for easy detection.

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List of publications

1. **Modh H** (85%), Witt M (5%), Urmann K, Lavrentieva A, Segal E, Scheper T, Walter JG (10%). Aptamer-based detection of adenosine triphosphate via qPCR. Talanta. 2017 Sep 1;172:199-205.
2. **Modh H** (95%), Scheper T, Walter JG (5%). Detection of Ochratoxin A by aptamer-assisted real-time PCR-based assay (Apta-qPCR). Engineering in Life Sciences. 2017 Aug 1.
3. **Modh H** (90%), Scheper T, Walter JG (10%). Aptamer-modified magnetic beads in biosensing. Sensors. 2018 Mar 30;18(4):1041.

Oral presentation

1. Aptamer-based detection of small molecules at Analytica 2018, Munich

Poster presentations

1. **Modh H**, Scheper T, Walter JG. Aptamer-based assay for the detection of small molecules using qPCR (Apta-qPCR), September 29 2016, BMWZ Symposium, Hannover, Germany. (Best poster award)
2. **Modh H**, Witt M, Urmann K, Lavrentieva A, Segal E, Scheper T, Walter JG. Aptamer-based quantification of small molecules using qPCR (Apta-qPCR), February 12-16 2017, New Concepts in Biosensing, Dead Sea, Israel
3. **Modh H**, Witt M, Urmann K, Lavrentieva A, Segal E, Scheper T, Walter JG. Aptamer-based quantification of ATP and Ochratoxin A using qPCR, April 3 2017, Infotag Aptamer, DECHEMA, Frankfurt, Germany
4. **Modh H**, Scheper T, Walter JG. Detection of small molecules using Apta-qPCR, March 6 2018, Spring Meeting – Israel day, Leibniz House, Hannover, Germany

Curriculum Vitae

Personal data

Name	Harshvardhan Bharatkumar Modh
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Education

10/2014 - Present **Leibniz University Hannover, PhD in Chemistry**
Hannover, Germany

06/2011 - 07/2013 **National Institute of Pharmaceutical Education and Research, Mohali, M.Sc. (India)**
Punjab, India (Score: 9.64/10)

07/2007 - 04/2011 **Institute of Pharmacy, Nirma University, B. Pharm.**
Ahmedabad, India (Score: 8.48/10)

Work Experience

08/2013 - 03/2014 **Department of Microbiology, Research Fellow**
Delhi University, South Campus, Delhi, India

05/2010 - 06/2010 **Urocare hospital, Mehsana, Hospital Pharmacist**

Professional activities

Research group visit

- Dr. Akram Alian, Department of Biology, Technion – Israel Institute of Technology. Haifa, Israel (3 weeks)
- Prof. Ester Segal, Department of Biotechnology and Food Engineering, Technion – Israel Institute of Technology. Haifa, Israel (1 week)

Courses

- Marketing in Biopharmaceutical Industries, Prof. Dr. Oscar-Werner Reif, Sartorius, 2018
- Patents and Founding – from idea to the market, European Patent Office, 2017
- Quality management in the pharmaceutical industries, Dr. Frank Lammers from Sanofi-Aventis, 2017