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Development of aptamers for *in vivo* and *in vitro* biosensor applications

PhD Thesis by

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“An expert is a person who has found out by his own painful experience all the mistakes that one can make in a very narrow field.”

(Niels Bohr – as quoted by Edward Teller)

Preface

This thesis is written as a partial fulfillment of the requirements to obtain the PhD degree at the Technical University of Denmark (DTU), This thesis includes work carried out at The Novo Nordisk Center for Biosustainability from December 2011 until January 2015 under the supervision of Prof. Alex Toftgaard Nielsen.

From February 2012 until August 2012 I worked at the School of Chemistry and Molecular Biosciences, University of Queensland in Brisbane, Australia, under the supervision of Dr. Rakesh Naduvile Veedu and Prof. Alex Toftgaard Nielsen. The Novo Nordisk Foundations financed the work

Lasse Holm Lauridsen

Hørsholm, February 2015

Acknowledgements

Three years in the blink of an eye. I was on my way to do a PhD in, Brisbane Australia when I got the call to join The Novo Nordisk Foundation Center for Biosustainability. I said yes and with that came the fortunate experience of participating in the birth of The Novo Nordisk Foundation Center for Biosustainability. But even more important it lead me to meet a great amount of extremely talented people, all of which I have to thank for making these three years incredibly inspiring and academically challenging. None mentioned, none forgotten.

I have to thank my family and friends for getting me through this PhD, especially Anne, my soul mate and mother to our children. It has been three intense years were we have seen considerable changes to our lives. You have been the calming voice in my ear when things were touch and I am going to, try my hardest to repay and earn the trust and commitment you give to me, for as long as you'll have me.

To my parents who have been incredibly attentive and always helpful, thank you. For taking care of Anne and Frederik in China for two months, while I was off to Denmark to start my PhD. For always going out of their way to make sure that we have everything we need.

To my two extremely talented younger brothers, whom I a long time ago stopped trying to compete with. Thank you for continuously calling and checking, thank you for hanging out and helping out.

Lastly, I want to thank my supervisor Prof. Alex Toftgaard Nielsen. For always being an inspired and inspiring mentor. Even when faced with establishing a large research group of his own, he has remained dedicated and focused on the success of this project. I think of him as a great mentor and a great friend. I could not ask for a better collaborator in the future projects to come.

Thank you, to The Novo Nordisk Foundation for funding.
Thank you all,

Abstract

The nylon in the clothes we wear; the paracetamol in the painkillers we eat; and LEGO® blocks we may buy for our children – they all are manufactured from chemical material based on *petroleum*. Most of us are blissfully unaware of how our dependency on oil, transcends the use of fuel-based transportation. Nylon is made from adipic acid made from *petroleum*. Paracetamol is made from phenol, which is made from *petroleum* and LEGO® bricks are made up of acrylonitrile, butadiene, and styrene all *petroleum* based monomers. To reduce our dependency on oil many pharmaceuticals, nutraceuticals and building block *chemicals* are now being *sustainably produced* in bacterial cell-factories. The development of new bacterial cell-factories is a difficult and expensive process, in part due to time required to screen for and optimize productions strains. A new promising way of reducing the development time is generating new and faster ways of screening and optimizing using biosensors.

In this thesis we develop new functional biological recognition modules for biosensors. These DNA- and RNA-based recognition modules are called aptamers and are developed to interact with targets of choice. Aptamers are developed through a laborious process; which suffers from high error-rates and, therefore, the process has undergone significant improvements. Here we present two new versions of aptamer development schemes that have been used to identify aptamers against snake venom toxin (with a possible pharmaceutical application) and small molecule food additives (for optimization production in cell factories). Additionally, the characterization an all-polymer physicochemical biosensor is presented for the detection of antibiotics in food products.

These results have lead to the ongoing development of a high-throughput all-polymeric biosensor device at DTU Nanotech and also resulted in extended funding of 3M DKK from the Danish National Innovation Foundation, Biosyntia and The Technical University of Denmark to advance the use of aptamers and biosensors in cell-factory development.

Resumé (Danish abstract)

De fleste mennesker aner ikke hvordan vores afhængighed af råolie transcenderer brugen af benzin- og dieseldrevet transport. Nylon i vores påklædning, paracetamol i vores smertestillende medicin og de LEGO® byggesæt, vi køber til vores børn, er alle baseret på forarbejdning af råolie. Nylon er baseret på adipinsyre, som er kemisk fremstillet fra råolie. Paracetamol er fremstillet fra phenol som er et derivat af råolie, og LEGO® klodser er fremstillet af acrylnitril, butadien og styren, som alle er fremstillet fra råolie. For at reducere vores afhængighed af råolie bliver mange lægemiddelstoffer, kosttilskud og grund-kemikalier nu produceret i bakterielle celle-fabrikker. Udviklingen af celle-fabrikker er en udfordrende, langvarig og dyr proces, dels på grund af den omfattende tid brugt på at screene og optimere cellelinjer til produktion af biokemikalier. En ny lovende måde at reducere udviklingstiden på celle-fabrikker er ved at anvende biosensorer til hurtigere at kunne screene og optimere cellelinjer.

I denne afhandling udvikler vi funktionelle biologiske moduler til 'bio-delen' af biosensorerne. Disse DNA- og RNA-baserede funktionelle moduler kaldes aptamerer og udvikles typisk ved en iterativ og langvarig proces, der ofte ender med ikke funktionelle aptamerer. Aptamer-udviklingsprocessen (SELEX) har derfor været igennem flere fornyelser. Her præsenterer vi to nye alternativer til SELEX samt udviklingen af aptamerer, der binder til et slangetoksin (der har lægemiddelpotentiale) og fødevaretilsætningsstoffer, der udgør lovende kandidater for udvikling af bæredygtige produktionsruter i celle-fabrikker. Ydermere, præsenterer vi karakteriseringen af en ny biosensortype til identificering af antimikrobielle stoffer i fødevarer.

Disse resultater har resulteret i en fortsat indsats imod videreudviklingen af denne biosensortype på DTU Nanotech og ligeledes resulteret i ydereligere finansiering på 3 mio. DKK fra Innovationsfonden, Biosyntia og DTU, til videre udvikling af aptamer biosensorteknologi til brug i forbindelse med udviklingen af cellefabrikker.

Publications

---------- Included in this thesis ----------

Lauridsen LH, Doessing HB, Long KS, Nielsen AT (2015) A Capture-SELEX strategy for multiplexed selection of structure-switching RNA aptamers against small molecules.

Daprà J, **Lauridsen LH**, Nielsen AT, Rozlosnik N (2013) Comparative study on aptamers as recognition elements for antibiotics in a label-free all-polymer biosensor. Biosens Bioelectron 43: 315–320. doi:10.1016/j.bios.2012.12.058.

Lauridsen LH, Shamaileh HA, Edwards SL, Taran E, Veedu RN (2012) Rapid one-step selection method for generating nucleic acid aptamers: development of a DNA aptamer against α-bungarotoxin. PLoS One 7: e41702. doi:10.1371/journal.pone.0041702.

Lauridsen LH, Veedu RN (2012) Nucleic acid aptamers against biotoxins: a new paradigm toward the treatment and diagnostic approach. Nucleic Acid Ther 22: 371–379. doi:10.1089/nat.2012.0377.

---------- Not included in this thesis ----------

Tannenberg RK, Shamaileh H Al, **Lauridsen LH**, Kanwar JR, Dodd PR, et al. (2013) Nucleic acid aptamers as novel class of therapeutics to mitigate Alzheimer's disease pathology. Curr Alzheimer Res 10: 442–448.

Lauridsen LH, Rothnagel JA, Veedu RN (2012) Enzymatic recognition of 2'-modified ribonucleoside 5'-triphosphates: towards the evolution of versatile aptamers. Chembiochem 13: 19–25. doi:10.1002/cbic.201100648.

Crouzier L, Dubois C, Edwards SL, **Lauridsen LH**, Wengel J, et al. (2012) Efficient reverse transcription using locked nucleic acid nucleotides towards the evolution of nuclease resistant RNA aptamers. PLoS One 7: e35990. doi:10.1371/journal.pone.0035990.

Content

Introduction and outline

Increased awareness of global warming and fossil fuel supply, security, and prices, has led to a paradigm shift in perceived routes to commodity chemical production and energy generation. The human population continues to grow significantly, and this increases the need for further growth both in the production of chemicals but also in the requirements for nutrients and protein. Accordingly, the majority of the world community has now set challenging targets for reductions in greenhouse gas emissions to be achieved in part through the development of sustainable routes to chemicals, fuels, and energy. One way of securing sustainable production of biochemicals is by utilizing bacterial cells that can be altered into microscale factories converting cheap and abundant biomass into added value chemicals and pharmaceuticals, reducing the dependency on petroleum based chemical industry and oil.

The Novo Nordisk Foundation Center for Biosustainability (mouthful; from now on CFB) is an institute under the Technical University of Denmark committed to accelerating cell factory development by state-of-the-art tools for metabolic engineering.

This thesis contains some of the results obtained during my three years as a PhD student at the Novo Nordisk Foundation Center for Biosustainability and the Department of Systems biology and the Technical University of Demark. The bulk of the work is dedicated to selecting nucleic acid aptamers. These aptamers can then be used in either *in vitro* biosensor for measuring extracellular concentrations of ligands or in *in vivo* genetic sensor elements that measure the intracellular concentration of a ligand. When we started this quest, the NNF Center for Biosustainability was a smaller operation with limited equipment access. As the center grew so did the project outline and directions were changed. This thesis will probably illustrate that fact, but for good measure here is a chronological walkthrough of how things evolved and how this is reflected in the thesis.

In 2010, Dietrich *et al.* pinpointed the bottleneck of cell factory development as the screening of strain libraries. Genetic diversity can today be generated at an unprecedented speed and magnitude, however we are precluded from harnessing

that ability because standard screening methods only allow a limited throughput that does not match the diversity that can be generated (Dietrich et al. 2010). Typically, bacterial cell factories are designed by rational engineering of biosynthetic pathways and changes in the pathway are interrogated with low throughput liquid or gas chromatography methods that normally have a throughput of hundreds per day. In 2009, Wang *et al.* managed to harness the diversity that could be created with multiplex genome engineering by applying a screening method for the colored product lycopene. Screening more than 4 billion combinatorial variants per day, and isolating variants with a fivefold improvement in lycopene production rate within just 3 days (Wang et al. 2009). However, not all compounds that are interesting to produce in bacterial cell factories are colored like the red tomato-pigment lycopene. Knowing your luck, what you want to produce is likely an inconspicuous compound that has no color or florescent activity to screen for. In reality, most metabolites and secondary metabolites will not be colorful and readily 'screenable' by e.g. colorimetric assays or florescence-activated cell sorting. Therefore, there is an obvious need address the bottleneck surrounding the screening limitations.

This project started out with the idea of using aptamers combined with a physiochemical sensor from DTU Nanotech to detect various metabolite concentrations in batch fermentations. The overall goal was to avoid the use of low throughput analytical equipment for screening purposes and turn to higher throughput nanotechnology solutions to provide the quantitative output. For that to be realized we needed a large number of novel aptamers. Aptamers are single stranded nucleic acid molecules that are selected from huge combinatorial libraries of DNA or RNA to bind specific ligands using a method called Systematic Evolution of ligands by Exponential Enrichment (SELEX). These ligands can be anything from cell surface glycoproteins, enzymes, and carbohydrates to small organic molecules. Aptamers will be introduced in **chapter 1** *(Aptamers)*, but for now a key message is that they can be used as recognition elements in physiochemical sensors (Ferguson et al. 2013) or as custom parts for intracellular sensors like ribozymes (Tang and Breaker 1997). Both of which can be used for screening small molecule compounds in the setting of cell factory production of commodity chemicals (Schallmey et al. 2014).

Aptamer development is an iterative challenging and time consuming method and has an alarmingly low success rate (<30%) (Famulok and Mayer 2014). Therefore a significant number of permutation to the original SELEX (systematic evolution of ligand by exponential enrichment) strategy have been developed during its lifetime of nearly 25 years (Tuerk and Gold 1990; Ellington and Szostak 1990; Ozer et al. 2014). This thesis contains two different permutations of the SELEX technology. The first focuses on the rapid identification of aptamers against a snake toxin using a single-round selection protocol and the other a strategy that allows the aptamer ligand to remain in solution during selection

In **chapters 2 and 3** a brief overview of the permutations to the SELEX technology is given and used to introduce **article 1 and 2** that are appended to this thesis. The first paper of this thesis work concerns the rapid development of an α-bungarotoxin aptamer using an novel approach, which was a collaboration with Dr. Rakesh N. Veedu at the University of Queensland, Australia. **Article 2** deals with the development of an aptamer selection technique that allows parallel selection of aptamers against multiple ligands in solution. These aptamers are destined to be used in *in vitro* and *in vivo* sensor that are introduced in the two following chapters.

Chapter 4 *(Aptamers in synthetic riboswitches)* deals with the adaptation of aptamers into riboswitches. Followed by **chapter 5** *(Aptamers for affinity biosensor devices)* concerns the application of aptamers in physiochemical biosensors for label-free detection of ligands. Included in this chapter are two papers stemming from thesis work, one of which was a collaboration with Professor Noemi Rozlosnik at the Technical University of Denmark, Department of Nanotechnology, and the other a review on toxin detection using aptamers and physicochemical biosensors.

Finally in **chapter 5** *(Conclusions and perspectives)* concluding remarks and future perspectives will be presented followed by **chapter 8** *(Publications)*

1 Aptamers

1.1 On the origin of aptamers by means of *in vitro* selection

Bacteriophage Qβ is a RNA phage that infects *E. coli*. The RNA genome of the bacteriophage contains three open reading frames and one of these is an enzyme subunit that takes part in replicating the RNA genome. In the mid 1960s, Sol Spiegelman isolated the Qβ replicase to characterize how the RNA was replicated. He soon realized that the three fundamental processes of Darwinian evolution – amplification, mutation and selection – could be applied to a population of ribonucleic acids (RNA) *in vitro* (Spiegelman et al. 1965; Mills et al. 1967). In his classic test tube evolution experiment (figure 1) Sol Spiegelman found that the RNA genome template replicated by the Qβ RNA replicase in serial transfer experiments decreased in size and ultimately lead to a smaller RNA species (~15 %) with improved replication rate (15-fold). The notion that an iterative system with an RNA template and a bacteriophage RNA replicase, with its intrinsic error rate, would produce a shorter, faster replicating RNA template was a milestone in *in vitro* evolution. In this experiment Spiegelman isolated an RNA species that was evolving towards the minimal binding sequence of the Qβ replicase RNA binding domain. In that sense, it could be claimed that Sol Spiegelman isolated the first aptamer 25 years before the term aptamer was first used. In this seminal work Spiegelman and coworkers commented on the inability of the of the new shorter RNA species to form infective virus particles and went on to propose the usage of the shorter fragments as decoys that might interfere with virus replication. However, Spiegelman also stated:

"It should not escape the attention of the reader that … other selective stresses can be imposed on the system to generate RNA entities which exaggerate other molecular features".

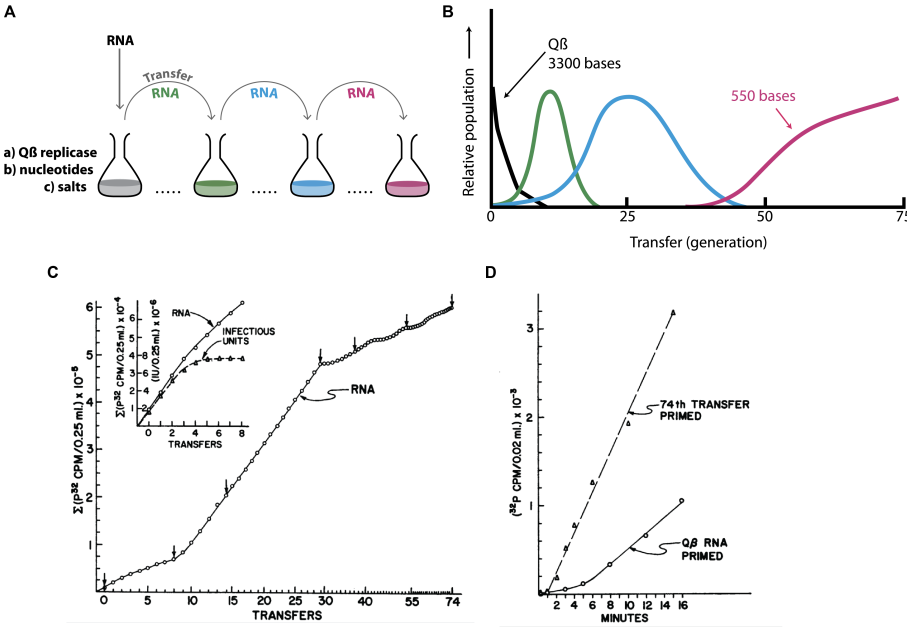


Figure 1.1: The advent of *in vitro* selection - evolution in a test tube.
(A) Schematic view of the original serial transfer experiment published by Mills, Peterson and Spiegelman in 1967. (B) Evolution of RNA molecules adapted from (Stryer et al. 2002). RNA that replicate more rapidly were generated from Qβ RNA by exerting selective pressure. The green and blue curves correspond to species of intermediate size that accumulated and then became extinct in the course of the experiment. (C) The progress of the serial transfer experiment was monitored by measuring the amount of incorporation of [α-32P]-labeled uridine 5'-triphosphate (UTP) into polynucleotides with each transfer. Qβ RNA evolves to faster replicating species resulting in higher concentrations of RNA in each transfer. Arrows indicate increase in selective pressure (decreased incubation time). (D) The comparison of RNA replication rates for native Qβ RNA and RNA from the 74th transfer shows the increased replication rate of the *in vitro* evolved minivariant RNA.

The Qβ evolution system was subsequently used to evolve minivariants of Qβ RNA that exhibited high amplification efficiencies, even when deprived of cytidine triphosphate (Levisohn and Spiegelman 1969) or exposed to the intercalating agent ethidium bromide (Saffhill et al. 1970; Kramer et al. 1974), by imposing additional challenges to the replicating RNA molecules. The high degree of substrate selectivity limits the use of the Qβ evolution system to cognate RNA and, therefore, the real breakthrough in *in vitro* evolution came with the development of the polymerase chain reaction (PCR). PCR allowed almost any sequence of DNA (or RNA if reverse

transcribed into complementary DNA) to be amplified, consequently opening up the possibility of selecting RNA molecules based on other properties other than their ability to be amplified by a replicase.

In 1990, only four years after the invention of the polymerase chain reaction (PCR) (Saiki et al. 1985; Mullis et al. 1986), two research groups independently reported a technique describing the isolation of oligonucleotides with predefined functions. Ellington and Szostak used a method they called "*in vitro* selection" to generate "aptamers"; binders that exhibited dissociation constants in the upper μM range towards organic dyes (Ellington and Szostak 1990). Tuerk and Gold described a method they called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) to isolate "RNA ligands" towards bacteriophage T4 DNA polymerase (Tuerk and Gold 1990). Just as the original serial transfer experiment concocted by Spiegelman, these two groups had used an iterative process of selection and mutation during amplification to generate functional RNA aptamers (Figure 1.2).

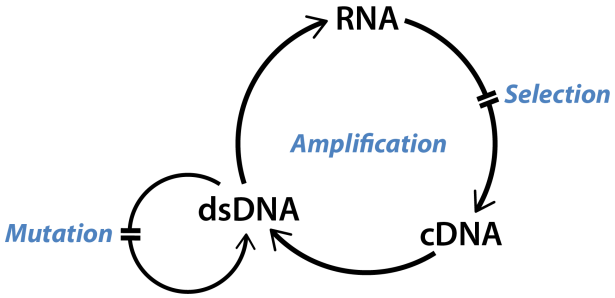


Figure 1.2: The general principle of *in vitro* evolution
A representation of the *in vitro* evolution of functional RNA. Adapted from (Joyce 2007). The selected RNA is amplified by reverse transcription and the resulting complementary DNA (cDNA) can be amplified by PCR to yield double stranded DNA (dsDNA), which in turn can be transcribed into RNA, thus completing a full cycle of selection. Mutagenesis during amplification of dsDNA can be used to maintain population diversity and secure exploration of additional sequence space.

1.2 Systematic Evolution of Ligands[1] by Exponential Enrichment

An aptamer in its most basic form is a string of nucleic acids that folds onto itself and creates a three-dimensional structure that stabilizes prolonged interaction with a ligand. Aptamers have been developed to specifically interact with a plethora of different ligands, from whole cells (Homann et al. 1999), proteins (Tuerk and Gold 1990), peptides (Fukuda et al. 2000), carbohydrates (Yang et al. 1998), to small inorganic molecules (Ellington and Szostak 1990). Aptamers are developed using a method called Systematic Evolution of ligands by Exponential Enrichment (SELEX).

SELEX is an iterative process of binding, partitioning and amplification (Figure 3). The process starts with a library of nucleic acids that traditionally contains on the order of 10^{16} – 10^{17} RNA molecules (~1-10 nmol) with different sequences. The library is most often a single stranded DNA (ssDNA) oligonucleotide with a randomized central region of 40 nucleotides (nt) flanked by primer regions. This allows the ssDNA library to be amplified by PCR between rounds of selection. If RNA aptamers are selected the library includes an RNA polymerase promoter sequence in order to generate single stranded RNA for each selection round.

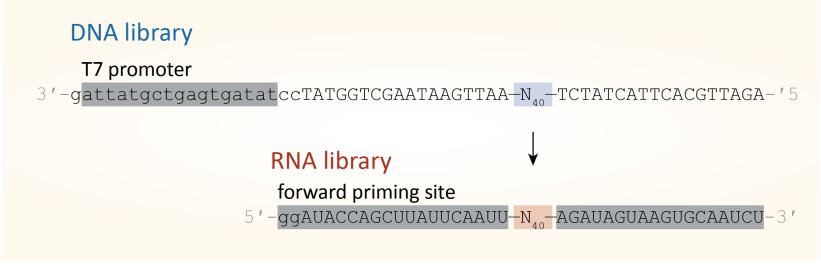


Figure 1.3: The SELEX library.
An example of a traditional SELEX library. The library contains two constant regions for PCR amplification. In this case 40 randomized positions are used leading to a theoretical sequence diversity of 1.2×10^24 DNA or RNA molecules. If RNA selections are carried out the DNA library contains a promoter sequence for RNA transcription.

[1] "Ligands" refers to aptamers. However for the sake of clarity, in this thesis the word ligand is used to describe the aptamer target, whether it being a protein or a small inorganic molecule.

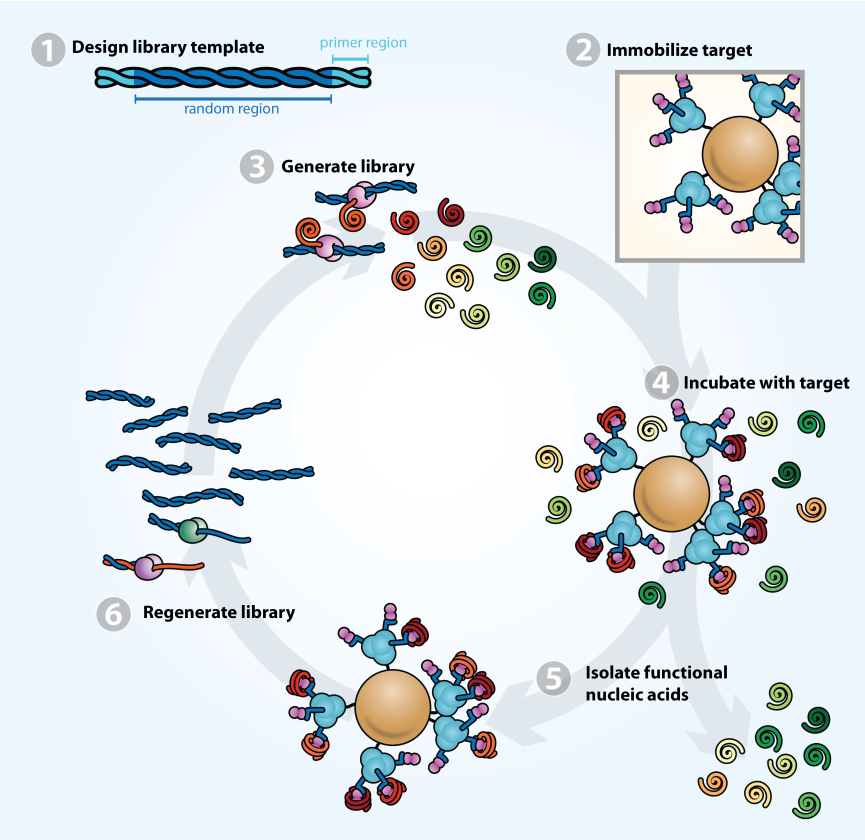


Figure 1.4: SELEX.
The first step in aptamer selection is designing and chemically synthesizing the DNA library (1). This library can be used directly in selection, or transcribed into an RNA library (3). In the case of RNA aptamer selection the RNA is incubated with a target that has been immobilized on solid support (2). Careful characterization of immobilization is important for selection success. The solid support helps partitioning the functional (binding) RNA molecules from the non-functional RNA by enabling stringent washing of the solid matrix (5). Subsequently, the binding aptamers are recovered from the matrix and a new functional pool is regenerated traditionally through reverse transcription, PCR and RNA transcription.

After preparing the pool of putative aptamer candidates, one proceeds to incubate the pool with a ligand of choice. The ligand of choice is either free in solution of immobilized on a surface that allows partitioning. After incubation the aptamers that bind the ligand are separated from the non-binding sequences. The binding sequences are recovered and amplified for a new round of selection. The procedure

is re-iterated, usually employing increasingly stringent selection parameters until the pool of nucleic acid converges on a few sequence families that can be cloned, sequenced and studied for binding.

2 SELEX modifications

A pronounced advantage of the SELEX technique is its adaptability. The basic SELEX experiment only requires standard molecular biology tools to be realized. It has been claimed, that aptamers can be raised against virtually any target (Gold et al. 2012); however, often the requirement for a large number of iterations is associated with the risk of experimental bias precluding the overall successful outcome of the selection. As a consequence, aptamer selections are subject to what might be conceived as an unacceptably low success rates, however meticulously planned. In our hands, the overall chance of positive outcome in aptamer selections is less than 50 % for the aptamer selections presented in this thesis. Even internationally established aptamer groups report success rates of less than 30 % (Famulok and Mayer 2014). Additionally, success rates are very ligand specific. The aptagenic potential of ligands significantly influence the ability to successfully develop aptamers (Carothers et al. 2010). This, together with the design of library, the maintenance of pool integrity and the selection conditions are an integral part of ensuring SELEX success. To date, one of the major focuses of aptamer selection has been the continuous quest to deliver higher success rates and improve upon the original SELEX technology. There have been significant improvements to the SELEX method since it was developed 25 years ago (reviewed by Stoltenburg et al. 2007; Gopinath 2007; Ozer et al. 2014). In this thesis, only the most important developments with direct relevance to the performed experiments are mentioned in depth. Table 1 gives an overview of the abundance of SELEX schemes that have been developed over the years.

Early adaptations of the SELEX focused on how selectivity could be improved; including negative selection rounds against the naked separation matrix to remove non-specific binders from the nucleic acid pool (Negative SELEX) or against ligands with structural similarities to the target ligand (Counter-SELEX) (Table 2.1). Later adaptations focus more on modifications addressing separation efficiency, improving throughput and post-selection analysis (McKeague and Derosa 2012).

Method	Description	Ref.
Atomic force microscopy (AFM)-SELEX	AFM-SELEX uses a dynamic atomic force microscopy tip to pick up and visualize aptamer-target complexes. This SELEX requires only one round of selection.	
Automated SELEX	This SELEX uses automated systems for the procedure to reduce the time and labour required.	
Blended SELEX	In this technique, a lead chemical compound is attached covalently or non-covalently to a nucleic acid library. Each nucleic acid conjugate in the starting library is a variant of the chemical compound moiety and allows up to 10^{15} variants of the small molecule to be screened for the most active of these composite assemblies.	
Cell-SELEX	Cell-SELEX generates aptamers that can bind specifically to a cell of interest. Commonly, a cancer cell line is used as the target to generate aptamers that can differentiate that cell from other cancers or normal cells.	
Capillary electrophoresis (CE)-SELEX	The separation of bound and nonbound oligonucleotides is performed using capillary electrophoresis.	
Chimeric SELEX	Chimeric SELEX uses two or more different oligonucleotide libraries for production of chimeric aptamers with more than one wanted feature or function. Each of the parent libraries will be selected first to a distinct feature; the resulting aptamers are then fused together.	
Conditional SELEX	This SELEX uses regulator molecules during the selection, thus, allowing aptamer binding to the target to be regulated.	
Counter selection/ subtractive SELEX	This technique employs additional rounds of SELEX to remove sequences that bind to similar target structures.	
Covalent/ Crosslinking SELEX	This process is used to select aptamers that contain reactive groups which are capable of covalent linking to a target protein.	
Deconvolution SELEX	Deconvolution SELEX is used to generate aptamers for complex targets. Typically selection is performed on mixtures (or a cell). Once aptamers have been generated, a second part of SELEX involves discriminating which aptamers bind to which parts of the complex mixture.	
Electrophoretic mobility shift assay (EMSA)-SELEX	The partitioning step of SELEX occurs through the use of electrophoretic mobility shift assay (EMSA) at every round.	
Expression cassette SELEX	This is a special form of blended SELEX that involves transcription factors and optimizes aptamer activity for gene therapy applications.	
Fluorescence-activated cell sorting (FACS) SELEX	This SELEX makes use of fluorescence-activated cell sorting to differentiate and separate aptamer-bound cells.	
FluMag SELEX	Here the library is modified with fluorescein instead of radiolabels for quantification purposes. Additionally, the target is immobilized to magnetic beads instead of agarose.	
Genomic SELEX	The SELEX library is constructed from an organism's genome and target proteins and metabolites from the same organism are used to elucidate meaningful interactions.	
Indirect SELEX	The target used in the selection is not the aptamer binder; however, it becomes required for aptamer binding to the new target.	
In vivo SELEX	In vivo SELEX uses transient transfection in an iterative procedure in cultured vertebrate cells to select for RNA-processing signals.	
Multivalent aptamer isolation (MAI) SELEX	This process is used to generate aptamer pairs for a given target.	

continued …

Method	Description	Ref.
Microfluidics SELEX	This SELEX uses microfluidic technologies, creating an automatic, and miniature SELEX platform for fast aptamer screening.	
Monolex	Monolex involves a single affinity chromatography step, followed by physical segmentation of the affinity material, to obtain the highest affinity aptamers.	
Multiplexed massively parallel SELEX	This allows analysis of large numbers of transcription factors in parallel through the use of affinity-tagged proteins, bar-coded selection oligonucleotides, and multiplexed sequencing.	
Multi-stage SELEX	Multistage SELEX is a modified version of chimeric selex. Here, the fused aptamer components then go through an additional selection with all the targets.	
Negative selection	An additional step, performed typically at the beginning of selection, removes sequences that have an affinity for the selection matrix.	
Next generation SELEX	This SELEX uses designed oligonucleotide libraries that tile through a pre-mRNA sequence. The pool is then partitioned into bound and unbound fractions, which are quantified by a two-color microarray.	
Non-SELEX (NECEEM)	This process involves repetitive steps of partitioning with no amplification steps.	
Photo SELEX	Aptamers bearing photo-reactive groups that can photo cross-link to a target and/or photo activate a target molecule are used.	
Primer-free SELEX	This SELEX involves removal of the primer-annealing sequences from the library prior to selection, preventing unwanted primer-based secondary structures.	
Serial analysis of gene expression (SAGE) or high- throughput SELEX	SAGE SELEX links oligomers from SELEX with longer DNA molecules that can be efficiently sequenced.	
Spiegelmer technology	The aptamer selection is performed with the natural D-nucleic acids but on the opposite enantiomer of the chiral target molecule. After sequencing, the aptamers are synthesized as L-isomers for binding to the desired enantiomer of the target.	
Slow off-rate modified aptamers (SOMAmer)	The selection is performed with oligonucleotide libraries that are uniformly functionalized at the 5′-position resulting in high-quality aptamers.	
Tailored SELEX	This is an integrated method to identify aptamers with only 10 fixed nucleotides through ligation and removal of primer binding sites within the SELEX process.	
Target expressed on cell surface (TECS) SELEX	Recombinant proteins on the cell surface are used directly as the selection target.	
Tissue-SELEX	This method is for generating aptamers capable of binding to tissue targets.	
Toggle-SELEX	The selection is performed on different targets in alternating rounds.	
Yeast Genetic SELEX	This method optimizes in vitro selected aptamers by creating a library of degenerate aptamers and performing a secondary selection in vivo using a yeast three (one)-hybrid system.	

Table 2.1: Adaptations of SELEX.
Table adopted with permission from (Stoltenburg et al. 2007).

With imminent success in mind, the first consideration in any SELEX experiment is how to secure efficient separation between functional and non-functional nucleic acid species in the aptamer library. One of the most common means of separation in aptamer selection is the immobilization of target on filter-, resin-, or magnetic bead-based separation matrix. The immobilization may either be a covalent coupling of the target through standard coupling chemistries employing free amine-, thiol-, hydroxyl-, or carboxyl groups on the target molecule. Or the target – in case of proteins – can be tagged and immobilized on an affinity resin (e.g. Ni-NTA, Glutathione, Amylose resins, etc.). Immobilization of aptamer target on solid support offers a simple solution to the separation of functional from non-functional nucleic acids. Once a working immobilization strategy has been developed for the target, fine-tuning of selection stringency requires minimal effort and no specialized equipment (Ozer et al. 2014).

Another vital reason for choosing an immobilization strategy is scarcity of target. Magnetic bead based selections are ubiquitously used in aptamer selection, because of their ease of handling, established protocols and most importantly low amount of target needed. While filter- and column based methods previously required substantially more target to reach high density than magnetic bead based approaches (Stoltenburg et al. 2005). Furthermore, selections involving solid support have the added advantage of being able to change between selection in steady-state equilibrium and non-equilibrium conditions induced by extensive washing of the matrix. This allows for the selection of aptamers based on kinetic parameters such as slow off-rates (Gold et al. 2010).

Tuning the availability of target, however, comes with a few caveats. Firstly, if pre-immobilization is required the density of target can have substantial influence on the success of aptamer selections. High target density can induce cooperative non-specific binding of nucleic acids to multiple neighboring targets and reduce the enrichment of true aptamers in a selections cycle (Ozer et al. 2013). Whereas, low target concentration may facilitate increased selection of background binding aptamers (Wang et al. 2012). The latter is especially relevant when post-selection immobilization is used as a separation technique (i.e. nitrocellulose filter binding and affinity resin capture). These methods often require high background to target ration on order to efficiently capture aptamer:ligand complexes. This was a problem

especially in the early SELEX experiments were nitrocellulose filters were used as matrices to partition bound RNA/protein hybrids from unbound RNA. With the use of any kind of solid support for ligand immobilization there is a chance that aptamers with affinity towards the matrix rather than target are isolated. Especially when selection RNA aptamers using the filter biding separation technique (Shi et al. 2002). In literature there are multiple reports of G-rich sequences being overrepresented in aptamer selections using this filtration method (Tuerk and Gold 1990; Shi et al. 1997). Introducing a negative selection against the separation matrix can ameliorate this, but will not eliminate the tendency of these sequences to dominate the selection in the later cycles, even alternating the types of matrix may not be sufficient to prevent unspecific binding if the difference between matrices is not sufficient to discriminate against binding by less specific interactions like hydrophobic interaction (Shi et al. 2002). Recent advances in micro column based SELEX now reduce the amount of resin needed in aptamer selections (Szeto et al. 2014); however the technology is currently limited to research groups with specialized equipment.

While changing the solid support during selection avoids unwanted enrichment of background binding aptamers it also increases the time required for selections to be performed. This is because each immobilization reaction has to be carefully analyzed. Therefore, selection of aptamers against targets in solution has the ability to circumvent aptamers against matrix and added time used to evaluate immobilization. This will be introduced later in this thesis. SELEX has undergone many changes since its inception in 1990. Correspondingly, the library of nucleic acids used for these changed SELEX protocols has undergone scrutiny to identify the optimal design for obtaining functional aptamers.

2.1 Design of the SELEX Library

The design of starting sequence library has great impact on the outcome of functional nucleic acid selections and the overall success of SELEX. As mentioned earlier, the commonly used recombinant aptamer libraries have a 30-60 nucleotide random region flanked by priming regions and are derived from chemical synthesis. However, they may also be derived from genomic DNA (Vu et al. 2012) and from transcriptomic sources (Pagano et al. 2014; Fujimoto et al. 2012). The type of target and the intended use of the aptamer post-selection are factors that typically dictate the design of the library. Generally, libraries used in aptamer selections are shorter than libraries used to select for catalytically active nucleic acids (e.g. ribozymes) (Bartel and Szostak 1993). Short libraries have a sequence space sampling advantage, however, longer regions allow for more structural diversity. As an example, the original selection of the class I RNA ligase used a random tract of 220-nt (Bartel and Szostak 1993), while an isoleucine aptamer was isolated from a library with just 22 random positions (Lozupone et al. 2003).

There is no consensus in library design that states the optimal length of the random part of an aptamer library and only a few experimental investigations have tried to ascertain the difference in outcome in aptamer selection resulting from different size random region in an aptamer library. Huang and coworkers found that when using a mixture of libraries containing 30-, 60-, 100-, and 140 nucleotide random regions, only the shorter 30- and 60-nt libraries were maintained during the selection due to replicative disadvantage of larger pools. However, it was still argued that aptamer libraries containing a 60-nt region where superior to 30-nt (Huang et al. 2000; Coleman and Huang 2002). Later, Yarus and coworkers repeated the isoleucine aptamer selection with libraries (26-, 50-, 70-, and 90-nt). In discordance with the intuitive notion that longer pools should have higher occurrence of small sequence motifs, quite surprisingly, pools with length 26- and 90-nt performed equally poor in the isolation of isoleucine motifs, consequently labeling the mid-range pools as optimal for selecting aptamers with the previously identified motif (Legiewicz et al. 2005).

Others have tried to quantify the suitability of aptamer libraries design via computational approaches. From computational analysis of 141 different aptamers

(2000 sequences), it has been suggested that random region length does not affect the overall thermodynamic stability of resulting aptamers. Surprisingly, it was also reported that constant regions of libraries did not participate significantly in aptamer secondary structure, however the smaller the random region gets, the more likely it is that the flanking primer sites contribute to aptamer secondary structure, possibly decreasing coverage of sequence space and impeding the chance of finding high-affinity aptamers (Cowperthwaite and Ellington 2008). In our experience and from the results in this thesis, the priming regions often take part in the overall secondary structure of the aptamer candidates. This is also in concordance with previous reports from other groups (Shi et al. 2007; Ozer et al. 2014). Several primer-free selection protocols that have minimal or absent constant regions have been developed, however, these methods still remain an uncommon application of SELEX, and their limited use still beckons the question of the importance and superiority of removing constant regions (Pan et al. 2008). In fact, simply adding oligonucleotides complementary to the priming regions in the selection step has been shown to yield aptamers that do not include constant regions in the binding specificity (Nutiu and Li 2005).

Ultimately, the canonical design of random regions in aptamer libraries is still a subject of discussion. While some research groups claim that 60-nt random regions provide the optimal condition for successful aptamer discovery (Coleman and Huang 2005), others advocate for consistently using parallel selections with varying aptamer library sizes (Velez et al. 2012), which in turn might increase experimental complexity and prohibit the parallel sampling of multiple targets.

One interesting modification of the conventional uniform randomization of the random region is the use of structurally biased random regions. Davis and Szostak observed that incorporating a specific stem-loop into the random region as a nucleation point for increased structure resulted in a better performing library in the selection of guanosine triphosphate (GTP) (Davis and Szostak 2002). Recently, the uses of random regions biased for stem loop formation have been used to expedite the isolation aptamers against streptavidin, immunoglobulin G (IgG) and VEGF (Ruff et al. 2010). An interesting addition to the story of structurally biased libraries is the observation that aptamers selected from structurally rigid libraries tend to rely less on the concentration of divalent cations for functionality (Ruff et al. 2010). This might aid

the *in vitro* isolation of aptamer that are functional in conditions that are relevant for *in vivo* use (Carothers et al. 2010). Selecting vivo functional aptamers are an important when aptamers are used as sensor domains in RNA regulators like riboswitches (as discussed in section 4.3 p. 53). Because many catalytic RNAs that are functional *in vivo* display higher order junctions as a common structural feature, Lou *et al.* used computational design of libraries with increased tendencies to for 4- and 5-way junctions (Luo et al. 2010). The library was confirmed to display increased content of higher-order junctions, but failed to effectively increase throughput or affinity of isolated aptamers compared to a regular selection against ATP.

3 Expediting aptamer development

Until very recently, the standard operating procedure for most aptamer selections seems to have been: *single library, single target, single type of separation* and more iterations than one can shake a stick at. However, as mentioned earlier, the most recent advances in aptamer selection focus more on throughput. In essence, every aptamer selection has multiple targets if you consider separation matrix and different epitopes on a single proteinaceous target.

This thesis features two selection articles. **Article 1** is a selection against snake toxin with a high aptagenic potential based on its size and overall surface charge distribution (presence of positive-charged regions). **Article 2** features selection against smaller molecules that do not poses the aptagenic potential of a large positively charged protein. One key difference that will be elaborated on later is the immobilization strategy employed in the two selections.

3.1 Single round selection

As mentioned earlier, the multiple iterations of SELEX allow for unwanted experimental biases to occur. One such experimental bias is the unwanted influence non-specific PCR amplification. The conventional source of PCR by-product is primer-primer hybridization, however, in SELEX libraries the large heterogeneity in the random region also permits unwanted primer-random region hybridizations that skew the overall outcome of PCR. Aptamer selections have been reported to fail due to failure of maintaining pool integrity through selection rounds (Tolle et al. 2014). Usually, the overamplification of the aptamer pool results in larger dsDNA fragments that appear as high molecular smears upon electrophoresis. The nature of these calamitous PCR products have been identified as stemming from inadvertent hybridization of primer and random region between library members and subsequent enzymatic extension. In addition, the propensity to form unwanted by-product is highly library specific, and can presently only be determined by empirical observations (Tolle et al. 2014). Another PCR-related issue in aptamer selection pertains to the *de novo* selection of aptamers containing modified nucleotides.

Single round selections are a concerted effort to avoid the introduction of PCR bias into selection outcome, while simultaneously improving throughput of aptamer selections. Additionally, the absence of multiple rounds of selection, has enabled the commercial exploitation of aptamer technology and avoid patent issues (Dua et al. 2008). Nitsche *et al.* proposed the first application of single round selection, MonoLex. In MonoLex, the aptamer library is passed through an affinity column with immobilized target and then vigorously washed to remove non-specific and low-affinity interactions.

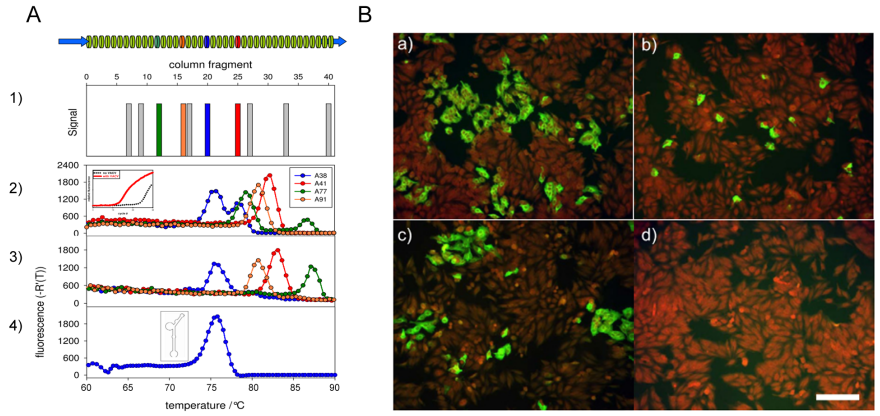


Figure 3.1: MonoLex selection of aptamers against Vaccinia virus particles. Original work as presented in (Nitsche et al. 2007). Results for the selection of ssDNA aptamers using an affinity column with immobilized inactivated Vaccinia virus. (A1) The column was cut into 40 segments and analysis of DNA content show localization into distinct regions in the column (A2) Analysis of pool shows. (Reprinted with permission from publisher).

The affinity column is then cut into discrete segments and the aptamers present in each segment are analyzed for affinity. Affinity chromatography should separate individual aptamers into in theoretical plates based on their affinity. Hence, higher affinity aptamers are expected to inhabit the top of the affinity column, whereas, lower affinity interactions with slower on-rates or faster-off rates are found closer to the bottom of the column. However, in the study by Nitsche *et al.* the distribution of aptamers was not found to follow this hypothesis, since the identified aptamer with the highest affinity did demonstrate the highest retention on the column. Ultimately, the selection was performed on solid support and the affinity measurement was performed in solution, whether this change is the source of discrepancy or differences could be attributed to cooperative binding to solid support remains uncertain. Nonetheless, this method has been used to select DNA aptamers against β-1-adrenoceptor autoantibodies (Haberland et al. 2011) and Vaccinia virus (Nitsche et al. 2007).

In this thesis, one such method has been used to isolate a DNA aptamer against α-bungarotoxin (Figure 3.2). This method utilizes the immobilization of target on a glass coverslip that can be subjected to microscopy evaluation for nonspecific binding and subsequent increased stringency washing steps. The method has been tested on two different targets: α-bungarotoxin (α-BuTx) and green fluorescent protein (GFP), of which only the first was a success. GFP has previously been shown to be a particularly difficult protein to select aptamers against failing, in one instance (Stanlis and McIntosh 2003) and requiring a massive 27 rounds of SELEX in another (Shui et al. 2012), however, ultimately arriving at an aptamer structure with no interference from the constant regions. The limited surface complexity of β-barrel structure of the GFP may be the influencing factor, why this ubiquitously used protein has eluded the aptamer community so efficiently. Evidently, the method of selection via immobilization on glass slides and on array allow for optical interrogation of the level of nonspecific binding if the library is labeled with a fluorescent dye. Currently, one of the more successful custom aptamer selection companies BasePair Bio uses the method of target immobilization on glass slide. This allows an array of targets to be selected and massively parallel aptamer selection to be performed by incubating the target array with one bulk solution of library. The selected sequences are then linked with target array spots by ligation of a target specific sequence tag (Jackson 2012).

Comparable efforts to the include single round selections using on-filter DNaseI-digestion to enrich aptamers protected by larger ligands (Liu et al. 2012), selection using Atomic Force Microscopy (Peng et al. 2007), isolating aptamers against Kallikrein-related peptidase 6 (KLK6) (Arnold et al. 2012) and recombinant human growth hormone (rHGM) (Çalik et al. 2010) by stringent washing in high-salt and temperature gradients.

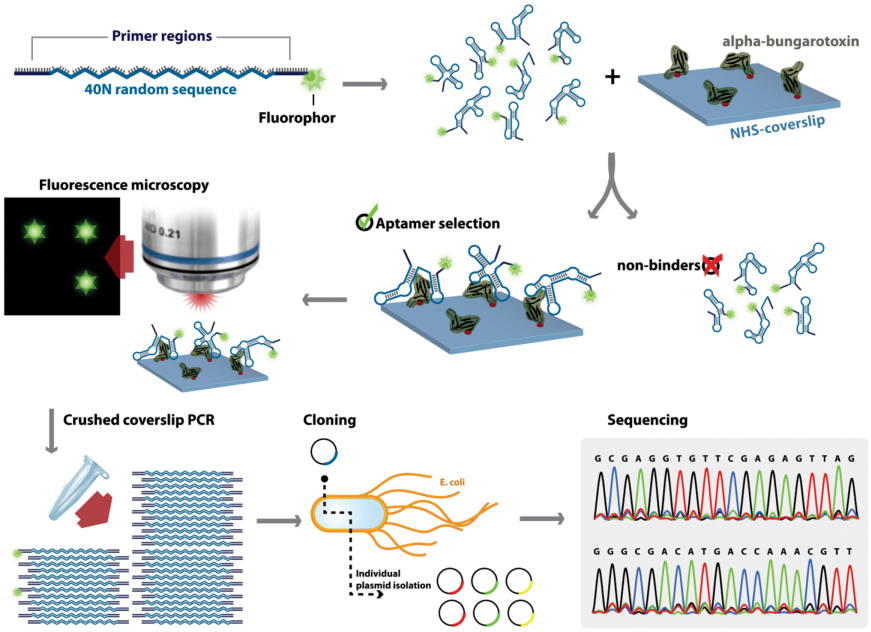


Figure 3.2: Single round selection method.
A schematic overview of the single step selection protocol used to develop an ssDNA aptamer against α-bungarotoxin. A fluorescently labeled aptamer library is incubated with target immobilized on an *N*-hydroxysuccinimide acid (NHS) functionalized coverslip. The cover slip is extensively washed and binding sequences are eluted from the crushed coverslip by heating. PCR fragments are cloned and sequenced to obtain aptamer sequences.

The absence of iterative rounds of selection requires a different approach to sequence analysis. Traditionally, the abundance of a full-length sequence at the end of a multiple round selection is used as a measure of selective enrichment. In single round selections, the recurrence of a structural sub-motif is the measure to which enrichment is benchmarked, not the full length sequence. In a single round selection,

the enrichment factor is of great importance to the overall successful outcome of selection. In theory, applying a sequence diversity of 10^{16} requires an enrichment factor of 10^{10} to be attainable for high-throughput sequencing. Enrichment factors this high require the target of interest to have increased aptamer binding potential. Aptamer binding potential "aptagenic potential" has been linked to the isoelectric point (pI) of a protein. Because DNA and RNA carry a net negative charge on their phosphate backbones high-affinity interaction might be stabilized with targets carrying a net positive surface charge (pI > pH).

Parallel selection of aptamers against platelet derived growth factor B (PDGF-BB; pI=9.3), thrombin (pI=8,3) and apolipoprotein E3 (ApoE; pI=5,3) revealed that after 3 round of selection bulk dissociation constant (K_d) of aptamer pools were lower for ApoE, compared to PDGF-BB and thrombin (Ahmad et al. 2011) (Figure 3.3). In our one round selection, α-BuTx (pI=7,9) and rGFP (est. pI=6,0) were selected at pH 7.4, resulting a net positive surface charge of α-BuTx and a net negative charge of GFP.

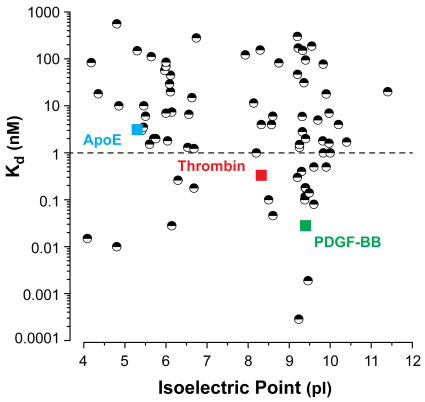


Figure 3.3: Equilibrium dissociation constant versus on Isoelectric point.
The plot shows the relationship between protein pI and aptamer K_d for 75 aptamers with validated binding affinity taken from published literature. Additionally, the three selected aptamers against platelet derived growth factor B (PDGF-BB; pI=9.3), thrombin (pI=8,3) and apolipoprotein E3 (ApoE; pI=5,3) from (Ahmad et al. 2011). For the selected aptamers there is an apparent inverse relationship between protein pI and aptamer K_d. The proteins were identified in the UniProt database and isoelectric points were determined from the Compute pI/Mw tool at ExPASy. (reprinted with permission from (Ahmad et al. 2011).

Based on results from an additional 75 aptamers against proteins the result presented in Figure 3.3 lead Ahmed *et al.* to claim an inverse relationship between protein pI and aptamer K_d. The data is to be taken with a grain of salt since the K_d -values may have been obtained at different pH using different affinity determination tools. This can affect dissociation constants by orders of magnitudes (Hianik et al. 2007; Daniel et al. 2013). However, the fact that there is an observable inverse relationship between protein aptamers selected by only three rounds of SELEX under similar conditions might imply that the hypothesis is true. In that case, overall charge availability might be even more important in single step selections that are deprived of the option of increasing selection pressure through iterative evolution. Recently, the single round selection of aptamers has been combined with high-throughput sequencing analysis to allow an intuitively more robust method of yielding aptamers in a single selection round. Next-generation sequencing undoubtedly become an integral part of future permutations to the SELEX technology.

3.2 Next-generation sequencing in aptamer evolution

Next-generation sequencing (NGS) has revolutionized metabolic engineering (Mitchell 2011), structure probing (Lucks et al. 2011) and is equally important for the analysis of sequence space in aptamer selections. SELEX traditionally relies on multiple rounds of selection and maturation to identify enriched aptamer candidates. With the advent of next-generation sequencing came the possibility of monitoring SELEX progression through the selection rounds in a more detailed way (Cheng et al. 2013). Also used in this thesis, the intricate knowledge of how a pool evolves allows for further optimization of the SELEX approach and gives key insights into the mechanistic effects of increased selection pressure (Ameta et al. 2014) or the interplay between target-specific and non-specific sequences during selection (Cho et al. 2010).

Essentially, next-generation sequencing has the potential to reduce the amount of iterations needed for successful selection of aptamers, given that enrichment of certain sequences can be observed earlier in the process and even studied through round-to-round enrichment. Additionally, these high-throughput sequencing methods might give increased insight into the many complications that might arise from bias in PCR or transcription reactions. To that end, Zimmermann *et al.* conducted the first experimental investigation of PCR bias in genomic SELEX (Zimmermann et al. 2010). Genomic SELEX uses RNA derived from genomic DNA as selection input to discover new regulatory RNA species. Zimmermann *et al.* compared the sequence output of 9 rounds of SELEX without target to a previous selection against Hfq protein. They found favoritism for shorter oligonucleotides with overall tendency of less secondary structural stability in selected pools vs. initial libraries. Additionally they noted a nucleotide bias towards adenosines, but concluded that this did not seem to hamper the evolution of nucleotides responsible for binding affinity of Hfq aptamers. Favoritism of adenosines might be a concern if structural rigidity is sought, as structural rigidity is often mentioned in the context of a higher number of GC pairs. But also if it were due to an intrinsic restriction of the enzymes utilized in SELEX, which would lead to an inherent inability to sample sequence space properly. However, enrichment of adenosines is not observed in all SELEX experiments as described by Thiel *et al.* in 2011 (Thiel et al. 2011). In their selections they saw increase in purine bases during selection without target resulting a pool with a lower

average minimum free energy (ΔG) which means higher structural stability. This study employed a small random library with 20 random bases. It is an often seen feature of NGS type SELEX experiments that overall structural change or change in nucleotide distribution is given merit in predicting the outcome of future selections. Since there also are publications suggesting enrichment of especially thymine in SELEX experiment, the overall importance of such observations remain to be elucidated and seem to be very library-specific.

One genuinely useful discovery after utilization of NGS in SELEX is that the most highly enriched sequences in selection not necessarily constitute the best binders as they have to be seen in a context of enzymatic amplification. In one such example, streptavidin aptamers were evolved over 10 iterations of the SELEX protocol and analyzed by NGS. Sequencing of each round of SELEX showed that specific sequence species dominate different rounds of selection and that no notable enrichment of affinity is seen after the 3rd round of SELEX (Schütze et al. 2011). The authors claim to have increased selective pressure albeit only slightly with no real change in rounds 2-9. Therefore, affinity might have been better had the selective pressure been incrementally increased. Nonetheless, the notion that highest enrichment provides highest affinity is now well known not to be the case (Cho et al. 2010). Especially, since a large number of sequence populations can be sustained at a high level of enrichment by virtue of their 'amplify-ability' (Cho et al. 2010). This highlights how arbitrary sequences, simply by virtue of PCR bias, affinity to partitioning matrix or sequencing bias, can appear enriched in selections utilizing only endpoint sequencing and why SELEX is still prone to failure for some targets. Instead, the round-to-round fold enrichment seems to have become the new choice for the targeted use of NGS in multiple round selections. Aptamers selected against PDGF-BB (a highly aptagenic target) that were identified based on their fold enrichment, exhibited up to 8-fold greater affinity than the aptamers found using total enrichment.

In 2011, Hoon *et al.* proposed a novel selection strategy that combined high-throughout sequencing and single step selection against thrombin (Hoon et al. 2011). Thrombin is an excellent choice for proof-of-concept selections since it repeatedly has been shown to allow high affinity interaction with DNA aptamers even to different epitope sites (Bock et al. 1992; Tasset et al. 1997). Aptamer Selection by k-mer

Analysis of Sequences (ASKAS), yielded a novel human thrombin aptamers in a single round of selection, which were predicted to bind thrombin with a K_d's in the nanomolar to micromolar range, The isolated motif were exclusively consistent of guanosine and thymine residues, exactly like the original aptamer isolated by Bock *et al.* The ASKAS method uses frequency of varied length nucleotide motifs in determining the binding motif of a selected aptamer. Hoon *et al*. averaged sequence counts of each of the top ten most represented 15- to 33-mers respectively, and saw a two-fold increase in occurrences of 15-mers over 16-mers. The sudden increase in occurrences lead to the identification and characterization of five 15- and 16-mers with affinity towards thrombin. Interestingly, all the aptamers identified were homopolymers of guanosine and thymine, which emphasizes the structural favorability of the G-quadroplex in binding thrombin (Macaya et al. 1993). All in all, the method developed by Hoon *et al.* represented a novel fast, promising and cost-effective way of selecting DNA aptamers. Since we were looking for ways of generating a large amount of aptamers in one experiment, the simple and straightforward ASKAS methodology fitted well with our overall needs.

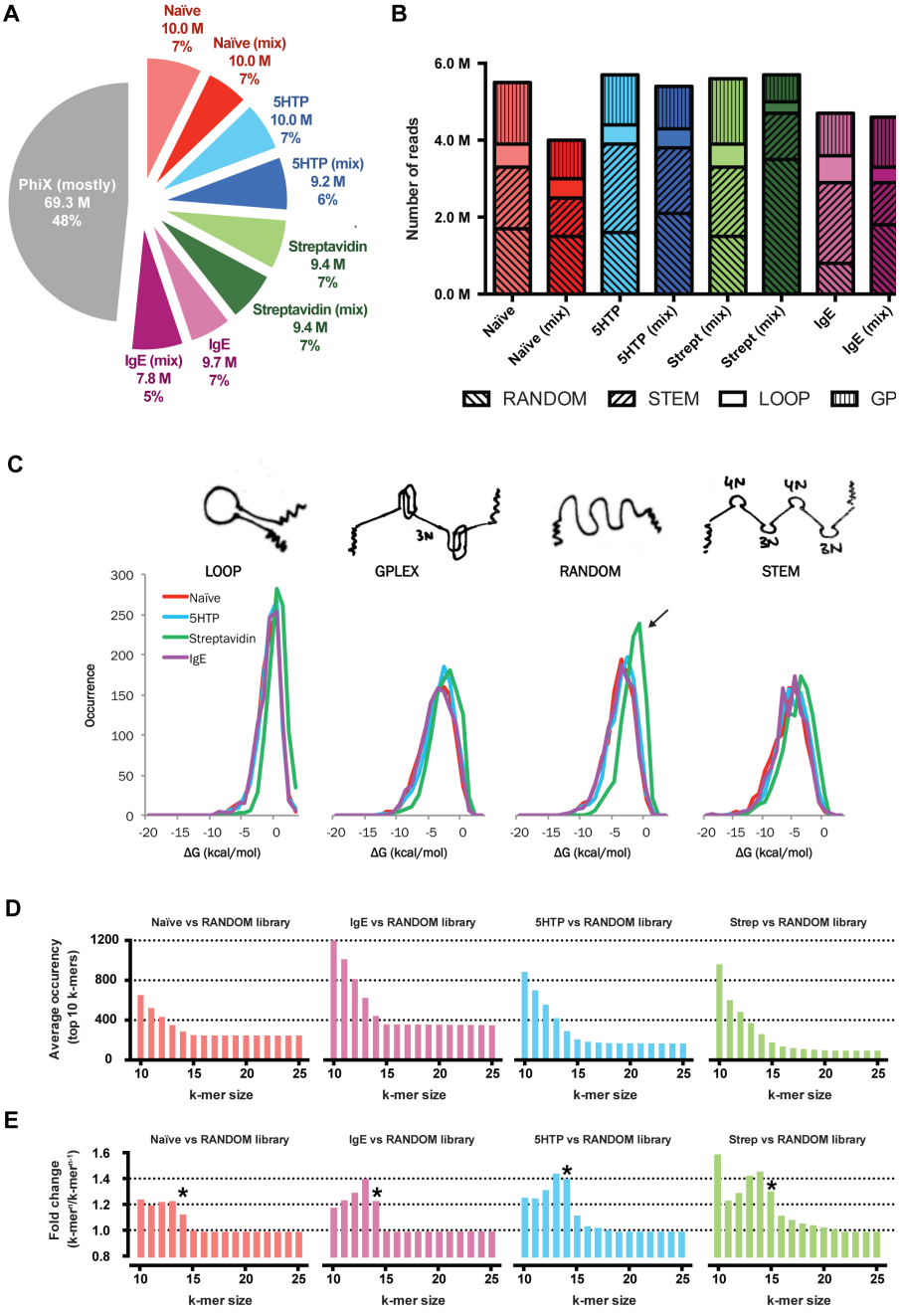
Spurred on by the apparent success of the ASKAS analysis, as part of this thesis the approach was adopted to select DNA aptamers against three different targets: Immunoglobulin E (IgE), Streptavidin and 5-hydroxy-tryptohan (5HTP). IgE and Streptavidin aptamers have previously been reported and, therefore, served as potentially good candidates for successful selection, while 5HTP is an interesting candidate for microbial production (Lin et al. 2014). Furthermore, we sought to expand the analysis of ASKAS feasibility to different library designs. Therefore, we included four different libraries including the original loop library used in the original paper. The library designs for our combined next-generation sequencing approach (aptly name NGS SELEX) were inspired by the previously presented results from Ruff *et al.* and Yoshida *et al.*, who used structurally constrained libraries with increased potential for stem-loop- and G-quadroplex formation, respectively (Ruff et al. 2010; Yoshida et al. 2009) (Figure 3.4 C).

In this investigation, the total number of selections was 15. We have yet to find a comparable effort to investigate the selection of aptamers with this many simultaneous parallel selections. The 15 selections were a result of a complete combination of all targets and all ligands (3 x 5); an additional selection against each

target with a mixture of all the libraries was designed as a study of inter-library competition. A selection scheme was devised where every selection was spiked with small amounts of aptamers that had been previously reported against streptavidin (Qian et al. 2009) and IgE (Wiegand et al. 1996; Jing and Bowser 2011). To verify the enrichment of aptamers in selections with their cognate ligands each selection contained on the order of 60,000 aptamer molecules spiked into the 10^{14} library DNA molecules. After selection, each library was barcoded and amplified with PCR primers containing illumine sequencing adaptors, designed to obviate the need for additional amplification during library. The sequence data was subjected to similar analytical procedure as proposed by Hoon *et al.*

Initially, we identified the need for very stringent quality filtering parameters since sequencing results were littered with homo-polymeric nucleotide regions, which grossly affected k-mer analysis. After stringent quality filtering, the number of reads per selection were 500K to 3.5M reads (less than originally intended and less than the originally demonstrated 15 M in Hoon *et al.*). Another key feature of the original paper is exceptionally low recurrence of 15 and 16-mers. In the original ASKAS paper (based on 15 M reads), the average occurrence of the top ten most enriched sequence motifs were 40 and 98 times for 15- and 16-mers, respectively. In our experiment occurrences were higher for and even lower amount of sequence space (Figure 3.4D). This skewing of data meant that the enrichment of control aptamers was difficult to ascertain and the overall selection enrichment of aptamers was difficult to distinguish from enrichment through PCR (possibly due to amplification bias).

Figure 3.4: Representative results of NGS SELEX.
(A) The overall read count of the NGS SELEX experiment, roughly 20 M reads per target and Naïve selection (red). To create the mandatory sequence diversity for HiSeq® a PhiX diversity standard was used at 50 % spike (70 M reads). (B) Sequence numbers based on library type. (C) Minimum free energies of 1000 randomly picked sequences from selections and folded using UNAFOLD software. Selection against streptavidin, using the fully randomized library, lead to a change in overall secondary structure distribution. (D) Average occurrence of top ten k-mers size 10 to 25 bases showed no significant difference between naïve (red) and selected pools (purple, blue, green). (E) Fold change of data presented in 4D shows increase in fold change between k-mers of 15 and 16 bases.


A
Naïve
10.0 M
7%
Naïve (mix)
10.0 M
7%
5HTP
10.0 M
7%
5HTP (mix)
9.2 M
6%
Streptavidin
9.4 M
7%
Streptavidin (mix)
9.4 M
7%
IgE
9.7 M
7%
IgE (mix)
7.8 M
5%
PhiX (mostly)
69.3 M
48%
B
Number of reads
6.0 M
4.0 M
2.0 M
0.0 M
Naïve
Naïve (mix)
5HTP
5HTP (mix)
Strept (mix)
Strept (mix)
IgE
IgE (mix)
RANDOM
STEM
LOOP
GP
C
LOOP
GPLEX
RANDOM
STEM
Naïve
5HTP
Streptavidin
IgE
Occurrence
ΔG (kcal/mol)
D
Naïve vs RANDOM library
IgE vs RANDOM library
5HTP vs RANDOM library
Strep vs RANDOM library
Average occurency
(top 10 k-mers)
k-mer size
E
Naïve vs RANDOM library
IgE vs RANDOM library
5HTP vs RANDOM library
Strep vs RANDOM library
Fold change
k-mer size

In a combinatorial oligonucleotide library with 40 random positions, the theoretical diversity is 4^40 = 1.2E24. All libraries were synthesized with forced distribution of 25% per nucleotide. Theoretically, this means that a selection that only uses a single round will not allow a specific sequence to be sampled more than once. We therefore tried removing sequences from each positive selection (IgE, streptavidin and 5HTP) that also were present in the negative selection (Naïve), and reducing the sequence count of each sequence to one, yielding a dataset with unique sequences. This would more accurately portray the theoretically scenario of single-step selection. However, after exhaustive screening of the 15 selections and comparing them to their naïve counterparts, no radical changes in k-mer occurrence were found as initially suggested by Hoon *et al.* Nevertheless, even though the initial trial to verify the applicability of this method failed to generate fruitful results, single-round selection still is a promising addition to the SELEX family. More detail will be given in section 6.2 – On the future of single round selections.

Single round selections might offer a quick fix to the time aspect of selecting multiple new aptamers; it does not, however, address another key issue – immobilization. All single round selections and most of the 'NGS-turbocharged' variants of SELEX, have required immobilization of target. As previously mentioned, target immobilization can have significant effects on the successful outcome of selection. Even more importantly, immobilization favors larger targets and is not necessarily applicable for small molecules. While, some of the interesting biological pharmaceuticals are larger peptides and proteins being produced in microbial hosts, most compounds of biotechnological interest are small molecules with a limited amount of functional groups that can be used for immobilization to solid support. Therefore, there is a need for a robust alternative allowing the selection of aptamers against small molecules.

3.3 Selection of aptamers towards small molecules in solution

Often, biologically and environmentally interesting molecules that are targets for biosensors are small molecules. The small size of these molecules, however, signifies a key challenge in aptamer selections. As the likelihood of obtaining a synthetic aptamer correlates positively with the size of the chosen target (Carothers et al. 2010), the selection of aptamers against small molecules is particularly challenging. While aptamers interact with their targets mainly via specific hydrogen bonds, electrostatic complementarity, and π-π stacking (Hermann and Patel 2000), smaller targets possess fewer chemical groups that can stabilize binding. Molecular shape complementarity is another major specificity factor that may also be difficult to realize with targets that exhibit high conformational disorder due to multiple rotatable bonds (Carothers et al. 2010). As discussed earlier, the aptamer selection protocol most often calls for immobilization of target. In this case the desired ligand must be carefully evaluated for the presence of suitable groups (e.g. carboxylic acids, primary amines, or various carbohydrate moieties) for chemical coupling to solid support and also evaluated for overall stability during the matrix immobilization procedure. Small molecular targets are particularly affected by chemical alterations, which often grossly change their size, shape, and charge distribution, and may sometimes result in their suboptimal (i.e. for aptamer selection purposes) orientation against the matrix. Altogether, these issues make it difficult to obtain an aptamer with high selectivity and/or affinity towards small molecules. For this reason, selection against free targets in solution is preferred whenever possible.

Very few adaptations of the SELEX technology enable the selection against targets in solution. When selecting aptamers against small molecules and using an immobilization strategy there is a possibility that the aptamer only recognizes the immobilized ligand and not the solubilized version. As previously mentioned, in-solution binding has previously been secured by eluting aptamers off affinity columns with high concentrations of target ligand (Geiger 1996). However, this still does not obviate the need for immobilization, which sometimes can be a laborious addition to the already extensive SELEX protocol. Since separation (or partitioning) of functional and non-functional aptamers is the most important step in SELEX, researchers have reverted to immobilization of libraries on magnetic beads for the selection against

target ligands in solution (Rajendran and Ellington 2003). Just as ligands in solution have been used to elute the aptamers off solid support, so has the ligand solution been used to elute nucleic acids off beads as a targeted strategy to evolve molecular beacons (Rajendran and Ellington 2003). Originally, molecular beacon referred to nucleic acid DNA probes that allowed detection of complementary strands of DNA. Molecular beacons traditionally have a quencher and fluorophor pair that is separated by a structure that switches upon binding of a complementary small oligonucleotide (ligand), thus eliciting concentration-dependent fluorescent signal upon binding of target probe. Jhaveri and Ellington were the first to report a direct selection of fluorescent aptamers intended for the use structure switching reporting aptamers. In this example, ATP was immobilized to the surface of agarose beads and, while successful in finding switching aptamers, their results showed that a significant number of them failed to elicit any fluorescent change upon target binding (Jhaveri and Ellington 2001).

In 2003, the Ellington group published a SELEX protocol for structure-switching DNA aptamers designed for the use as molecular beacons. Here, the target, an oligonucleotide probe, was in solution and used to directly identify molecular beacons (Rajendran and Ellington 2003). In this case the DNA library was hybridized to solid support using a capture oligonucleotide containing a quencher. The result was highly active molecular beacons directly selected to elicit a certain response – structure switching. However, the elicited structure switching was based on canonical interactions of base pairs and not proven for non-nucleic acid targets. Motivated by their work Nutiu *et al.* in 2005 adapted the work to directly select signaling-aptamers against small molecules, identifying DNA-based molecular beacons with affinity towards adenosine triphosphate and guanosine triphosphate (Nutiu and Li 2005). This approach was later adapted to RNA with the intent of selecting molecular beacons against tobramycin (Morse 2007). Although Morse found signaling aptamers that responded to tobramycin the highly efficient switching as found by Nutiu *et al.* could not be replicated.

In 2012 came the first adaptation of the library capture approach directed at pharmaceutical compounds in a multiplexed fashion. Capture-SELEX is a variant of the *in vitro* selection process that is well suited for isolating aptamers against small soluble target molecules (Stoltenburg et al. 2012). In contrast to classical SELEX,

where aptamer candidates are selected by passing the oligonucleotide library over the immobilized target molecule, the oligonucleotide library in Capture-SELEX is immobilized by annealing to a bead-bound capture probe and aptamer candidates are eluted with a solution of the solvated target. Any library member that undergoes gross conformational changes upon binding the target will potentially dissociate from the capture probe. The liberated aptamer candidates are then collected, amplified, and used in the next selection round. This process repeats until the pool has been sufficiently enriched for putative aptamers. As the target remains solubilized throughout the selection process, the elution of aptamer candidates lends itself well to multiplexing using several targets. The method was first reported to only yield DNA aptamers against kanamycin A (MW: 681 Da) from a mixture containing kanamycin A, sulfacarbamide (MW: 215 Da), sulfamethoxazole (MW: 253 Da), and sotalol (MW: 272 Da) after 13 rounds of selection (Stoltenburg et al. 2012).

In **article 2,** the adaptation of this method to RNA is presented, together with next generation sequencing data to follow the pool evolution, as well as the the introduction of biolayer interferometry to characterize selected aptamers. The motivation for enabling the selection of RNA-based aptamers is that such aptamers may be more readily converted into riboswitches, which can be used for regulation *in vivo*. Figure 3.5 gives an overview of the RNA Capture-SELEX approach presented in **article 2.** This approach was used in an attempt to isolate aptamers against Carminic acid, Rebaudioside A and 5-hydroxy tryptophan (5HTP).

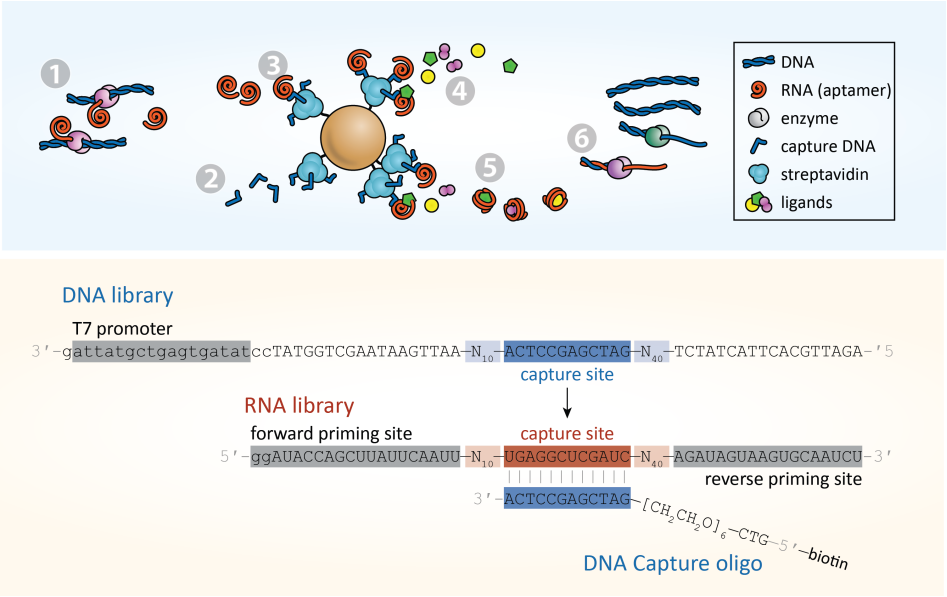


Figure 3.5: Rationale behind RNA Capture-SELEX.
(Upper panel): An overview of the RNA Capture-SELEX approach. 1: A biotinylated capture oligonucleotide is immobilized onto paramagnetic streptavidin beads. 2: The pre-folded RNA library is docked onto the immobilized capture oligonucleotide. 3: Following extensive washing of the beads, one or more ligands are added. 4: Putative structure-switching aptamers, i.e. RNAs that bind the ligand and are released from the beads in the process, are collected from the supernatant. (Lower panel): Design of the libraries used in this thesis. The DNA library is transcribed to yield the corresponding RNA library. The biotinylated capture oligomer binds the RNA library via its capture site flanked by 10 upstream and 40 downstream randomized positions (N10, N40). The forward and reverse priming sites are used for reverse transcription and PCR.

In the present work, we decided to develop aptamers towards three small molecules, including carminic acid, rebaudioside A and 5-hydroxy tryptophan, which are all of industrial interest. Carminic acid is one of the most widely used red food-coloring agents. It can be isolated from scale insects, such as the cochineal, which typically grows on the prickly pear cacti. This makes the production of large quantities both cumbersome, expensive and time consuming (Dapson 2007). A controlled biological process for production of the dye is attractive, however, so far the complete biosynthesis pathway has not been elucidated. Having an aptamer or riboswitch for carminic acid would potentially enable screening for enzymes required for its production, and seeing as fermented carmine has been in development for a few

years at Christian Hansen A/S the interest is tangible. Rebaudioside A is a steviol glycoside and one of the major constituents in *Stevia rebaudiana* plant. Since rebaudioside A is 200 times sweeter than sugar, it is an attractive sweetener in low carbohydrate foods. Stevia has become approved as a food additive in many countries, and the market is therefore rapidly growing. The Stevia plant contains a mix of many different steviol glycosides, and rebaudioside A is often purified to high purity (Pawar et al. 2013), resulting in a relatively high production cost. Production of rebaudioside A has not yet beet reported in microbial production organisms but is in last-phase development at the company Evolva. 5-hydroxy tryptophan is an intermediate in the production of a range of human hormones, including serotonin, melatonin and others. For the potential production of such compounds, it is important to optimize the availability of the precursor 5-hydroxy tryptophan. Production has so far been demonstrated in *E. coli*, albeit with relatively low yields (Lin et al. 2014).

The results of RNA captures-SELEX can be found in **article 2.** In short we managed to run two parallel selections each containing 3 targets constituting 6 parallel selections. Apart from the Selections against Carminic Acid, Rebaudioside A and 5HTP we performed selections on Streptomycin, Kanamycin A and Colistin, the latter are still under evaluation. We managed to select ligand responsive RNA in 8 rounds of RNA Capture-SELEX. The pool was split after 7 rounds of iteration with mixed ligands, and the 8^{th} round was conducted in three selections with one ligand. Next-generation sequencing allowed us to study the evolution of aptamers through round 3 to 8, and even more importantly allowed us to assign ligand specificity to certain sequence types. The selection of aptamer with mixed ligand solution yielded ligand specific aptamers with limited cross-reactivity.

It is a significant quality of the RNA Capture-SELEX approach that it allows simultaneous selection against multiple targets in solution. In selections requiring immobilization this is only possible with specialized equipment as mentioned before (Szeto et al. 2014). Capture-SELEX requires no specialized equipment and for now this technique still remains the most easily adaptable solution to multiplex aptamer discovery.

Recently, a similar method to the Capture-SELEX approach has been used to isolate aptamers against cortisol. Cortisol is a glucocorticoid hormone tightly associated with stress-response in humans and therefore involved in many pathological conditions,

making it clinically important to monitor human cortisol levels (Gatti et al. 2009). The aptamer identified showed enhance cortisol binding and showed equilibrium dissociation constants in the lower micromolar range (Martin et al. 2014). Another recent adaptation is the use of microfluidic SELEX technology to select aptamers against xanthine. The specific method employs not immobilizaition of the small molecule, but uses encapsulation in sol-gel as measure of partitioning. Sol-gel is a silicate material that contains both nanoscale and microscale pores. The general idea is that small molecules are trapped in the nanoscale pores, while aptamer can freely flow through the sol-gel in the microscale pores. Aptamers are retained within the sol-gel medium by their interaction with the entrapped small molecule (Bae et al. 2013). The aptamer identified using this process showed ligand specific micromolar binding affinity with xanthine. Microfluidic Sol-gel SELEX is an interesting addition to the field of small molecule aptamer selection, however, still needs to establish itself as more than a proof-of-concept method and, furthermore, requires acces to sol-gel and microfluidic chip technology (Ahn et al. 2011).

One possible end-use of aptamers selected for their switching behavior is as intracellular biosensors. These ribosensors also known as riboswitches are introduced in the next chapter.

4 Aptamers in synthetic riboswitches

Many species of bacteria have the ability to synthesize all the complex molecules required for growth, however, they can also scavenge the environment for these molecules, obviating the need for *de novo* synthesis of said compounds. Since *de novo* synthesis of complex molecules is energetically costly, it comes with an evolutionary advantage to be able to regulate and repress the synthesis of genes required to make the complex molecules, thereby conserving energy. A regulatory response to external stimuli requires a means for sensing the concentration of a specific molecule and regulating the expression of genes. Normally, proteins that interact with DNA or RNA control expression (or repression) of relevant biosynthesis pathways are often essential parts of these critical regulatory tasks. The *trp* repressor of *Escherichia coli* that binds the *trp* operon DNA operator in the presence of tryptophan exemplifies this. At high concentration of tryptophan (trp), the *trp* repressor binds tryptophan and changes conformation that allows it to represses transcription of the five structural genes in the *trp* operon *trpE*, *trpD*, *trpC*, *trpB*, and *trpA,* involved in tryptophan synthesis.

An interesting additional mechanism of second manner of regulation in the *trp* operon is attenuation. Upstream of the structural genes involved in tryptophan production is the leader sequence that has two important features: An attenuator site, that forms a transcription terminator preventing the RNA polymerase from producing full-length mRNA and a site with two consecutive tryptophan codons (Bertrand et al. 1976).

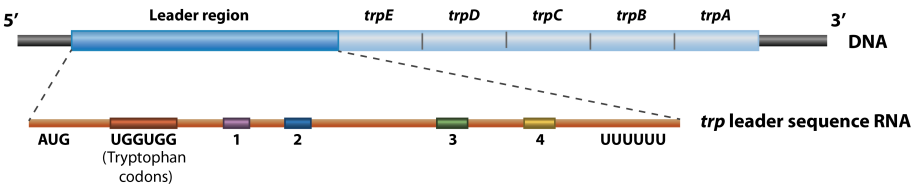


Figure 4.1:The leader region and the structural genes of the *trp* operon.
When the leader region of the *trp* operon is transcribed the transcript contains distinct functional regions: A start codon (AUG) for translation initiation, a site with tryptophan repeats, 4 regions of increased base complementarity and a distal poly-uridine stretch that causes transcription termination (see figure **Figure 4.3**).

In low concentrations of tryptophan, tryptophan charged tRNA is in short supply, therefore, when the ribosome translates the leader peptide and meets the tryptophan codon repeats it stalls momentarily (Yanofsky 1981). The stalling of the ribosome allows the downstream mRNA to rearrange its secondary structure, thereby, removing the transcription terminator sequence and allowing the RNA polymerase to produce full-length mRNA (Lee and Yanofsky 1977). In short, low levels of tryptophan allow transcription and translation of tryptophan biosynthesis genes. Modifying transcription factors like the tryptophan repressor permits the targeted regulation of tryptophan production and could be used in the subsequent design of bacterial cell factories (Tribe and Pittard 1979).

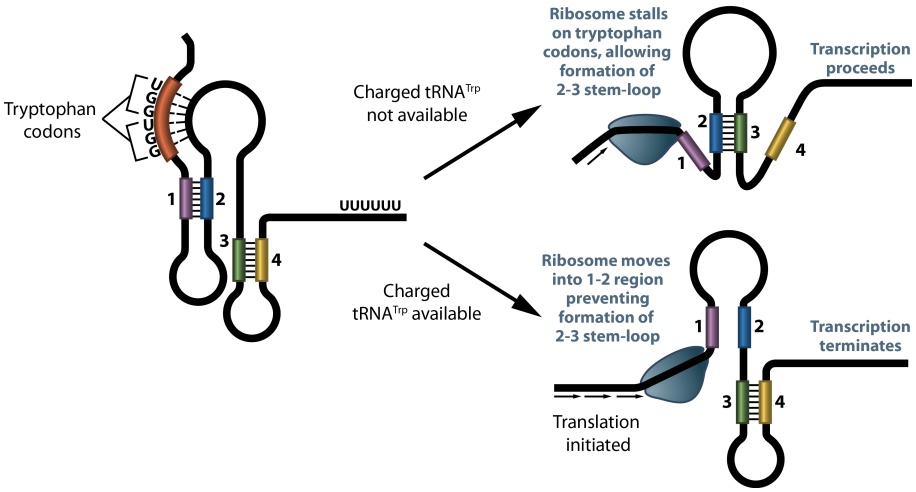


Figure 4.2: Attenuation mechanism of the *trp* operon in *E. coli*
During transcription the mRNA originating from the leader region of the *trp* operon folds, so that regions 1 and 2 base pair as well as 3 and 4. When the RNA polymerase starts synthesizing the poly-U stretch it pauses, due to increased recruitment time of consecutive uredines. In prokaryotes, translation occurs simultaneously with transcription. A ribosome attaches to the mRNA and translated the mRNA until it reaches the two consecutive tryptophan (trp) codons. When the ribosome pauses at the trp codons. If tryptophan is scarce the ribosome pauses for an extended amount of time allowing the mRNA to refold and regions 2 and 3 to hybridize instead. This decreases the structural constrain on the region were the RNA polymerase is paused so that it readily transcribes the rest of the mRNA without terminating. Leading to expression of tryptophan synthesis genes. Conversely, if sufficient intracellular tryptophan (charged tRNA) is present the ribosome continues into the 1-2 region and prevents the formation of the anti-terminator (2-3) maintaining the terminator motif (3-4) causing transcription termination and preventing up-regulation of tryptophan synthesis.

When its was discovered that the vitamins thiamin, riboflavin and cobalamin inhibited their respective biosynthetic genes, efforts were made to identify the proteins involved in repression of the biosynthesis pathways (Serganov and Nudler 2013). The search for the protein factors was futile, however, it was found that conserved mRNA sequences were instrumental in the regulation (Gelfand 1999; Stormo and Ji 2001; Miranda-Ríos et al. 2001) and that the three vitamin derivatives: Thiamine pyrophosphate (TPP) (Mironov et al. 2002; Winkler et al. 2002), flavin mononucleotide (Mironov et al. 2002) and adenosylcobalamin (AdoCbl) (Nahvi et al. 2002) interacted directly with mRNA of the B_1- , B_2- and B_{12}-vitamin operons. In essence, this confirmed that RNA, sans protein, is able to regulate the downstream expression of genes by binding small molecules. The small molecule binding stabilizes the conformation of an evolutionary conserved RNA sensor (aptamer) and induces a switch in folding of downstream RNA region (expression platform), forming a structure that affects transcription or translation of the mRNA (Figure 4.3). This, in its most basic form this, describes the riboswitch.

Riboswitches control numerous metabolic pathways in prokaryotes (reviewed by Breaker 2012). The modular nature of riboswitches (i.e. aptamer and expression domain), allows for the aptamer domain to be interchanged with a heterologous aptamers expanding the regulatory potential beyond the naturally occurring riboswitches. Aptamers have been proven to undergo adaptive recognition of ligands – a feature that lends itself well to the construction of synthetic riboswitches (Patel et al. 1997; Hermann and Patel 2000). A prime example is the theophylline aptamer (Jenison et al. 1994), which has proven to be exceptionally suitable for inducing structural change when its cognate ligand is bound. This has lead to then application of theophylline synthetic RNA switches in over 10 different host systems (Chang et al. 2012).

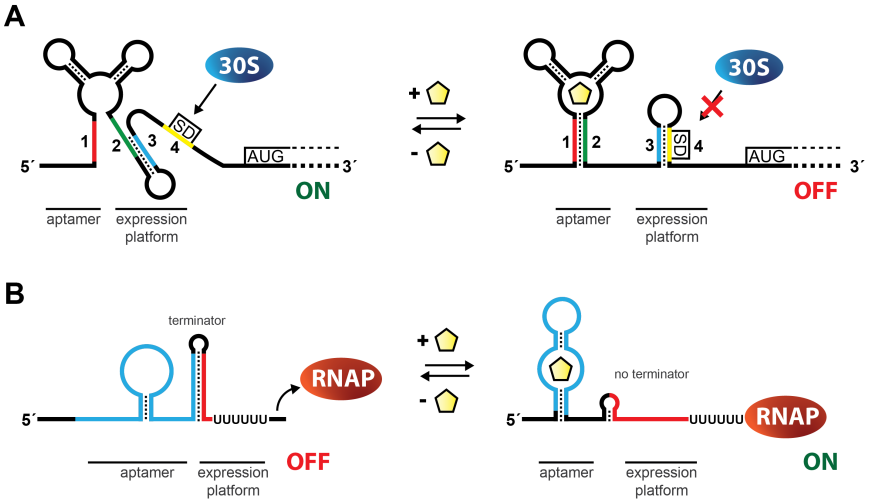


Figure 4.3: Translational and transcriptional regulation by riboswitches.
Two mechanisms of regulation in riboswitches. (A) Regulation of translation initiation: In the absence of ligand the aptamer domain and the expression platform interact by forming a stem loop structure (2:3 hybridization). The Shine-Dalgarno (SD) sequence is accessible to the 30S ribosomal subunit and translation can be initiated. Upon ligand binding (yellow pentagon) the riboswitch undergoes structural rearrangement to favor a different stem loop formation (3:4 hybridization), which sequesters the SD sequence and inhibits translation. (B) Regulation of transcription termination: In the absence of ligand, the complementary 3′ part is base-paired with the aptamer forming a terminator structure; thus, RNA polymerase (RNAP) dissociates and transcription are blocked. Upon ligand binding, terminator structure formation is inhibited and transcription can proceed, resulting in expression of the reporter gene. (Redrawn with permission, from (Wittmann and Suess 2012; Groher and Suess 2014)).

4.1 Screening synthetic riboswitches that regulate ribosome binding

Figure 4.3 (A) shows one of the most common mechanisms of riboswitch function in bacteria, which is changing the accessibility of the ribosome binding site (Groher and Suess 2014). The regulation of translation is extensively used in screening novel synthetic riboswitches. The earliest adaptations of synthetic riboswitch development in eukaryotic hosts were based on inserting RNA aptamers into the 5´ untranslated region (UTR) of mRNA, often hundreds of nucleotides in length. The increased structural rigidity of aptamer:ligand complexes become an obstacle for the ribosomal translation and results in the cessation of expression (Werstuck and Green 1998;

Harvey et al. 2002; Suess 2003; Hanson et al. 2003). In prokaryotes, however, the ribosome binding site is located on average 5-13 nucleotides from the start codon (Kozak 2005). This, allows no space for inserting multiple aptamers in tandem, like as in the case for eukaryotic regulation by synthetic riboswitches towards tobramycin and kanamycin A, where up to three repeating aptamer domains were inserted in the 5´UTR. The application of aptamers selected by SELEX in prokaryotes needed a different strategy.

The aforementioned theophylline riboswitch was selected by introducing a stem-loop at varying distances from the RBS of a constitutively expressed *xylR* gene. After identifying stem loop structure that did not affect XylR expression, the theophylline aptamer was fused to the stem loop. In this way Suess and colleagues showed theophylline dependent repression of xykA::lacZ fusion gene (Suess et al. 2004), proving that a riboswitch based on a theophylline aptamer could be generated to control protein translation in then Gram-positive bacterium *Bacillus subtilis*. In a parallel effort, Desai and Gallivan in 2004, published the selection of a theophylline responsive riboswitch in *E. coli* that could also regulate LacZ expression (Desai and Gallivan 2004). But what made this effort all the more interesting was the additional use of the selected aptamer for controlling the expression of a chloramphenicol resistance gene. Using the coupling of riboswitch function with cell survival, Desai and Gallivan were able to perform selections of theophylline responsive switches in a background of nonfunctional switches (Desai and Gallivan 2004). The overall goal of this paper was to couple survival of a bacterium with the ability to uptake or synthesize a desired small-molecule. However, in order to achieve that goal, it was speculated that the 8-fold induction of the theophylline needed to be improved in order for efficient selection to be attained. As a result, a number of high throughput riboswitch screening systems based on enzymatic reporting (Lynch et al. 2007), FACS (Fowler et al. 2008; Lynch and Gallivan 2009), motility (Topp and Gallivan 2008), and dual selection (Nomura and Yokobayashi 2007; Muranaka et al. 2009).

Recently, a lysine responsive riboswitch coupled to tetracycline resistance was used to enrich for *E. coli* bacteria that have increased lysine production. This effectively demonstrates the long-term goals mentioned by Desai and Gallivan almost 10-years earlier. Using the dual selection strategy of Nomura and Yokobayashi (2007), a synthetic riboswitch based on the L-tryptophan aptamer (Majerfeld and Yarus 2005)

was also created. This synthetic riboswitch coupled the production of tryptophan with tetracycline resistance and it was demonstrated that strains carrying the tryptophan "riboselector" (WTR1) had higher growth rates than the same strain with a deletion of the *aroG* gene (WTR0). This deletion is known to decrease metabolic flux towards tryptophan production. In the absence of riboselector, the two strains showed comparable populations sizes in mixed cultures, leading the authors to predict that the tryptophan riboswitch could be used for engineering *E. coli* for increased tryptophan production (Yang et al. 2013). The overall feasibility of this claim has not yet been proven by an actual selection of a high titer strain producing tryptophan, nevertheless, the long-term of using riboswitches that regulate ribosome binding for selecting improved production organisms seems more within reach.

4.2 Synthetic transcriptional regulators

Resembling the function of the mRNA attenuator located on the *trp* operon, riboswitches can also be based on intrinsic termination. Recently, a theophylline riboswitch based on transcription termination in *E. coli* was developed (Wachsmuth et al. 2013). The theophylline aptamer was used as sensor domain followed by a spacer sequence complementary to the to the 3´-end of the aptamer and a poly-uridine stretch (Figure 4.4 A). The riboswitch was engineered with an inverse folding algorithm to construct stem-loop structures that had two mutually exclusive folds. In absence of theophylline, the favored structure included a transcription terminator, whereas in the presence of ligand the aptamer structure would be favored (Busch and Backofen 2006). Most remarkably, generating a few putative candidates, and testing only six, facilitated the discovery of a single theophylline responsive riboswitch with a 6,5-fold increase in gene expression (Wachsmuth et al. 2013). In a impressive effort, the Robert Batey lab investigated the modularity of existing riboswitch expression platforms from four transcription regulating riboswitches in combination with seven naturally occurring riboswitch aptamers and two *in vitro* selected aptamers (theophylline and tetracycline) (Ceres et al. 2013b, 2013a; Trausch and Batey 2015). Chimeric riboswitches were identified and the results suggest a kind of modularity, which will be interesting to see transferred onto other less-proven aptamer domains. Similarly, using inverse RNA folding holds great potential for *de novo* design of transcription regulating riboswitches, but is currently limited by the requirement for well-characterized aptamer domains (Findeiß et al.

2015). However, inverse folding knowledge might be helpful in designing pre-structured libraries for structure switching aptamers targeted for use in transcription regulating riboswitches.

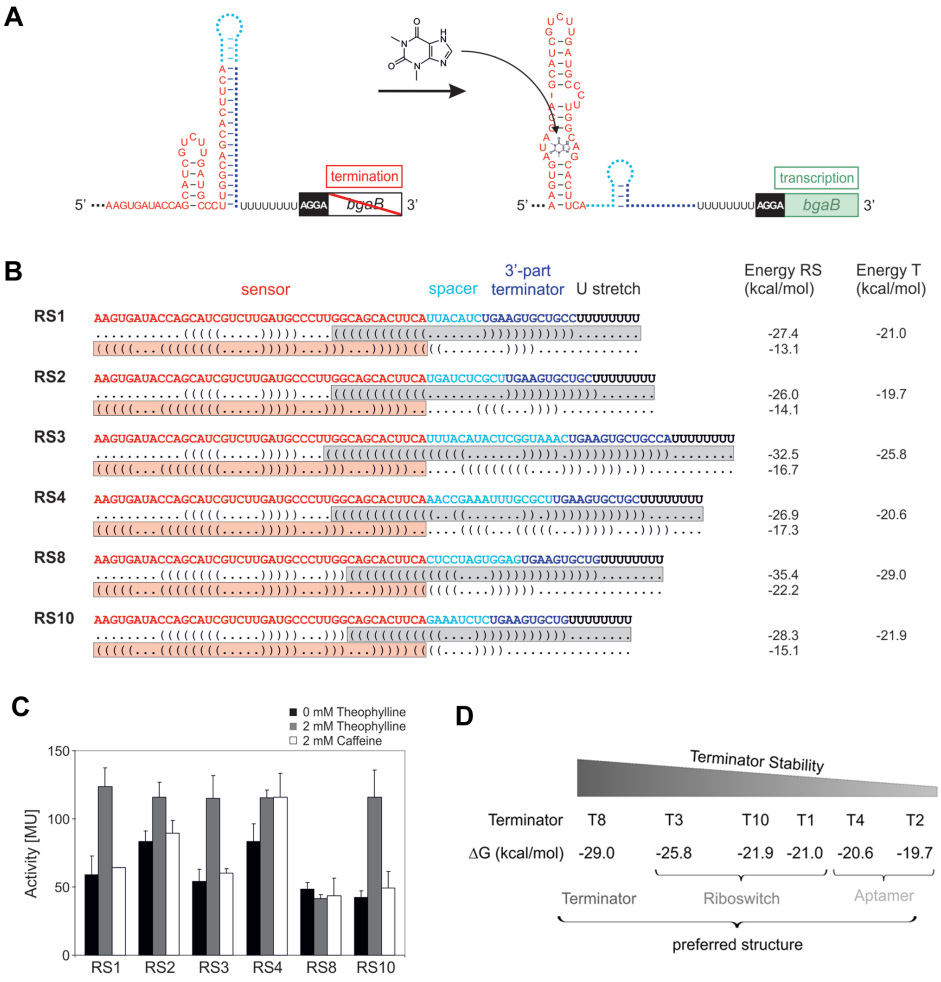


Figure 4.4: *In silico* design of transcription regulating theophylline riboswitches. (A) The design strategy for developing theophylline-dependent transcription regulators. The TCT8-4 aptamer (red) was fused to a short spacer region (blue), and 3' sequence complementary to the aptamer and a U stretch (black). (B) Tested constructs with secondary fold in dot bracket notation and free energy of predicted active and inactive folds. (C) Activity testing based on β-galactosidase assay in presence and absence of 2mM theophylline. (D) Possible correlation between terminator stability and activity (Wachsmuth et al. 2013).

4.3 Not all aptamers make good riboswitches

In principle, it should possible to generate synthetic riboswitches using any aptamer that binds non-toxic, cell permeable targets using the high throughput screening technologies presented in this chapter. To that end, more than 60 small-molecule binding RNA's have been characterized from *in vitro* selections (Chang et al. 2014). However, most novel riboswitch applications are limited to a handful of aptamer domains, including the theophylline aptamer, which can be used as sensing domains for riboswitches (Chang et al. 2012).

Generally, *in vitro* selected aptamers currently being used in riboswitches might provide us with hints as to what makes a good aptamer. Not surprisingly, structural change upon ligand binding is a common feature of aptamers in riboswitch sensing domains. NMR studies of theophylline, malachite green and neomycin aptamers suggest profound changes in overall structure as ligand binds (Zimmermann et al. 1997; Baugh et al. 2000; Duchardt-Ferner et al. 2010). Generally, the overall effects of ligand binding are described as leading from a more open relaxed state in the absence of binding to rigid structure upon binding.

The tetracycline aptamer has been mutated to understand the effects of affinity on regulatory potential (Xiao et al. 2008). Here it was shown that even a small decrease in binding affinity (from 770 pM to 3 nM) resulted in half the regulation efficiency; indicating that affinity might have influence on adaptability. High affinity is however by no means a guarantee for switch functionality. This is substantiated by the finding that high affinity aptamers from a neomycin selection, which did not show significant structural rearrangement, were found to be inactive as riboswitch sensing elements (Weigand et al. 2011). Interestingly, the tryptophan riboswitch presented by Yang *et al.* was based on at tryptophan aptamer Trp 70-727 that had a high dissociation constant of 16 μM, implying that high affinity is not a sole requirement and that the degree of conformational change is even more important.

Depending on application, the on-rate (k_{on}) of an aptamer can be important for its use in riboswitches. For instance in the transcriptional regulator it might be important for the ligand to bind fast if it competes with the processivity of an RNA polymerase. Additionally, ligands for eukaryotic riboswitches have been hypothesized to compete

with RNA associated protein, necessitating the fast on-rate of ligand before being blocked by formation of the ribonucleotprotein (Wittmann and Suess 2012).

The overall characteristics of aptamers suited for ‘riboswitchification’ could possibly be met by rationally designing SELEX strategies for structure switching RNA aptamers with fast on-rates.

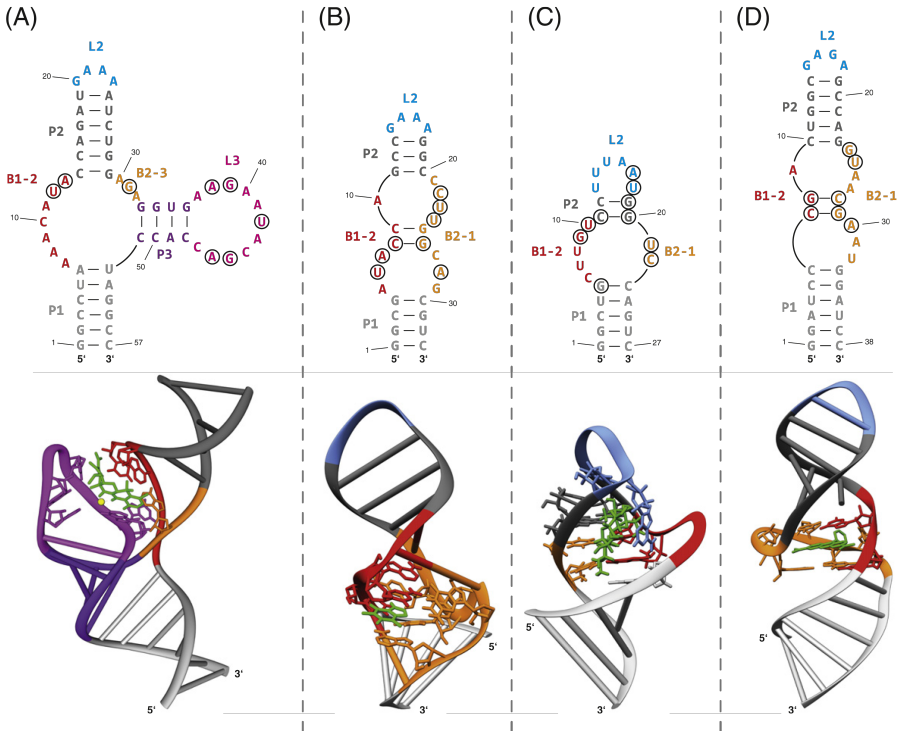


Figure 4.5: Aptamers in use as synthetic riboswitches.
In vitro selected aptamers used in both prokaryotic (theophylline) as well as eukaryotic (tetracycline, neomycin and malachite green) riboswitches. The aptamers are, (A) Tetracycline, (B) theophylline, (C) neomycin and (D) malachite green. Structural features like bulges, B loops, L and stems, P are color-coded, however, are not meant as highlighted features necessary for an aptamers ability to exert riboswitch functionality (adapted from (Groher and Suess 2014) with permission).

5 Aptamers for affinity biosensor devices

As mentioned in the introduction to this thesis, one of the overall goals was to apply aptamers as recognition elements for *in vitro* biosensors (physicochemical). In this thesis one such implementation is shown in **article 3** were a label-free all-polymer biosensor developed in collaboration with DTU Nanotech was used to measure antibiotics in solution, by impedance spectroscopy (Daprá et al. 2013).

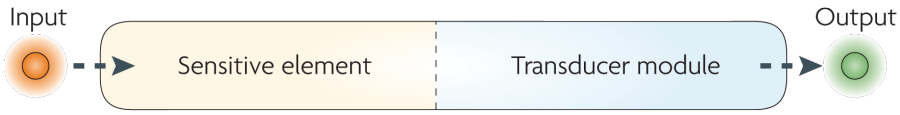


Figure 5.1: The basic sensor design principle.
In the case of vitro biosensors the sensitive element being an aptamer or other bio-recognition elements (e.g. monoclonal antibodies) senses and the physiochemical transducer module converts the biochemical event into an electrical output. Adopted with permission from (Khalil and Collins 2010).

5.1 Why label-free aptasensors

A multitude of sensing strategies have been developed combining aptamers with various transducers to measure concentration of a give ligand without the need to label the sensitive element (here, aptamer) with redox-probes or fluorescent moieties (Mascini 2009). The wider application of biosensors is sometimes challenged by the relatively high cost of producing labeled recognition elements. Because aptamers frequently undergo significant conformational changes when recognizing their ligands (Patel et al. 1997; Hermann and Patel 2000) the internal labeling might affect the overall binding affinity and result in a changed limit of detection (LOD). For example the labeling of RNA and DNA aptamers recognizing adenosine lead to a decrease in binding affinity from ~6 μM to ~300 μM for the RNA aptamer and ~30 μM for the DNA aptamer (Jhaveri et al. 2000). This is a frequent observation in aptamers that rely in conformational transduction for signaling and highlights the unpredictable element of adopting aptamers to labeled bio-sensing approaches (Cho et al. 2009).

5.2 Why not antibodies? The advantage of being an aptamer

Biosensors gain the functionality through their recognition elements. Antibodies have long been the preferred functional unit of biosensors given their dominance in the market of interaction chemistry. Aptamers have been developed to specifically interact with a plethora of different ligands and based on their capability to act as high affinity, selective, ligand recognition elements, aptamers have, therefore, been likened to antibodies. Aptamers are now emerging as viable substitutes for antibodies in biosensor applications because of a number of advantages that grants them a much wider field of application. As mentioned earlier in this thesis, aptamers can be generated entirely *in vitro*, thereby, eliminating the need for immunizations of animals as traditionally needed to raise antibodies. Furthermore, this allows selection against highly toxic compound incompatible with the live host systems, or for targets that carry no immunogenic potential. Aptamers are mostly 'short' oligonucleotides (< 100 nt), which allow them to be synthesized by standard chemical synthesis and site-specifically functionalized with various functional groups and specific moieties such as biotin, carboxyl, amino, thiol, fluorophore and quenchers without risking the integrity of aptamer binding ability. The ease of synthesis and functionalization has lead to the aptamer becoming a widely used tool for functionalization of biosensors. Furthermore, nonspecific adsorption phenomena are usually less pronounced in nucleic acid interfaces as compared to protein interfaces and, lastly, aptamers allow re-folding upon denaturing, while most proteins irreversibly denature upon heating (Willner and Zayats 2008).

5.3 Impedance spectroscopy – a short introduction

There are several kinds of electrochemical biosensors based on measurement of resistance, oxidation or reduction potential or current. The later amperometric biosensor that measures current is perhaps the most well known biosensor of all, as it is the functional base of the well known and first glucose biosensor (Wang 2008). The impedance biosensor is a fourth electrochemical biosensor type, and should not be confused with the conductometric biosensor that measures resistance. The impedance biosensor utilizes impedance spectroscopy, which is a powerful tool for analyzing the complex electrical resistance of a system and is especially sensistive to changes at the electrode surface. Due to its label-free nature and high sensitivity to

surface properties impedance detection is particularly suited for investigating binding events in the field of biosensors.

One way of understanding impedance spectroscopy may be to think of impedance as the equivalent of the resistance in a direct current (DC) system extended to an alternating current (AC). In DC circuits the resistance (R) describes the opposition to the flow of electrons and is defined in Ohm's law as the ratio of voltage (V) across an object to the current (I) through it.

$$R \equiv \frac{V}{I} \qquad \text{(Equation 1)}$$

In alternating current (AC) circuits the direction of electric flow is altered at a given frequency to provide a sinusoidal system. Ideally, resistance is independent of frequency and when the voltage (V) is highest so is the current flow (I). The two are said to be in phase. However, in real world applications electrical circuits where alternating current (AC) is applied the opposition to flow of electrons is highly dependent on the frequency and time, governed by capacitors or electrode processes such as diffusion. Therefore, impedance (Z) is used to describe an opposition to electron flow in AC circuits. Impedance is a combination of resistor forces known from the traditional DC circuits (Z_{real} or real part) and capacitor/inductor contribution in an AC circuit (imaginary part of impedance or Z_{im}). As a consequence of the introduction of capacitance and inductance the current can not only differ in amplitude but can also show a phase shift ϕ. Therefore, at a given time point impedance is expressed by Equation 2.

$$Z = \frac{V(t)}{I\,(t)} = \frac{V_0 \sin(2\pi f t)}{I_0 \sin(2\pi f t + \phi)} \qquad \text{(Equation 2)}$$

Electrochemical impedance spectroscopy (EIS) is measured over a large subset of different frequencies f. Depending on the frequency applied to the AC circuit different contributions to overall system impedance are prominent. At high frequencies the bulk resistance is the most prominent factor on impedance since the resistance of the capacitor is low. At low frequency, however, the double layer capacitors dominate the contribution to the overall impedance. Consequently, the capacitor located at the ionic double layer on the electrode surface can be investigated by looking at the low frequency interval. Generally, these effects are

presented in either Nyquist or Bode plots. These plots illustrate the spectrum of electrochemical impedance as a function of frequency. The most common graphical representation of impedance data is the Nyquist plot. A typical Nyquist plot usually depicts a semi circle in the high frequency area associated with solution resistance. The width of the semi circle is related to the charge-transfer resistance or ohmic resistance. In **article 3**, the Nyquist plot is used to characterize the aptamer sensor system, building an equivalent circuit of resistors, capacitors, inductors or constant phase elements or Warburg resistances. These circuit elements have characteristics that affect the behavior of the system. Resistors are constant and refer to material characteristics and others are frequency dependent (i.e. inductors block high frequencies and capacitors only let high frequencies pass). All of these circuit elements influence the shape of the impedance spectrum and hint to the processes that govern the interaction that is studied. The in depth explanation of the part of the equivalent system is beyond this introduction but is reviewed in (Orazem 2008).

5.4 Detection of antibiotics as a proof-of-concept

Physicochemical biosensors based on aptamers are becoming increasingly popular in the field of nanotechnology. With the intent of using aptamers against small molecules and cellular metabolites selected by the novel aptamer selection techniques developed through this thesis, we decided to investigate the feasibility of detection of small molecule antibiotics using an all polymer biosensor in development at DTU Nanotech.

In the study we used previously identified aptamers against kanamycin A and ampicillin as model aptamers. This was done both as a proof-of-principle experiment, but also because of the general need for robust and cheap biosensor devices to detect antibiotic contamination in the environment. This addresses the increasing emergence of pathogens resistant towards important antibiotics. Multidrug resistant pathogens like *Staphylococcus aureus* and *Mycobacterium tuberculosis* are increasingly being reported. It is estimated that no less than 25,000 patients die each year in the EU as a consequence of infection by these drug resistant phenotypes. This constitutes an major risk to human health since developing new antibiotics attract little attention from the pharmaceutical industry, with very few or no late state clinical trials of new antibiotics against gram-negative bacteria. Antibiotics are

increasingly being used as a preventive measure in the aqua- and agricultural business, which increases environmental exposure. As a consequence, bacterial pathogens are continuously exposed to sub-lethal amount of antibiotics, allowing them to evolve antibiotic resistance in the environment before infecting a host. An important measure to limit the use of antibiotics and thereby decreasing the environmental load is to advance the field of antibiotic sensors (Daprá et al. 2013).

In our case we used a novel all-polymer biosensor for the detection of kanamycin A and ampicillin in phosphate buffered and spiked milk samples. The biosensor enabled detection of picomolar amounts (LOD: 34.9 pg/ml for ampicillin and 4.8 ng/ml for kanamycin A), which is directly comparable to, or lower than, other biosensor devices previously described for kanamycin and ampicillin detection (Huet et al. 2010). Additionally, the impedance measurement also gave us information about a distinction between the to aptamers mode of action. Impedance upon immobilization of aptamer gave a rise in impedance as expected for interactions perturbing the ionic double layer at the electrode interphase. However, interestingly we observed to distinct and reproducible responses to aptamer:ligand interaction. An increase in impedance was seen with the kanamycin binding and a decrease in impedance was observed for ampicillin binding at a frequenzy of 501 mHz. Injecting aptasensors with non-cognate ligands resulted in no observable change in impedance. The observed distinct differences in impedance at a fixed frequency may in the future enable the more precise determination of how these aptamers interact with their ligands.

This is the first approach that presented label-free antibiotic detection with impedance spectroscopy in an all-polymer device. Most electrochemical biosensors use electrodes fabricated at high cost using materials like gold or platinum (Kerman et al. 2003). These electrode substrates provide excellent properties for conductivity and good environmental stability, however, they require costly clean room fabrications facilities and are not easily recyclable, unlike biodegradable polymer electrodes (Rozlosnik 2009). Conductive polymers, therefore, constitute a promising alternative for transducing a binding event between an analyte and a biological recognition element. This sensor is currently being developed as a parallelized setup for detection of tryptophan for production strain development.

6 Conclusion and perspectives

6.1 Perspectives in biochemical production

The total market for petrochemicals is predicted to approach 3.0 trillion dollars in 2015, leaving a significant economical and environmental incentive to find alternative approaches. In 2012, the total US bioeconomy was 350 billion dollars with a growth of 7%. The production of biochemicals accounts for a significant and growing fraction of the bioeconomy, with a global market of 32 billion dollars in 2010 that is expected to grow to 76 billion dollars in 2017. This expected growth is partly due to the advances made in the field of synthetic biology and metabolic engineering for generation of novel products and production organisms.

A significant number of chemicals have been identified, both because they are biologically feasible to produce and because they have a property that renders them easy to polymerize or convert into other more complex molecules using standard organic chemistry (Werpy and Petersen 2004). Their small size and relatively simple structure is characteristic is of these chemicals. Production of such chemicals from fermentable sugars at competitive prices requires significant optimization of the production organism in order to achieve the highest possible conversion yield, since the carbon source often accounts for a very significant fraction of the production cost.

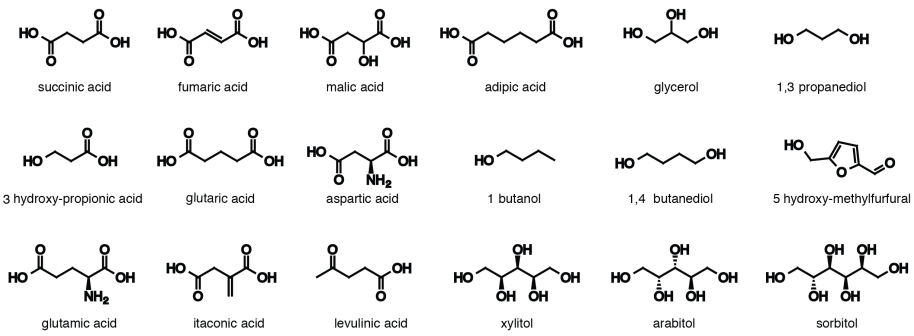


Figure 6.1: Small molecules of interest in biochemical production

Therefore, efficient screening devices have great potential during the development of cell-factories. In figure 6.1, is an overview of some of the building block chemicals in demand today that potentially are being investigated for the production in bacterial

cell-factories. All of the compounds have functional groups that allow immobilization to solid support (i.e. -OH, -COOH, -NH_2), however immobilizing these compounds might remove functional groups that could interact with an aptamer leading to reduced likelihood of finding specific aptamers against these targets. None of the targets above are great for aptamer development; however aptamers with affinities towards inconspicuous targets like ethanolamine and glycine have been reported (Mann et al. 2005; Butler et al. 2011). Other chemicals of higher value, such as food and feed additives, nutraceuticals and pharmaceuticals are also targets for biological production. These might be of higher structural complexity than the molecules in figure 6.1 and more likely to be suitable target for DNA and RNA biosensor development.

The structure and metabolic pathways for such compounds are often more complicated or unknown, making it more challenging to optimize the production. Examples of this are carminic acid and rebaudioside A for which a riboswitch could help elucidate optimal enzymes for microbial production via phenotypic selection.

6.2 On the future of the single round selection of aptamers

Single round selections still constitute a rarity in aptamer selection but might find extended use in the future. One application is within the selection of aptamers with base- and ribose modifications, such as 2´-fluoro ribose modifications (Rhie et al. 2003) or SOMAmers® (Gold et al. 2010). Aptamers with these modification can elicit improved stability and affinity (Keefe et al. 2010; Lauridsen et al. 2012). Chemically modified bases can be incorporated into aptamers post-SELEX but might impede function. Using modified nucleotides directly in selection can obviate this, but modified bases are more difficult to amplify. It has been proven that some modified bases are not sustained during selection cycles, making it virtually impossible to select aptamers containing these beneficial modifications (Doessing et al. 2012). These complications would be avoided if it were possible to identify aptamers without multiple cycles of selection and amplification. However, identification will still require amenability to sequencing and, therefore, enzymatic extension and maybe also PCR amplification.

For the selection of unmodified aptamers single round selection is still at the proof-of-concept stage. In this thesis, we have tried to move out of that stage, by attempting

selections with new target and new libraries. For the time being single cycle selection seem to require a highly aptagenic target (Hoon et al. 2011), a small library (Kupakuwana et al. 2011) or a significant amount of sequences from Next-generation sequencing with limited PCR bias. The most obvious application is targeted re-selections that employ libraries with limited diversity. These libraries with fewer than 16 randomized bases enable most of the sequence space to be evaluated by next generation sequencing. (Kupakuwana et al. 2011). Especially, since the aptagenic potential of a target is difficult to evaluate and isoelectric point is not a safe way to estimate potential outcome of selection since spatial localization of positive and negative charges might also be of increased importance in selection, at least based on previously isolated aptamers a difference in pI from 5.5 (IgE) to 9.7 (Angiogenin) yielded the same K_d value probably by virtue of partitioning efficiency and selection pressure in the SELEX experiment (Figure 6.2).

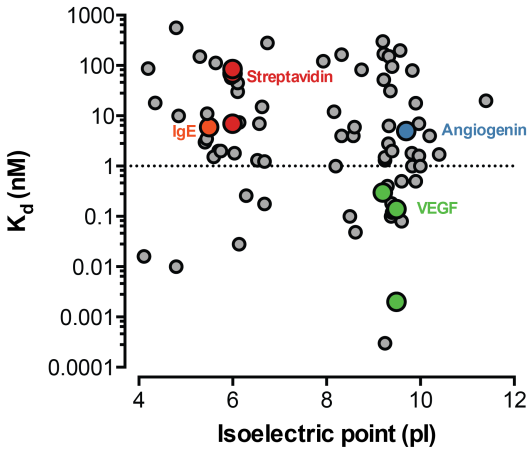


Figure 6.2: Isoelectric points of proteinaceous aptamer targets.
A more detailed view of aptamer K_d versus isoelectric point. Redundant selections against streptavidin (red) and VEGF (green) illustrate the apparent clustering of these targets in respect to aptagenic potential. Conversely, aptamers against IgE (orange) and Angiogenin (blue) with very different pI were isolated and have the same K_d.

One difficulty to our NGS SELEX approach for the single round aptamer selections was the amount of DNA material required from commercial vendors for Illumina sequencing, which necessitated considerable amplification by PCR. This might be solved with the advent of new preparation-free true single molecule sequencing

technologies like Helicos or Nanopore sequencing, however, it is still very uncertain what these platforms will offer in the future. The question that remains is whether a few additional rounds of selection would be preferable to only one. Especially since, the inclusion of next-generation sequencing in most SELEX experiments now offer the possibility to observe sequence enrichment at an earlier point in selection.

With respect to the single cycle selection used in **article 1**, the simultaneous selection and visualization during aptamer selection enables direct verification of nucleic acid binding. This may be further improved by employing arraying technique to create arrays of targets for the simultaneous selection against multiple targets. However, this will not increase the success-rate for finding an aptamer against a specific target.

6.3 On the future of RNA Capture-SELEX

One of the key challenges in development of novel aptamers is the throughput of selection. Recently, the Capture-SELEX technology has been reviewed and the high number of selection rounds needed to obtain aptamers was mentioned as a key disadvantage (Ozer et al. 2014). Our results show that the number of selection rounds required for successful RNA Capture-SELEX (eight, in the present case) is in line with classical SELEX and might even be improved by employing the splitting of multiplex pools into single-ligand selections and/or next-generation sequencing.

One general thing that can be said for all the presented small molecule selections that do not employ ligand immobilization is that the overall observed affinity of isolated aptamers is in the micromolar range. If aptamers from RNA Capture-SELEX are to be used in functional riboswitches the overall affinity may in some cases need to be higher than what we observe. One way this could be accomplished is to use a lower concentration of ligand in the elution step. However, for now the proof-of-principle suggests that RNA Capture-SELEX can be used to isolate aptamers with specificity from multiplexed samples with different targets. Also, the use of next-generation sequencing of multiple selections rounds, combined with splitting of the pools (into single ligand selections) enables an additional dimension of information to be added to the sequencing outcome: The simultaneous evaluation of apparent affinity based on enrichment and the allocation target-sequence relationship based

on allotment of sequences in the final split selections. Currently, the affinities of the aptamers are being thoroughly validated before submitting **article 2.**

Capture-SELEX was interesting to us because it allowed the selection of structure switching aptamers against small molecule targets in solution. Structure switching is an important feature in both impedance sensing and especially riboswitch function as discussed. RNA Capture-SELEX could complement the development of synthetic riboswitches and increase likelihood of finding aptamers that function in *in vivo* applications. As a part of this, we briefly considered adding a Shine-Dalgarno (SD) sequence to the capture region to aid the further engineering of riboswitches, however, the optimal location and sequence context of an SD may be better determined through subsequent screening, and we furthermore wanted to keep the number of experimental variables to a minimum. Another more direct way of utilizing the RNA capture probe based results is the direct *in vitro* selection of transcriptional attenuators. As mentioned transcriptional attenuators are RNA elements that consist of an aptamer region and stem-loop followed by a string of uracil bases. Inactivation of the trp-attenuator has previously been shown to increase tryptophan biosynthesis in a production strain. Transcription terminators are therefore viable targets for improving metabolic flux in bacterial cell factories (Gu et al. 2012). Recently the thiamine pyrophosphate riboswitch was converted into a transcriptional terminator by Wachsmuth et al. (2013) using the aptamer domain of the riboswitches and engineering the terminator domain based on secondary structure predictions. Combining the knowledge of synthetic transcription terminators and our RNA Capture-SELEX approach, a library for direct selection of transcriptional terminators can be envisioned. The capture probe in this instance would constitute one half of the RNA terminator hairpin and the aptamer library would constitute the other part of the terminator. After successful selection of aptamer sequences that show ligand induced release from the capture probe, the complete RNA element can be constructed by combining the selected aptamer and capture sequence with various loop designs or tetra-loop sequences. This approach could obviate the need for introducing random sequences as spacers between aptamer domain and the genetic marker to be used in the screening for functional and selectable switches.

We firmly believe that the affinities of aptamers isolated using RNA Capture-SELEX can be tuned by decreasing the ligand concentration in solution, just as it has been

shown in a recent publication mentioning a comparable effort to select DNA aptamers (Martin et al. 2014). Another interesting aspect of RNA Capture-SELEX is the possibility to tune selection pressure not only in terms of ligand concentration but also in terms of capture-affinity. One approach to modifying the aptamer affinity could be the incorporation of modified nucleotides that enhance hybridization efficiency such as locked nucleic acids (Doessing et al. 2012) in the capture oligonucleotide. This could potentially be used to increase the thermodynamic threshold for RNA release from the beads and therefore constitute a novel technique to confer increased selection pressure in the system without having to decrease ligand concentration or change capture probe length.

6.4 Emerging new tools for aptamer characterization

The SELEX method has been through many perturbations as discussed in this thesis. Especially, within the last five years, the expansion of the aptamer selection technique has been apparent. This is especially driven by next-generation sequencing (Szeto and Craighead 2014). However, emerging technologies within high-throughput aptamer characterization platforms could have an equal impact on aptamer technology progress. We have used bio-layer interferometry to rapidly screen the kinetic structure switching profiles of over 400 clones in relation to the RNA Capture-SELEX approach. Previously, thousands of sequence variants of 235 aptamer candidates isolated by microfluidic SELEX have been screened on microarrays (Cho et al. 2013). Another attempt termed HiTS-RAP employs the Illumina sequencing platform to determine RNA protein interaction during sequencing. This allowed the RNA affinity profiling of millions of GFP aptamers (Tome et al. 2014). However, these high-throughput methods require labeling of target with fluorescent probes and are, therefore, not yet feasible for small molecules. However, with the advent of RNA molecules that mimic GFP and the subsequent enabling of fluorescently visualizing small molecules, this might become plausible in the future (Paige et al. 2011).

6.5 Screening for production phenotype

An important issue to address that has not been discussed thoroughly in its entirety in this thesis is the potential construction of cell-factories. We have justified the

choice of selecting aptamers with micromolar affinities with the expected notion that an intracellular environment in a cell factory contains high titers of the compound, thus requiring a riboswitch with high specificity, but also a dynamic range of structure change correlating with this high titer. Cell factories are often designed to export the desired compound of interest and might therefore not achieve high intracellular concentration of compound, especially not if the compound of interest is toxic at high concentrations. While it may be possible to change the dynamic range of the riboswitch-based regulatory system, the developed technologies may be particularly suited for identification of novel production pathways and for identifying other regulatory elements in the cell that affects production. In some cases, it may also be envisioned that intracellular riboswitch based sensor systems may be used for screening of optimized exporter systems.

6.6 Conclusion

There is considerable interest in using synthetic riboswitches as biosensors to facilitate the development of production strains. However, there is a serious lack of aptamers against small molecules of interest in biochemical production, and no effective ways of developing these aptamers to function in such settings. In order for RNA based intracellular sensors to achieve the potential as screening devices for chemical production, relevant aptamer technologies have to mature. In this thesis an aptamer selection scheme that potentially decreases the gap between *in vivo* riboswitch and *in vitro* aptamer has been presented. The method has resulted in aptamers with specificity towards rebaudioside A and carminic acid. Additionally, an aptamer against α-bungarotoxin has been presented. The clinical validation of which still remains to be elucidated at the University of Queensland. Finally, we have characterized an all-polymeric biosensor that now is being further developed to allow high-throughput screening of amino acid production in a production strain.

This is research will be continuously developed based on a recent grant from the Danish National Innovation Foundation in collaboration with Biosyntia and The Technical University of Denmark.

Cheers.

7 References

Ahmad KM, Oh SS, Kim S, McClellen FM, Xiao Y, Soh HT. 2011. Probing the limits of aptamer affinity with a microfluidic SELEX platform. *PLoS One* **6**: e27051.

Ahn J-Y, Jo M, Dua P, Lee D-K, Kim S. 2011. A sol-gel-based microfluidics system enhances the efficiency of RNA aptamer selection. *Oligonucleotides* **21**: 93–100.

Ameta S, Winz M-L, Previti C, Jäschke A. 2014. Next-generation sequencing reveals how RNA catalysts evolve from random space. *Nucleic Acids Res* **42**: 1303–10.

Arnold S, Pampalakis G, Kantiotou K, Silva D, Cortez C, Missailidis S, Sotiropoulou G. 2012. One round of SELEX for the generation of DNA aptamers directed against KLK6. In *Biological Chemistry*, Vol. 393 of, pp. 343–353.

Bae H, Ren S, Kang J, Kim M, Jiang Y, Jin MM, Min IM, Kim S. 2013. Sol-gel SELEX circumventing chemical conjugation of low molecular weight metabolites discovers aptamers selective to xanthine. *Nucleic Acid Ther* **23**: 443–9.

Bartel DP, Szostak JW. 1993. Isolation of new ribozymes from a large pool of random sequences. *Science* **261**: 1411–1418.

Baugh C, Grate D, Wilson C. 2000. 2.8 A crystal structure of the malachite green aptamer. *J Mol Biol* **301**: 117–28.

Bertrand K, Squires C, Yanofsky C. 1976. Transcription termination in vivo in the leader region of the tryptophan operon of Escherichia coli. *J Mol Biol* **103**: 319–337.

Bock LC, Griffin LC, Latham JA, Vermaas EH, Toole JJ. 1992. Selection of single-stranded DNA molecules that bind and inhibit human thrombin. *Nature* **355**: 564–566.

Breaker RR. 2012. Riboswitches and the RNA world. *Cold Spring Harb Perspect Biol* **4**: a003566.

Busch A, Backofen R. 2006. INFO-RNA--a fast approach to inverse RNA folding. *Bioinformatics* **22**: 1823–31.

Butler EB, Xiong Y, Wang J, Strobel SA. 2011. Structural basis of cooperative ligand binding by the glycine riboswitch. *Chem Biol* **18**: 293–8.

Çalik P, Balci O, Özdamar TH. 2010. Human growth hormone-specific aptamer identification using improved oligonucleotide ligand evolution method. *Protein Expr Purif* **69**: 21–28.

Carothers JM, Goler J a, Kapoor Y, Lara L, Keasling JD. 2010. Selecting RNA aptamers for synthetic biology: investigating magnesium dependence and predicting binding affinity. *Nucleic Acids Res* **38**: 2736–47.

Ceres P, Garst AD, Marcano-Velázquez JG, Batey RT. 2013a. Modularity of select riboswitch expression platforms enables facile engineering of novel genetic regulatory devices. *ACS Synth Biol* **2**: 463–72.

Ceres P, Trausch JJ, Batey RT. 2013b. Engineering modular "ON" RNA switches using biological components. *Nucleic Acids Res* **41**: 10449–61.

Chang AL, McKeague M, Liang JC, Smolke CD. 2014. Kinetic and equilibrium binding characterization of aptamers to small molecules using a label-free, sensitive, and scalable platform. *Anal Chem* **86**: 3273–3278.

Chang AL, Wolf JJ, Smolke CD. 2012. Synthetic RNA switches as a tool for temporal and spatial control over gene expression. *Curr Opin Biotechnol* **23**: 679–88.

Cheng C, Chen YH, Lennox KA, Behlke MA, Davidson BL. 2013. In vivo SELEX for Identification of Brain-penetrating Aptamers. *Mol Ther Nucleic Acids* **2**: e67.

Cho EJ, Lee J-W, Ellington AD. 2009. Applications of aptamers as sensors. *Annu Rev Anal Chem (Palo Alto Calif)* **2**: 241–64.

Cho M, Soo Oh S, Nie J, Stewart R, Eisenstein M, Chambers J, Marth JD, Walker F, Thomson J a, Soh HT. 2013. Quantitative selection and parallel characterization of aptamers. *Proc Natl Acad Sci U S A* **110**: 18460–5.

Cho M, Xiao Y, Nie J, Stewart R, Csordas AT, Oh SS, Thomson J a, Soh HT. 2010. Quantitative selection of DNA aptamers through microfluidic selection and high-throughput sequencing. *Proc Natl Acad Sci U S A* **107**: 15373–8.

Coleman TM, Huang F. 2005. Optimal random libraries for the isolation of catalytic RNA. *RNA Biol* **2**: 129–136.

Coleman TM, Huang F. 2002. RNA-catalyzed thioester synthesis. *Chem Biol* **9**: 1227–1236.

Cowperthwaite MC, Ellington AD. 2008. Bioinformatic analysis of the contribution of primer sequences to aptamer structures. *J Mol Evol* **67**: 95–102.

Daniel C, Roupioz Y, Gasparutto D, Livache T, Buhot A. 2013. Solution-Phase vs Surface-Phase Aptamer-Protein Affinity from a Label-Free Kinetic Biosensor. *PLoS One* **8**: 1–6.

Daprá J, Lauridsen LH, Nielsen AT, Rozlosnik N. 2013. Comparative study on aptamers as recognition elements for antibiotics in a label-free all-polymer biosensor. *Biosens Bioelectron*.

Dapson RW. 2007. The history, chemistry and modes of action of carmine and related dyes. *Biotech Histochem* **82**: 173–187.

Davis JH, Szostak JW. 2002. Isolation of high-affinity GTP aptamers from partially structured RNA libraries. *Proc Natl Acad Sci U S A* **99**: 11616–21.

Desai SK, Gallivan JP. 2004. Genetic screens and selections for small molecules based on a synthetic riboswitch that activates protein translation. *J Am Chem Soc* **126**: 13247–54.

Dietrich J a, McKee AE, Keasling JD. 2010. High-throughput metabolic engineering: advances in small-molecule screening and selection. *Annu Rev Biochem* **79**: 563–590.

Doessing H, Hansen LH, Veedu RN, Wengel J, Vester B. 2012. Amplification and re-generation of LNA-modified libraries. *Molecules* **17**: 13087–13097.

Dua P, Kim S, Lee D-KK. 2008. Patents on SELEX and therapeutic aptamers. *Recent Pat DNA Gene Seq* **2**: 172–86.

Duchardt-Ferner E, Weigand JE, Ohlenschläger O, Schmidtke SR, Suess B, Wöhnert J. 2010. Highly modular structure and ligand binding by conformational capture in a minimalistic riboswitch. *Angew Chem Int Ed Engl* **49**: 6216–9.

Ellington AD, Szostak JW. 1990. In vitro selection of RNA molecules that bind specific ligands. *Nature* **346**: 818–22.

Famulok M, Mayer G. 2014. Aptamers and SELEX in Chemistry & Biology. *Chem Biol* **21**: 1055–1058.

Ferguson BS, Hoggarth D a, Maliniak D, Ploense K, White RJ, Woodward N, Hsieh K, Bonham AJ, Eisenstein M, Kippin TE, et al. 2013. Real-time, aptamer-based tracking of circulating therapeutic agents in living animals. *Sci Transl Med* **5**: 213ra165.

Findeiß S, Wachsmuth M, Mörl M, Stadler PF. 2015. Design of Transcription Regulating Riboswitches. In *Methods in enzymology* (ed. Donald H. Burke-Aguero), Vol. 550 of, pp. 1–22, Elsevier Inc.

Fowler CC, Brown ED, Li Y. 2008. A FACS-based approach to engineering artificial riboswitches. *Chembiochem* **9**: 1906–11.

Fujimoto Y, Nakamura Y, Ohuchi S. 2012. HEXIM1-binding elements on mRNAs identified through transcriptomic SELEX and computational screening. *Biochimie* **94**: 1900–1909.

Fukuda K, Vishnuvardhan D, Sekiya S, Hwang J, Kakiuchi N, Taira K, Shimotohno K, Kumar PK, Nishikawa S. 2000. Isolation and characterization of RNA aptamers specific for the hepatitis C virus nonstructural protein 3 protease. *Eur J Biochem* **267**: 3685–3694.

Gatti R, Antonelli G, Prearo M, Spinella P, Cappellin E, De Palo EF. 2009. Cortisol assays and diagnostic laboratory procedures in human biological fluids. *Clin Biochem* **42**: 1205–1217.

Geiger A. 1996. RNA aptamers that bind L-arginine with sub-micromolar dissociation constants and high enantioselectivity. *Nucleic Acids Res* **24**: 1029–1036.

Gelfand M. 1999. A conserved RNA structure element involved in the regulation of bacterial riboflavin synthesis genes. *Trends Genet* **15**: 439–442.

Gold L, Ayers D, Bertino J, Bock C, Bock A, Brody EN, Carter J, Dalby AB, Eaton BE, Fitzwater T, et al. 2010. Aptamer-based multiplexed proteomic technology for biomarker discovery. *PLoS One* **5**: e15004.

Gold L, Janjic N, Jarvis T, Schneider D, Walker JJ, Wilcox SK, Zichi D. 2012. Aptamers and the RNA world, past and present. *Cold Spring Harb Perspect Biol* **4**: 1–10.

Gopinath SCB. 2007. Methods developed for SELEX. *Anal Bioanal Chem* **387**: 171–82.

Groher F, Suess B. 2014. Synthetic riboswitches - A tool comes of age. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms*.

Gu P, Yang F, Kang J, Wang Q, Qi Q. 2012. One-step of tryptophan attenuator inactivation and promoter swapping to improve the production of L-tryptophan in Escherichia coli. *Microb Cell Fact* **11**: 30.

Haberland A, Wallukat G, Dahmen C, Kage A, Schimke I. 2011. Aptamer neutralization of beta1-adrenoceptor autoantibodies isolated from patients with cardiomyopathies. *Circ Res* **109**: 986–992.

Hanson S, Berthelot K, Fink B, McCarthy JEG, Suess B. 2003. Tetracycline-aptamer-mediated translational regulation in yeast. *Mol Microbiol* **49**: 1627–1637.

Harvey I, Garneau P, Pelletier J. 2002. Inhibition of translation by RNA-small molecule interactions. *RNA* **8**: 452–463.

Hermann T, Patel DJ. 2000. Adaptive recognition by nucleic acid aptamers. *Science* **287**: 820–5.

Hianik T, Ostatná V, Sonlajtnerova M, Grman I. 2007. Influence of ionic strength, pH and aptamer configuration for binding affinity to thrombin. *Bioelectrochemistry* **70**: 127–133.

Homann M, Goringer HU, Göringer HU. 1999. Combinatorial selection of high affinity RNA ligands to live African trypanosomes. *Nucleic Acids Res* **27**: 2006–2014.

Hoon S, Zhou B, Janda KD, Brenner S, Scolnick J. 2011. Aptamer selection by high-throughput sequencing and informatic analysis. *Biotechniques* **51**: 413–6.

Huang F, Bugg CW, Yarus M. 2000. RNA-catalyzed CoA, NAD, and FAD synthesis from phosphopantetheine, NMN, and FMN. *Biochemistry* **39**: 15548–15555.

Huet AC, Delahaut P, Fodey T, Haughey S a., Elliott C, Weigel S. 2010. Advances in biosensor-based analysis for antimicrobial residues in foods. *TrAC - Trends Anal Chem* **29**: 1281–1294.

Jackson G. 2012. Methods for simultaneous generation of functional ligands. EP2411807 A2.

Jenison RD, Gill SC, Pardi A, Polisky B. 1994. High-resolution molecular discrimination by RNA. *Science (80-)* **263**: 1425–1429.

Jhaveri SD, Ellington AD. 2001. In vitro selection of RNA aptamers to a protein target by filter immobilization. *Curr Protoc Mol Biol* **Chapter 24**: Unit 24.3.

Jhaveri SD, Kirby R, Conrad R, Maglott EJ, Bowser M, Kennedy RT, Glick G, Ellington AD. 2000. Designed signaling aptamers that transduce molecular recognition to changes in fluorescence intensity. *J Am Chem Soc* **122**: 2469–2473.

Jing M, Bowser MT. 2011. Isolation of DNA aptamers using micro free flow electrophoresis. *Lab Chip* **11**: 3703–9.

Joyce GF. 2007. Forty years of in vitro evolution. *Angew Chem Int Ed Engl* **46**: 6420–36.

Keefe AD, Pai S, Ellington A. 2010. Aptamers as therapeutics. *Nat Rev Drug Discov* **9**: 537–50.

Kerman K, Kobayashi M, Tamiya E. 2003. Recent trends in electrochemical DNA biosensor technology. *Measurement Science and Technology*, February 1.

Khalil AS, Collins JJ. 2010. Synthetic biology: applications come of age. *Nat Rev Genet* **11**: 367–79.

Kozak M. 2005. Regulation of translation via mRNA structure in prokaryotes and eukaryotes. *Gene* **361**: 13–37.

Kramer FR, Mills DR, Cole PE, Nishihara T, Spiegelman S. 1974. Evolution in vitro: sequence and phenotype of a mutant RNA resistant to ethidium bromide. *J Mol Biol* **89**: 719–736.

Kupakuwana G V, Crill JE, McPike MP, Borer PN. 2011. Acyclic identification of aptamers for human alpha-thrombin using over-represented libraries and deep sequencing. *PLoS One* **6**: e19395.

Lauridsen LH, Rothnagel JA, Veedu RN. 2012. Enzymatic recognition of 2'-modified ribonucleoside 5'-triphosphates: towards the evolution of versatile aptamers. *Chembiochem* **13**: 19–25.

Lee F, Yanofsky C. 1977. Transcription termination at the trp operon attenuators of Escherichia coli and Salmonella typhimurium: RNA secondary structure and regulation of termination. *Proc Natl Acad Sci U S A* **74**: 4365–9.

Legiewicz M, Lozupone C, Knight R, Yarus M. 2005. Size, constant sequences, and optimal selection. *RNA* **11**: 1701–1709.

Levisohn R, Spiegelman S. 1969. Further extracellular Darwinian experiments with replicating RNA molecules: diverse variants isolated under different selective conditions. *Proc Natl Acad Sci U S A* **63**: 805–811.

Lin Y, Sun X, Yuan Q, Yan Y. 2014. Engineering bacterial phenylalanine 4-hydroxylase for microbial synthesis of human neurotransmitter precursor 5-hydroxytryptophan. *ACS Synth Biol* **3**: 497–505.

Liu Y, Wang C, Li F, Shen S, Tyrrell DLJ, Le XC, Li X-FF. 2012. DNase-mediated single-cycle selection of aptamers for proteins blotted on a membrane. *Anal Chem* **84**: 7603–7606.

Lozupone C, Changayil S, Majerfeld I, Yarus M. 2003. Selection of the simplest RNA that binds isoleucine. *RNA* **9**: 1315–1322.

Lucks JB, Mortimer SA, Trapnell C, Luo S, Aviran S, Schroth GP, Pachter L, Doudna JA, Arkin AP. 2011. Multiplexed RNA structure characterization with selective 2'-hydroxyl acylation analyzed by primer extension sequencing (SHAPE-Seq). *Proc Natl Acad Sci U S A* **108**: 11063–8.

Luo X, McKeague M, Pitre S, Dumontier M, Green J, Golshani A, Derosa MC, Dehne F. 2010. Computational approaches toward the design of pools for the in vitro selection of complex aptamers. *RNA* **16**: 2252–62.

Lynch SA, Desai SK, Sajja HK, Gallivan JP. 2007. A high-throughput screen for synthetic riboswitches reveals mechanistic insights into their function. *Chem Biol* **14**: 173–84.

Lynch SA, Gallivan JP. 2009. A flow cytometry-based screen for synthetic riboswitches. *Nucleic Acids Res* **37**: 184–92.

Macaya RF, Schultze P, Smith FW, Roe JA, Feigon J. 1993. Thrombin-binding DNA aptamer forms a unimolecular quadruplex structure in solution. *Proc Natl Acad Sci U S A* **90**: 3745–3749.

Majerfeld I, Yarus M. 2005. A diminutive and specific RNA binding site for L-tryptophan. *Nucleic Acids Res* **33**: 5482–93.

Mann D, Reinemann C, Stoltenburg R, Strehlitz B. 2005. In vitro selection of DNA aptamers binding ethanolamine. *Biochem Biophys Res Commun* **338**: 1928–34.

Martin J a., Chávez JL, Chushak Y, Chapleau RR, Hagen J, Kelley-Loughnane N. 2014. Tunable stringency aptamer selection and gold nanoparticle assay for detection of cortisol. *Anal Bioanal Chem* **406**: 4637–4647.

Mascini M. 2009. *Aptamers in Bioanalysis*. ed. M. Mascini. John Wiley & Sons, Inc., Hoboken, NJ, USA.

McKeague M, Derosa MC. 2012. Challenges and opportunities for small molecule aptamer development. *J Nucleic Acids* **2012**: 748913.

Mills DR, Peterson RL, Spiegelman S. 1967. An extracellular Darwinian experiment with a self-duplicating nucleic acid molecule. *Proc Natl Acad Sci U S A* **58**: 217–224.

Miranda-Ríos J, Navarro M, Soberón M. 2001. A conserved RNA structure (thi box) is involved in regulation of thiamin biosynthetic gene expression in bacteria. *Proc Natl Acad Sci U S A* **98**: 9736–9741.

Mironov AS, Gusarov I, Rafikov R, Lopez LE, Shatalin K, Kreneva RA, Perumov DA, Nudler E. 2002. Sensing small molecules by nascent RNA: A mechanism to control transcription in bacteria. *Cell* **111**: 747–756.

Mitchell W. 2011. Natural products from synthetic biology. *Curr Opin Chem Biol* **15**: 505–15.

Morse DP. 2007. Direct selection of RNA beacon aptamers. *Biochem Biophys Res Commun* **359**: 94–101.

Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H. 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harb Symp Quant Biol* **51 Pt 1**: 263–73.

Muranaka N, Sharma V, Nomura Y, Yokobayashi Y. 2009. An efficient platform for genetic selection and screening of gene switches in Escherichia coli. *Nucleic Acids Res* **37**: e39.

Nahvi A, Sudarsan N, Ebert MS, Zou X, Brown KL, Breaker RR. 2002. Genetic control by a metabolite binding mRNA. *Chem Biol* **9**: 1043–1049.

Nitsche A, Kurth A, Dunkhorst A, Pänke O, Sielaff H, Junge W, Muth D, Scheller F, Stöcklein W, Dahmen C, et al. 2007. One-step selection of Vaccinia virus-binding DNA aptamers by MonoLEX. *BMC Biotechnol* **7**: 48.

Nomura Y, Yokobayashi Y. 2007. Reengineering a natural riboswitch by dual genetic selection. *J Am Chem Soc* **129**: 13814–13815.

Nutiu R, Li Y. 2005. In vitro selection of structure-switching signaling aptamers. *Angew Chem Int Ed Engl* **44**: 1061–5.

Orazem ME. 2008. *Electrochemical impedance spectroscopy*. John Wiley & Sons.

Ozer A, Pagano JM, Lis JT. 2014. New Technologies Provide Quantum Changes in the Scale, Speed, and Success of SELEX Methods and Aptamer Characterization. *Mol Ther Nucleic Acids* **3**: e183.

Ozer A, White BS, Lis JT, Shalloway D. 2013. Density-dependent cooperative non-specific binding in solid-phase SELEX affinity selection. *Nucleic Acids Res* **41**: 7167–7175.

Pagano JM, Kwak H, Waters CT, Sprouse RO, White BS, Ozer A, Szeto K, Shalloway D, Craighead HG, Lis JT. 2014. Defining NELF-E RNA Binding in HIV-1 and Promoter-Proximal Pause Regions. *PLoS Genet* **10**.

Paige JS, Wu KY, Jaffrey SR. 2011. RNA mimics of green fluorescent protein. *Science* **333**: 642–6.

Pan W, Xin P, Clawson GA. 2008. Minimal primer and primer-free SELEX protocols for selection of aptamers from random DNA libraries. *Biotechniques* **44**: 351–360.

Patel DJ, Suri AK, Jiang F, Jiang L, Fan P, Kumar RA, Nonin S. 1997. Structure, recognition and adaptive binding in RNA aptamer complexes. *J Mol Biol* **272**: 645–664.

Pawar RS, Krynitsky AJ, Rader JI. 2013. Sweeteners from plants-with emphasis on Stevia rebaudiana (Bertoni) and Siraitia grosvenorii (Swingle). *Anal Bioanal Chem* **405**: 4397–4407.

Peng L, Stephens BJ, Bonin K, Cubicciotti R, Guthold M. 2007. A combined atomic force/fluorescence microscopy technique to select aptamers in a single cycle from a small pool of random oligonucleotides. *Microsc Res Tech* **70**: 372–81.

Qian J, Lou X, Zhang Y, Xiao Y, Tom Soh H. 2009. Generation of highly specific aptamers via micromagnetic selection. *Anal Chem* **81**: 5490–5495.

Rajendran M, Ellington AD. 2003. In vitro selection of molecular beacons. *Nucleic Acids Res* **31**: 5700–5713.

Rhie A, Kirby L, Sayer N, Wellesley R, Disterer P, Sylvester I, Gill A, Hope J, James W, Tahiri-Alaoui A. 2003. Characterization of 2′-fluoro-RNA aptamers that bind preferentially to disease-associated conformations of prion protein and inhibit conversion. *J Biol Chem* **278**: 39697–39705.

Rozlosnik N. 2009. New directions in medical biosensors employing poly(3,4-ethylenedioxy thiophene) derivative-based electrodes. *Anal Bioanal Chem* **395**: 637–645.

Ruff KM, Snyder TM, Liu DR. 2010. Enhanced functional potential of nucleic acid aptamer libraries patterned to increase secondary structure. *J Am Chem Soc* **132**: 9453–64.

Saffhill R, Schneider-Bernloehr H, Orgel LE, Spiegelman S. 1970. In vitro selection of bacteriophage Q-beta ribonucleic acid variants resistant to ethidium bromide. *J Mol Biol* **51**: 531–539.

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N. 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**: 1350–4.

Schallmey M, Frunzke J, Eggeling L, Marienhagen J. 2014. Looking for the pick of the bunch: High-throughput screening of producing microorganisms with biosensors. *Curr Opin Biotechnol* **26**: 148–154.

Schütze T, Wilhelm B, Greiner N, Braun H, Peter F, Mörl M, Erdmann V a, Lehrach H, Konthur Z, Menger M, et al. 2011. Probing the SELEX process with next-generation sequencing. *PLoS One* **6**: e29604.

Serganov A, Nudler E. 2013. A decade of riboswitches. *Cell* **152**: 17–24.

Shi H, Fan X, Ni Z, Lis JT. 2002. Evolutionary dynamics and population control during in vitro selection and amplification with multiple targets. *RNA* **8**: 1461–1470.

Shi H, Fan X, Sevilimedu A, Lis JT. 2007. RNA aptamers directed to discrete functional sites on a single protein structural domain. *Proc Natl Acad Sci U S A* **104**: 3742–3746.

Shi H, Hoffman BE, Lis JT. 1997. A specific RNA hairpin loop structure binds the RNA recognition motifs of the Drosophila SR protein B52. *Mol Cell Biol* **17**: 2649–2657.

Shui B, Ozer A, Zipfel W, Sahu N, Singh A, Lis JT, Shi H, Kotlikoff MI. 2012. RNA aptamers that functionally interact with green fluorescent protein and its derivatives. *Nucleic Acids Res* **40**: 1–11.

Spiegelman S, Haruna I, Holland IB, Beaudreau G, Mills D. 1965. The synthesis of a self-propagating and infectious nucleic acid with a purified enzyme. *Proc Natl Acad Sci U S A* **54**: 919–27.

Stanlis KKH, McIntosh JR. 2003. Single-strand DNA aptamers as probes for protein localization in cells. *J Histochem Cytochem* **51**: 797–808.

Stoltenburg R, Nikolaus N, Strehlitz B. 2012. Capture-SELEX: Selection of DNA Aptamers for Aminoglycoside Antibiotics. *J Anal Methods Chem* **2012**: 415697.

Stoltenburg R, Reinemann C, Strehlitz B. 2005. FluMag-SELEX as an advantageous method for DNA aptamer selection. *Anal Bioanal Chem* **383**: 83–91.

Stoltenburg R, Reinemann C, Strehlitz B. 2007. SELEX-A (r)evolutionary method to generate high-affinity nucleic acid ligands. *Biomol Eng* **24**: 381–403.

Stormo GD, Ji Y. 2001. Do mRNAs act as direct sensors of small molecules to control their expression? *Proc Natl Acad Sci U S A* **98**: 9465–7.

Stryer L, Berg JM, Tymoczko JL. 2002. Biochemistry. New York, NY.

Suess B. 2003. Conditional gene expression by controlling translation with tetracycline-binding aptamers. *Nucleic Acids Res* **31**: 1853–1858.

Suess B, Fink B, Berens C, Stentz R, Hillen W. 2004. A theophylline responsive riboswitch based on helix slipping controls gene expression in vivo. *Nucleic Acids Res* **32**: 1610–4.

Szeto K, Craighead HG. 2014. Devices and approaches for generating specific high-affinity nucleic acid aptamers. *Appl Phys Rev* **1**.

Szeto K, Reinholt SJ, Duarte FM, Pagano JM, Ozer A, Yao L, Lis JT, Craighead HG. 2014. High-throughput binding characterization of RNA aptamer selections using a microplate-based multiplex microcolumn device. *Anal Bioanal Chem* **406**: 2727–32.

Tang J, Breaker RR. 1997. Rational design of allosteric ribozymes. *Chem Biol* **4**: 453–459.

Tasset DM, Kubik MF, Steiner W. 1997. Oligonucleotide inhibitors of human thrombin that bind distinct epitopes. *J Mol Biol* **272**: 688–98.

Thiel WH, Bair T, Wyatt Thiel K, Dassie JP, Rockey WM, Howell CA, Liu XY, Dupuy AJ, Huang L, Owczarzy R, et al. 2011. Nucleotide bias observed with a short SELEX RNA aptamer library. *Nucleic Acid Ther* **21**: 253–63.

Tolle F, Wilke J, Wengel J, Mayer G. 2014. By-Product Formation in Repetitive PCR Amplification of DNA Libraries during. *PLoS One* **9**: e114693.

Tome JM, Ozer A, Pagano JM, Gheba D, Schroth GP, Lis JT. 2014. Comprehensive analysis of RNA-protein interactions by high-throughput sequencing-RNA affinity profiling. *Nat Methods* **11**.

Topp S, Gallivan JP. 2008. Random walks to synthetic riboswitches--a high-throughput selection based on cell motility. *Chembiochem* **9**: 210–3.

Trausch JJ, Batey RT. 2015. Design of Modular " Plug- and-Play " Expression Platforms Derived from Natural Riboswitches for Engineering Novel Genetically Encodable RNA Regulatory Devices. In *Methods in enzymology* (ed. D.H. Burke), Vol. 550 of, pp. 41–71, Elsevier Inc.

Tribe DE, Pittard J. 1979. Hyperproduction of tryptophan by Escherichia coli: genetic manipulation of the pathways leading to tryptophan formation. *Appl Environ Microbiol* **38**: 181–190.

Tuerk C, Gold L. 1990. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science (80-)* **249**: 505–510.

Velez TE, Singh J, Xiao Y, Allen EC, Wong OY, Chandra M, Kwon SC, Silverman SK. 2012. Systematic evaluation of the dependence of deoxyribozyme catalysis on random region length. *ACS Comb Sci* **14**: 680–687.

Vu MMK, Jameson NE, Masuda SJ, Lin D, Larralde-Ridaura R, Lupták A. 2012. Convergent evolution of adenosine aptamers spanning bacterial, human, and random sequences revealed by structure-based bioinformatics and genomic SELEX. *Chem Biol* **19**: 1247–54.

Wachsmuth M, Findeiß S, Weissheimer N, Stadler PF, Mörl M. 2013. De novo design of a synthetic riboswitch that regulates transcription termination. *Nucleic Acids Res* **41**: 2541–2551.

Wang HH, Isaacs FJ, Carr PA, Sun ZZ, Xu G, Forest CR, Church GM. 2009. Programming cells by multiplex genome engineering and accelerated evolution. *Nature* **460**: 894–898.

Wang J. 2008. Electrochemical glucose biosensors. In *Electrochemical Sensors, Biosensors and their Biomedical Applications*, pp. 57–69.

Wang J, Rudzinski JF, Gong Q, Soh HT, Atzberger PJ. 2012. Influence of Target Concentration and Background Binding on In Vitro Selection of Affinity Reagents. *PLoS One* **7**: 1–8.

Weigand JE, Schmidtke SR, Will TJ, Duchardt-Ferner E, Hammann C, Wöhnert J, Suess B. 2011. Mechanistic insights into an engineered riboswitch: a switching element which confers riboswitch activity. *Nucleic Acids Res* **39**: 3363–72.

Werpy T, Petersen G. 2004. *Top Value Added Chemicals from Biomass Volume I — Results of Screening for Potential Candidates from Sugars and Synthesis Gas Top Value Added Chemicals From Biomass Volume I : Results of Screening for Potential Candidates*.

Werstuck G, Green MR. 1998. Controlling gene expression in living cells through small molecule-RNA interactions. *Science* **282**: 296–298.

Wiegand TW, Williams PB, Dreskin SC, Jouvin MH, Kinet JP, Tasset D. 1996. High-affinity oligonucleotide ligands to human IgE inhibit binding to Fc epsilon receptor I. *J Immunol* **157**: 221–30.

Willner I, Zayats M. 2008. Electrochemical Aptasensors. In *Aptamers in Bioanalysis*, pp. 61–86, John Wiley & Sons, Inc.

Winkler W, Nahvi A, Breaker RR. 2002. Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression. *Nature* **419**: 952–6.

Wittmann A, Suess B. 2012. Engineered riboswitches: Expanding researchers' toolbox with synthetic RNA regulators. *FEBS Lett* **586**: 2076–83.

Xiao H, Edwards TE, Ferré-D'Amaré AR. 2008. Structural basis for specific, high-affinity tetracycline binding by an in vitro evolved aptamer and artificial riboswitch. *Chem Biol* **15**: 1125–37.

Yang J, Seo SW, Jang S, Shin S-I, Lim CH, Roh T-Y, Jung GY. 2013. Synthetic RNA devices to expedite the evolution of metabolite-producing microbes. *Nat Commun* **4**: 1413.

Yang Q, Goldstein IJ, Mei HY, Engelke DR. 1998. DNA ligands that bind tightly and selectively to cellobiose. *Proc Natl Acad Sci U S A* **95**: 5462–5467.

Yanofsky C. 1981. Attenuation in the control of expression of bacterial operons. *Nature* **289**: 751–758.

Yoshida W, Mochizuki E, Takase M, Hasegawa H, Morita Y, Yamazaki H, Sode K, Ikebukuro K. 2009. Selection of DNA aptamers against insulin and construction of an aptameric enzyme subunit for insulin sensing. *Biosens Bioelectron* **24**: 1116–20.

Zimmermann B, Gesell T, Chen D, Lorenz C, Schroeder R. 2010. Monitoring genomic sequences during SELEX using high-throughput sequencing: neutral SELEX. *PLoS One* **5**: e9169.

Zimmermann GR, Jenison RD, Wick CL, Simorre J-P, Pardi A. 1997. Interlocking structural motifs mediate molecular discrimination by a theophylline-binding RNA. *Nat Struct Biol* **4**: 644–649.

8 Publications

This chapter includes some of the articles prepared as part of this PhD project. The articles are presented in the following order.

Article 1

Lauridsen LH, Shamaileh HA, Edwards SL, Taran E, Veedu RN (2012) Rapid one-step selection method for generating nucleic acid aptamers: development of a DNA aptamer against α-bungarotoxin. PLoS One 7: e41702. doi:10.1371/journal.pone.0041702.

Article 2

Lauridsen LH, Doessing HB, Long KS, Nielsen AT (2015) A Capture-SELEX strategy for multiplexed selection of structure-switching RNA aptamers against small molecules. RNA *in preparation*.

Article 3

Daprà J, **Lauridsen LH**, Nielsen AT, Rozlosnik N (2013) Comparative study on aptamers as recognition elements for antibiotics in a label-free all-polymer biosensor. Biosens Bioelectron 43: 315–320. doi:10.1016/j.bios.2012.12.058.

Article 4

Lauridsen LH, Veedu RN (2012) Nucleic acid aptamers against biotoxins: a new paradigm toward the treatment and diagnostic approach. Nucleic Acid Ther 22: 371–379. doi:10.1089/nat.2012.0377.

Article 1

Rapid one-step selection method for generating nucleic acid aptamers: development of a DNA aptamer against α-bungarotoxin

OPEN ACCESS Freely available online

PLoS one

Rapid One-Step Selection Method for Generating Nucleic Acid Aptamers: Development of a DNA Aptamer against α-Bungarotoxin

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Abstract

Background: Nucleic acids based therapeutic approaches have gained significant interest in recent years towards the development of therapeutics against many diseases. Recently, research on aptamers led to the marketing of Macugen®, an inhibitor of vascular endothelial growth factor (VEGF) for the treatment of age related macular degeneration (AMD). Aptamer technology may prove useful as a therapeutic alternative against an array of human maladies. Considering the increased interest in aptamer technology globally that rival antibody mediated therapeutic approaches, a simplified selection, possibly in one-step, technique is required for developing aptamers in limited time period.

Principal Findings: Herein, we present a simple one-step selection of DNA aptamers against α-bungarotoxin. A toxin immobilized glass coverslip was subjected to nucleic acid pool binding and extensive washing followed by PCR enrichment of the selected aptamers. One round of selection successfully identified a DNA aptamer sequence with a binding affinity of 7.58 μM.

Conclusion: We have demonstrated a one-step method for rapid production of nucleic acid aptamers. Although the reported binding affinity is in the low micromolar range, we believe that this could be further improved by using larger targets, increasing the stringency of selection and also by combining a capillary electrophoresis separation prior to the one-step selection. Furthermore, the method presented here is a user-friendly, cheap and an easy way of deriving an aptamer unlike the time consuming conventional SELEX-based approach. The most important application of this method is that chemically-modified nucleic acid libraries can also be used for aptamer selection as it requires only one enzymatic step. This method could equally be suitable for developing RNA aptamers.

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Introduction

In recent years, nucleic acids-based therapy has attracted significant interest for the treatment of many diseases. It comprises several approaches based on nucleic acid as the active component, which include antisense [1,2], ribozymes [2], short interfering RNA (siRNA) [2–4], microRNA (miRNA) [5,6], and aptamers [7–11]. Aptamer technology is one of the most promising and important approaches for therapeutic development. Aptamers are short single-stranded (ss) DNA or RNA oligonucleotides that can bind to its target with high affinity and specificity due to their ability to adopt three-dimensional shapes in solution. These structured sequence-specific ssDNA or RNA aptamers can specifically bind to a myriad of different targets [11]. Coupled with superior storage stability and easy solid-phase production steps, aptamers have become prime candidates for therapeutic and diagnostic applications. Furthermore, research on aptamers led to the marketing of Macugen®, an inhibitor of vascular endothelial growth factor (VEGF) for the treatment of age related macular degeneration (AMD) [12].

Traditionally, aptamers are generated by a process referred to as SELEX (Systematic Evolution of Ligands by Exponential enrichment) [13–15]. This method is very time consuming and involves several enzymatic steps. Aptamers composed of natural DNA and RNA pose some serious limitations such as poor nuclease resistance and decreased binding affinity. To combat these problems, chemically modified nucleotides are used. However, the use of modified nucleotides in SELEX is very limited because of their poor enzymatic recognition capabilities, demanding an alternative approach for aptamer selection [16,17]. In addition, the repetitive nature of the SELEX procedure increases the risk of inducing mutations during the enzymatic amplifications steps and consequently losing important sequence information before isolating the high binding aptamers [18].

We have selected α-bungarotoxin (7984 Da) as the target in our study. α-Bungarotoxin is one of the toxic components of krait snake (*Bungarus multicinctus*) venom. The toxin binds irreversibly and competitively to the acetylcholine receptor found at the neuromuscular junction, causing paralysis, respiratory failure and ultimately death in the victim. Krait snakebite is lethal and often encountered in Southeast Asia and the Indian subcontinent. The treatment often becomes difficult unless the snake is properly spotted and also noted that there is frequently little or no pain at the site of a krait bite, which can lead to false-reassurance to the victim [19,20].

Results

One-step Selection on a Glass Coverslip

Our approach of one-step aptamer selection is schematically illustrated in Figure 1. First, the target α-bungarotoxin was covalently linked to the surface of a glass coverslip which was coated with ***N***-hydroxysuccinimide (NHS) functionalized polyethylene glycol (PEG) for reaction with primary amines of the target. The non-linked sites were blocked with a deactivation buffer supplied by the manufacturer. The coverslip is very thin and fragile that makes it difficult to work with. In our experience, it was best to handle the coverslip on a piece of Parafilm. The nucleic acid library was first incubated with a deactivated coverslip without the target peptide to remove the sequences that have affinity to the surface (negative selection). The remaining unbound pool was collected and added on to the coverslip immobilized with α-bungarotoxin and incubated overnight in a humidifying chamber. The coverslip was then washed with a large excess of the binding buffer and dried by gently blowing N_2 gas. The aptamer binding to the target was monitored directly by fluorescence microscopy. The absence of high background fluorescence and the appearance of localized fluorescent spots indicated a successful aptamer selection against α-bungarotoxin (Figure 2A).

After the initial observation by fluorescence showing aptamer binding to the toxin, the bound aptamers were eluted by crushing the glass coverslip well and soaking in water in a 1.5 mL Eppendorf tube followed by vortexing and heating at 90°C for ten minutes. The supernatant was collected and PCR amplification yielded a single band on an agarose gel corresponding to the expected library size (Figure 2B). The purified PCR product was later cloned into *E.coli* and fifty five positive clones were collected and the purified individual plasmid DNA was sequenced. The obtained crude sequences were screened and only those sequences, which retained the initial library design with correct primer binding regions, were considered for further analyses. Although most sequences contained a particular motif -GGGC-, we have only selected the repeating sequences with the motif -GGGC- for binding studies, which are listed in Table 1.

Aptamer Binding Affinity Determination

The binding interaction between the selected aptamer and α-bungarotoxin was determined by surface Plasmon resonance (SPR)-based Biacore 3000 instrument. The selected aptamer sequences were chemically synthesized which contained a biotin tag at 5′-end. The biotinylated aptamer sequences were then immobilized on a sensor chip SA (streptavidin coated chip, GE life sciences). Varying concentrations of α-bungarotoxin (500 nM – 50 μM) was then passed through the surface immobilized with the aptamer. SPR data showed that the aptamer sequence of clone 24 & 51 can bind to α-bungarotoxin with a K_D of 7.5 μM (Figure 3A). In contrast, aptamer sequences of clones 15, 22 & 42; 28 & 45 and 1 & 16 showed negligible binding (data not shown). We have also performed non-specific binding analyses using a non-sense target, insulin. There was no binding observed as expected, even at higher concentrations.

Discussion

Highly sensitive and specific nucleic acid aptamers against α–bungarotoxin can be extremely useful as a diagnostic and therapeutic against krait snake envenomation. Aptamers offer a unique alternative to the existing antibodies against the toxins which are much larger, easily degraded, expensive and require the use of animals for a laborious time consuming production [9,21]. On the other hand, aptamers are easily synthesized and can be modified with reporter molecules or other nucleotide analogues to enhance their properties like nuclease resistance. Aptamers also adopt unique sequence-dependent three-dimensional shapes, so they are in a sense, shape libraries that may bind to a target based on their sequence, conformation and/or charge through interactions with binding pockets, hydrogen bonds, stacking of aromatic rings, van der Waals forces, or a combination of these [21]. Aptamers are normally developed by SELEX, an *in vitro* evolution process developed around twenty years ago. Since the invention, several SELEX protocols employing different selection approaches have been developed [13,22]. The majority of these modified protocols still require repetitive selection and enrichment steps for selecting high affinity aptamers. Considering the increased interest in aptamer technology globally that rival antibody approach, a simplified selection, possibly in one-step, technique is required for developing aptamers in limited time period and to avoid repeated enzymatic steps that might induce sequence mutations. In this direction, there are few reports showing the selection of aptamers in a single step [23–25]. One approach was called MonoLEX in which a DNA aptamer was selected against the Vaccinia virus [23]. The technique involved an affinity chromatography step followed by subsequent physical partitioning of the affinity resin and PCR amplification of the bound aptamers. Another method to develop aptamers in one step was by using a combination of atomic force and fluorescence microscopy called NanoSelection [24]. However, the major problem with this approach was that the selection was carried out with a very small pool of randomized preselected thrombin binding aptamer oligonucleotides unlike a conventional SELEX library and there was no further report on aptamer selection against new targets by this approach. Krylov and co-workers reported a Non-SELEX selection of aptamers using capillary electrophoresis by which they were able to develop aptamer with affinity in the low micro molar range [26].

We have developed a one-step method for rapid selection of aptamers that is simple, cheap and user-friendly. For successful aptamer selection, three key steps that need to function optimally; 1) efficient separation of the target bound aptamers, 2) enzymatic amplification by PCR and 3) regeneration of the selected single-stranded aptamers. One major highlight of our method is that it eliminates the need for repeated enzymatic amplification and regeneration of the single-stranded aptamer that always complicate and affect the selection success. In one step selection method, we have adopted an easy to use glass coverslip for immobilizing the target for selecting aptamers by monitoring fluorescence under a microscope to ensure that we are in fact selecting and processing the target bound aptamer. One-step selection of aptamers on a glass coverslip is an easy way for generating aptamers even for a small biomolecular target like α-bungarotoxin (7984 Da) used in this study. The target immobilization on to the coverslip was verified by using an Alexa Fluor 555 dye-labeled α-bungarotoxin

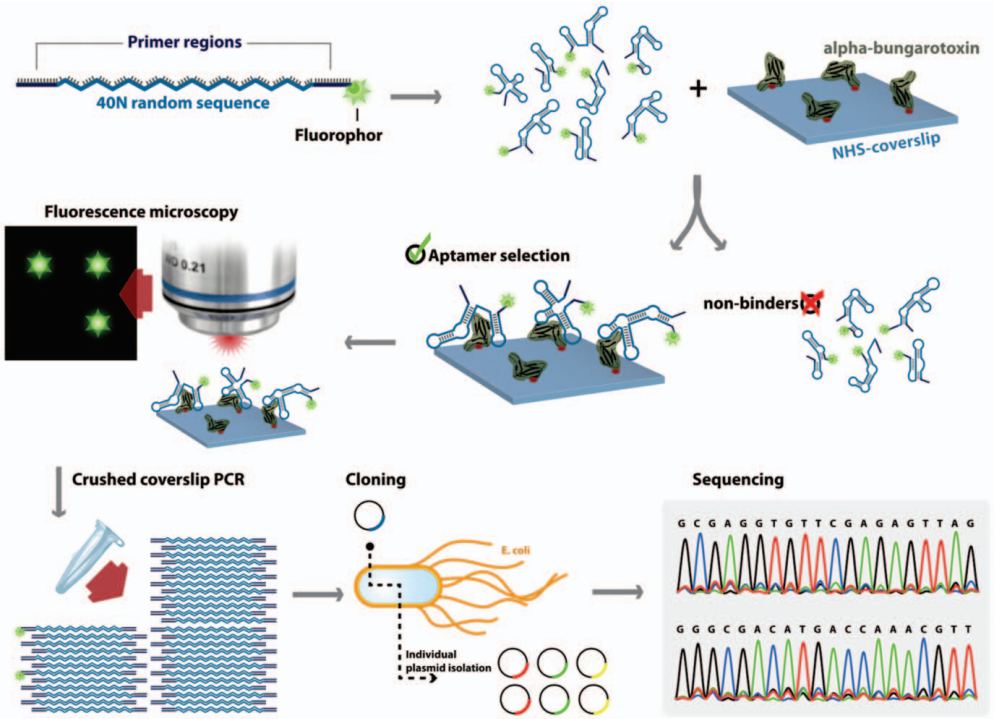

Figure 1. Single-step selection method. FAM-labeled oligonucleotide library containing a 40 nt random region was incubated with a target immobilized on a glass coverslip. Unbound sequences were discarded by extensive washing followed by fluorescence microscopy. The coverslip was later crushed and eluted the bound sequences by heating in water. The selected aptamers were amplified by PCR and cloned into *E.coli* and the purified individual plasmid DNA was sequenced.
doi:10.1371/journal.pone.0041702.g001

followed by fluorescence microscopy (Figure S1B). A large pool of fluorescein (FAM) dye–labeled DNA oligonucleotides (100 μL of 40 μM, contains 2.4×10^{15} members, the protocol allows using much larger sized libraries, however, this may increase the background fluorescence and force non-specific binding) was then incubated with the target-immobilized coverslip. We speculate that a stringent washing procedure employed might also account to a great extent for the successful aptamer identification by this approach followed by fluorescence microscopy. The coverslip was later viewed under a microscope and observed the green spots indicating that we have selected an aptamer. A control experiment was performed to make sure that we have selected the aptamers against the intended target peptide immobilized on to the coverslip. For this purpose, we used the Alexa Flour 555-labeled α-bungarotoxin and FAM-labeled DNA library and performed the selection as mentioned. Later, we did overlay the images. A target specific selection should turn to an orangish-yellow color upon overlay. If not, both the target and the aptamer will remain as red and green spots respectively. In our case, we have seen very distinct orangish-yellow spots upon overlay reassuring the target specific aptamer selection (Figure S1). Based on this experiment, we believe that the aptamer was indeed selected specifically to α-bungarotoxin.

Negative selection using a deactivated coverslip is also a necessary step prior to the actual aptamer selection against the target. However, it is important to mention that some of the most potent binders to α-bungarotoxin that might also have affinity for the coverslip surface will be removed by this step even before the actual aptamer selection. But, in this selection protocol this negative selection is required mainly to avoid the non-specific surface binding aptamers. Majority of the glass surface binding aptamers can be removed by this step and other week non-specific binders can subsequently be washed away by very stringent washing procedure. To further verify this, we have performed one selection without performing negative selection using a deactivated glass coverslip. But in this case, we observed an increased amount of green fluorescence on the coverslip indicating nonspecific selection of the aptamers against the glass surface (data not shown). The binding affinity ($K_D = 7.52$ μM) of the identified aptamer (from clone 24 & 51, Table 1) against α-bungarotoxin is not very high. The binding affinity can be improved by truncating the selected aptamer based on its predicted structure by mfold web server (Figure 3B) [27]. Although we cannot generalize, we believe that using our method, the binding affinity can be improved by adopting various strategies. Using a larger target protein (>15 kD) may improve the probability in selecting high affinity aptamers to

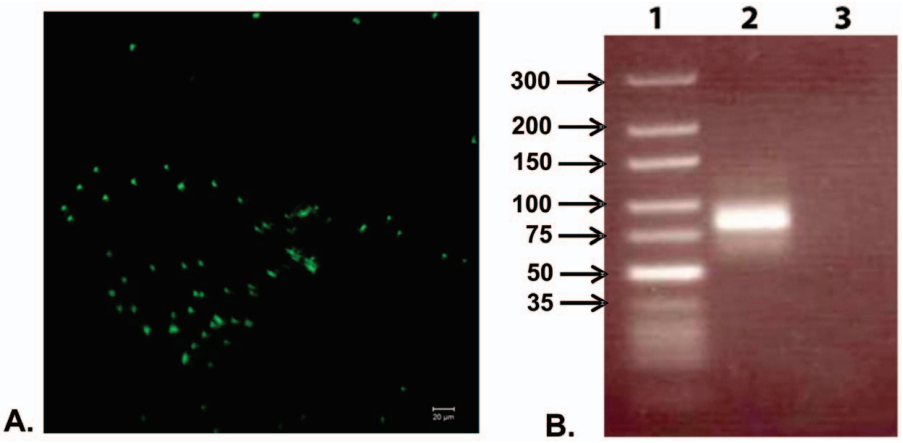

Figure 2. One-step selection and amplification of the aptamer candidates. A. The observed fluorescence on the toxin immobilized glass coverslip after washing. The glass coverslip was washed with extensive amounts of binding buffer, effectively removing all nonspecific adhesion to the coverslip. The pattern of highly localized fluorescent dots and the absence of background smear provide an indication of successful selection; B. PCR amplification of the eluted bound sequence. Lane 1: Marker DNA, lane 2: Amplified product from the eluted DNA aptamers, lane 3: PCR amplification without using a template DNA (negative control).
doi:10.1371/journal.pone.0041702.g002

some extent because of their structural diversity and immobilization efficiency on the coverslip. We also foresee that by combining capillary electrophoresis [26,28] and our one-step protocol may yield high affinity aptamers, ie, the target bound aptamer candidates are first separated by capillary electrophoresis followed by incubation on the target immobilized coverslip to remove all week binders monitored by fluorescence microscopy. It is noteworthy to mention that our selection protocol allows aptamer selection within 24 hrs, much shorter than a month long conventional SELEX selection procedure.

In summary, we have demonstrated a simple, rapid one-step selection protocol for developing nucleic acid aptamers. Using this method, one DNA aptamer against α-bungarotoxin was identified with a dissociation constant of 7.5 μM. This method opens up new ways for aptamer selection in a very limited time at low cost. So far, the use of important chemically-modified nucleotides in aptamer selection is limited due to their poor enzymatic recognition capabilities. Our approach may prove useful especially for using modified nucleotides containing library-based aptamer selection. Although we reported a DNA aptamer selection using this approach, it could equally be suitable for developing RNA aptamers.

Table 1. Aptamer sequences obtained from the selected clones.

Clones	Sequences
15, 22 & 42	ATCATGTCTTTTCGGGATGGGCAAGAAGGGAAATAATGC
28 &45	AGAAACGTAGCGGTAACTGCTAGAATGCGCCGAGAGAGCG
24 & 51	GCGAGGTGTTCGAGAGTTAGGGGCGACATGACCAAACGTT
1 & 16	AGGGCACAGAGAAGAAGTCGTGGATTTGAATGGTTTTGGT

Only the sequences from the random regions are shown.
doi:10.1371/journal.pone.0041702.t001

Materials and Methods

All DNA oligonucleotide sequences were purchased from Integrated DNA technologies (Coralville, USA). The *N*-hydroxysuccinimide activated glass coverslip was purchased from MicroSurfaces Inc (supplied by Stratech Scientific, Australia). Phusion DNA polymerase was purchased from New England Biolabs (supplied by Genesearch Australia). NucleoSpin® Extract II kit for PCR clean up was purchased from Macherey-Nagel (supplied by Scientifix Australia). For cloning the PCR product, the plasmid pCR®-Blunt was purchased from Invitrogen, Australia. Plasmid DNA was extracted and purified using a QIAprep Miniprep Kit (purchased from Qiagen, Australia). Fluorescence microscopy was performed using a Laser Scanning Microscope (LSM) 710 from Zeiss and a 20x objective at an excitation wavelength of 488 nm and laser power of 1 mW.

The designed nucleic acid library contained a 40nt random region flanked by two primer binding regions (5′-GGACAG-GACCACACCCAGCG-40nt-GGCTCCTGTGTGTCGCTTTGT-FAM-3′) and the library was labeled with a fluorescent tag (FAM) and purchased from IDT in 1 μmol scale. The library was dissolved in 1×PBS containing 5 mM $MgCl_2$ to yield a concentration of 40 μM and denatured by heating at 90°C for 5 min followed by cooling in ice. Before use, the library was kept at room temperature for 15 min to adjust the temperature.

Single-step Selection on Glass Coverslip

The *N*-hydroxysuccinimide activated glass coverslip was treated as per the protocol recommended by the manufacturer. In short, 25 μL of the α-bungarotoxin (125 μM) was dissolved in 30 μL PBS containing 10% glycerol and applied drop by drop on to the NHS activated coverslip which was placed on a piece of Parafilm® M and incubated for 30–40 min at 24°C in a humidifying chamber. The glass coverslip was then washed with 3×200 μL washing buffer (1×PBS containing 0.05% Tween 20) by adding

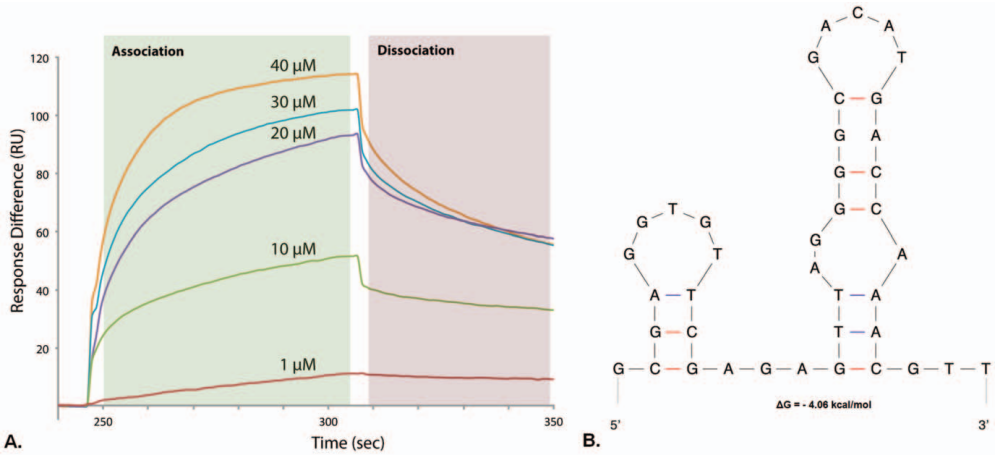

Figure 3. Aptamer characterization. A. Binding specificity of the aptamer obtained from clone 24 & 51 by surface plasmon resonance (SPR) technique using Biacore 3000. Various concentrations of α-bungarotoxin (1, 10, 20, 30, and 40 μM) were passed through the aptamer which was immobilized on a streptavidin coated sensor chip. The obtained sensorgrams demonstrate the aptamer binding to α-bungarotoxin; B. Predicted structure of the obtained aptamer using the DNA folding platform from mfold web server [26].
doi:10.1371/journal.pone.0041702.g003

droplets to a piece of Parafilm® M and then by gently placing the coverslip upside down soaking the coverslip in 200 μL washing buffer and waiting for 5 min at 24°C. The coverslip was then placed in 300 μL deactivation buffer (supplied by the manufacturer) to block any unreacted groups for 35 minutes in a humidifying chamber. The coverslip were subsequently washed two times with PBS containing 0.05% Tween 20. After this immobilization step, 120 μL of the prepared library was incubated for 30 min on an inactivated coverslip without α-bungarotoxin immobilization (negative selection). Then, 100 μL of the counter-selected nucleic acid library was transferred to the α-bungarotoxin immobilized coverslip and incubated overnight at 24°C in a humidifying chamber. Extensive washing was then performed with 4 mL washing buffer (5×800 μL), followed by drying the coverslip by gently blowing nitrogen gas. The backside of the coverslip was cleaned with a clean tissue soaked in ethanol before fluorescent imaging by microscopy.

The toxin bound aptamers were later eluted by crushing the glass coverslip in an Eppendorf tube followed by heating in Milli-Q water at 90°C for 20 minutes. The resulting solution was centrifuged at 14000 rpm and the supernatant was collected for the subsequent PCR amplification step. In short, the PCR mixture was prepared in a total volume of 50 μL by adding 10 μL 5× Phusion HF buffer (included in the Phusion DNA polymerase kit), 4 μL of dNTPs (400 μM), 28 μL of two times distilled water, 1.5 μL of forward primer (50 μM), 1.5 μL of reverse primer (50 μM), 5 μL of template (supernatant) and 0.5 μL of Phusion DNA polymerase (250 U/μL). The reaction mixtures were gently vortexed and then amplified using a thermal cycler (S1000™ Thermal cycler, Bio-Rad). A 25-cycle PCR consisted of denaturation at 98°C for 10 seconds, annealing at 55°C for 15 seconds and extension at 72°C for 25 seconds. After the polymerase reactions, gel-loading buffer (included in Ultra Low DNA size marker kit from Fermentas, supplied by VWR Australia) was added (1.5 μL) and the products were analysed by 4% agarose gel electrophoresis followed by UV-photography. The PCR product was later purified by using the NucleoSpin® Extract II kit.

Cloning and Sequencing

Purified PCR products (50 ng) were cloned into *E.coli* using a pCR®-Blunt (Invitrogen) vector according to the manufacturer's instructions. Plasmid DNA was extracted using QIAprep (Qiagen) and sequenced by the Australian Genome Research Facility (AGRF, Brisbane, Australia).

K_D Determination by SPR

The binding affinities of the selected aptamers against α-bungarotoxin were analyzed by Surface Plasmon Resonance using a Biacore 3000 Instrument (GE) at 24°C. Streptavidin immobilized sensor chip SA (GE, for Biacore 3000) was used for all measurements of kinetics and 1×PBS binding buffer (137 mM NaCl, 2.7 mM KCl, 4.2 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.005% Tween 20) was used as the running buffer. The sensor chip was preconditioned with 1 mL of the running buffer at a flow rate of 100 μL/min. Then, the 5′-biotinylated aptamer sequences were immobilized on the chip as recommended by manufacturer's manual. Varying concentrations of α-bungarotoxin was then injected at a flow rate of 30 μL/min for 2 min. The surface was regenerated by treating with 10 mM NaOH. The K_D values were calculated on the basis of a 1:1 Langmuir binding model by fitting the association and dissociation rates using the Biacore 3000 evaluation software.

Supporting Information

Figure S1 Fluorescence-based characterization of the α-bungarotoxin aptamer selection by one-step protocol. A. An overlay image (orangish-yellow spot indicating aptamer binding to α-bungarotoxin) of the immobilized α-bungarotoxin labeled with Alexa Fluor 555 (red colour) and the toxin bound FAM-labeled DNA aptamer (green fluorescence); B. Character-

ization of the α-bungarotoxin labeled with Alexa Fluor 555 (red colour) immobilized on a glass coverslip; C. α-bungarotoxin bound FAM-labeled DNA aptamer (green fluorescence).
(TIF)

References

1. Crooke ST (2008) Antisense Drug Technology: Principles, Strategies, and Applications. Boca Raton: CRC Press. 848 p.
2. Opalinska JB, Gewirtz AM (2002) Nucleic-acid therapeutics: basic principles and recent applications. Nat Rev Drug Discov 1: 503–514.
3. Mello CC, Conte D (2004) Revealing the world of RNA interference. Nature 431: 338–342.
4. Hannon GJ (2002) RNA interference. Nature 418: 244–251.
5. Ambros V (2001) microRNAs: tiny regulators with great potential. Cell 107: 823–826.
6. Bartel D (2004) MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116: 281–297.
7. Tuerk C, Gold L (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 249: 505–510.
8. Ellington AD, Szostak JW (1990) *In vitro* selection of RNA molecules that bind specific ligands. Nature 346: 818–822.
9. Jayasena S (1999) Aptamers: An emerging class of molecules that rival antibodies in diagnostics. Clin Chem 45: 1628–1650.
10. Famulok M, Mayer G, Blind M (2000) Nucleic acid aptamers - From selection in vitro to applications in vivo. Accounts Chem Res 33: 591–599.
11. Nimjee SM, Rusconi CP, Sullenger BA (2005) Aptamers: An emerging class of therapeutics. Annu Rev Med 56: 555–583.
12. Gragoudas ES, Adamis AP, Cunningham ET Jr, Feinsod M, Guyer DR (2004) Pegaptanib for neovascular age-related macular degeneration. N Engl J Med 351: 2805–2816.
13. Gopinath S (2007) Methods developed for SELEX. Anal Bioanal Chem 387: 171–182.
14. Klug S, Famulok M (1994) All you wanted to know about SELEX. Mol Biol Rep 20: 97–107.
15. Stoltenburg R, Reinemann C, Strehlitz B (2007) SELEX - A (r)evolutionary method to generate high-affinity nucleic acid ligands. Biomol Eng 24: 381–403.
16. Lauridsen LH, Rothnagel JA, Veedu RN (2012) Enzymatic recognition of 2'-modified ribonucleoside 5'-triphosphates: towards the evolution of versatile aptamers. Chembiochem 13: 19–25.
17. Keefe AD, Cload ST (2008) SELEX with modified nucleotides. Curr Opin Chem Biol 12: 448–456.
18. Hoon S, Zhou B, Janda KD, Brenner S, Scolnick J (2011) Aptamer selection by high-throughput sequencing and informatic analysis. Biotechniques 51: 413–416.
19. Ha TH, Hojer J, Trinh XK, Nguyen TD (2010) A controlled clinical trial of a novel antivenom in patients envenomed by Bungarus multicinctus. J Med Toxicol 6: 393–397.
20. Nirthanan S, Gwee MC (2004) Three-finger alpha-neurotoxins and the nicotinic acetylcholine receptor, forty years on. J Pharmacol Sci 94: 1–17.
21. Hermann T, Patel DJ (2000) Adaptive recognition by nucleic acid aptamers. Science 287: 820–825.
22. Aquino-Jarquin G, Toscano-Garibay JD (2011) RNA aptamer evolution: two decades of SELEction. Int J Mol Sci 12: 9155–9171.
23. Nitsche A, Kurth A, Dunkhorst A, Panke O, Sielaff H, et al. (2007) One-step selection of Vaccinia virus-binding DNA aptamers by MonoLEX. BMC Biotechnol 7: 48.
24. Peng L, Stephens B, Bonin K, Cubicciotti R, Guthold M (2007) A combined atomic force/fluorescence microscopy technique to select aptamers in a single cycle from a small pool of random oligonucleotides. Microsc Res Techniq 70: 372–381.
25. Fan M, Mcburnett SR, Andrews CJ, Allman AM, Bruno JG, et al. (2008) Aptamer selection express: A novel method for rapid single-step selection and sensing of aptamers. J Biomol Tech 19: 311–321.
26. Berezovski M, Musheev M, Drabovich A, Krylov SN (2006) Non-SELEX Selection of Aptamers, J Am Chem Soc 128: 1410–1411.
27. Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31: 3406–3415.
28. Mendonsa SD, Bowser MT (2004) In Vitro Evolution of Functional DNA Using Capillary Electrophoresis. J Am Chem Soc 126: 20–21.

Author Contributions

Conceived and designed the experiments: RNV. Performed the experiments: LHL HAS SLE ET. Analyzed the data: RNV. Contributed reagents/materials/analysis tools: RNV. Wrote the paper: RNV LHL.

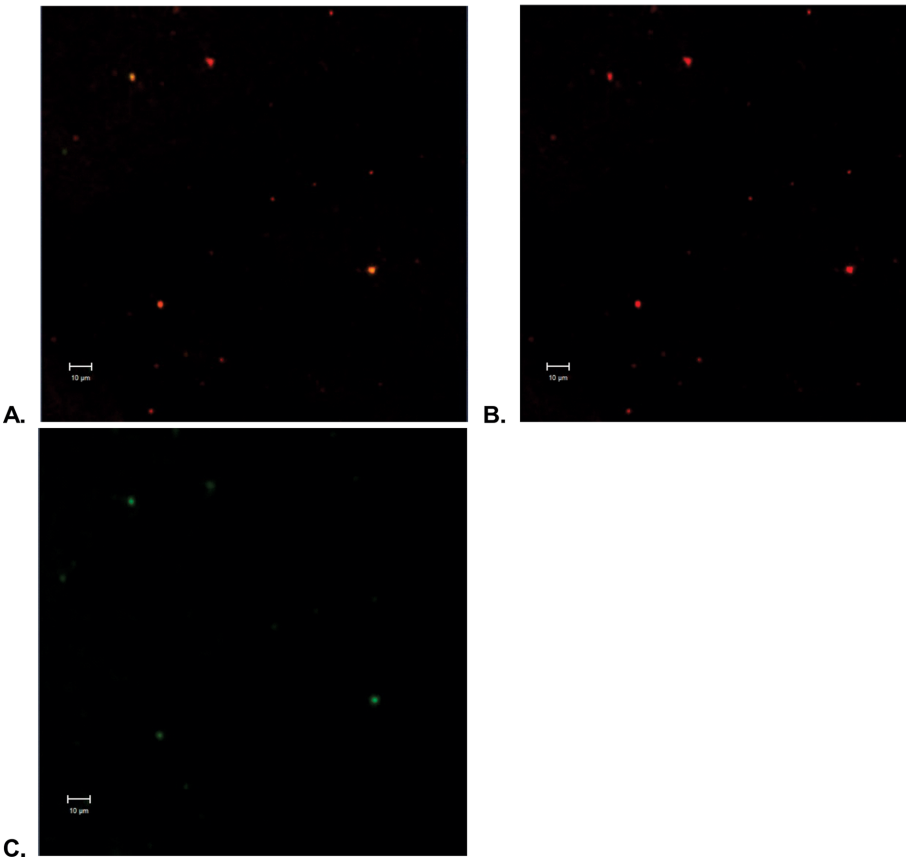

Figure S1: Fluorescence-based characterization of the α-bungarotoxin aptamer selection by one-step protocol.

A. An overlay image (orangish-yellow spot indicating aptamer binding to α-bungarotoxin) of the immobilized α-bungarotoxin labeled with Alexa Fluor 555 (red colour) and the toxin bound FAM-labeled DNA aptamer (green fluorescence); B. Characterization of the α-bungarotoxin labeled with Alexa Fluor 555 (red colour) immobilized on a glass coverslip; C. α-bungarotoxin bound FAM-labeled DNA aptamer (green fluorescence). doi:10.1371/journal.pone.0041702.s001

Article 2

A Capture-SELEX strategy for multiplexed selection of structure-switching RNA aptamers against small molecules

A Capture-SELEX strategy for multiplexed selection of structure-switching RNA aptamers against small molecules

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ABSTRACT

The Systematic Evolution of Ligands by Exponential Enrichment (SELEX) technology has been used for over two decades to generate aptamers that recognize a cornucopia of targets. However, selection of aptamers that recognize small organic molecules have not seen the same attention as larger targets like proteins. This is in part due to the difficulty of immobilizing small molecules on solid support for efficient separation during SELEX. Here, we present a novel variant of Capture-SELEX, which uses an RNA library to yield structure-switching RNA aptamers against small organic molecules in solution. We have successfully implemented this strategy for simultaneously selecting aptamers with specificity towards the food-coloring agent carminic acid as well as a steviol glycoside rebaudioside A. Next gen sequencing was used to investigate progression of the SELEX process, and also enabled determination of target-sequence relationship. A high-throughput aptamer screening platform based on bio-layer interferometry was developed, thereby enabling screening of large clone libraries of aptamer candidates. The same system was used to interrogate aptamer affinity, resulting in an apparent K_d of 47.6 µM for carminic acid and 130 µM for rebaudioside A. The RNA Capture-SELEX strategy enables sampling of multiple targets in a single selection, thereby effectively increasing throughput. In addition, the absence of immobilization enables easy parallelization and adaptation to other targets in solution with even higher affinities.

Keywords: SELEX; RNA aptamer; small molecule; next-generation sequencing; Biolayer interferometry.

INTRODUCTION

Aptamers are single-stranded oligonucleotides that adopt defined tertiary structures, allowing them to recognize and bind specific targets with high affinity and selectivity. These properties make aptamers particularly useful as biosensor elements and components of riboswitches that regulate gene expression.

Most aptamers are discovered through an *in vitro* selection process called SELEX (Tuerk and Gold 1990; Ellington and Szostak 1990). A combinatorial library, typically containing 10^9-10^{15} unique sequences, is incubated with the target of interest, and unbound sequences are discarded to enrich the pool for putative aptamers. The enriched pool is then amplified, and may be subjected to repeated rounds of selection until the pool reaches the desired affinity. This process may eventually yield individual aptamers with affinities in the nano- or picomolar range.

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The fact that the selection is carried out *in vitro* confers a significant advantage over custom antibody production by allowing the use of compounds that are otherwise difficult to target for reasons such as toxicity or rapid biodegradation.

Additionally, RNA aptamers may be further engineered into synthetic riboswitches that can have regulatory functions in the cell (Lynch et al. 2007; Lynch and Gallivan 2009; Link and Breaker 2009).The advent of synthetic biology has led to an increased interest in creating strains for the production of value-added compounds, and the term 'cell factories' is used to describe strains that have been engineered to produce specific compounds in yields that make them viable on an industrial scale. Metabolic engineering of such strains requires careful optimization of each step in the biosynthetic pathway as well as its interaction with the native metabolism of the host organism. This generally necessitates the use of directed evolution, targeted mutagenesis, and the construction of recombinant libraries, followed by high-throughput screening for the desired phenotype. The

biosynthesis of some compounds is easily detected from their color or auto-fluorescence, but the majority is not readily detectable and requires time-consuming extraction and analytical methods, which significantly lowers throughput. High-throughput screens of such compounds are therefore often based on enzyme-coupled assays, growth complementation, or biosensor-driven reporter production (Dietrich et al. 2010). Although some biological compounds have naturally occurring enzymes or regulators, such as transcription factors that may be engineered into biosensors, many compounds have no known regulator, prompting researchers to look for custom-tailored solutions. Raising aptamers specific to the target compound(s) and subsequently engineering them into riboswitches enables, in principle, the regulation of gene expression in response to the target compounds. The ability to regulate gene expression in response to higher titers of industrially interesting compounds has been used recently to select for tryptophan production phenotypes in selection assays (Yang et al. 2013).

Creating novel riboswitches is, however, a challenging task and the most straightforward approach is to build upon the structure-switching properties of an existing RNA aptamer. Aptamers typically undergo a conformational change upon binding to their targets (Nutiu and Li 2005; Vallée-Bélisle and Plaxco 2010). When an aptamer is part of a larger transcript this rearrangement may either break or establish new interactions with its flanking sequences and thus mask or unmask nearby elements, such as ribosomal binding sites, (anti-)terminators, or splicing sites. The overall effect is an impact on gene expression that is either increased or decreased in response to the concentration of the target molecule. Rational design of these interactions is currently not feasible and the chosen aptamer is therefore often recombined with one or more random sequence elements and subsequently screened for the desired activity *in vivo* (Lynch et al. 2007; Lynch and Gallivan 2009; Suess 2003; Muranaka et al. 2009; Topp and Gallivan 2008). By using an aptamer with a pre-established conformational rearrangement upon target binding, the prospects of realizing a synthetic riboswitch with the desired properties are improved.

As the likelihood of obtaining a synthetic aptamer correlates positively with the size of the chosen target (Carothers et al. 2010), the selection of aptamers against small molecules is particularly challenging. While aptamers interact with their targets mainly via specific hydrogen bonds, electrostatic complementarity, and π- π stacking (Hermann and Patel 2000), smaller targets possess fewer chemical groups that can stabilize binding. Molecular shape complementarity is another major specificity factor that may also be difficult to realize with targets that exhibit high conformational disorder due to multiple rotatable bonds (Carothers et al. 2010). When the aptamer selection protocol calls for target immobilization, the desired ligand must be carefully evaluated for the presence of suitable chemical groups (e.g. carboxylic acids, primary amines, or various carbohydrate moieties) and its overall stability during the matrix coupling reaction. Small molecular targets are particularly susceptible to chemical alterations, which often grossly affect their size, shape, and charge distribution, and may sometimes result in their suboptimal (i.e. for aptamer selection purposes) orientation against the matrix. Altogether, these issues make it difficult to obtain an aptamer with high selectivity and/or affinity towards small molecules. For this reason, selection against free targets in solution is preferred whenever possible.

Capture-SELEX is a variant of the *in vitro* selection process that is well suited for isolating aptamers against small soluble target molecules (Stoltenburg et al. 2012). In contrast to classical SELEX, where aptamer candidates are selected by passing the oligonucleotide library over the immobilized target molecule, the oligonucleotide library in Capture-SELEX is immobilized by annealing to a bead-bound capture probe and aptamer candidates are eluted with a solution of the solvated target. Any library member that undergoes gross conformational changes upon binding the target will potentially dissociate from the probe. The liberated aptamer candidates are then collected, amplified, and used in the next selection round. This process repeats until the pool has been sufficiently enriched for putative aptamers. As the target remains solubilized throughout the selection process, the elution of aptamer candidates lends itself well to multiplexing using several targets. The method was first reported to yield DNA aptamers against kanamycin A (MW: 681 Da) from a mixture containing kanamycin A, sulfacarbamide (MW: 215 Da), sulfamethoxazole (MW: 253 Da), and sotalol (MW: 272 Da) (Stoltenburg et al. 2012). However, by adopting the protocol to use RNA, Capture-SELEX could become a powerful tool for discovering structure-switching RNA aptamers and thus shorten the path towards creating synthetic riboswitches.

Here, we present a novel variant of Capture-SELEX, which uses an RNA library to yield structure-switching RNA aptamers against small organic molecules. Following 8 rounds of RNA Capture-SELEX, we have successfully selected aptamers against carminic acid, a food-coloring agent, as well as rebaudioside A, a steviol glycoside. Next-generation sequencing of the libraries from the various rounds indicates that the selected pools contain a wide array of structurally diverse sequences. Furthermore, we have developed a high-throughput screening platform based on bio-layer interferometry, which allowed us to rapidly obtain quasi-kinetic parameters for 288 clones of putative aptamer clones. This investigation expands the toolbox for developing RNA aptamers suited for engineering into riboswitches with sensitivity towards small metabolites and foreign compounds.

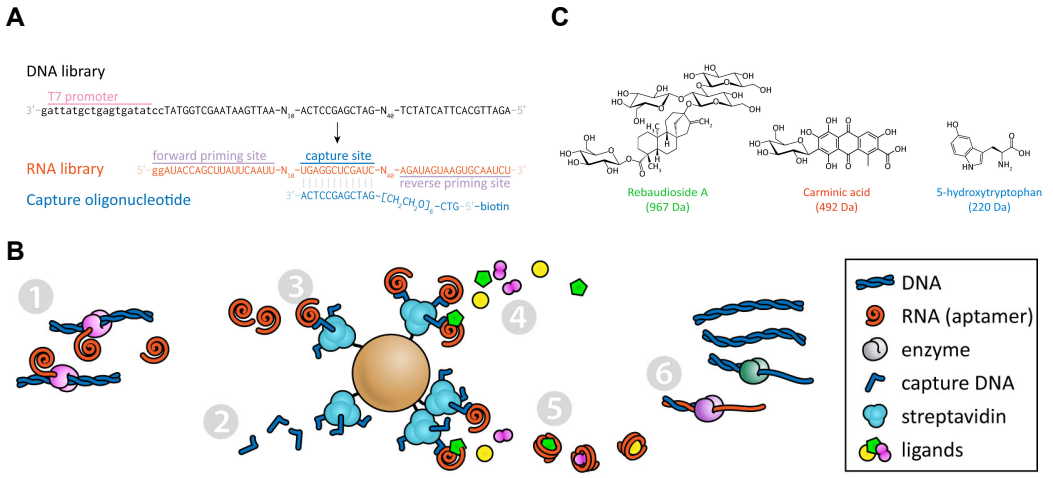

FIGURE 1: **Rationale of RNA Capture-SELEX.** (A) Design of the libraries used. The DNA library is transcribed to yield the corresponding RNA library. The reverse complement of the DNA library used by Stoltenburg et al. (2012) for Capture-SELEX was extended at the 3′-end with two C nucleotides (lowercase letters) and a T7 promoter sequence to enable in vitro transcription. The biotinylated capture oligomer binds the RNA library via its capture site flanked by 10 upstream and 40 downstream randomized positions (N10, N40). The forward and reverse priming sites are used for reverse transcription and PCR. (B) Overview of the RNA Capture-SELEX approach. 1: A biotinylated capture oligonucleotide is immobilized onto paramagnetic streptavidin beads. 2: The pre-folded RNA library is docked onto the immobilized capture oligonucleotide. 3: Following extensive washing of the beads, one or more ligands are added. 4: Putative structure-switching aptamers, i.e. RNAs that bind the ligand and are released from the beads in the process, are collected from the supernatant. (C) Structures of the target ligands being used. Rebaudioside A, carminic acid, and 5-hydroxytryptophan are small (<1000 Da) organic molecules with several groups available for hydrogen bonding and π-π stacking interactions.

RESULTS

In vitro selection by RNA Capture-SELEX

The current bottleneck in the development of synthetic riboswitches is the selection of RNA aptamers, with the most pertinent issues being the challenges of proper target immobilization, low success rates (Famulok and Mayer 2014), and overall time consumption. Capture-SELEX offers some attractive advantages over other *in vitro* selection methods, namely the ability to select aptamers with a defined, central region that undergoes significant rearrangement upon binding their ligand; the opportunity to use unmodified small molecular targets during the selection; and the ease of multiplexing selections with several targets.

The scope of Capture-SELEX was expanded to include *in vitro* selection of RNA aptamers by extending the reverse complement of the DNA library used by Stoltenburg et al. (2014) to include an upstream T7 promoter sequence (Fig. 1A). *In vitro* transcription of this DNA library yielded an RNA library of the same sequence composition as that used in the seminal work, i.e. a central 12-nucleotide capture site flanked by 10 upstream and 40 downstream fully randomized positions and appropriate forward and reverse priming sites for reverse transcription and PCR. The estimated complexity of this library was 1.3×10^{30} unique sequences. One advantage of basing the RNA library on the previously published sequence was that the capture oligonucleotide, primers, and PCR conditions could be used with only minor modifications. The latter was especially important, as the erroneous amplification of artifacts is a common problem in SELEX (Musheev and Krylov 2006), and Stoltenburg et al. had already demonstrated that this design could sustain library amplification over 13 rounds of Capture-SELEX.

An overview of the RNA Capture-SELEX approach is shown in Fig. 1B. The DNA library was transcribed to yield the corresponding RNA library (Fig. 1B, step 1). Following purification, the RNA was heat-denatured and allowed to fold in selection buffer. The RNA was then incubated overnight with streptavidin beads previously coated with the biotinylated capture oligonucleotide. During this incubation the capture sequence of the RNA would hybridize to the immobilized capture oligonucleotide (Fig. 1B, steps 2-3). After extensive washing of the beads to remove unbound RNA, including a brief incubation at slightly elevated temperature (15 min at 28 °C) to reduce nonspecific binding, the background level of RNA release in the absence of ligands was determined (see Materials and Methods for details). The beads were then incubated in a mixture of the chosen ligands (Fig. 1B, step 4), causing RNA species with affinity towards

A

Selection round	Amounts: Beads	Amounts: Library (nmol)	Target: 5HTP (0.5 mM)	Target: CarmA (0.33 mM)	Target: RebA (0.5 mM)
1	1×10^9	10.0	+	+	+
2	1×10^8	1.6	+	+	+
3	1×10^8	2.7	+	+	+
4	1×10^8	1.5	+	+	+
5	1×10^8	2.4	+	+	+
6	1×10^8	0.6	+	+	+
7	1×10^8	0.5	+	+	+
		Split			
5HTP – 8	1×10^8	0.5	+		
CarmA – 8	1×10^8	0.5		+	
RebA – 8	1×10^8	0.5			+

B

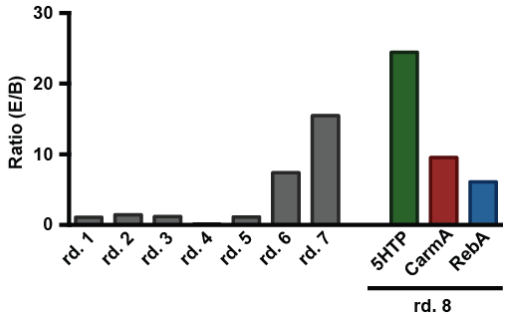

FIGURE 2: Selection overview. (A) The table shows input amount of RNA and the amount of beads used for preparing for Capture SELEX. Also the amount of ligand in each round is listed and the separation of ligands in three different selections after round 7. (B) Progress of selection was measured in terms of ligand induced RNA elution of beads [E] versus background elution whitout the presence of ligand [B]. After 7 rounds the pool was split into three separate selections each containing only one of the three ligands: Carminic Acid (CarmA), Rebaudioside A (RebA) and 5-hydroxy-tryptophan (5HTP.)

any of the ligands to bind them. RNA molecules undergoing a significant conformation change involving the capture site would eventually dissociate from the beads, and these putative structure-switching aptamers were collected from the eluate (Fig. 1B, step 5). Following reverse transcription, the extent of dissociation was determined by quantitative PCR, and the corresponding DNA library amplified to yield a template for the next round of RNA Capture-SELEX (Fig. 1B, step 6).

One of the advantages of maintaining the ligand(s) in solution, rather than the classical approach of immobilization on a solid support, is that it is relatively straightforward to incubate the RNA with a mixture of several ligands. This improves the overall chances of obtaining an aptamer and obviates concerns regarding the compatibility of coupling chemistries, the impact of necessary modifications to the ligand, and cross reactivity. Three ligands were chosen for evaluating RNA Capture-SELEX: Rebaudioside A (RebA), a steviol glycoside; carminic acid (CarmA), a food-coloring agent; and L-5-hydroxytryptophan (5HTP), a hormonal precursor and dietary supplement (Fig. 1C). These compounds were selected based on interest in their bio-based production and thus the need for sensitive screens during development of cell factories. Their molecular weights span from 967 Da (RebA) to 220 Da (5HTP), with larger size indicative of more rotatable bonds, suggesting that RebA in particular might show significant conformational disorder in solution. However, some properties are shared, such as the presence of one or more glycosylations (CarmA, RebA) and aromatic ring systems (5HTP, CarmA). These small ligands would be challenging to immobilize, both regarding potential loss of important chemical groups or stereogenic centers, and also maintaining their orientation. All ligands have above millimolar solubilities, making them well suited as eluents in Capture-SELEX. RebA and 5HTP were both used at 0.5 mM each, while CarmA was used at 0.33 mM, as higher concentrations were found to impact pH beyond the capacity of the selection buffer. Importantly, the compounds were neither found to interact with each other or with the selection matrix, and inhibitory effects on PCR or fluorescence readout were not observed at the concentrations used (data not shown).

Selection of aptamers through RNA Capture-SELEX.

In total 8 rounds of RNA Capture-SELEX were performed as described above. As in the seminal work a continuous a selection pressure was maintained by using the same concentration of ligand throughout the 8 selection rounds. In the first round 10 nmol RNA was added to 1×10^9 streptavidin beads coated with CASE004 capture oligonucleotide. The amount of beads used in the first round have a theoretical RNA capture capacity of 3 nmol. In all subsequent rounds 1×10^8 beads were used maintaining the theoretical RNA available for selection in round 2-8 at 300 pmol. As previously mentioned the amount of ligand used were 330 µM for carminic acid and 500 µM for 5HTP and Rebaudioside A in 300 µL; representing a theoretical 1,330 time surplus of ligand to RNA in selection rounds were ligands were added as a mixture. This surplus should effectively prevent eluted aptamers from reattaching to the capture oligonucleotide and influencing the overall selection outcome. In round 3 and 4 shorter aborted transcripts started to appear in the RNA pool. These parasitic sequences featured a defined length of 97 nt, which is 20 nt shorter than the expected PCR amplicon size. Gel purification and removal of parasitic sequences between round 4 and 5 alleviated the unwanted accumulation of spurious RNA product.

Progress after each round RNA Capture-SELEX was assessed by comparing the amount of eluted RNA after ligand addition to the background elution without ligand. An aliquot of RNA pools from background and ligand elution steps reverse transcription quantitative PCR was used to determine the concentration of RNA in each step. Figure 2B shows the ratio between ligand induced and background elution. The first few rounds the elution ratio was ~1; with the amount of eluted RNA in background and ligand steps below 5 pmol. Background elution increased in the first 5 round of selection (Fig. S1, Supplementary), therefore the amount of loaded RNA was decreased. At six iterations of aptamer evolution the amount of RNA eluted from the beads when ligand was added started to increase significantly in the selection. After two rounds (6 and 7) with significant increase in eluted RNA (16.6 and 19.9 pmol) and stable background elution, the aptamer pool was divided into three separate single-ligand selections for round 8, while maintaining the bead:RNA ratio (Fig. 2A). The splitting provided a measure of ligand specific elution in the combined pool. The highest response to additions of ligand was observed for the pool where rebaudioside A was added. 105 pmol RNA was eluted from the beads in round 8 upon adding rebaudioside A. This constitutes 57.12% of the added RNA in the selection (based on the quantification of RNA in the background elution, three heat elution steps and RNA in the penultimate washing step before background elution). This 24.5-fold difference in ligand eluted RNA to background hinted that the library of RNA contained clones of highly responsive Rebaudioside A aptamers. In order to further understand the evolution of aptamers in solution pools of dsDNA from rounds 3 to 8 and the naïve library were sequenced using NGS.

Identification of selected aptamers through next-generation sequencing

RNA derived from the individual selection rounds 3 to 8, as well as the unselected DNA library were converted into barcoded cDNA libraries, pooled and subjected to next-generation sequencing. The resulting sequences were trimmed to remove the flanking primer sites and subsequently quality filtered to remove sequences with more than 10 % of base-calls below a Phred quality factor of 20. Quality filtering removed up to 20% of total reads from each pool. Sequencing of the RNA pools yielded a total of 17 million high quality reads, where the amount of reads per pool varied from 1.4 million reads for the unselected library, 1.3-1.4 million for selection rounds 3-5 to 1.8-2.2 million for selection rounds 6-8. The extent of sequence diversity was investigated using a custom Perl script to determine the number of times each sequence occurred prior to selection and after three to eight rounds. In concurrence with the increase in ligand specific elution of RNA as seen in the qPCR data (Fig. 2B), the diversity of the RNA pool decreases dramatically from selection round 4 to 8. Sequencing data from selection round 4 yielded 1.41 million unique sequences from a total of 1.44 million (97.8% sequence diversity). In comparison, sequencing data from selection round 8 (eluted with RebA) yielded only 98,255 unique sequences out of 1.91 million total sequences (5.1% sequence diversity). In Figure 3A, the total number of sequences is compared to the number of unique sequences obtained from each selection round. In selection round 4, the 1000 most common sequences, '1 k', make up 0.51% of the total sequences, and in the subsequent rounds 5, 6, and 7 this increases to 11.7%, 55.3% and 76.6%, respectively. The first signs of decreasing diversity begin to emerge between round 4 and 5, and the overall pool composition changes noticeably in round 5.

In the following round, the change in conditional elution upon ligand addition was also evident in the eluate:background ratio as seen in Figure 2A. Following selection round 7, the pool was divided into three equal parts. For selection round 8 each individual portion was then eluted with only one of the three ligands. This resulted in the specific enrichment of certain sequences in each of these 3 pools compared to round 7. The round-to-round enrichment of the 11 specific sequences is shown in Figure 3C. Sequences RebA_01, RebA_02, and RebA_03 were greatly enriched when eluted with rebaudioside A but much less so with the other ligands. The sequences RebA_01, RebA_02, and RebA_03 exhibited respectively 28.0-fold, 14.3-fold and 16.6-fold enrichment in frequency from round 7 to 8. Similarly, the pools eluted with 5-hydroxytryptophan or carminic acid also resulted in the ligand-specific enrichment of certain sequences. In contrast to the RebA-enriched sequences, however, these CarmA- and 5HTP-enriched sequences were also enriched in the preceding pools. The sequences CarmA_01, CarmA_02, 5HTP_01, and 5HTP_02 comprised a total of 9.7% of sequences from selection round 6 and 14.1% of sequences from selection round 7. This indicates that ligand-specific sequences had been enriched during the selection and also allowed for a preliminary assignment to ligand-specific sequence families, a key advantage of splitting multiplexed pools in late selection stages. Additionally, the most prominent sequences in the early selection rounds did not dominate the final pools (data not shown).

Figure 3D shows the three most abundant sequences from the next-generation sequencing analysis of each pool from selection round 8. In general, there was found to be a significant enrichment of adenosine homopolymers in the selected sequences. Through further sequence analysis, it was not possible to identify pool-specific conserved sequence clusters or structural motifs in each of the pools of round selection 8. An analysis of positional base composition in the selected pools versus the

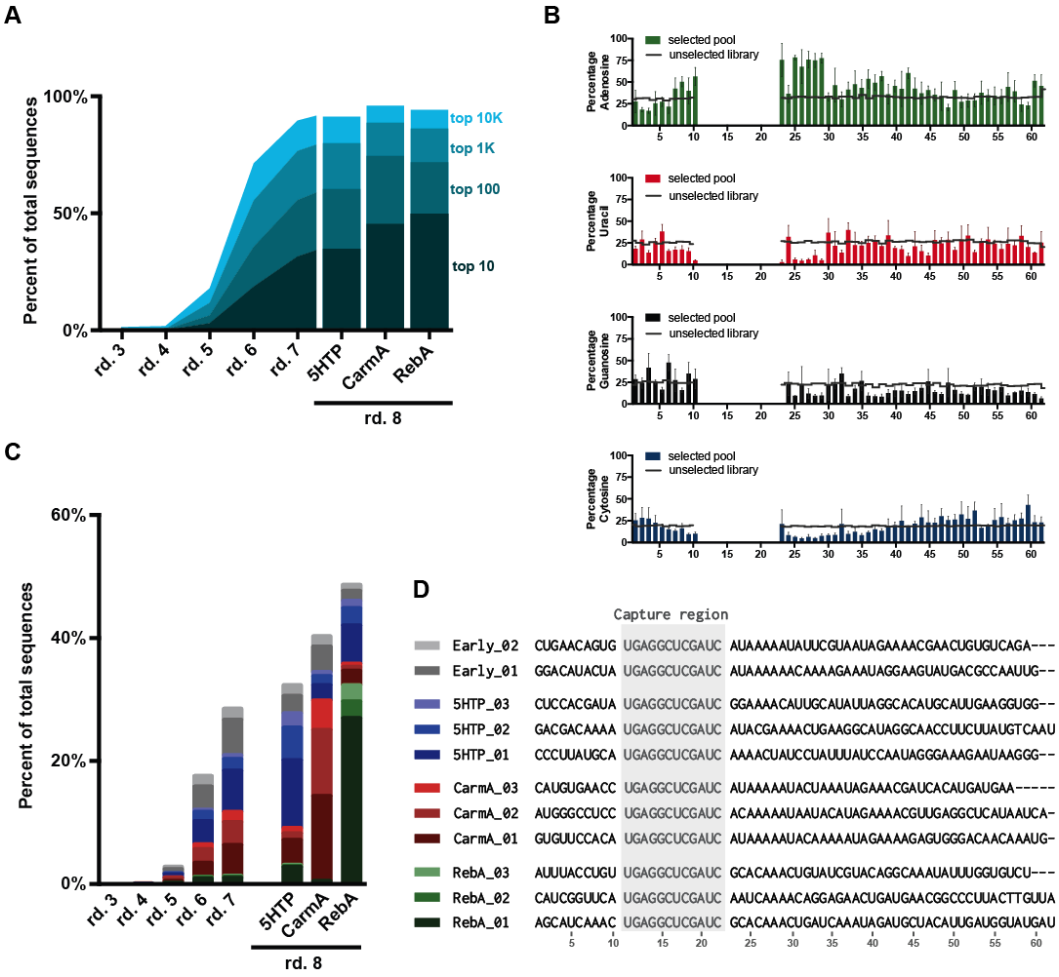

FIGURE 3: Next-generation data analysis of selected aptamer pools. (A) Sequence diversity of the RNA pool from selection rounds 3-8 shown as the fraction of the total sequencing material falling within the top 10/100/1,000/10,000 most frequent unique sequences in each round. (B) Nucleotide distribution at each position of the randomized core from the unselected DNA library (line) compared to RNA pools subjected to 8 rounds of RNA Capture-SELEX (bars with standard deviation). (C) Specific sequences that eventually dominated the selections. Sequences are color coded with respect to the pool where they are most frequently represented. (D) Core region of select sequences from panel C. The region used for binding the capture oligonucleotide is highlighted in gray.

naïve library was therefore performed as shown in Figure 3B. Many of the sequences were found to have deletions of one or more bases in the random region. This creates a challenge when analyzing the base composition of each position in the selected RNA because the position of conserved capture probe might shift in response to these deletions in the final curated data set. Therefore, we used a custom perl script to isolate all sequences with a conserved capture region as well as 10 base positions at the 5' end and 40 positions at the 3' end. The percentage of each base at all positions of the 62 nt cutout sequence is plotted in Figure 3B; the Figure shows the overall distribution of bases in 6.06 million combined reads from selection round 8 pools, which have been selected with different ligands. The base distribution of each of the selection round 8 pools was calculated and used to establish the mean and standard deviation of base distribution. A significant enrichment of adenosines especially at the positions flanking the capture region of the RNA was observed. This suggests an experimental bias in the selection method that favors single stranded regions with limited base pairing potential. Likely resulting in struc-

tural flexibility around the capture region of the RNA library.

In previous capture SELEX experiments performed with DNA, mutations in the capture site have been observed and speculated to be a factor that might influence the overall outcome of aptamer selection (Stoltenburg et al. 2012). In the present RNA-based approach, the beads are loaded with a significant excess of RNA library relative to available bead capture sites. This resulted in the retention of the 12 nt capture site in all of the most abundant RNA sequences in every pool that has been subjected to next generation sequencing. As a consequence, only 8 sequences have mutations or deletions in the capture site throughout the entire data set for RNA eluted with rebaudioside A, which contains 1.91 million sequences.

Screening for ligand responsive aptamers using bio-layer interferometry

The three RNA pools resulting from elution with any of the three individual ligands in selection round 8 were each dominated by different RNA sequences, indicating ligand-specific enrichment of each pool. In order to study the ligand-specific elution of these putative aptamers, we developed an assay based on bio-layer interferometry (BLI). BLI is technique that provides real-time, label-free association and dissociation kinetics of a ligand onto a sensor coated with an appropriate bio-layer. We used BLI to detect both the rate of binding of RNA pools from selection rounds 4-8 by the immobilized capture oligonucleotide and the subsequent rate of RNA release by the addition of ligand(s) (Fig. 4A). The BLI sensorgrams showed similar loading of each RNA pool, as the selection progressed through rounds 4-7, and elution of the RNA by addition of 1 mM ligands remained modest (Fig. 4B). The latter was surprising, as quantitative PCR data had clearly showed a major increase in the amount of eluted RNA in rounds 5-7. The RNA pool that was eluted with RebA in selection round 8 exhibited the highest loading level of all the tested pools and also showed markedly improved elution, releasing as much as 63% of the RNA in 200 seconds. The RNA pool eluted with carminic acid showed the second highest response to addition of its ligand (47%). The RNA pool eluted with 5HTP did not show any improvement in loading and elution characteristics over the earlier selection round 5.

High-throughput screening of selected libraries

Based on the promising results from the next-generation sequencing analysis and the BLI dissociation assay, three sequences from each final pool were chemically synthesized and evaluated individually using the BLI assay. While the three sequences originating from the RebA pool showed ligand-induced release from the capture probe, the six sequences from the CarmA and 5HTP RNA pools did not (data not shown). Rather than the more costly approach of chemically synthesizing a larger subset of putative aptamers from each of the sequence

pools from round 8, the PCR products from the three pools were cloned into plasmid vectors and transformed into *E. coli*. Crude *in vitro* transcripts generated from

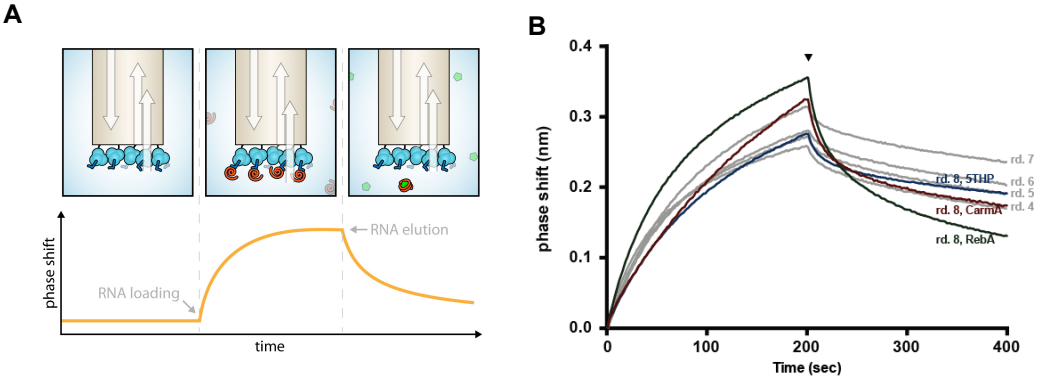

FIGURE 4: Response of RNA pools to ligands. (A, left panel) Bio-layer interferometry (BLI) is a label-free technique for studying molecular interations in real-time. An optical fiber coated with a bio-layer is immersed in the analyte solution and white light is directed down the fiber. Light reflected back from the tip of the fiber interferes with light reflected from the interface between the bio-layer (streptavidin and capture oligomer, blue) and the surrounding solution, resulting in a distinct interference pattern. *(A, center panel)* When RNA (red) binds to the bio-layer the optical thickness at the tip is increased, and the interferometric profile shifts in proportion to the extent of binding. *(A, right panel)* When ligand (green) is added it binds to and releases the RNA, thereby reducing the optical thickness. *(A, bottom)* The measurements are presented as a sensorgram indicating the interferometric phase shift as a function of time. *(B)* Bio-layer interferometry sensorgrams of eluted RNA from RNA Capture-SELEX rounds 4-8. The arrow marks the addition of ligands. The data shown are means of triplicate measurements.

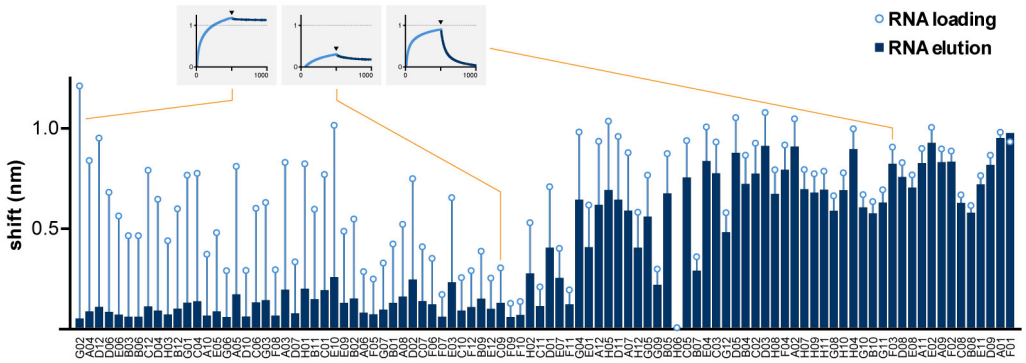

Figure 5: Screening reveals a population of RebA-responsive RNA clones. BLI endpoint measurements of loading and ligand-specific elution of RNA from 96 clones isolated from the RebA library. The BLI sensors were loaded with crude *in vitro* transcripts for 500 seconds, followed by elution with 1 mM RebA. The data are shown in decreasing order of loading:elution ratio. Inserts: Illustration of sensorgrams of select RNAs with (left) desirable loading and elution kinetics, (center) poor loading, or (right) poor elution. Arrows indicate the addition of ligand.

plasmid isolates from randomly picked colonies were then screened using the BLI assay. Fig. 5 shows endpoint measurements of loading and subsequent RebA-induced release of RNA from 96 clones from the RebA library. More than 40% of the sequences exhibited high binding (>0.5 nm interferometric shift), as well as high ligand-induced elution (>50% of binding). Surprisingly, some sequences showed poor binding to the capture oligonucleotide. This was remarkable, considering that the sequences had been through 8 rounds of selection, which should have removed any such sequences. Screening of RNA from 96 clones from the CarmA library yielded 8 putative aptamers, whereas screening of RNA from 96 clones from the 5HTP library failed to identify RNAs that could be eluted with 5HTP.

Putative aptamers show ligand-specific and concentration dependent kinetics

8 promising candidates from each of the RebA and CarmA libraries were used to further establish their specificity and kinetic responses. Purified *in vitro* transcripts from the corresponding plasmid isolates were assayed by BLI using varying concentrations (0.0625-1.0 mM) of ligands. Figure 6A and B show the kinetic profiles of aptamers 'RebA E04' and 'CarmA D02' in the presence of varying concentrations of their respective ligands; kinetic profiles of the remaining aptamers can be found in Supplementary Information. 1 mM CarmA or 5HTP had little effect on the binding of RebA aptamers (Fig. 6A), whereas 1 mM RebA yielded a rapid, but limited release of CarmA aptamers (Fig. 6B). All 8 RebA aptamer candidates showed marked concentration-dependent release of RNA from the capture oligonucleotide upon addition of RebA, while only 3 of the 8 CarmA aptamer candidates showed concentration-dependent release upon addition of CarmA. These three aptamers all corresponded to the 21st most prominent sequence in the next-generation sequencing data set. This suggests that the most prominent sequences in the final pool surprisingly did not fulfill our criteria for functional aptamers.

The normalized BLI profile of each aptamer eluted from the capture oligonucleotide was fitted to a model for single-phasic exponential dissociation to allow the extrapolation of the amount of aptamer released at equilibrium. Following the methodology by Stoltenburg et al. (2012), the resulting data were then fitted to a single-site saturation binding curve to yield the dissociation constant, K_d. Fig. 6C and D show fitted saturation binding plots for RebA and CarmA aptamers. Most of the RebA aptamers were found to have dissociation constants in the mid-micromolar range (130-305 µM), although the K_d of one aptamer was in the low millimolar range (1188 µM). Because the three CarmA aptamers were identical they were treated as triplicates, resulting in a derived dissociation constant of 47.6 µM.

DISCUSSION

In this work, the Capture SELEX strategy (Stoltenburg et al. 2012), a combination of FluMag-SELEX (Stoltenburg et al. 2005) and the *in vitro* selection of structure-switching aptamers (Nutiu and Li 2005), has been expanded to enable the parallel selection of multiple selective RNA aptamers in a mixture of ligands. Using a synthetic oligonucleotide library with a capture region located within the randomized region, and a mixture of ligands, novel RNA aptamers towards rebaudioside A and carminic acid, ligands with hitherto no reported aptamers have been identified. The aptamers were tested for ligand-dependent responsiveness using a new affinity assay

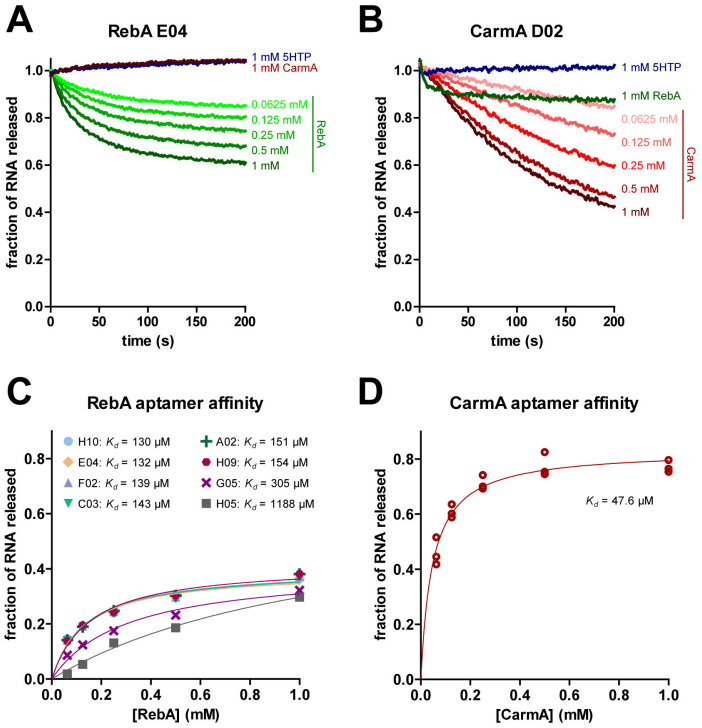

FIGURE 6: Determining the ligand affinity of select RebA and CarmA aptamers. (A, B) Sensorgrams showing the release of aptamers 'RebA E04' and 'CarmA D02' upon addition of varying concentrations of ligands. The data were normalized against aptamer dissociation in buffer only and fitted to an exponential decay model, which allowed the release of aptamers at equilibrium to be extrapolated. (C, D) The amounts of released aptamers at equilibrium in the presence of varying concentrations of ligands were fitted to a single-site saturation binding model to yield the respective dissociation constants, K_d. The three identical CarmA aptamers were treated as triplicates.

based on bio-layer interferometry that allows for the efficient screening of hundreds of RNA aptamers. This work augments the RNA aptamer selection toolbox and addresses key challenges of existing selection techniques. Furthermore it demonstrated the fruitful combination of Capture-SELEX using RNA pools and subsequent analysis of the enriched pools using next-generation sequencing, as well as the use of bio-layer interferometry to measure the ligand-specific dissociation of aptamers selected.

General use of RNA Capture-SELEX

The selections described here yielded aptamers against 2 out of 3 ligands, whereas Stoltenburg et al. only found aptamers against 1 of 4 ligands using the DNA based capture SELEX approach (Stoltenburg et al. 2012). This difference may be due to the nature of the selected ligands, but it could also be a result of the separation of the RNA pool into three pools, each of which was eluted with a single ligand in round 8 to enrich for aptamers specific to that specific ligand. The ligands that have yielded aptamers in Capture-SELEX experiments thus far are glycosylated compounds (RebA and CarmA; this study) and an aminoglycoside (kanamycin A; seminal study), whereas those that failed to yield aptamers are significantly smaller and less water-soluble aromatic compounds (5HTP, sulfacarbamide, sulfamethoxazole, and sotalol). Stoltenburg et al. (2012) speculated that hydrophilicity and the presence of several amino groups could account for the apparent preference of kanamycin, however, neither RebA nor CarmA are amines. The results presented here suggest that hydrophobicity and molecular size in particular are major determinants for the outcome of a Capture-SELEX experiment. Very small ligands (<300 Da) typically only have few groups that may engage in chemical interactions, conceivably requiring the aptamer to form a structurally more complex binding pocket, which may be very rare or even absent from the oligonucleotide library being used.

A relatively high apparent Kd of the identified aptamers was observed towards both RebA and CarmA. This could potentially be the result of maintaining a constant high ligand concentration throughout the selections. This decision was based on the goal of isolating aptamers with a higher concentration window for conformational change, which is desired for aptamers based sensors used in assays or selections with higher titers of ligands. Using a more traditional approach of decreasing the concentration of ligand may likely yield aptamers with lower estimated Kd´-values from the BLI measurements, but may also decrease the number of different aptamer candidates that successfully change conformation given the fact that any ligand must compete with the affinity of a 12 nt RNA:DNA hybridization. One interesting aspect of RNA Capture SELEX is the possibility to tune selection pressure not only in terms of ligand concentration but also in terms of capture-affinity. One approach to modifying the aptamer affinity could be the incorporation of modified nucleotides that enhance hybridization efficiency such as locked nucleic acids (Doessing et al. 2012) in the capture oligonucleotide. This could potentially be used to increase the thermodynamic threshold for RNA release from the beads and therefore constitute a novel technique to confer increased selection pressure in the system without having to decrease ligand concentration or change capture probe length.

Although negative selection was not employed in the selection strategy used here, aptamers responsive to specific ligands were nevertheless isolated from a selection using a mixed ligand environment. A counter-selection is easily incorporated into the RNA Capture SELEX strategy. It could be employed as an additional step after background elution and include multiple ligands as well, provided they are soluble in selection buffer.

Ligand solubility is a key factor in the success of the RNA Capture SELEX approach. Some ligands with lower solubility may be dissolved in organic solvents like DMSO and diluted to working concentration in selection buffer considering the effect of solvent on hybridization efficiency (Juang and Liu 1987). Although the amount of ligands needed is dependent on the number of selection rounds, RNA Capture SELEX requires larger amount of ligands in comparison to immobilization-based aptamer selection techniques. Typically, a 10-round selection with 1 mM ligand solution would require 5 mg of a 1 kDa ligand. However, the use of smaller ligands, less concentrated solutions and smaller elution-volumes in the selection would decrease the amount of ligand required to the microgram range and allow for the use of more scarce and expensive targets.

The approach described here required 6 selection rounds to elicit an increase in the amount of RNA elution upon addition of ligands and was deemed completed after 8 selection rounds. This is typical for classical SELEX experiments. However, while the success rate of classical SELEX is generally low (<30%) (Famulok and Mayer 2014), Capture-SELEX enables several target compounds to be screened simultaneously (multiplexing), which improves the overall throughput. Another significant advantage is that the chosen target species does not need to be immobilized. This alleviates concerns regarding various coupling chemistries and their impact on the structure and final orientation of the target molecule, and it greatly simplifies the selection against compounds with short half-lives by obviating prior preparation of immobilized target stocks.

The issues of overall time consumption and target multiplexing have also been addressed by others, employing techniques such as capillary electrophoresis (Berezovski et al. 2006; Mosing and Bowser 2009; Yunusov et al. 2009), microfluidics (Qian et al. 2009; Lou et al. 2009; Cho et al. 2010; Ahmad et al. 2011), micro-arrays (Cho et al. 2013), deep sequencing of initial selection pools (Hoon et al. 2011; Schütze et al. 2011; Kupakuwana et al. 2011; Cho et al. 2010; Bayrac et al. 2011) atomic force microscopy (Miyachi et al. 2010), or various combinations hereof. In contrast to these approaches, RNA Capture-SELEX does not rely on specialized equipment or advanced data analysis. The present study employed quantitative PCR, chip-based capillary electrophoresis of DNA and RNA, as well as bio-layer interferometry of RNA pools and select aptamer clones. However, these techniques may be substituted by more traditional techniques, such as PCR dilution series, gel electrophoresis, and competitive binding experiments.

BLI and Next-generation sequencing for aptamer analysis and identification

Next-generation sequencing following rounds 3 to 8 provided a unique insight into the development of the RNA pools subjected to RNA Capture-SELEX. In this work, significant enrichment of homopolymer adenosine sequences adjacent to the capture region of the sequence pool was observed. Moreover, there was no significant mutation of the capture region itself that could result in decreased affinity towards the capture oligonucleotide, in contrast to the mutational changes reported by Stoltenburg et al. (2012). In the present work, the amount of DNA loaded onto the beads was higher than the theoretical capture capacity, which might account for this difference. BLI measurements showed that the initial pool has significantly lower affinity towards the capture oligonucleotide than the enriched pools (data not shown). This suggests that the RNA is also selected with respect to its affinity towards the capture oligonucleotide, and that the observed increase in homopolymer adenosines may be a prerequisite for increased structural flexibility around the capture region.

The ligand binding kinetics of selected RNA pools and 288 individual clones were screened using BLI on an 8-channel Octet RED96 apparatus. The developed assay monitored the initial binding and subsequent ligand-driven dissociation of the RNA from the capture oligonucleotide, which was immobilized on streptavidin-coated biosensors. This setup closely emulated the conditions during Capture-SELEX and allowed us to rank the isolated sequences for their potential use as aptamers, as well as observe the overall progress of selection. In contrast to other techniques relying on detection of adsorption (e.g. surface plasmon resonance or quartz crystal microbalances), the BLI-based assay used here is very simple, low-cost, and robust.

From RNA Capture-SELEX towards the development of synthetic riboswitches – future perspectives

RNA Capture-SELEX enabled the selection of RNA pools enriched for sequences that interact with rebaudioside A or carminic acid in a manner that causes them to dissociate from the capture oligonucleotide. Next-generation sequencing showed considerable variation in the final pools, suggesting that the selection had retained many unique potential aptamers rather than converging on a small subset of structures. We believe these pools are good starting points for the development of the functional riboswitches. The construction of synthetic riboswitches for genetic screening can be approached in several ways. Splicing of mRNA may be targeted by introducing an aptamer that sequesters a splice site (Kim et al. 2005; Weigand and Suess 2007), or ribozyme-aptamer chimeras called 'aptazymes', whose activity is dependent on ligand binding, may be used to regulate the half-life of the mRNA (Wieland and Hartig 2008) or to block access to the ribosomal binding site (RBS) (Ogawa and Maeda 2008). Blocking of the RBS may also be achieved by simply cloning an aptamer upstream of the binding site (Suess et al. 2004; Desai and Gallivan 2004), but, the optimal sequence context is ideally determined by fusing with a spacer of random length and/or sequence (Lynch et al. 2007; Lynch and Gallivan 2009; Davidson et al. 2013). A similar approach can be used to insert a pool enriched for aptamers upstream of a reporter gene, as reported by Weigand et al. (2008). In that study it was found that riboswitches isolated in this manner were highly underrepresented in the original pool, underscoring that all aptamers do not necessarily make good riboswitches and emphasizing the utility of screening recombinant libraries for clones with the desired riboswitch activity.

While it is relatively straightforward to generate large recombinant libraries of putative riboswitches, recombination of a randomized spacer with a converged pool of RNA molecules may considerably increase the sequence space that is explored. In the case of the RNA pool eluted with RebA in round 8, recombination of the pool with a randomized spacer would predominantly result in recombinants of the most frequently occurring species (constituting 27% of the total pool; data not shown). However, the second-most frequent species constitutes 6.2% of the total pool (data not shown), and its recombination with a spacer would therefore result in 4.3-fold fewer recombinants. The correspondingly lower coverage of the sequence space of the randomized spacer could result in the potential loss of functional riboswitches, and this effect becomes increasingly predominant as the frequency of each unique sequence in the pool decreases. In general, combination of a converged pool with a randomized spacer is highly skewed towards the most abundant species within the pool. If the aim is to screen a high number of putative aptamers for their function as riboswitches, a less converged pool can be used with the caveat that each putative aptamer may only recombine with a small fraction of the available spacer sequences. These are aspects that will be investigated in future work.

MATERIALS AND METHODS

Library preparation

4 nmol of the single-stranded DNA template library (CASE001, desalted 5′-AGATTGCACTTACTA-TCT$(N)_{40}$GATCGAGCCTCA$(N)_{10}$AATTGAATAAGC-TGGTATCCTATAGTGAGTCGTATTAG-3′, IDT) was annealed with 8 nmol primer (CASE003, 5′-CTAATA-CGACTCACTATAGGATACCAGCTTATTCAATT-3′, IDT) for T7 transcription; subsequent selection rounds used 0.6-3.2 µg double-stranded PCR product as template. The template was transcribed for 15-30 min at 37 ° C using the TranscriptAid T7 High Yield Transcription kit (1 ml, Thermo Scientific). The reaction was filtered through a 0.22 µm Corning Costar Spin-X filter (Sigma-Aldrich), and the RNA was extracted twice with TRIzol and chloroform, precipitated with isopropanol, and redissolved in RNase-free water. The RNA was then treated with DNase I (1.25 units/µg DNA, Fermentas), extracted, precipitated, and redissolved again in RNase-free water. The integrity of the RNA was assessed on a BioAnalyzer capillary electrophoresis sRNA chip (Agilent Technologies), and yield was measured using a Qubit (Life Technologies); initial yield was 34 nmol and 1-3 nmol in subsequent rounds.

Capture-SELEX

10^9 streptavidin-coated paramagnetic M-270 Dynabeads (Life Technologies) were washed twice with 1 ml RNase-free 0.1 M NaOH/0.05 M NaCl and once with 0.1 M NaCl in accord with the manufacturer's instructions to

remove ribonucleases. The beads were then washed three times in 0.5 ml B&W buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 2 M NaCl), re-suspended in 1 ml B&W buffer with 6 nmol biotinylated docking oligonucleotide (CASE004, HPLC-purified 5′-biotin-GTC-[hexaethylene glycol]-GATCGAGCCTCA-3′, IDT), and incubated for 1 hr with gentle nutation. Unbound docking oligonucleotide was removed by washing the beads three times with 0.5 ml B&W buffer and three times with 0.5 ml Strehlitz buffer (20 mM Tris-HCl, pH 7.4; 2 mM $MgCl_2$; 5 mM KCl; 1 mM $CaCl_2$; 100 mM NaCl; and 0.005% (v/v) Tween-20). The beads were then re-suspended in 1 ml Strehlitz buffer to 10^9 beads/ml and stored at 4 °C until needed.

Most of the RNA (initial round: 10 nmol; subsequent rounds: 0.5-2.7 nmol) was denatured for 8 min at 90 °C, fast-cooled for 10 min on a 5 °C cold block, and relaxed for 1 min at room temperature. The RNA was added to 10^9 beads (10^8 beads in subsequent rounds) coated with docking oligonucleotide, along with 300 units of RiboLock RNase inhibitor (Thermo Scientific), and incubated overnight at 21 °C with gentle shaking. Unbound RNA was removed by washing the beads nine times with 0.5 ml Strehlitz buffer (hereafter with 0.1 unit/µl RiboLock). Unspecific binding was reduced by incubating in 0.5 ml Strehlitz buffer for 15 min at 28 °C with gentle shaking, followed by washing seven times with 0.5 ml Strehlitz buffer. Background elution of the RNA was assessed by incubating the beads in 300 µl Strehlitz buffer for 45 min at 21 °C with gentle shaking, and recovering the supernatant for analysis. Potential aptamer candidates were eluted by incubating the beads with 300 µl of Strehlitz buffer with a mixture of the targets (0.33 mM carminic acid, 0.5 mM rebaudioside A, 0.5 mM 5-hydroxytryptophan; Sigma-Aldrich), or the individual targets (round 8 only). Elution was for 45 min at 21 °C with gentle shaking, after which the supernatant was recovered and stored at −20 °C. Finally, we performed 3 successive heat elutions (5 min at 80 °C) in 300 µl Strehlitz buffer each to assess the quality and quantity of the RNA remaining on the beads.

Quantitation of eluates by RT-qPCR

Reverse transcription and quantitative PCR (RT-qPCR) on aliquots of the eluates was used to monitor the progress of the Capture-SELEX, as well as checking for contaminating DNA. RNA was annealed to reverse primer CASE002 (5′-AGATTGCACTTACTATCT-3′, IDT) and reverse transcribed using RevertAid Premium Reverse Transcriptase (Thermo Scientific) according the manufacturer's instructions, except that RiboLock was included at 1 unit/µl. qPCR was with primers CASE002 and CASE003 and using Phusion DNA polymerase in HF buffer supplemented with 1x EvaGreen (Biotium) and ROX reference dye (Thermo scientific). The qPCR was run on an Mx3005P qPCR system (Agilent Technologies; 1 min at 98 °C; 40 cycles of: 30 s at 98 °C, 30 s at 51 °C, 30 s at 72 °C; 5 min at 72 °C). ROX was used for signal normalization.

Transcription templates for the following round were prepared by precipitating all the eluted RNA with ethanol and glycogen (Fermentas), followed by reverse transcription as above. 20 µl pilot PCR reactions were run as above, except without dyes. The reactions were manually sampled every 2 cycles, and analyzed on a BioAnalyzer High Sensitivity DNA chip to determine the optimal cycle number (3-8 cycles) to avoid over-amplification. Large-scale PCR reactions (160 parallel reactions) of the remainder of the cDNA libraries were concentrated with Amicon Ultra 30K centrifugal filters (Merck Millipore), purified on NucleoSpin PCR Clean-up columns (Macherey-Nagel), and quantitated by Qubit. Aliquots of the DNA were stored for later analysis by next-generation sequencing and the remainder was used for transcribing an RNA library for the following selection round.

Blunt end cloning of aptamer pools

To isolated clones for screening aptamer libraries were blunt-end cloned into pJET 1.2 vector (Thermo Fisher Scientific) and transformed into XL1-Blue Subcloning-Grade Competent Cells (Agilent) using standard protocols. To obtain dsDNA for T7 transcription reaction 96 colonies per target pool were picked for colony PCR using pJET1.2 Forward Sequencing Primer or pJET1.2 Reverse Sequencing Primer and PCR product verified on a LabChipGX II (Perkin Elmer).

Sequencing analysis

Selected pools from round 3 to 8 were sequenced on a MiSeq using standard library prep procedures. The sequencing run was spiked with 5 % PhiX diversity library to avoid constant regions to interfere with the quality of the reads. A total of 17 M reads were obtained and quality filtered to remove sequences with more than 10 % of base-calls below a Phred quality factor of 20.

Biolayer Interferometry

Screening assay

5 µl transcription reactions were performed on 1µL of purified T7prep-PCR product using TranscriptAid kit (Thermo Fisher Scientific). After 4h of incubation at 37°C the T7 reaction was DNAse treated and diluted to 200 µl in selection buffer, 22 randomly picked transcripts from each pool were verified using a bioanalyzer small RNA chip. Analyses were conducted at 25°C on an Octet RED 96 system (FortéBio) using 96-well microplates

(Greiner Bio-One) in selection buffer. Streptavidin-coated tips were activated with biotinylated capture probe at 1µM in selection buffer over 300 s. RNA was bound to the capture probe by dipping 600 s in crude diluted T7 reaction. Screening for ligand-induced elution was done with 48 clones per plate dipping in 1mM target solution for 600 s. This was followed by a 3-step 5s re-generation in regeneration buffer (50 mM NaOH, 1M NaCl). Subsequently the tips where loaded again with RNA to study the dissociation in buffer with no ligand (600s). This was used to subtract unspecific dissociation from target-induced dissociation.

Kinetic assay

RNA was prepared using the TranscriptAid kit (Thermo Fisher Scientific) and dsDNA from individually picked clones for the screening assay. RNA was purified using Direct-zol™ RNA miniprep kit according to standard procedure and RNA fidelity was analyzed using Bioanalyzer™ Small RNA chip and quantified by Qubit™. Streptavidin-coated tips were activated with biotinylated capture probe at 1µM in selection buffer over 300 s. 10 ng RNA in a total volume of 200 µL selection buffer was used to load the RNA onto the activated tips over 200 s. Following incubation with RNA solution tips were dipped in selection buffer with or without ligand. The specificity of RebA clones were tested against 1 mM carminic acid and 1 mM 5HTP and CarmA clones were tested against 5HTP and Rebaudioside A at 1mM. The kinetic analysis was done in dilution series with 1mM to 0,0625 mM target. All steps were conducted at 25°C.

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REFERENCES

Ahmad KM, Oh SS, Kim S, McClellen FM, Xiao Y, Soh HT. 2011. Probing the limits of aptamer affinity with a microfluidic SELEX platform. *PLoS One* **6**: e27051.

Bayrac AT, Sefah K, Parekh P, Bayrac C, Gulbakan B, Oktem HA, Tan W. 2011. In vitro Selection of DNA Aptamers to Glioblastoma Multiforme. *ACS Chem Neurosci* **2**: 175–181.

Berezovski M V, Musheev MU, Drabovich AP, Jitkova J V, Krylov SN. 2006. Non-SELEX: selection of aptamers without intermediate amplification of candidate oligonucleotides. *Nat Protoc* **1**: 1359–69.

Carothers JM, Goler J a, Kapoor Y, Lara L, Keasling JD. 2010. Selecting RNA aptamers for synthetic biology: investigating magnesium dependence and predicting binding affinity. *Nucleic Acids Res* **38**: 2736–47.

Cho M, Soo Oh S, Nie J, Stewart R, Eisenstein M, Chambers J, Marth JD, Walker F, Thomson J a, Soh HT. 2013. Quantitative selection and parallel characterization of aptamers. *Proc Natl Acad Sci U S A* **110**: 18460–5.

Cho M, Xiao Y, Nie J, Stewart R, Csordas AT, Oh SS, Thomson J a, Soh HT. 2010. Quantitative selection of DNA aptamers through microfluidic selection and high-throughput sequencing. *Proc Natl Acad Sci U S A* **107**: 15373–8.

Davidson ME, Harbaugh S V, Chushak YG, Stone MO, Kelley-Loughnane N. 2013. Development of a 2,4-dinitrotoluene-responsive synthetic riboswitch in E. coli cells. *ACS Chem Biol* **8**: 234–41.

Davis JH, Szostak JW. 2002. Isolation of high-affinity GTP aptamers from partially structured RNA libraries. *Proc Natl Acad Sci U S A* **99**: 11616–21.

Desai SK, Gallivan JP. 2004. Genetic screens and selections for small molecules based on a synthetic riboswitch that activates protein translation. *J Am Chem Soc* **126**: 13247–54.

Dietrich J a, McKee AE, Keasling JD. 2010. High-throughput metabolic engineering: advances in small-molecule screening and selection. *Annu Rev Biochem* **79**: 563–590.

Doessing H, Hansen LH, Veedu RN, Wengel J, Vester B. 2012. Amplification and re-generation of LNA-modified libraries. *Molecules* **17**: 13087–13097.

Ellington AD, Szostak JW. 1990. In vitro selection of RNA molecules that bind specific ligands. *Nature* **346**: 818–22.

Famulok M, Mayer G. 2014. Aptamers and SELEX in Chemistry & Biology. *Chem Biol* **21**: 1055–1058.

Hermann T, Patel DJ. 2000. Adaptive recognition by nucleic acid aptamers. *Science* **287**: 820–5.

Hoon S, Zhou B, Janda KD, Brenner S, Scolnick J. 2011. Aptamer selection by high-throughput sequencing and informatic analysis. *Biotechniques* **51**: 413–6.

Juang JK, Liu HJ. 1987. The effect of DMSO on natural DNA conformation in enhancing transcription. *Biochem Biophys Res Commun* **146**: 1458–1464.

Kim D, Gusti V, Pillai SG, Gaur RK. 2005. An artificial riboswitch for controlling pre-mRNA splicing. *RNA* **11**: 1667–77.

Kupakuwana G V, Crill JE, McPike MP, Borer PN. 2011. Acyclic identification of aptamers for human alpha-thrombin using over-represented libraries and deep sequencing. *PLoS One* **6**: e19395.

Link KH, Breaker RR. 2009. Engineering ligand-responsive gene-control elements: lessons learned from natural riboswitches. *Gene Ther* **16**: 1189–201.

Lou X, Qian J, Xiao Y, Viel L, Gerdon AE, Lagally ET, Atzberger P, Tarasow TM, Heeger AJ, Soh HT. 2009. Micromagnetic selection of aptamers in microfluidic channels. *Proc Natl Acad Sci U S A* **106**: 2989–94.

Lynch S a, Desai SK, Sajja HK, Gallivan JP. 2007. A High-Throughput Screen for Synthetic Riboswitches Reveals Mechanistic Insights into Their Function. *Chem Biol* **14**: 173–184.

Lynch S a, Gallivan JP. 2009. A flow cytometry-based screen for synthetic riboswitches. *Nucleic Acids Res* **37**: 184–92.

Miyachi Y, Shimizu N, Ogino C, Kondo A. 2010. Selection of DNA aptamers using atomic force microscopy. *Nucleic Acids Res* **38**.

Mosing RK, Bowser MT. 2009. Isolating aptamers using capillary electrophoresis-SELEX (CE-SELEX). *Methods Mol Biol* **535**: 33–43.

Muranaka N, Sharma V, Nomura Y, Yokobayashi Y. 2009. An efficient platform for genetic selection and screening of gene switches in Escherichia coli. *Nucleic Acids Res* **37**: e39.

Musheev MU, Krylov SN. 2006. Selection of aptamers by systematic evolution of ligands by exponential enrichment: Addressing the polymerase chain reaction issue. *Anal Chim Acta* **564**: 91–96.

Nutiu R, Li Y. 2005. In vitro selection of structure-switching signaling aptamers. *Angew Chem Int Ed Engl* **44**: 1061–5.

Ogawa A, Maeda M. 2008. An artificial aptazyme-based riboswitch and its cascading system in E. coli. *Chembiochem* **9**: 206–9.

Qian J, Lou X, Zhang Y, Xiao Y, Tom Soh H. 2009. Generation of highly specific aptamers via micromagnetic selection. *Anal Chem* **81**: 5490–5495.

Schütze T, Wilhelm B, Greiner N, Braun H, Peter F, Mörl M, Erdmann V a, Lehrach H, Konthur Z, Menger M, et al. 2011. Probing the SELEX process with next-generation sequencing. *PLoS One* **6**: e29604.

Stoltenburg R, Nikolaus N, Strehlitz B. 2012. Capture-SELEX: Selection of DNA Aptamers for Aminoglycoside Antibiotics. *J Anal Methods Chem* **2012**: 415697.

Stoltenburg R, Reinemann C, Strehlitz B. 2005. FluMag-SELEX as an advantageous method for DNA aptamer selection. *Anal Bioanal Chem* **383**: 83–91.

Suess B. 2003. Conditional gene expression by controlling translation with tetracycline-binding aptamers. *Nucleic Acids Res* **31**: 1853–1858.

Suess B, Fink B, Berens C, Stentz R, Hillen W. 2004. A theophylline responsive riboswitch based on helix slipping controls gene expression in vivo. *Nucleic Acids Res* **32**: 1610–4.

Topp S, Gallivan JP. 2008. Random walks to synthetic riboswitches--a high-throughput selection based on cell motility. *Chembiochem* **9**: 210–3.

Tuerk C, Gold L. 1990. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **249**: 505–10.

Vallée-Bélisle A, Plaxco KW. 2010. Structure-switching biosensors: inspired by Nature. *Curr Opin Struct Biol* **20**: 518–26.

Weigand JE, Sanchez M, Gunnesch E-B, Zeiher S, Schroeder R, Suess B. 2008. Screening for engineered neomycin riboswitches that control translation initiation. *RNA* **14**: 89–97.

Weigand JE, Suess B. 2007. Tetracycline aptamer-controlled regulation of pre-mRNA splicing in yeast. *Nucleic Acids Res* **35**: 4179–85.

Wieland M, Hartig JS. 2008. Improved aptazyme design and in vivo screening enable riboswitching in bacteria. *Angew Chem Int Ed Engl* **47**: 2604–7.

Yang J, Seo SW, Jang S, Shin S-I, Lim CH, Roh T-Y, Jung GY. 2013. Synthetic RNA devices to expedite the evolution of metabolite-producing microbes. *Nat Commun* **4**: 1413.

Yunusov D, So M, Shayan S, Okhonin V, Musheev MU, Berezovski M V, Krylov SN. 2009. Kinetic capillary electrophoresis-based affinity screening of aptamer clones. *Anal Chim Acta* **631**: 102–7.

A Capture-SELEX strategy for multiplexed selection of structure-switching RNA aptamers against small molecules

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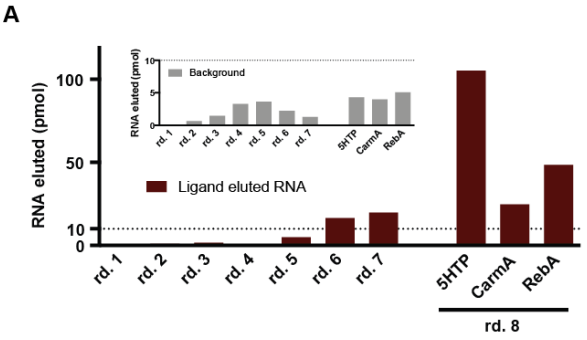
Supplementary information



B

Selection round	RNA eluted with Background (pmol)	Ligand (pmol)
1	1.81×10^{-3}	1.992×10^{-3}
2	0.66	0.97
3	1.46	1.75
4	3.29	0.38
5	3.46	4.90
6	2.25	16.60
7	1.12	19.82
	Split	
5HTP – 8	4.30	105.27
CarmA – 8	3.98	24.74
RebA – 8	5.09	48.60

Figure S1: RNA elution. The figure shows the amount of RNA eluted of the beads in 45 min without the presence of ligand (grey) and in the presence of traget (red).

Article 3

Comparative study on aptamers as recognition elements for antibiotics in a label-free all-polymer biosensor

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Comparative study on aptamers as recognition elements for antibiotics in a label-free all-polymer biosensor

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ABSTRACT

We present an all-polymer electrochemical microfluidic biosensor using Topas® as substrate and a conductive polymer bilayer as electrode material. The conductive bilayer consists of tosylate doped poly(3,4-ethylenedioxythiophene) (PEDOT:TsO) and the hydroxymethyl derivative PEDOT-OH:TsO, which was covalently functionalized with two aptamer probes with affinity to ampicillin or kanamycin A, respectively. Using electrochemical impedance spectroscopy (EIS) we were able to detect ampicillin in a concentration range from 100 pM to 1 μM and kanamycin A from 10 nM to 1 mM. The obtained EIS spectra were fitted with an equivalent circuit model successfully explaining the impedance signal. Real samples from regular ultra-high temperature treated low-fat milk spiked with ampicillin were successfully tested to assess the functionality of the sensor with real samples. In conclusion, we have demonstrated the applicability of the newly developed platform for real time, label-free and selective impedimetric detection of commonly used antibiotics. Additionally it was possible to detect ampicillin in a milk sample at a concentration below the allowed maximum residue limit (MRL) in the European Union.
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1. Introduction

Detection, identification and quantification of pathogens and chemical agents are crucial for water and environmental analysis, clinical diagnosis, food safety, and biodefence. The existing immunological or nucleic acid technologies are mostly time consuming and require both sophisticated equipment as well as highly trained personnel, hence increasing the analysis costs. Another limitation of these techniques lies in their nature, which only allows the detection of certain types of analytes.

Therefore, new assay technologies are continuously emerging, and among these, the biosensor technology is the area with fastest growth (Lazcka et al., 2007). Ideally, the assays used in a detection system should enable the detection and quantification of a broad range of different molecules. Furthermore, the technique should provide reliable, real time, on-site, user-friendly, and inexpensive detection with adequate sensitivity, specificity and reproducibility.

Label-free biosensors for in situ measurements with high sensitivity and high specificity are of significant interest for the development of diagnostic devices (Cosnier, 2003; Gerard et al., 2002; Liao et al., 2006).

1.1. Impedimetric biosensors

Impedance spectroscopy is a powerful method to analyse the complex electrical resistance of a system and it is sensitive to surface phenomena and changes of bulk properties. Electrochemical detection using electrochemical impedance spectroscopy (EIS) is advantageous because of its label-free and reagentless character and high sensitivity, as recently reviewed by Pänke et al. (2008). Thus impedance detection is particularly suited to follow binding events in the field of biosensors.

The tools that allow for specific detection (generally referred to as affinity tools) are highly selective binders of the target. At present, antibodies of mammals are the best characterised and most widely used affinity tools, i.e. biological recognition elements in biosensor platforms. However, antibodies do have their limitations: they are sensitive to pH changes and can easily be inactivated (e.g. through elevated temperatures, proteases) (Binz et al., 2005; Hoogenboom, 2005). Moreover, producing antibodies is generally difficult and expensive.

1.2. Aptamers as recognition elements

As alternatives to antibodies, aptamers have recently attracted increasing attention due to their capability to bind a wide range of targets: nucleic acids, proteins, metal ions and other molecules

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with high affinity and sensitivity (Hong et al., 2012; Song et al., 2012b).

Aptamers are peptides or oligonucleotides (RNA or single-stranded DNA; ssDNA) that bind to a specific target molecule. The aptamers typically fold into a three-dimensional structure, whose conformation is changing upon ligand binding. Aptamer-like structures can also be found in nature: riboswitches in bacteria and eukaryotes control translation depending on ligand binding (Winkler et al., 2002).

Novel aptamers can be developed using a process called systematic evolution of ligands by exponential enrichment (SELEX) (Ellington and Szostak, 1990; Tuerk and Gold, 1990). It enables the selection of high-affinity nucleic acid sequences from a random pool of candidates.

The oligonucleotide aptamers can easily be modified with signal moieties and can be produced at low cost. Up to now, a variety of assays have been successfully developed for aptamer-based analysis of biomolecules (Centi et al., 2007; Guo et al., 2008; Wu et al., 2011; Yan et al., 2010).

Efforts have been made to develop aptamer-based biosensors (aptasensors) for sandwich assays in recent years. Although these biosensors could detect the target, the detection limits still need to be improved (Wang et al., 2007; Zuo et al., 2009).

1.3. Detection of antibiotics

The development of novel detection systems for antibiotics is of particular importance. The emergence of microorganisms resistant towards important antibiotics occurs at alarmingly high rates. Infections with multidrug-resistant pathogens have been reported for a range of potentially lethal bacteria including *Staphylococcus aureus* (Klevens et al., 2007) and *Mycobacterium tuberculosis* (Centers for Disease Control and Prevention (CDC), 2006), leaving few or no options for treatment. In the European Union (EU), it is estimated that 25 000 persons die every year from infections with antibiotic-resistant micro-organisms (White et al., 2011). Development of novel antibiotics, however, is attracting little attention from the pharmaceutical industry, and for gram-negative pathogens there are few or no late stage clinical trials of new drugs (Septimus and Kuper, 2009). Besides their use for treating human infections, antibiotics are also utilised as growth promoters in agri- and aquaculture (Cabello, 2006; Witte, 1998).

Due to their short generation time, bacteria can quickly evolve resistance towards all known antibiotics if these are present in sub-lethal concentrations in the environment (Gullberg et al., 2011). To minimise the development of resistance towards antibiotics, it is, therefore, of critical importance to limit both the use and the release of antibiotics into the environment.

Detection of different antibiotics, however, is challenging due to the varied chemical structure of the compounds and the lack of available simple spectroscopic or electrochemical assays.

Recently, aptamers targeting a range of antibiotics, including tobramycin (González-Fernández et al., 2011), kanamycin (Song et al., 2011), ampicillin (Song et al., 2012a) and chloramphenicol (Mehta et al., 2011) have been developed.

1.4. Design of the aptasensor

Polymer-based microfluidic systems meet the requirements of disposable devices for low sample consumption, cost efficiency, reliability, and fast response time, which make the systems ideal for rapid analysis. However, most biosensors for electrochemical detection involve metallic electrodes (Kerman et al., 2004). To avoid oxidation or participation in electrochemical reactions, noble metals such as gold or platinum are usually employed. These materials have a number of disadvantages, such as high (and still increasing) market prices or comparably low biocompatibility. Apart from that they also require very costly fabrication methods.

Conductive polymers offer very suitable properties to master the specialized task of transducing a binding event between an analyte and a biological probe. They have been used as alternative to traditional electrode materials because of the additional advantageous properties of inexpensive electrode fabrication and easy electrode functionalization (Kiilerich-Pedersen et al., 2011; Rozlosnik, 2009).

Because of their excellent compatibility with biological samples, polypyrrole (PPy) and poly(3,4-ethylenedioxythiophene) (PEDOT) have repeatedly been used for sensors in biological environments (Balamurugan and Chen, 2007; Bidan et al., 1999; Dubois et al., 2005; Kiilerich-Pedersen et al., 2011; Sarma et al., 2009; Vidal et al., 1999; Xie et al., 2009).

Even though pyrrole is the less expensive monomer compared to 3,4-ethylenedioxythiophene (EDOT), and PPy shows equally good air stability, PEDOT was used exclusively in this study for its superior stability in phosphate buffers and for its higher conductivity (Groenendaal et al., 2000; Syritski et al., 2005; Yamato et al., 1995).

PEDOT can be processed in different ways. Popular polymerization methods are electropolymerization (Kros et al., 2002; Lima et al., 1998; Shi et al., 2008; Sotzing et al., 1996; Xie et al., 2009; Yamato et al., 1995) or chemical oxidation polymerization in liquid (Aasmundtveit et al., 1999; Hansen et al., 2006, 2007a, 2007b; Winther-Jensen and West, 2006) as well as in vapor phase (Bhattacharyya and Gleason, 2011; Le et al., 2005; Winther-Jensen and West, 2004). Chemical oxidation is advantageous because it does not require a conductive substrate.

Micropatterning of conductive polymers is regularly done using cleanroom-based photolithographic techniques. However, although such processes are versatile, they are not well-suited for fabricating inexpensive biosensor platforms due to their costs. As an alternative, we have recently developed a simple micropatterning procedure, which is based on contacting PEDOT thin films with a micro-structured agarose stamp soaked in a solution of aqueous hypochlorite and a non-ionic detergent (Hansen et al., 2007b; Lind et al., 2012). Where contacted, PEDOT is removed and the underlying substrate exposed. By applying a cyclic-olefin-copolymer (COC) substrate we were able to fabricate nucleotide-functionalized PEDOT microelectrodes on a COC background with a low degree of unspecific binding of DNA, in a simple and inexpensive manner. This sensor has been used to specifically detect picomolar concentrations of antibiotics.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany) if not stated otherwise.

2.1.1. Chip material

The substrate and the top part of the microfluidic chip were fabricated from Cyclic Olefin Copolymer (COC) Topas® grade 5013 (T_g at 130 °C, TOPAS Advanced Polymers GmbH, Germany).

2.1.2. Electrode material

For the electrodes, the conducting polymer poly(3,4-ethylene-di-oxy-thio-phene) doped with tosylate (PEDOT:TsO) was used.

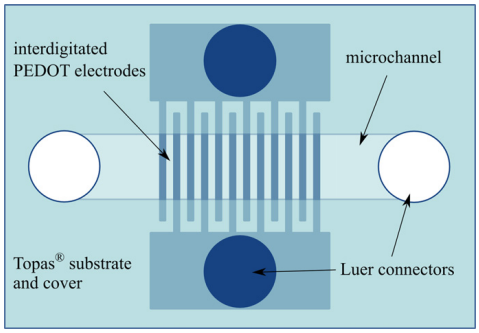

Fig. 1. Schematic drawing of the assembled microfluidic chip with interdigitated PEDOT:TsO electrodes. Drawing is not to scale.

2.1.3. Antibiotics and aptamers

In this study, we used short high-affinity aptamers against kanamycin A and ampicillin presented by Song et al. (2011, 2012a, 2012b), respectively. The DNA aptamers were synthesized by Integrated DNA Technologies (Denmark) as high performance liquid chromatography (HPLC) purified and lyophilized ssDNA functionalized with a 5′-amino modified C_6 linker. Their sequences from 5′ to 3′ are:

Ampicillin	GCG GGC GGT TGT ATA GCG G
Kanamycin A	TGG GGG TTG AGG CTA AGC CGA

2.2. Chip fabrication

The all-polymer microfluidic flow system was fabricated from Topas® polymer using a bilayer composite of PEDOT:TsO and PEDOT-OH:TsO conducting polymer as electrodes (Fig. 1).

Both the top and the bottom part of the chip were made by injection moulding using Topas® pellets.

2.2.1. Conducting polymer film deposition

Conductive thin films of PEDOT:TsO were made by spin coating the reaction solution containing the monomer and thermal activation according to the following procedure:

About 260 μL Baytron C (40% $Fe^{(III)}$ tosylate in butanol), 80 μL butanol, 6 μL pyridine and 8.8 μL EDOT were thoroughly mixed and spun on a 50.8 mm Topas® 5013 disc with 1000 rpm for 60 s. Coated polymer discs were then heated to 70 °C for 5 min. The inhibitor pyridine evaporated and the progress of polymerization was observable by a colour change from yellow to green. After rinsing the discs with de-ionized water to remove excess reactants and by-products, they obtained the characteristic blue colour of PEDOT:TsO. A second polymer layer was applied in the same way using the monomer ((2,3-dihydrothieno[3,4-*b*][1,4]-dioxin-2-yl)methanol) also known as hydroxymethyl-EDOT or EDOT-OH instead of EDOT. This yielded a PEDOT-OH:TsO coating of the same thickness, where the hydroxymethyl group of each monomer provides a possible grafting site for later functionalization.

2.2.2. Microelectrode preparation

Interdigitated microelectrodes were fabricated by agarose stamping, as described in detail by Hansen et al. (2007b) and more recently by Lind et al. (2012).

In short, a stamp was prepared by casting a 10% w/w agarose gel into a mould with a relief of the electrode design etched in silicon. After solidification, the stamp was soaked in the etching solution and applied manually to the PEDOT:TsO/PEDOT-OH:TsO bilayer.

For etching a 1–1.5% w/v solution of sodium hypochlorite in water containing 0.1% v/v of the surfactant TritonX100 was used. The stamping time depends on the thickness of the conductive polymer layer or bilayer, respectively. To etch through approximately 200 nm we needed between 45 and 60 s. After stamping, over-oxidized PEDOT was removed by washing with water. The conducting polymer layers were re-doped by immersion in 4% w/v $Fe^{(III)}$ tosylate solution for a few seconds. During this process, the colour of the polymer changed from dark to light blue indicating the conversion from a reduced state to a more conductive oxidized state with higher degree of doping. The stamp design comprised interdigitated electrodes with a width of 20 μm. The spacing between the single wires was the same as their width.

2.2.3. Chip assembly

The chip consisted of two parts: the flat Topas® disc with the microelectrodes and an injection molded Topas® cover disc with Luer connectors for simple connection to the electronic equipment and exchange of liquids. Both parts were assembled with a 150 μm thick sheet of transfer adhesive (ARcare 90106, Adhesive Research Ireland). Cavities were cut into the tape by laser ablation to create microfluidic channels. To ensure homogeneous and tight sealing around the channels, the assembled discs were pressed with a force of 500 N at a bonding temperature of 75 °C for 5 min. Pressure was released after the chip had cooled down to 40 °C. A schematic drawing of the chip is presented in Fig. 1.

2.2.4. Electrode functionalization

Succinic acid was grafted onto surface hydroxymethyl groups by filling the channels with 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer at pH 4.0 with 50 mM of the coupling agent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and succinic anhydride for 30 min.

The new surface carboxylic acid groups were activated with 50 mM EDC and 40 mM NHS (*N*-hydroxy succinimide) in MES buffer (0.1 M, pH=4.0) for 5 min. After washing with MES buffer, the 5′-amino modified aptamer (100 nM in MES) was added to form a stable amide bond.

After a reaction time of 3 h, the channels were rinsed with several millilitres of binding buffer (DPBS; Dulbecco's phosphate buffered saline, containing 5 mM $MgCl_2$).

To verify the success of the immobilization, the grafting method was tested with fluorescently labeled DNA. Control experiments without activation reagents showed that no non-specifically adsorbed DNA remained after washing. Fluorescence micrographs are shown in Fig. S1 in the electronic supplementary information.

2.3. Electrochemical impedance measurements

Electrochemical impedance spectroscopy (EIS) was conducted on chips with and without immobilized aptamers. A potentiostat with integrated frequency generator (Zahner IM6, Zahner Elektrik GmbH, Germany) was connected to the electrodes by spring-loaded (Pogo Pin) contacts and the impedance of the system was measured with wide spectrum sweeps in a frequency range from 100 kHz to 200 mHz (logarithmic scale with 10 points per decade for frequencies <66 Hz and four points per decade for frequencies >66 Hz). After the baseline was recorded, the target was

added in increasing concentrations and the change of the impedance signal monitored.

3. Results and discussion

3.1. Film characterization

As has been reported in previous publications, the conductivity of both PEDOT:TsO and PEDOT-OH:TsO is, compared to other conjugated polymers, very high. Surface conductivity values of up to 700 S/cm have been found for PEDOT:TsO (Hansen et al., 2007b; Winther-Jensen and West, 2006), while for tosylate doped hydroxymethyl-PEDOT a value of 83 S/cm has been reported (Hsiao et al., 2010).

Our findings for PEDOT:TsO were 424 ± 57.2 S/cm and for PEDOT-OH:TsO 81 ± 10.7 S/cm. The double layer composite showed a conductance of 275 ± 61.2 S/cm. The conductances of our polymer films are, therefore, within the expected range.

3.2. Micropatterning

After stamping, the actual width of the micro-electrode wires was determined by optical microscopy. Despite being designed with a width of 20 μm, the mean width of the wires was measured to be 9.7 ± 2.2 μm. The large deviation from the original wire size can be explained by diffusion processes occurring during stamping. Shortening the exposure time would certainly reduce the effect; however, then the etching of the desired areas would not be complete, resulting in residual conductivity between the wires. Uniform pressure control by automation of the stamping process would improve the reproducibility significantly.

3.3. Impedimetric characterization of the aptasensor

In this study, we show that the above-described all-polymer biosensor is a sensitive and selective platform for detection of different antibiotics. We have chosen already published aptamers against kanamycin A and ampicillin for our experiments (Song et al., 2011, 2012a).

The immobilization of the aptamers alone provoked an increase in the impedance, as was expected due to the increased charge transfer resistance at the electrode/liquid interface.

Binding of the target molecules to the corresponding aptamer caused a further increase in the impedance measured at frequencies below 1 Hz. A logarithmic correlation of analyte concentration and impedance response was found for a broad dynamic range and both analytes. In the following, the individual aptasensors will be discussed in more detail.

Analyte concentrations starting from 10 pM were tested with ampicillin-aptamer. Fig. 2 shows the relative change of absolute impedance with increasing concentrations. The first statistically significant ($p < 0.05$) signal was observed at 100 pM. Further increase of analyte concentration showed a fairly linear dependence in the logarithmic plot.

As described above, the patterning of the chips was subject to uncertainties, which resulted in variation in the width of the electrodes in the range of several micrometers.

There was a very good correlation between impedance change and wire size (see Fig. S2 in the electronic supplementary information), so that a better reproducibility can be expected from electrodes fabricated with higher accuracy.

With a dissociation constant $k_D = 78.8$ nM the kanamycin A aptamer establishes a weaker bond to its target than the ampicillin aptamer ($k_D = 13.4$ nM) (Song et al., 2011, 2012a). It is, therefore, likely that an aptasensor based on this probe would be less sensitive. As shown in Fig. 2 our findings verify this presumption. Typically a concentration of 10 nM was necessary to obtain a statistically significant increase ($p < 0.05$) of the impedance modulus. Lower concentrations, starting from 10 pM, caused little or no effect on the signal. We believe that the relatively low limit of detection (LOD) and wide dynamic range of the biosensor is also due to specific device properties and not exclusively related to affinity of the probes used in this study. In addition, sensors and assays for Ochratoxin A (OTA) have shown that using the same aptamer in different assays and sensors can result in over a 1000-fold differences in LOD (Lauridsen and Veedu, 2012). The lower detection limit found with the ampicillin aptamer indicates that a high affinity for target compounds will facilitate higher devise sensitivity.

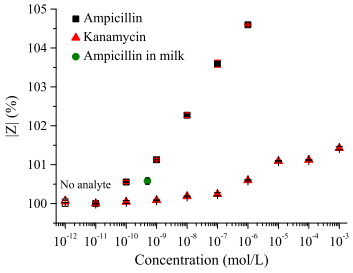

Fig. 2. A typical impedance response for increasing concentrations of ampicillin or kanamycin, respectively. The empty symbols illustrate the measured baseline level before the first injection. The green circle represents the obtained impedance change in the case of 500 mol/L ampicillin in milk sample. The error bars show the average of the signal noise. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

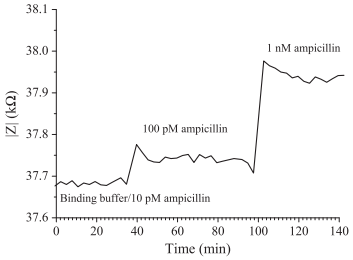

Fig. 3. Increase of impedance at a frequency of 356 mHz is shown after addition of the analyte in different concentrations.

Control experiments, where the antibiotics were reacted with the mismatching aptamers, gave no changes in the impedance signal compared to the baseline. After that, injecting the matching antibiotics into the biosensor resulted in the expected increase of impedance. This shows that the immobilization of the aptamers on the electrodes did not affect the selectivity of the aptamers towards their targets.

With the described experimental setup (see Section 2.3) we were able to obtain one full impedance spectrum in 2:35 min. Fig. 3 shows the impedance data at a single frequency (356 mHz), which was taken from the full spectra. As can be seen in the graph, the impedance change occurs within the sampling time, i.e. within 2:35 min and stabilizes at a new level. We have also conducted impedance measurements at a single frequency, where

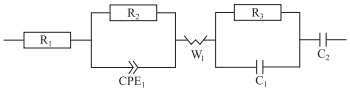

Fig. 4. Equivalent circuit model for fitting the experimental spectra (see description in the text).

the instrument-determined minimum sampling time was ca. 17 s (data not shown), but also this higher temporal resolution was insufficient to determine the reaction rate of the target-aptamer complex formation. However, this shows that the analysis time is reasonably short for a number of real-life applications.

EU regulation no. 675/92 (as amendment to regulation no. 2377/90) lays down a maximum residue limit (MRL) for ampicillin of 4 μg/L in milk, which is equivalent to a concentration of 11.45 nM. Our experiments shown above proved that we were able to detect much lower concentrations of ampicillin in buffer solution. In order to prove the reliability of the sensor detecting analytes in real samples, a 10% solution of ultra-high temperature treated (UHT) low fat milk in DPBS containing 5 mM $MgCl_2$ was prepared and spiked with 500 pM ampicillin. Impedance spectra were measured as before. In these experiments, we were able to measure an increase of impedance of $0.58 \pm 8\%$. These data are in well accordance with the concentration dependence reported above. We can, therefore, claim that our sensor is capable of reproducibly detecting an ampicillin concentration in milk that is far below the MRL set by the EU.

3.4. Equivalent circuit modeling of the EIS results

An equivalent circuit model fitting the electrical properties of the sensor is shown in Fig. 4. This model was used to simulate impedance spectra while changing certain parameters to understand the effects observed in our experiments; it satisfactorily fits the experimental data over the measured frequency range.

In the model, R_1 is the intrinsic resistance of the electrodes. The parallel resistance (R_2) and the constant phase element (CPE_1) with coefficients P_1 and n_1 represent the electrode–solution interface, where R_2 is the charge transfer resistance and CPE_1 the electrical double layer on the surface. The following Warburg impedance (W_1) indicates the diffusion limited electrochemical processes specified by the coefficient A_{w1}. The impedance spectrum includes a semicircle portion at high frequencies corresponding to bulk solution resistance (R_3) and the geometrical capacitance (C_1) of the interdigitated electrodes. Finally, the capacitance C_2 is the limiting capacitance, which reflects the limited solid state diffusion of charges inside the conducting polymer.

The intrinsic resistance of the electrode (R_1), the solution resistance (R_3) and electrode geometrical capacitance (C_1) are not changing during the aptamer-target binding process. The Warburg coefficient (A_{w1}) represents the impedance due to the diffusion of charges to and from the electrode surface. The temperature and the viscosity of the solution were constant during the binding process; however, conformational changes and rearrangements of the surface bound aptamers during complex formation are likely to alter the electrode surface area available for charge transfer, influencing A_{w1}. Modifications of the electrode surface can inflict changes to the electrolytic double layer at the electrode/electrolyte interface with its non-ideal capacitance (reflected as the constant phase element CPE_1) and the reaction rate of the transfer of charges between electrode and electrolyte (expressed as the charge transfer resistance R_2). Both factors are dependent on the change of the ionic concentration near the electrode surface, which is influenced by the binding event of an analyte molecule to an immobilized aptamer. We can, therefore, assume that both the constant phase element, the

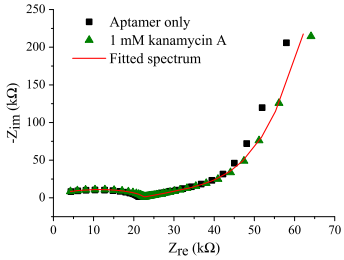

Fig. 5. Nyquist plots from impedance spectra before (■) and after (▲) addition of kanamycin A. In the low frequency region (right side) of the spectra a clear shift towards higher resistance is visible. The solid red line represents a simulated spectrum based on the fitting parameters from Table 1. The curve is calculated from the values after analyte addition. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

Table 1
Comparison of parameters corresponding to elements in the equivalent circuit model fitted to the spectra shown in Fig. 5.

Parameter	Aptamer only (error/%)	Kanamycin (error/%)	Unit
C_1	1.59×10^{-10} (2.8)	1.58×10^{-10} (2.3)	F
C_2	4.11×10^{-6} (2.3)	4.06×10^{-6} (3.0)	F
R_1	3.34×10^{2} (41.3)	3.07×10^{2} (46.7)	Ω
R_2	2.51×10^{4} (7.8)	2.41×10^{4} (9.3)	Ω
R_3	2.06×10^{4} (0.8)	2.16×10^{4} (0.8)	Ω
A_{w1}	1.44×10^{4} (12.8)	2.13×10^{4} (9.0)	$\Omega/\sqrt{s}$
P_1	5.75×10^{-6} (6.2)	6.80×10^{-6} (6.9)	$1/\Omega$
n_1	0.58 (1.9)	0.57 (2.2)	–

charge transfer resistance and the Warburg element will be the most interesting parameters for investigations of target binding to surface-bound probes. The surface coverage with aptamers could have an influence on the sensitivity of such a device as described here, but the size of the antibiotics is much less that than the aptamers, therefore, it is not likely that steric hindrance would affect the results.

Impedance spectra recorded at different stages of the experiment were fitted with the "EIS Spectrum Analyser" software.[1] An example of the fitted curves is shown together with two measured spectra represented as a Nyquist plot in Fig. 5. The curve was calculated from parameters which were fitted to the impedance data obtained from the kanamycin aptasensor after the analyte was added. The result indicates a good agreement with the measured data, especially in the lower frequency range.

A comparison of parameters obtained from fitting the model to the data recorded before analyte addition and after injection of kanamycin A at a concentration of 1 mM is made in Table 1. As expected, only slight changes were observed for the capacitances C_1 and C_2 as well as the material or solution related resistances R_1 and R_3. Elements describing the electrode/liquid interface experienced a larger change. The strongest deviation from the original value was found for the Warburg coefficient A_{w1}.

4. Conclusion

We have successfully developed the first prototype of an all-polymer electrochemical biosensor using Topas® as microfluidic

[1] A.S. Bondarenko and G.A. Ragoisha, http://www.abc.chemistry.bsu.by/vi/analyser/.

system and a bilayer of tosylate doped poly(3,4-ethylenedioxythiophene) (PEDOT:TsO) and its hydroxymethyl derivative PEDOT-OH:TsO as electrode material covalently functionalized with aptamers. The newly developed inexpensive biosensor has high sensitivity and selectivity for the tested targets, which makes it a promising platform for detection of analytes from complex samples by a fast, label-free and reliable method. The device was tested with real samples from milk, where it was possible to detect ampicillin in concentrations below the MRL defined in EU regulation no. 675/92.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at http://dx.doi.org.10.1016/j.bios.2012.12.058.

References

Aasmundtveit, K.E., Samuelsen, E.J., Pettersson, L.A.A., Inganäs, O., Johansson, T., Feidenhans'l, R., 1999. Synthetic Metals 101 (1–3), 561–564.
Balamurugan, A., Chen, S.-M., 2007. Analytica Chimica Acta 596 (1), 92–98.
Bhattacharyya, D., Gleason, K.K., 2011. Chemistry of Materials 23 (10), 2600.
Bidan, G., Billon, M., Livache, T., Mathis, G., Roget, A., Torresrodriguez, L., 1999. Synthetic Metals 102 (1–3), 1363.
Binz, H., Amstutz, P., Plückthun, A., 2005. Nature Biotechnology 23 (10), 1257–1268.
Cabello, F.C., 2006. Environmental Microbiology 8 (7), 1137–1144.
Centers for Disease Control and Prevention (CDC), March 2006. MMWR. Morbidity and Mortality Weekly Report 55(11), 301–305.
Centi, S., Tombelli, S., Minunni, M., Mascini, M., 2007. Analytical Chemistry 79 (4), 1466–1473.
Cosnier, S., 2003. Analytical and Bioanalytical Chemistry 377 (3), 507–520.
Dubois, M.-P., Gondran, C., Renaudet, O., Dumy, P., Driguez, H., Fort, S., Cosnier, S., 2005. Chemical Communications (Cambridge) (34), 4318–4320.
Ellington, A.D., Szostak, J.W., 1990. Nature 346 (6287), 818–822.
Gerard, M., Chaubey, A., Malhotra, B., 2002. Biosensors and Bioelectronics 17 (5), 345–359.
González-Fernández, E., de-los Santos-Álvarez, N., Lobo-Castañón, M.J., Miranda-Ordieres, A.J., Tuñón-Blanco, P., 2011. Biosensors and Bioelectronics 26 (January (5)), 2354–2360.
Groenendaal, L., Jonas, F., Freitag, D., Pielartzik, H., Reynolds, J.R., 2000. Advanced Materials 12 (7), 481.
Gullberg, E., Cao, S., Berg, O.G., Ilbäck, C., Sandegren, L., Hughes, D., Andersson, D.I., 2011. PLoS Pathogens 7 (July (7)).
Guo, W., Yuan, J., Li, B., Du, Y., Ying, E., Wang, E., 2008. Analyst 133 (9), 1209–1213.
Hansen, T.S., West, K., Hassager, O., Larsen, N.B., 2006. Synthetic Metals 156 (18–20), 1203.
Hansen, T.S., West, K., Hassager, O., Larsen, N.B., 2007a. Journal of Micromechanics and Microengineering 17 (5), 860.
Hansen, T.S., West, K., Hassager, O., Larsen, N.B., 2007b. Advanced Materials 19 (20), 3261.
Hong, P., Li, W., Li, J., 2012. Sensors 12 (2), 1181–1193.
Hoogenboom, H., 2005. Nature Biotechnology 23 (9), 1105–1116.
Hsiao, A.-E., Tuan, C.-S., Lu, L.-H., Liao, W.-S., Teng, W.-J., 2010. Synthetic Metals 160 (21–22), 2319.
Kerman, K., Kobayashi, M., Tamiya, E., 2004. Measurement Science and Technology 15 (2).
Kiilerich-Pedersen, K., Poulsen, C.R., Jain, T., Rozlosnik, N., 2011. Biosensors and Bioelectronics, 386–392.
Klevens, R.M., Morrison, M.A., Nadle, J., Petit, S., Gershman, K., Ray, S., Harrison, L.H., Lynfield, R., Dumyati, G., Townes, J.M., Craig, A.S., Zell, E.R., Fosheim, G.E., McDougal, L.K., Carey, R.B., Fridkin, S.K., 2007. JAMA—Journal of the American Medical Association 298 (15), 1763–1771.
Kros, A., Nolte, R.J.M., Sommerdijk, N.A.J.M., 2002. Journal of Polymer Science. Part A—Polymer Chemistry 40 (6), 738.
Lauridsen, L., Veedu, R., 2012. Nucleic Acid Therapeutics. 22 (6), 371–379, http://dx.doi.org/10.1089/nat.2012.0377.
Lazcka, O., Campo, F.J.D., Muñoz, F.X., 2007. Biosensors and Bioelectronics 22 (7), 1205–1217.
Le, T.T., Kim, D.W., Lee, Y., Nam, J.D., 2005. Accessed online 25.11.2010 〈http://gralib.hcmuns.edu.vn/gsdl/collect/hnkhbk/index/assoc/HASH01f3/8a663927.dir/doc.pdf〉.
Liao, W., Guo, S., Zhao, X., 2006. Frontiers in Bioscience 11, 186–197.
Lima, A., Schottland, P., Sadki, S., Chevrot, C., 1998. Synthetic Metals 93 (February (1)), 33–41.
Lind, J.U., Acikgoz, C., Daugaard, A.E., Andresen, T.L., Hvilsted, S., Textor, M., Larsen, N.B., 2012. Langmuir 28 (15), 6502–6511.
Mehta, J., Van Dorst, B., Rouah-Martin, E., Herrebout, W., Scippo, M.-L., Blust, R., Robbens, J., 2011. Journal of Biotechnology 155 (4), 361–369.
Pänke, O., Balkenhohl, T., Kafka, J., Schäfer, D., Lisdat, F., 2008. Biochemical Engineering & Biotechnology 109, 195–237.
Rozlosnik, N., 2009. Analytical and Bioanalytical Chemistry 395 (3), 637–645.
Sarma, A.K., Vatsyayan, P., Goswami, P., Minteer, S.D., 2009. Biosensors and Bioelectronics 24 (8), 2313–2322.
Septimus, E.J., Kuper, K.M., 2009. Clinical Pharmacology & Therapeutics 86 (September (3)), 336–339.
Shi, Y., Luo, S., Fang, W., Zhang, K., Ali, E., Boey, F., Ying, J., Wang, J., Yu, H., Li, L., 2008. Organic Electronics 9 (5), 859.
Song, K.-M., Cho, M., Jo, H., Min, K., Jeon, S.H., Kim, T., Han, M.S., Ku, J.K., Ban, C., 2011. Analytical Biochemistry 415 (2), 175–181.
Song, K.-M., Jeong, E., Jeon, W., Cho, M., Ban, C., 2012a. Analytical and Bioanalytical Chemistry 402 (6), 2153–2161, http://dx.doi.org/10.1007/s00216-011-5662-3.
Song, K.-M., Lee, S., Ban, C., 2012b. Sensors 12 (1), 612–631.
Sotzing, G.A., Reynolds, J.R., Steel, P.J., 1996. Chemistry of Materials 8 (April (4)), 882–889.
Syritski, V., Gyurcsányi, R.E., Öpik, A., Tóth, K., 2005. Synthetic Metals 152 (1–3), 133–136.
Tuerk, C., Gold, L., 1990. Science 249 (4968), 505–510.
Vidal, J.C., Méndez, S., Castillo, J.R., 1999. Analytica Chimica Acta 385 (1–3), 203–211.
Wang, J., Wang, L., Liu, X., Liang, Z., Song, S., Li, W., Li, G., Fan, C., 2007. Advanced Materials 19 (22), 3943–3946.
White, A.R., on behalf of the BSAC Working Party on The Urgent Need: Regenerating Antibacterial Drug Discovery and Development, Blaser, M., Carrs, O., Cassell, G., Fishman, N., Guidos, R., Levy, S., Powers, J., Norrby, R., Tillotson, G., Davies, R., Projan, S., Dawson, M., Monnet, D., Keogh-Brown, M., Hand, K., Garner, S., Findlay, D., Morel, C., Wise, R., Bax, R., Burke, F., Chopra, I., Czaplewski, L., Finch, R., Livermore, D., Piddock, L. J.V., White, T., 2011. Journal of Antimicrobial Chemotherapy 66(9), 1948–1953.
Winkler, W., Nahvi, A., Breaker, R.R., 2002. Nature 419 (October (6910)), 952–956.
Winther-Jensen, B., West, K., 2004. Macromolecules 37 (12), 4538–4543.
Winther-Jensen, B., West, K., 2006. Reactive and Functional Polymers 66 (5), 479–483.
Witte, W., 1998. Science 279 (5353), 996–997.
Wu, S., Duan, N., Wang, Z., Wang, H., 2011. Analyst 136, 2306–2314.
Xie, H., Luo, S.-C., Yu, H.-H., 2009. Small 5 (22), 2611–2617.
Yamato, H., Ohwa, M., Wernet, W., 1995. Journal of Electroanalytical Chemistry 397 (1–2), 163–170.
Yan, X., Cao, Z., Lau, C., Lu, J., 2010. Analyst 135, 2400–2407.
Zuo, X., Xiao, Y., Plaxco, K., 2009. Journal of the American Chemical Society 131 (20), 6944–6945.

Article 4

Nucleic acid aptamers against biotoxins: a new paradigm toward the treatment and diagnostic approach.

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Nucleic Acid Aptamers Against Biotoxins: A New Paradigm Toward the Treatment and Diagnostic Approach

Lasse Holm Lauridsen[1,2] and Rakesh N. Veedu[1]

Nucleic acid aptamers are short single-stranded DNA or RNA oligonucleotides that can bind to their targets with very high affinity and specificity, and are generally selected by a process referred to as systematic evolution of ligands by exponential enrichment. Conventional antibody-based therapeutic and diagnostic approach currently employed against biotoxins pose major limitations such as the requirement of a live animal for the *in vivo* enrichment of the antibody species, decreased stability, high production cost, and side effects. Aptamer technology is a viable alternative that can be used to combat these problems. Fully sequestered *in vitro*, aptamers eliminate the need for a living host. Furthermore, one of the key advantages of using aptamers instead of antibodies is that they can be selected against very weakly immunogenic and cytotoxic substances. In this review, we focus on nucleic acid aptamers developed against various biotoxins of plant, microorganism, or animal origin and show how these can be used in diagnostics (e.g., biosensors) and therapy.

Introduction

Biotoxins, ranging from small molecules to macromolecular proteins, are commonly produced by living organisms (Proft, 2009). The occurrence of high levels of toxins in one's body poses significant risk that could ultimately lead to the death of the victim. This growing public health problem demands innovative and convenient technologies for detecting the levels of these toxins rapidly in order to fast-track the treatment process. Different molecular characteristics of the toxins have made it difficult to rely on a particular method for toxin detection. However, the method of choice that is frequently employed is based on immunoassays, which are time consuming and susceptible to temperature deviations. Although the currently available methodologies are sensitive and selective, mainly by using antibodies, there is still a need for simple, rapid, and cost-effective alternatives for toxin detection. Antibodies have been the gold standard in recognizing toxins for several decades. One limitation to traditional antibody-mediated toxin recognition is the dependence on *in vivo* enrichment of the antibody species. The introduction of a toxin into the bloodstream of any living animal creates an ethical problem that morally obligates us to seek alternative methods for producing life-saving medicines. Furthermore, antibody-based approach also constitutes an expensive laborious procedure for producing antibodies. In addition to possessing short shelf lives, the batch-to-batch reproducibility of the antibodies can often be less than satisfactory. Nucleic acid aptamers are another recent class of molecules that can be used as an alternative to the conventional antibody-mediated toxin detection and therapy.

Aptamers are short single-stranded RNA or DNA oligonucleotides that can bind to a target with very high affinity and specificity (Ellington and Szostak, 1990; Tuerk and Gold, 1990; Jayasena, 1999; Hermann and Patel, 2000; Nimjee et al., 2005; Famulok et al., 2007). In solution, the nucleotides form intramolecular interactions that fold the molecule into a complex 3-dimensional shape that allows the aptamer to bind tightly to the target molecule (Fig. 1). Aptamers can be developed for various targets with structures ranging from small molecules to complex proteins. Aptamer technology can be applied in diagnostics over therapeutics to nanotechnology. Aptamers are generated by a process referred to as systematic evolution of ligands by exponential enrichment (SELEX, Fig. 2) (Klug and Famulok, 1994; Gopinath, 2007; Stoltenburg et al., 2007). Although aptamers serve a function similar to that of antibodies in molecular recognition, they do posses several advantages. One distinct advantage is that they can be chemically synthesized and modified to improve nuclease resistance and delivery efficacy. With a significantly low production cost compared with antibodies, aptamers can be selected and identified by screening very large libraries (ca 10^{15} unique members) with different conformations, and the selection can be optimized to generate aptamers that suit specific needs and applications. Due to their small size, aptamers can avoid immunological responses and show favorable biodistribution (Healy et al., 2004; Nimjee et al., 2005).

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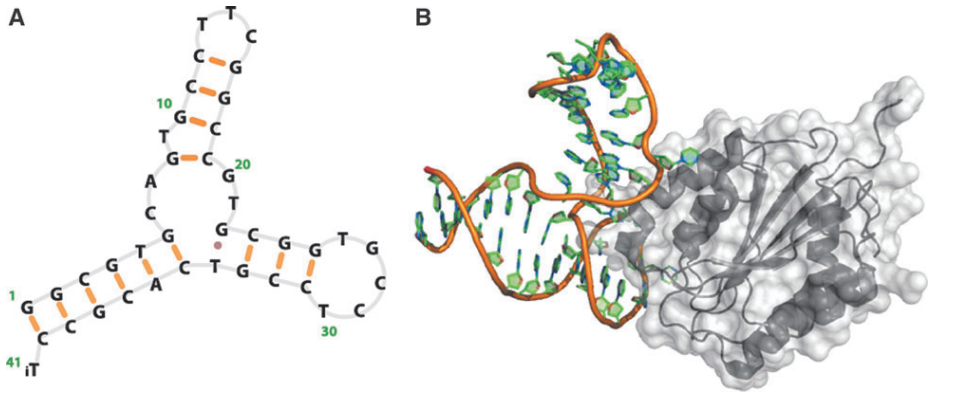

FIG. 1. Structural representations of a nucleic acid aptamer bound to its specific target. The exemplified structure shows **(A)** secondary structure of the ARC1172 aptamer and **(B)** the ARC1172 aptamer bound to Von Willebrandt Factor (Huang et al., 2009). Data were obtained from the Protein Data Bank; pdb ID: 3HXO and redrawn using Pymol software.

In this review, we focus on nucleic acid aptamers developed against various toxins of plant, microorganism, or animal origin and their usefulness in diagnostic and therapeutic development.

Bacterial Toxins

Botulinum neurotoxin

Clostridium botulinum produces one of the most potent neurotoxins known to man (Cartee and Monheit, 2011). Botulinum neurotoxins (BoNTs) are characterized by a heavy 100 kDa amino acid (aa) chain that is involved in the internalization of the toxin into neurons. The heavy chain (Hc) is linked by a disulphide bond to a 50 kDa light chain (Lc)

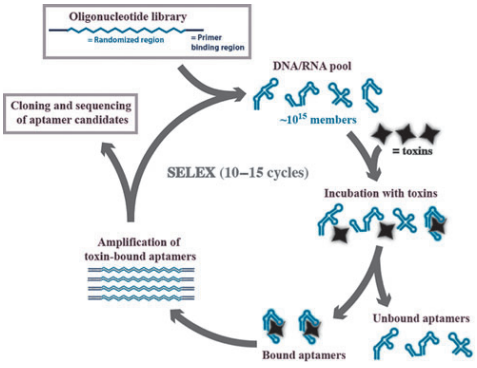

FIG. 2. SELEX technology principle. A chemically synthesized combinatorial oligonucleotide library pool is subjected to incubation with a toxin target; the binding motifs are then separated and then enzymatically amplified and enriched. After 10–15 rounds of enrichment, the individual aptamers are identified by sequencing.

zinc endopeptidase that cleaves soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins synaptosomal-associated protein 25 (SNAP-25), syntaxin, and synaptobrevin. This prevents vesicle docking and acetylcholine release at the neuromuscular junction leading to severe flaccid respiratory paralysis. Although the toxin is widely used for treating dermal wrinkles, it also has a reported Please expand abbreviated term LD_{50} LD_{50} of around 1 ng/kg, making it one of the most potent bacterial toxins (Gill, 1982). Current treatment relies on respiratory support and treatment with antitoxin, a polyclonal mixture of antibodies isolated from a host injected with toxin. No treatments exist for the reversal of the flaccid muscle paralysis caused by BoNTs. The current diagnostic tools include mouse bioassay for sybtyping BoNTs, which can take several days, or immunaffinty assays that take up to several hours for obtaining a measurable readout (Ferreira et al., 2003). A mathematical modeling of the deliberate release of small amounts of BoNTs in a single milk-processing facility estimated that several hundred thousand casualties could be observed in a matter of days (Wein and Liu, 2005).

Tok and Fisher (Tok and Fischer, 2008) first reported a nucleic acid aptamer developed against Botulinum toxin. They used aldehyde-inactivated toxin and a short peptide fragment (aa 1177–1195) of the heavy chain in a single microbead-based selection assay. This method used only a single target-functionalized bead for the aptamer selection. The aptamers obtained by this approach bound the toxoid with low nanomolar affinities ($K_d = 3$ nM) and the peptide fragment with low micromolar affinity ($K_d = 1.09$ μM). The aptamers for the Hc fragment (aa 1177–1195) competed with commercially available antibodies for the same epitope. It was also argued that this approach yielded aptamers in a single round since there was no observed change in bulk K_d measured by fluorescence anisotropy (Tok and Fischer, 2008). Recently, the aptamers generated by Tok and Fisher have been used in a lab-on-a-chip device combining antibodies and aptamers in an electrochemical detection system. As the aptamer binds the BoNT toxoid, it folds up and exposes a fluorescein tag that is recognized by a fluorescein antibody conjugated to

horseradish peroxidase. The measured signal allowed the detection of toxoid concentrations down to 40 pg/mL within minutes (Wei and Ho, 2009; Lillehoj et al., 2010; Wei et al., 2011). Later, Fan et al. published a novel method for selecting DNA aptamers against toxins. Using double-stranded DNA libraries of different lengths labeled with a quencher and a fluorophor, they selected aptamers against BoNT A light chain (Fan et al., 2008a). Very recently Bruno et al. performed a DNA SELEX experiment using BoNT A Lc funtionalized tosyl activated beads, and 60 DNA aptamer sequences were identified against BoNT subtype A light chain with binding affinities ranging from μM to nM (Bruno et al., 2012). Bruno et al. further performed an on-site quantification of BoNT A Lc using a Quantifluor™ handheld fluorometer. The reported detection limit was 1 ng/mL (Bruno et al., 2012), which was lower than the reported limit in the previously mentioned mathematical modelings by Wein and Liu (Wein and Liu, 2005).

To our knowledge no DNA aptamers have been evaluated for their therapeutic efficacy. However, the high binding affinities reported toward the light chain subdomain of BoNT indicate the potential for endopeptidase inhibition. On the other hand, using an automated selection protocol, 3 RNA aptamers were developed against Botulinum neurotoxin subtype A (Chang et al., 2010). The RNA aptamers selected against the light chain of BoNT A were able to bind and inhibit endopeptidase activity of Lc BoNT with dissociation constant (K_d) and half maximal inhibitory concentration (IC_{50}) values in the low nanomolar range. Enzyme kinetics and *in silico* docking studies confirmed that one of the aptamers exhibited noncompetitive inhibition of function, while the remaining two showed noncompetitive inhibition of enzyme function (Chang et al., 2010).

Anthrax toxin

Anthrax is an acute infection caused by the spore-forming gram-positive bacterium *Bacillus anthracis.* The pathogenicity of the bacterium is mainly due to the secretion of a tripartite protein complex referred to as the anthrax toxin. The toxin consists of a lethal factor (LF), an edema factor (EF) and a cell-binding protein component called the protective antigen (PA). PA binds to cell membrane receptors and is sub-sequentially cleaved to form a 63-kDa active species (PA63) that causes heptamerisation of PA63/receptor complexes. This cell-membrane complex is able to bind the remaining 2 enzymatic components (LF and EF) of the anthrax toxin protein with very high binding affinity (1–2 nM) and initiate internalization. In the cytosol, EF catalyzes the conversion of adenosine-5′-triphosphate to cyclic adenosine monophosphate (cAMP,) changing the water homeostasis and inhibiting macrophage function, while LF proteolytically cleaves certain mitogen-activated protein kinases, leading to apoptosis (Young and Collier, 2007). Therefore, aptamers binding to any of these toxin components may disrupt the interactions leading to the onset of anthrax or could be used in diagnostic applications.

The first aptamer against PA was a 2′-fluoro-RNA aptamer developed by Archemix (Wilson et al., 2004). Nitrocellulose filter selection was used to enrich aptamers binding the c-terminal domain with K_d values of around 500 nM (Wilson et al., 2004). Another 4 aptamers were generated using ultrafiltration as the means of separation. These 30-mer DNA aptamers were generated against the 63-kDa fragment of PA. The PA63 aptamers had an apparent K_d vlaue of 1–120 nM depending affinity assay. The aptamers shared a common 8-mer consensus 5′-d(CCGTAAGG)-3′. When truncated, the 8-mer exhibited an apparent K_d value of 1.99 nM but failed to display specificity towards PA63 as the K_d value was equally low (high affinity) for bovine serum albumin (BSA). The structure of the 30-mer exhibiting the highest affinity showed no prominent secondary structure. In the same year, Oh et al. tested the efficacy of two DNA aptamers reported by Choi et al. by a fluorescence-based assay (Choi et al., 2011; Oh et al., 2011). The tested aptamers facilitated the detection of 1 nM PA.

Staphylococcal enterotoxin B

Staphylococcal enterotoxins (SE, 23 to 29 kDa) produced by *Staphylococcus aureus* bind the major histocompatibility complex class II receptors with a K_d of 10–100 nM and crosslink to human leukocyte antigens (HLA-DR) or HLA-DQ and T-cell receptors (Herman et al., 1991). The SE toxins are classified as super antigens because of the fact that even picomolar concentrations can induce nonspecific oligoclonal activation of T-cells and massive cytokine release, without having to be internalized and presented via normal immune response mechanisms (Misfeldt, 1990; Herman et al., 1991). This results in profound inflammation of the gastrointestinal tract leading to vomiting, nausea, and cramping, often within a few hours of exposure. In some cases, the infection with *Staphylococcus aureus* can lead to allergic and autoimmune response, even toxic shock syndrome with a previously reported mortality rate of over 50 % (Crass and Bergdoll, 1986). Staphylococcal enterotoxin B (SEB) has been investigated thoroughly as a biowarfare agent, especially in 1960s. (Krakauer, 1999). One incident reported that the exposure of 15 individuals while testing the aerosolized SEB on monkeys, which ultimately ended up hospitalizing nine people.

In 2002, Bruno and Kiel used a magnetic bead-based aptamer selection approach to evolve DNA aptamers against SEB (Bruno and Kiel, 2002). Using the double-stranded DNA pool generated after 5 rounds of SELEX, they could detect SEB down to less than 10 pg using electrochemiluminescence with $Ru(bpy)_3^{2+}$. Using the enriched aptamer pool directly after *in vitro* selection for further assays is a rare procedure in aptamer development. The authors reasoned that this constituted the oligonucleotide equivalent of polyclonal antibodies used in antisera. We believe that this may be useful also in biosensors that normally use only one aptamer sequence, although several candidates that might have affinity to different regions on the same target are provided by the SELEX experiments. Purschke et al. reported a DNA aptamer (spiegelmer) against SEB (Purschke et al., 2003). The selection was conducted with a 25-mer truncation of the full-length (239 aa) SEB protein synthesized by solid-phase chemistry using only L-amino acids. The resulting mirror image of the SEB epitope was used in a magnetic bead-based selection approach resulting in DNA aptamers binding to the mirror image peptide. Resynthesizing the DNA aptamer with L-nuceotides yielded an L-DNA aptamer (spiegelmer) binding full-length SEB with a K_d of 420 nM, proving that the so-called spiegelmers can also be selected against larger proteins using carefully selected truncated peptide sequences. Very recently, a DNA aptamer against SEB has been reported by Degrasse

(Degrasse, 2012). The single-stranded DNA was reported to be able to precipitate SEB from a highly complex mixture of closely related enterotoxins (Degrasse, 2012).

Shiga toxin

Shiga toxin is produced by the *Shigella dysenteriae* toxin that shares many of the same pathological feats as the plant-derived ricin toxin (Spooner and Lord, 2012). Shiga toxin induced diarrhea-associated hemolytic uremic syndrome mainly affects the function of the kidneys and causes severe renal failure (Obrig and Karpman, 2012). Shiga toxins can also be found in some mutant cell lines of *Escherichia coli*, which posses a potential for extensive intoxication of humans due to their increased virulence (Hunt, 2010; Mora et al., 2011).

Fan et al. also reported an aptamer sequence for Shiga toxin. The Shiga toxin aptamer was used in a sensing system by employing double-stranded DNA labeled with a quencher and a fluorophor to detect Shiga toxin (Fan et al., 2008a). The sensitivity of the Shiga toxin aptamer was not tested but showed cross-reactivity with *Bacillus anthracis* spores and *Francisella tularensis* bacteria.

Cholera toxin

Cholera is caused by an infection with the toxigenic *Vibrio cholerae*. A life-threatening diarrheal disease is caused by both O1 and O139 serogroups of the bacterium producing the potent oligomeric protein named cholera toxin (Mandal et al., 2011). Cholera toxin causes the hypersecretion of electrolytes and fluid by interacting with intestinal mucosal cells and subsequent internalization resulting in elevated intracellular cAMP levels. The heightened levels of cAMP cause secretion of chloride and bicarbonate that draw water out into the small intestine (Mandal et al., 2011). Bruno and Kiel reported a DNA aptamer against cholera toxin in the same article about SEB aptamer development (Bruno and Kiel, 2002). By performing similar detection methodology as for SEB aptamers, they observed that the detection limit of the developed cholera toxin aptamer was between 10 and 40 ng (Bruno and Kiel, 2002). However, the sequence information of the developed aptamer was not disclosed in the article.

Mycotoxins

Ochratoxin A

Ochratoxin A (OTA) is a well-studied mycotoxin as a target for aptamer selection. In 2008, Cruz-Aguado and Penner reported the first aptamer-based mycotoxin detection system based on fluorescence polarization (Cruz-Aguado and Penner, 2008a). They selected a DNA aptamer that bound to OTA with a dissociation constant of 49 nM. Washing with different related compounds and wheat extract yielded an aptamer that bound specifically to OTA. The binding of the aptamer was closely associated with the concentration of divalent cations. Interestingly, changing 5 mM Mg^{2+} of the selection buffer to 10 mM Ca^{2+} resulted in a 4-fold increase in the affinity. Furthermore, the binding affinity did not change significantly with the complete removal of sodium or potassium ions, indicating no reliance on ionic strength of the assay solution (Cruz-Aguado and Penner, 2008a). The aptamer has been used to purify OTA from grain preparations (Cruz-Aguado and Penner, 2008b). Cruz-Aguado and Penner also reported that the aptamer was efficient enough to measure as low as 5 nM OTA using fluorescent polarization technique. The same aptamer has become a sort of gold-standard for many different OTA applications such as cyclic voltammetry-based detection assays (Kuang et al., 2010), electrochemiluminescent biosensor (Wang et al., 2010), colorimetric biosensors (Yang et al., 2011), fluorescent chromatographic strips (Wang et al., 2011a; Wang et al., 2011b), column extraction (Chapuis-Hugon et al., 2011), magnetic bead extraction (Wu et al., 2011b), electrochemical biosensors (Bonel et al., 2011), graphene biosensors (Sheng et al., 2011), luminescent nanoparticle assays (Wu et al., 2011b), impedimetric sensors (Prabhakar et al., 2011), exonuclease-assisted electrochemical detection (Tong et al., 2011), carbon nanotubes (Guo et al., 2011), fluorescence polarization (Kidd et al., 2011), DNAzyme biosensing (Yang et al., 2012), and rolling circle amplification aptasensing (Tong et al., 2012). Table 1 shows the listing of OTA aptamer applications and the achieved limit of detection (LOD) for all of the assays. The aptamers developed by Cruz-Aguado and Penner performed equally well as compared with the antibodies (Barthelmebs et al., 2011).

Fumonisin B1

Fumonisin B_1 (FB_1) produced by the fungus *Fusarium verticillioides* is a nephrotoxic, neurotoxic and hepatotoxic molecule that recently also has been attributed as a carcinogen in humans (Stockmann-Juvala and Savolainen, 2008). The toxin was first isolated and described from moldy corn. The fumonisins consist of 15 different subtypes grouped into 4 categories (A, B, C, and P), but the most prevalent of all fumonisins is B_1. Fumonisin B_1 aptamers have been developed by McKeague et al. in 2010 using FB_1 immobilized magnetic beads. Eighteen rounds of SELEX provided aptamers that bound FB_1 with affinities around 100 nM (McKeague et al., 2010).

Plant Toxins

Ricin toxin

Ricin is a potent toxin of the castor oil plant, which is isolated from the castor beans. A small dose of 500 μg is enough to kill an adult (Musshoff and Madea, 2009). Ricin toxin is a glycosylated heterodimer of 2 protein chains linked together with a disulfide bond. Ricin A chain (RTA) is a 32-kDa N-glycoside hydrolase that depurinates 28S RNA at position A4324. The position is called the sarcin-ricin loop and is important for the binding of elongation factors during protein synthesis. Hence, the loss of adenine moiety from position 4324 quickly and irreversibly abolishes ribosome function (Montanaro et al., 1973; Sperti et al., 1973). Ricin-filled metallic pellets discharged from the tip of an umbrella have been used as weapon in at least 8 assassinations attempts in history (Christopher et al., 1997). Although not always an effective toxin, the need for quick detection and therapeutic measures has signaled more research and development. Ricin sensing has been achieved in animal models (Clarke, 1953), antibodies (Poli et al., 1994; Narang et al., 1997; Delehanty and Ligler, 2002; Shyu et al., 2002; Ligler et al., 2003) and by mass spectroscopy (Darby et al., 2001). The development of novel ricin toxin antibodies is still a growing field in biosensing.

Given the strong history and use as harmful agent, ricin has become the most investigated plant toxin for aptamer

Table 1. Summary of Biotoxin Aptamers Developed in Recent Years

Target	*Reference*	*Aptamer*	K_d	*Detection Assay*	*LOD*
Bacterial toxins					
Botulinum	(Bruno et al., 2012)	RNA/2 (60*)	-	Fluorescence	1 ng/mL
	(Tok and Fischer, 2008)	DNA/8*	3 nM	-	-
	(Wei and Ho, 2009; Lillehoj et al., 2010; Wei et al., 2011)	Using aptamers from (Tok and Fischer, 2008)		Electrochemical	40 pg/mL
Anthrax	(Wilson et al., 2004)	DNA/150	500 nM		
	(Choi et al., 2011)	DNA/4	1 nM		
	(Oh et al., 2011)	Using aptamers from (Choi et al., 2011)		Fluorescence	1 nM
Enterotoxin B	(Bruno and Kiel, 2002)			Electrochemiluminescence	44 pg/mL
	(Purschke et al., 2003)	D-DNA/7	200 nM		
	(Degrasse, 2012)	DNA/1 (2*)	-		
Cholera	(Bruno and Kiel, 2002)			Electrochemiluminescence	176 ng/mL
Shiga Toxin	(Fan et al., 2008a)	DNA/1	-		
Plant toxins					
Ricin A	(Hesselberth et al., 2000)	RNA/5 (24)	7.3 nM		
	(Kirby et al., 2004)	Using aptamers from (Hesselberth et al., 2000)		Fluorescence	320 ng/mL
	(Cho et al., 2006)	Do.		Fluorescence	500 pg/mL
	(Haes et al., 2006)	Do.		Fluorescence	28 ng/mL
	(Tang et al., 2006)	DNA/31	58 nM		
	(Wang et al., 2012)	Using aptamers from (Tang et al., 2006)		Force spectroscopy	100 fM
Ricin B	(Lamont et al., 2011)	DNA/4	5 nm	Fluorescence	25 ng/mL
Abrin	(Tang et al., 2007)	DNA/8	28 nM	Colorimetric	1 nM
Mycotoxins					
Fumonisin B_1	(McKeague et al., 2010)	DNA/6	100 nM		
Ochratoxin A	(Cruz-Aguado and Penner, 2008a)	DNA/13	360 nM		
	(Cruz-Aguado and Penner, 2008b)	Using aptamers from (Cruz-Aguado and Penner, 2008a)		Fluorescence anisotropy	5 nM
	(Kuang et al., 2010)	Do.		Electrochemical	30 pg/mL
	(Wang et al., 2010)	Do.		Electrochemiluminescence	7 pg/mL
	(Yang et al., 2011)	Do.		Colorimetric	20 nM
	(Wang et al., 2011a)	Do.		Fluorescence	1.9 ng/mL
	(Wang et al., 2011b)	Do.		Fluorescence	1 ng/mL
	(Bonel et al., 2011)	Do.		Electrochemical	70 pg/mL
	(Sheng et al., 2011)	Do.		Fluorescence	21.8 nM
	(Wu et al., 2011a)	Do.		Luminescent nanoparticle	0.1 pg/mL
	(Prabhakar et al.)	Do.		Impedance	0.1 ng/mL
	(Tong et al., 2011)	Do.		Electrochemical	1 pg/mL
	(Guo et al., 2011)	Do.		Fluorescence	24.1 nM
	(Kidd et al., 2011)	Do.		Fluorescence polarization	
	(Yang et al., 2012)	Do.		Colorimetric	2.5 nM

The table gives a comparative overview of biotoxin aptamers and biosensor performance from references cited in this review. (* indicates sequences not shown in reference-article.)
K_d, dissociation constant; Do., ditto; LOD, limit of detection.

development. Ellington and co-workers first reported an RNA aptamer against the RTA domain (Hesselberth et al., 2000). The structure of an RNA aptamer reported by Hesselberth et al. has been used as a recognition tool in ricin-sensing applications (Hesselberth et al., 2000). Kirby et al. used another aptamer for its application in an electronic tongue array (Kirby et al., 2004). This assay consisted of a bead bound aptamer that captured ricin and was subsequently stained with anti-ricin antibody. This sandwich approach allowed for a LOD of 320 ng/mL. Remarkably, the beads could be stripped of ricin protein by denaturing aptamers with 7 M UREA buffer and reused for sensing (Kirby et al., 2004). The same group later published another setup in 2006 by utilizing glass slides coated with streptavidin or neutravidin to immobilize ricin aptamers. The assay was dependent on labeling of protein with a fluorescent dye and detected ricin in concentrations as low as 500 pg/μL (Cho et al., 2006). In 2006, Tang et al. developed two anti-ricin aptamers using two different selection approaches (Tang et al., 2006). They found that the 2 different methods provided high affinity binders with the best candidates having surprisingly high sequence similarity. Subsequent single molecule interaction studies have verified that the DNA aptamer had a slightly higher affinity, than the commercially available antibody (Wang et al., 2012). Superior *in vivo* stability was the key motivation for developing DNA aptamer instead of an RNA aptamer by Tang et al. However, Haes et al. demonstrated that a previously known RNA aptamer was able to sense ricin toxin down to 28 ng/μL in samples spiked with RNAse A (Haes et al., 2006).

In 2008, the RTA aptamer developed by the Hesselberth et al. was used in an *in vivo* study to test the inhibitory effect compared to the antibody against deglycosylated RTA (pRTA-IgG) (Fan et al., 2008b). They found that 200 ng/μL ricin aptamer reduced cell death from 80% to around 50%. The pRTA-IgG antibody generally performed better in the cell-death assays under the conditions used (Fan et al., 2008b). Single molecule recognition of ricin has also been achieved with the RNA aptamer dropping the detection limit to 100 fM. Interestingly, this unlabeled approach allowed measuring the actual interaction time between 2 ricin molecules binding to the same aptamer (i.e., time between binding, release, and rebinding). Ricin concentration was estimated from the interaction time as it was observed that the interval between first and second binding decreased from 35 to 2.6 minutes when ricin concentration was increased from 2.8 nM to 2.8 μM (Wang et al., 2012).

The structure of a DNA aptamer against ricin B-chain is recently published (Lamont et al., 2011). This aptamer was selected using tosyl-activated beads and after 8 rounds of selection, they identified that 93 out of 100 selected clones had the same sequence which is remarkable. The aptamer was used for sensing ricin toxin in juice and milk at a pH ranging from 2–7. In contrast, the commercially available enzyme-linked immunosorbent assay is performed only at neutral pH (Lamont et al., 2011).

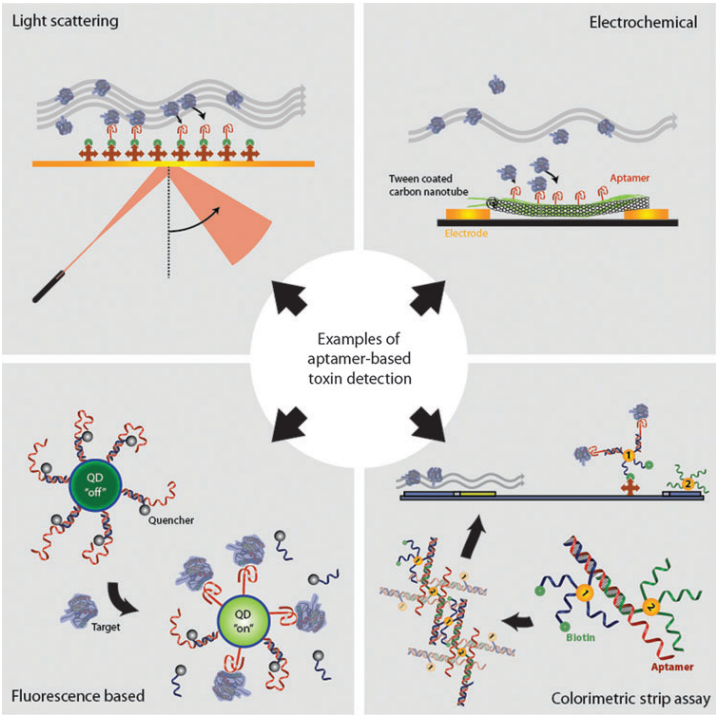

FIG. 3. Aptamer-based toxin detection assays. This figure was drawn by following the previously published assays (Guo et al., 2011; Wang et al., 2011a; Wang et al., 2011b).

Abrin toxin

Abrin is a class-2 ribosome inactivating protein. Abrin toxin uses the same pathogenic pathway as ricin toxin, however, ricin is 75 times more effective at depurinating A4324 (Dickers et al., 2003; Olsnes, 2004). Like ricin, abrin also possesses an enzymatically active A chain and a receptor-binding B chain. Abrin has been heavily investigated as a chemotherapeutic agent and also as a biological warfare agent. Tang et al. reported a DNA aptamer against abrin toxin (Tang et al., 2007). Since abrin features a galactose binding site, for aptamer development, it was immobilized on a galactose functionalized agarose beads. After 8 rounds of selection eight clones were sequenced. All 8 aptamer sequences had affinity to abrin in the low nanomolar range (28–130 nM). Using $[Ru(phen)2(dppz)]^{2+}$ as an intercalator allowed studying the binding of abrin. One truncated version showed concentration-dependent reduction in chemiluminescence of $[Ru(phen)2(dppz)]^{2+}$ upon binding. The assay facilitated the detection of a low amount of abrin toxin in complex serum samples and showed no cross-reactivity towards ricin or BSA (Tang et al., 2007).

Prospects of Biotoxin Aptamers

High levels of the above-mentioned biotoxins in humans can cause severe health problems that necessitate innovative detection technologies with high specificity and sensitivity. Table 1 highlights the development of aptamers against major biotoxins and their detection platforms. The existing traditional antibody-based methods are also selective and sensitive to some extent, however, there is still a need for simpler, more rapid and cost-effective approaches to produce the accurate results. Aptamer-based biosensor platforms generally show high sensitivities and specificities that could allow them to be competitive with existing detection methods. In Fig. 3, we summarized 4 efficient aptamer-based methods for rapid diagnosis of biotoxins. The performance of the developed aptamers under real-world conditions are yet to be seen although several of these existing aptamer platforms have been tested in various *in vitro* assays.

Conclusion

Nucleic acid aptamers can be viewed as molecular probes with a range of advantages that sometimes make them superior to traditional antibody-based technologies. In this report we have presented aptamers selected against naked toxins produced by living organisms. The presented aptamers are mainly used as biosensors for toxin detection, and their therapeutic efficacies have largely been unexplored. As aptamers are increasingly used in biosensor platforms and assays, we firmly believe that their therapeutic usefulness will also follow. Many of the aptamers presented herein will have direct applicability *in vivo* therapeutic assays and could potentially be used as therapeutic agents. With patent rights ending and more commercial ventures seeking to exploit the obvious advantages of aptamers as therapeutics and affinity ligands, aptamers will become increasingly present and hopefully live up to their predicted potential.

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Author Disclosure Statement

No competing financial interests exist.

References

BARTHELMEBS, L., JONCA, J., HAYAT, A., PRIETO-SIMON, B., and MARTY, J.L. (2011) Enzyme-linked aptamer assays (ELAAs), based on a competition format for a rapid and sensitive detection of Ochratoxin A in wine. Food Control **22,** 737–743.

BONEL, L., VIDAL, J.C., DUATO, P., and CASTILLO, J.R. (2011). An electrochemical competitive biosensor for ochratoxin A based on a DNA biotinylated aptamer. Biosens. Bioelectron. **26,** 3254–3259.

BRUNO, J.G., and KIEL, J.L. (2002). Use of magnetic beads in selection and detection of biotoxin aptamers by electrochemiluminescence and enzymatic methods. Biotechniques **32,** 178–180, 182–173.

BRUNO, J.G., RICHARTE, A.M., CARRILLO, M.P., and EDGE, A. (2012). An aptamer beacon responsive to botulinum toxins. Biosens. Bioelectron. **31,** 240–243.

CARTEE, T.V., and MONHEIT, G.D. (2011). An overview of botulinum toxins: past, present, and future. Clin. Plast. Surg. **38,** 409–426, vi.

CHANG, T.W., BLANK, M., JANARDHANAN, P., SINGH, B.R., MELLO, C., BLIND, M., and CAI, S. (2010). *In vitro* selection of RNA aptamers that inhibit the activity of type A botulinum neurotoxin. Biochem. Biophys. Res. Commun. **396,** 854–860.

CHAPUIS-HUGON, F., DU BOISBAUDRY, A., MADRU, B., and PICHON, V. (2011). New extraction sorbent based on aptamers for the determination of ochratoxin A in red wine. Anal. Bioanal. Chem. **400,** 1199–1207.

CHO, E.J., COLLETT, J.R., SZAFRANSKA, A.E., and ELLINGTON, A.D. (2006). Optimization of aptamer microarray technology for multiple protein targets. Anal. Chim. Acta **564,** 82–90.

CHOI, J.S., KIM, S.G., LAHOUSSE, M., PARK, H.Y., PARK, H.C., JEONG, B., KIM, J., KIM, S.K., and YOON, M.Y. (2011). Screening and characterization of high-affinity ssDNA aptamers against anthrax protective antigen. J. Biomol. Screen. **16,** 266–271.

CHRISTOPHER, L.G.W., CIESLAK, L.T.J., PAVLIN, J.A., EITZEN, E.M., (1997) Biological Warfare. JAMA, J. Am. Med. Assoc. **278,** 412–417.

CLARKE, E.G. (1953). The detection of ricin. J. Pharm. Pharmacol. **5,** 458.

CRASS, B.A., and BERGDOLL, M.S. (1986). Involvement of staphylococcal enterotoxins in nonmenstrual toxic shock syndrome. J. Clin. Microbiol. **23,** 1138–1139.

CRUZ-AGUADO, J.A., and PENNER, G. (2008a). Determination of ochratoxin a with a DNA aptamer. J. Agric. Food Chem. **56,** 10456–10461.

CRUZ-AGUADO, J.A., and PENNER, G. (2008b). Fluorescence polarization based displacement assay for the determination of small molecules with aptamers. Anal. Chem. **80,** 8853–8855.

DARBY, S.M., MILLER, M.L., and ALLEN, R.O. (2001). Forensic determination of ricin and the alkaloid marker ricinine from castor bean extracts. J. Forensic Sci. **46,** 1033–1042.

DEGRASSE, J.A. (2012). A single-stranded dna aptamer that selectively binds to *Staphylococcus aureus* enterotoxin B. PLoS One **7,** e33410.

DELEHANTY, J.B., and LIGLER, F.S. (2002). A microarray immunoassay for simultaneous detection of proteins and bacteria. Anal. Chem. **74,** 5681–5687.
DICKERS, K.J., BRADBERRY, S.M., RICE, P., GRIFFITHS, G.D., and VALE, J.A. (2003). Abrin poisoning. Toxicol. Rev. **22,** 137–142.
ELLINGTON, A.D., and SZOSTAK, J.W. (1990). *In vitro* selection of RNA molecules that bind specific ligands. Nature **346,** 818–822.
FAMULOK, M., HARTIG, J.S., MAYER, G., (2007) Functional aptamers and aptazymes in biotechnology, diagnostics, and therapy. Chem. Rev. **107,** 3715–3743.
FAN, M., MCBURNETT, S.R., ANDREWS, C.J., ALLMAN, A.M., BRUNO, J.G., and KIEL, J.L. (2008a). Aptamer selection express: a novel method for rapid single-step selection and sensing of aptamers. J. Biomol. Tech. **19,** 311–319.
FAN, S., WU, F., MARTINIUK, F., HALE, M.L., ELLINGTON, A.D., and TCHOU-WONG, K.M. (2008b). Protective effects of anti-ricin A-chain RNA aptamer against ricin toxicity. World J. Gastroenterol. **14,** 6360–6365.
FERREIRA, J.L., MASLANKA, S., JOHNSON, E., and GOODNOUGH, M. (2003). Detection of botulinal neurotoxins A, B, E, and F by amplified enzyme-linked immunosorbent assay: collaborative study. J. AOAC Int. **86,** 314–331.
GILL, D.M. (1982). Bacterial toxins: a table of lethal amounts. Microbiol. Rev. **46,** 86–94.
GOPINATH, S.C. (2007). Methods developed for SELEX. Anal. Bioanal. Chem. **387,** 171–182.
GUO, Z., REN, J., WANG, J., and WANG, E. (2011). Single-walled carbon nanotubes based quenching of free FAM-aptamer for selective determination of ochratoxin A. Talanta **85,** 2517–2521.
HAES, A.J., GIORDANO, B.C., COLLINS, G.E., (2006) Aptamer-based detection and quantitative analysis of ricin using affinity probe capillary electrophoresis. Anal. Chem. **78,** 3758–3764.
HEALY, J.M., LEWIS, S.D., KURZ, M., BOOMER, R.M., THOMPSON, K.M., WILSON, C., and MCCAULEY, T.G. (2004). Pharmacokinetics and biodistribution of novel aptamer compositions. Pharm. Res. **21,** 2234–2246.
HERMAN, A., KAPPLER, J.W., MARRACK, P., and PULLEN, A.M. (1991). Superantigens: mechanism of T-cell stimulation and role in immune responses. Annu. Rev. Immunol. **9,** 745–772.
HERMANN, T., and PATEL, D.J. (2000). Adaptive recognition by nucleic acid aptamers. Science **287,** 820–825.
HESSELBERTH, J.R., MILLER, D., ROBERTUS, J., and ELLINGTON, A.D. (2000). In vitro selection of RNA molecules that inhibit the activity of ricin A-chain. J. Biol Chem. **275,** 4937–4942.
HUANG, R.H., FREMONT, D.H., DIENER, J.L., SCHAUB, R.G., and SADLER, J.E. (2009). A structural explanation for the antithrombotic activity of ARC1172, a DNA aptamer that binds von Willebrand factor domain A1. Structure **17,** 1476–1484.
HUNT, J.M. (2010). Shiga toxin-producing Escherichia coli (STEC). Clin. Lab. Med. **30,** 21–45.
JAYASENA, S.D. (1999). Aptamers: an emerging class of molecules that rival antibodies in diagnostics. Clin. Chem. **45,** 1628–1650.
KIDD, A., GUIEU, V., PERRIER, S., RAVELET, C., and PEYRIN, E. (2011). Fluorescence polarization biosensor based on an aptamer enzymatic cleavage protection strategy. Anal. Bioanal. Chem. **401,** 3229–3234.
KIRBY, R., CHO, E.J., GEHRKE, B., BAYER, T., PARK, Y.S., NEIKIRK, D.P., MCDEVITT, J.T., and ELLINGTON, A.D. (2004). Aptamer-based sensor arrays for the detection and quantitation of proteins. Anal. Chem. **76,** 4066–4075.
KLUG, S.J., and FAMULOK, M. (1994). All you wanted to know about SELEX. Mol. Biol. Rep. **20,** 97–107.
KRAKAUER, T., (1999) Immune response to staphylococcal superantigens. Immunol. Res. **20,** 163–173.
KUANG, H., CHEN, W., XU, D., XU, L., ZHU, Y., LIU, L., CHU, H., PENG, C., XU, C., and ZHU, S. (2010). Fabricated aptamer-based electrochemical "signal-off" sensor of ochratoxin A. Biosens. Bioelectron. **26,** 710–716.
LAMONT, E.A., HE, L., WARRINER, K., LABUZA, T.P., and SREEVATSAN, S. (2011). A single DNA aptamer functions as a biosensor for ricin. Analyst **136,** 3884–3895.
LIGLER, F.S., TAITT, C.R., SHRIVER-LAKE, L.C., SAPSFORD, K.E., SHUBIN, Y., and GOLDEN, J.P. (2003). Array biosensor for detection of toxins. Anal. Bioanal. Chem. **377,** 469–477.
LILLEHOJ, P.B., WEI, F., and HO, C.M. (2010). A self-pumping lab-on-a-chip for rapid detection of botulinum toxin. Lab Chip **10,** 2265–2270.
MANDAL, S., MANDAL, M.D., and PAL, N.K. (2011). Cholera: a great global concern. Asian Pac. J. Trop. Med. **4,** 573–580.
MCKEAGUE, M., BRADLEY, C.R., DE GIROLAMO, A., VISCONTI, A., MILLER, J.D., and DEROSA, M.C. (2010). Screening and initial binding assessment of fumonisin b(1) aptamers. Int. J. Mol. Sci. **11,** 4864–4881.
MISFELDT, M.L. (1990). Microbial "superantigens". Infect. Immun. **58,** 2409–2413.
MONTANARO, L., SPERTI, S., and STIRPE, F. (1973). Inhibition by ricin of protein synthesis in vitro. Ribosomes as the target of the toxin. Biochem. J. **136,** 677–683.
MORA, A., HERRRERA, A., LOPEZ, C., DAHBI, G., MAMANI, R., PITA, J.M., ALONSO, M.P., LLOVO, J., BERNARDEZ, M.I., BLANCO, J.E., et al. (2011). Characteristics of the Shiga-toxin-producing enteroaggregative *Escherichia coli* O104:H4 German outbreak strain and of STEC strains isolated in Spain. Int. Microbiol. **14,** 121–141.
MUSSHOFF, F., and MADEA, B., (2009). Ricin poisoning and forensic toxicology. Drug testing and analysis 1, 184–191.
NARANG, U., ANDERSON, G.P., LIGLER, F.S., and BURANS, J. (1997). Fiber optic-based biosensor for ricin. Biosens Bioelectron 12, 937–945.
NIMJEE, S.M., RUSCONI, C.P., and SULLENGER, B.A. (2005). Aptamers: An emerging class of therapeutics. Annu. Rev. Med. **56,** 555–583.
OBRIG, T.G., and KARPMAN, D. (2012). Shiga toxin pathogenesis: kidney complications and renal failure. Curr. Top. Microbiol. Immunol. **357,** 105–136.
OH, B.N., LEE, S., PARK, H.Y., BAEG, J.O., YOON, M.Y., and KIM, J. (2011). Sensitive fluorescence assay of anthrax protective antigen with two new DNA aptamers and their binding properties. Analyst **136,** 3384–3388.
OLSNES, S. (2004). The history of ricin, abrin and related toxins. Toxicon **44,** 361–370.
POLI, M.A., RIVERA, V.R., HEWETSON, J.F., and MERRILL, G.A. (1994). Detection of ricin by colorimetric and chemiluminescence ELISA. Toxicon **32,** 1371–1377.
PRABHAKAR, N., MATHARU, Z., and MALHOTRA, B.D. (2011). Polyaniline Langmuir-Blodgett film based aptasensor for ochratoxin A detection. Biosens. Bioelectron. **26,** 4006–4011.
PROFT, T., ed. (2009). Microbial Toxins: Current Research and Future Trends. (Caister Academic Press, Norfolk, U.K.), pp. 1–192.

PURSCHKE, W.G., RADTKE, F., KLEINJUNG, F., and KLUSSMANN, S. (2003). A DNA Spiegelmer to staphylococcal enterotoxin B. Nucleic Acids Res. **31,** 3027–3032.

SHENG, L., REN, J., MIAO, Y., WANG, J., and WANG, E. (2011). PVP-coated graphene oxide for selective determination of ochratoxin A via quenching fluorescence of free aptamer. Biosens. Bioelectron. **26,** 3494–3499.

SHYU, H.F., CHIAO, D.J., LIU, H.W., TANG, S.S., (2002) Monoclonal antibody-based enzyme immunoassay for detection of ricin. Hybrid Hybridomics **21,** 69–73.

SPERTI, S., MONTANARO, L., MATTIOLI, A., and STIRPE, F. (1973). Inhibition by ricin of protein synthesis in vitro: 60 S ribosomal subunit as the target of the toxin. Biochem. J. **136,** 813–815.

SPOONER, R.A., and LORD, J.M. (2012). How ricin and Shiga toxin reach the cytosol of target cells: retrotranslocation from the endoplasmic reticulum. Curr. Top. Microbiol. Immunol. **357,** 19–40.

STOCKMANN-JUVALA, H., and SAVOLAINEN, K. (2008). A review of the toxic effects and mechanisms of action of fumonisin B1. Hum. Exp. Toxicol. **27,** 799–809.

STOLTENBURG, R., REINEMANN, C., and STREHLITZ, B. (2007). SELEX: a (r)evolutionary method to generate high-affinity nucleic acid ligands. Biomol. Eng. **24,** 381–403.

TANG, J., XIE, J., SHAO, N., and YAN, Y., (2006). The DNA aptamers that specifically recognize ricin toxin are selected by two *in vitro* selection methods. Electrophoresis **27,** 1303–1311.

TANG, J., YU, T., GUO, L., XIE, J., SHAO, N., and HE, Z. (2007). *In vitro* selection of DNA aptamer against abrin toxin and aptamer-based abrin direct detection. Biosens. Bioelectron. **22,** 2456–2463.

TOK, J.B., and FISCHER, N.O. (2008). Single microbead SELEX for efficient ssDNA aptamer generation against botulinum neurotoxin. Chem. Commun. (Camb.) **16,** 1883–1885.

TONG, P., ZHANG, L., XU, J.J., and CHEN, H.Y. (2011). Simply amplified electrochemical aptasensor of ochratoxin A based on exonuclease-catalyzed target recycling. Biosens. Bioelectron. **29,** 97–101.

TONG, P., ZHAO, W.W., ZHANG, L., XU, J.J., and CHEN, H.Y. (2012). Double-probe signal enhancing strategy for toxin aptasensing based on rolling circle amplification. Biosens. Bioelectron. **33,** 146–151.

TUERK, C., and GOLD, L. (1990). Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science **249,** 505–510.

WANG, B., GUO, C., CHEN, G., PARK, B., and XU, B. (2012). Following aptamer-ricin specific binding by single molecule recognition and force spectroscopy measurements. Chem. Commun. (Camb.) **48,** 1644–1646.

WANG, L., CHEN, W., MA, W., LIU, L., ZHAO, Y., ZHU, Y., XU, L., KUANG, H., and XU, C. (2011a). Fluorescent strip sensor for rapid determination of toxins. Chem. Commun. (Camb.) 47, 1574–1576.

WANG, L., MA, W., CHEN, W., LIU, L., ZHU, Y., XU, L., KUANG, H., and XU, C. (2011b). An aptamer-based chromatographic strip assay for sensitive toxin semi-quantitative detection. Biosens. Bioelectron. **26,** 3059–3062.

WANG, Z., DUAN, N., HUN, X., and WU, S. (2010). Electrochemiluminescent aptamer biosensor for the determination of ochratoxin A at a gold-nanoparticles-modified gold electrode using N-(aminobutyl)-N-ethylisoluminol as a luminescent label. Anal. Bioanal. Chem. **398,** 2125–2132.

WEI, F., BAI, B., and HO, C.M. (2011). Rapidly optimizing an aptamer based BoNT sensor by feedback system control (FSC) scheme. Biosens. Bioelectron. **30,** 174–179.

WEI, F., and HO, C.M. (2009). Aptamer-based electrochemical biosensor for Botulinum neurotoxin. Anal. Bioanal. Chem. **393,** 1943–1948.

WEIN, L.M., and LIU, Y. (2005). Analyzing a bioterror attack on the food supply: the case of botulinum toxin in milk. Proc. Natl. Acad. Sci. U. S. A. **102,** 9984–9989.

WILSON, C., EPSTEIN, D., CLOAD, S.T., MARSH, N., and HAMAGUCHI, N. (2004). WO2004085665-A2 Nucleic acid ligand to B. anthracis protective antigen. (Archemix Corp, Cambrigde, MA), pp. 1–144.

WU, S., DUAN, N., WANG, Z., and WANG, H. (2011a). Aptamer-functionalized magnetic nanoparticle-based bioassay for the detection of ochratoxin A using upconversion nanoparticles as labels. Analyst **136,** 2306–2314.

WU, X., HU, J., ZHU, B., LU, L., HUANG, X., and PANG, D. (2011b). Aptamer-targeted magnetic nanospheres as a solid-phase extraction sorbent for determination of ochratoxin A in food samples. J. Chromatogr. A. **1218,** 7341–7346.

YANG, C., LATES, V., PRIETO-SIMÓN, B., MARTY, J.L., and YANG, X. (2012). Aptamer-DNAzyme hairpins for biosensing of Ochratoxin A. Biosens. Bioelectron. **32,** 208–212.

YANG, C., WANG, Y., MARTY, J.L., and YANG, X. (2011). Aptamer-based colorimetric biosensing of Ochratoxin A using unmodified gold nanoparticles indicator. Biosens. Bioelectron. **26,** 2724–2727.

YOUNG, J.A.T., and COLLIER, R.J. (2007). Anthrax toxin: receptor binding, internalization, pore formation, and translocation. Annu. Rev. Biochem. **76,** 243–265.

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