GENERATION OF APTAMERS AGAINST THE GANGLIOSIDE-BINDING PROTEINS VIA SINGLE BEAD SELEX

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Summary

Botulinum neurotoxins (BoNTs) are widely regarded as the “poison of all poisons”. BoNTs are built upon two primary domains, the light chain (LC) and the heavy chain (HC). The LC constitutes a zinc metalloprotease which cleave the SNARE proteins, whereas the HC domain transports the LC into the neuronal cytosol *via* the acidic endosome. Numerous researchers have explored the design of potent BoNTs inhibitors targeting the LC domain. However, in nearly all cases, low cell uptake or high cytotoxicity was limiting factors preventing their potential for in vivo applications. Due to their mechanism of action, inhibitors targeting the HC domain would not need to cross cell membranes in order to reach their targets since they would interfere with BoNTs in the blood stream and thus prevent BoNTs uptake. Up to now, no rational approach for the identification of HC inhibitors has been reported. Such an approach is also considered challenging due to the necessity to address both neuroselective binding sites with so far unknown small molecule inhibitors. Therefore, the present project is focused on the development of macromolecular aptamer inhibitors ideally targeting both binding sites of the HC domain.

Aptamers are particularly attractive as potential antibody replacements due to their high binding specificities and affinities and improved accessibilities and stabilities. We have chosen the SELEX process to generate novel aptamers against the ganglioside binding peptide or protein reflecting the native membrane binding sites from the HC domain of BoNT/A. A single bead SELEX technique has been established successfully in the course of this project by using the 19mer ganglisoside binding peptide from the amino acid

sequence at one of the essential membrane binding site (gangalioside binding site) from BoNT/A HC domain as the first target. Aptamers were found with about 0.8uM binding affinity to this peptide after 3 cycles of SELEX. After the peptide SELEX process has been successfully established, the ganglioside binding protein (*gsbp*), which is part of the HC domain containing both essential binding sites for BoNT/A membrane recognition was used as the final target. This protein contained the native, active conformation and was a particular challenge to identify the optimal conditions to maintain this conformation during the whole SELEX process. Therefore, the SELEX procedure was further modified and adjusted to this very sensitive protein which facilitated the identification of novel aptamers that were able to interact with the protein surface. Aptamer with 5uM binding affinity was successfully identified after 5 cycles of SELEX. These aptamers will be very promising candidates to interact with BoNT/A and they might serve as valuable tools to allow a first proof-of-concept study and assess the therapeutic potential of blocking BoNT/A cell uptake, which has not been explored yet. Therefore, such aptamers pave the way to therapeutic aptamers displaying higher binding affinities which might serve as an efficient treatment of botulism at the early stage. The ability of these aptamers to prevent BoNT/A uptake will be in the focus of future investigations.

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Chapter 1. Introduction

1.1 Introduction of aptamers

Aptamers are single strand oligonucleotides which can bind to a target with high specificity and affinity. This innovative biotechnology tool has been developed for only 20 years. In 1989, the Nobel Prize of Chemistry was awarded to Professor Sidney Altman and Professor Thomas Cech for their discovery of catalytic properties of RNA which revealed that nucleic acids are not only information-containing molecules but also can bind to targets such as proteins and initiate and catalyze biological processes[1]. Inspired by this discovery, professor Larry Gold[2] and professor Jack W. Szostak[3] independently introduced the approach for the *in vitro* selection of RNA molecules that in principle can specifically interact with basically any protein. Later, this technique was called SELEX (systematic evolution of ligands by exponential enrichment) and the RNA molecules selected by this method were named aptamers，which is derived from the Latin word ‘‘aptus’’—which means *fitting* and the Greek word “meros’’ meaning *part*[3]. Two years later, the successful selection of single-stranded DNA (ssDNA) aptamers was also demonstrated by Szostak’s group[4] which revealed improved stability compared with RNA aptamers. This facile and efficient method to achieve ligands displaying high affinity and specificity for various proteins attracted plenty of research interests immediately. The targets of aptamers have been explored rapidly and the SELEX technique has also been further developed and optimized since the early times of aptamer research.

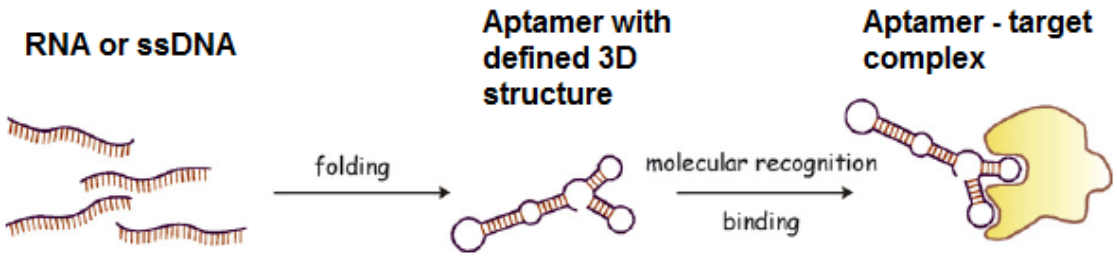


Figure. 1. Schematic representation of the architecture and binding capability of aptamers[5].

As short single-stranded oligonucleotides, aptamers can form complicated three-dimensional structures characterized by stems, loops, bulges, hairpins, pseudoknots, triplexes, or quadruplexes. Binding of aptamer to a target can attribute to multiple effects including the precise stacking of flat moieties such as aromatic rings, specific hydrogen bonding, and molecular shape complementarity with regard to electrostatic and van der Waals interactions[6]. Based on the large number of possible nucleotide sequences, the adopted structure of aptamers in principle can be diverse enough to bind to basically any biological target (Figure 1).

After the first report of aptamers binding to proteins, this principle was rapidly extended to many other targets including small bio-molecules, complexes and even the whole cells. In 1995, Szostak firstly reported an ATP-recognizing aptamer[7], and he even selected an aptamer with strong and specific recognition of the triphosphate of ATP in 2004[8]. After this discovery, many aptamers targeting small biomolecules have been reported, such as His-tag-specific aptamer[9], Coenzyme A aptamer [10], and mRNA aptamer[11]. Recently, the requirement for high cell specificity in cancer treatment encouraged the whole cell-based SELEX approach which generates aptamers recognizing a cancer cell

type in a highly specific manner [12, 13]. Cancer cell aptamers can selectively differentiate whole cancer cells from healthy. Compared with other known cancer makers such as e.g. folic acid receptors which are over expressed in cancer cells but to a lesser extent also present in normal cells, this technique paves the way to a personal cancer treatment with high specificity for the patient's tumor. These broad applications make aptamers technology particularly attractive for a broad range of applications in medicine but also in material science.

1.2 SELEX technique

After the first introduction of SELEX in 1990[2], numerous aptamers have been generated via this technique. Although many modern technologies were added to the SELEX process, the basic principle remained the same. A complete SELEX cycle involves three main processes: Incubation of the oligonucleotides pool with a target to allow binding; partitioning of binding aptamers from non-binding aptamers; and amplification of bound aptamers[14]. After the amplification, a new aptamer pool with preliminary affinity to the target will be obtained and this pool is used again for target binding (Figure 2). A successful SELEX normally involves 10 to 20 cycles.

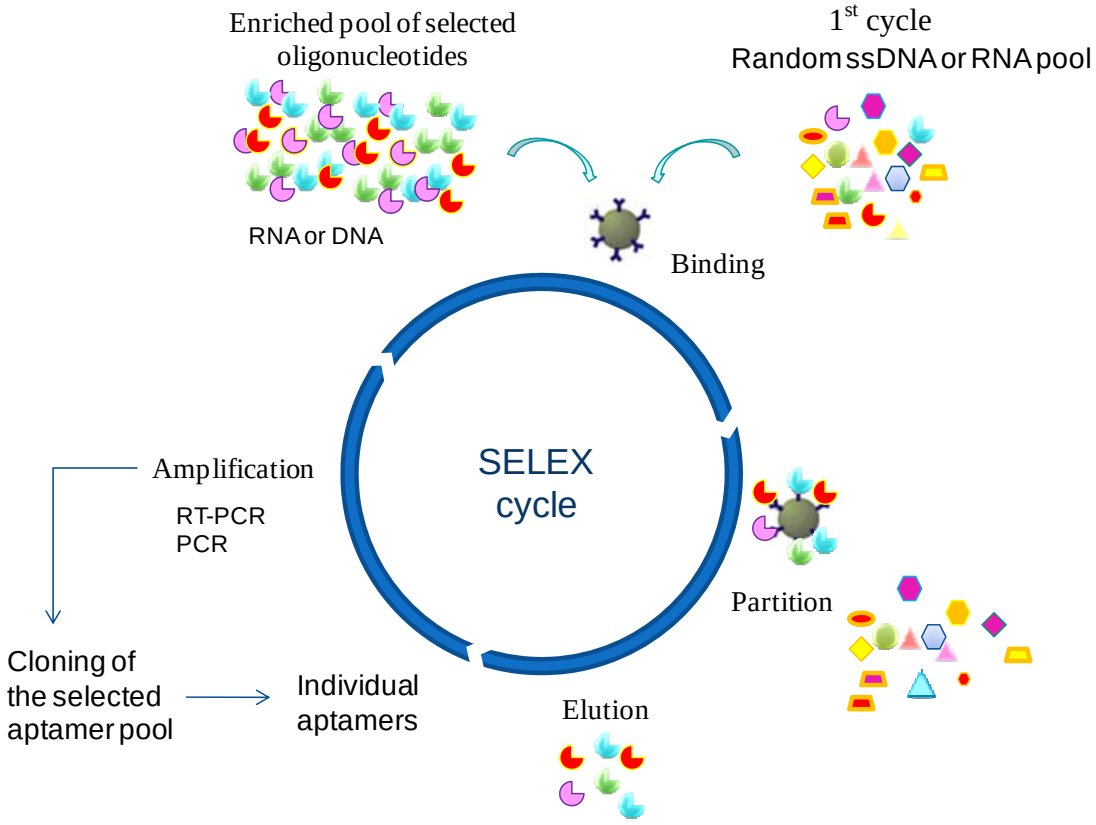


Figure. 2. *In vitro* selection of target-specific aptamers using SELEX technology.

Before starting the SELEX cycle, a random oligonucleotides pool with sufficient diversity but also some constant regions needs to be designed first. If the target doesn't have a known functional nucleic acid sequence or a bound structural motif, a random sequence pool is normally used to initiate the SELEX process. The length of the random sequence generally varies from 20nt to 80nt depending on the size of target and the degree of randomization required (Figure 3). On both ends of the random sequence, 18nt to 21nt constant regions for primers binding need to be introduced for Aptamer amplification. This random oligonucleotides library was chemically synthesized consisting of about 10^{13} to 10^{15} different sequences to ensure a successful SELEX[15]. In

a SELEX procedure which is aimed for identifying DNA aptamers, this library could be used without any pretreatments, whereas an *in vitro* transcription is needed for RNA aptamers.

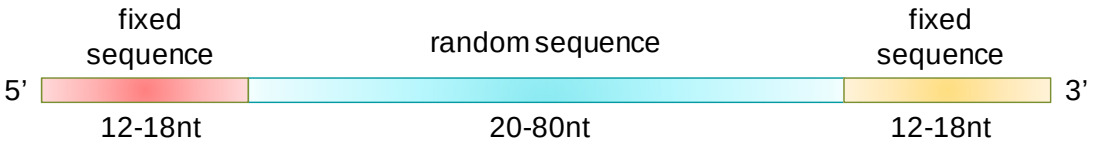


Figure 3. Design of a randomized oligonucleotides pool.

During the SELEX process, the randomized RNA or DNA pool is incubated with the target in an appropriate buffer and at constant temperature. Thereafter, the formed complexes need to be separated from unbound oligonucleotides. This is one of the most crucial steps in the SELEX process and there are various techniques available to achieve it. Traditional separation without target immobilization can be achieved by ultrafiltration using membrane filters with specific molecular weight cut-offs lower than the respective target-aptamer complex but higher than the unbound oligonucleotides[2, 16, 17]. However, membrane absorption of the complexes as well as unspecific interactions of oligonucleotides with the membrane often results in reduced selection efficiency. For protein targets, the most common methods nowadays are affinity column chromatography with the immobilized target protein. Proteins can be firstly equipped with His-tags or biotin units and immobilized on Ni-NTA or streptavidin columns either before SELEX or after incubation with the aptamers[18, 19]. Even though this method is quite convenient to operate, a significant amount of protein is usually needed in order to completely coat the whole surface area of the column, since otherwise non-specific

absorption of the oligonucleotides by the packing material is likely to occur. In recent years, several modern techniques have been applied in this step, e.g. Capillary Electrophoresis (CE)[20-22], Flow Cytometry, FC[12, 23], Electrophoretic Mobility Shift Assay, EMSA[24], Surface Plasmon Resonance, SPR[25] or centrifugation[26, 27]. Gopinath[14] has given a very helpful overview of all these different partition methods.

Partitioned target bound oligonucleotides are amplified by PCR (DNA SELEX) or RT-PCR (RNA SELEX). For DNA SELEX, the resulting double-stranded DNA from the PCR mixture has to be separated into a new ssDNA pool, which can be achieved by different approaches, such as asymmetric PCR or biotin-primer separation[28, 29]. Details are discussed in the experimental part. For RNA SELEX, an *in vitro* transcription and subsequent purification is needed. Then, this new pool of selected oligonucleotides is ready for use in the next SELEX cycle. After several selection and amplification cycles, the higher binding affinity sequences are enriched and lower binding affinity sequences are minimized due to binding competition.

Recently, some automatic SELEX techniques have also been reported, such as the automatic SELEX work station, which combines all SELEX steps together[30] as well as microfluidic channel based SELEX[31, 32]. Although the automatic SELEX considered as a dream in aptamer technology, the real automatic SELEX for broad target application is still not feasible at present. For each new target, a specific SELEX process needs to be established and optimized individually

1.3. Aptamers for therapeutic applications

Interactions between oligonucleotides and proteins with high affinity and specificity naturally occur in many biological processes such as transcription, translation or RNA interference. For example, protein conformation and function can be changed when binding to its oligonucleotide substrate. Based on the same concept, aptamers provide non-natural oligonucleotide-protein interactions, which theoretically can also modulate the protein's function. This therapeutic principle has been exploited in the past. Until June 2009, seven therapeutic aptamers were in clinical development and one of them has been already reached the market [Macugen^{TM}, anti-,macular degeneration drug] (Table 1)[33]. Among all these aptamer drugs in clinical trials, the common mechanism of their action is based on a specific binding property to a target protein and thereby blockade of the interaction of the particular protein with other proteins that function as competitive inhibitors.

Table 1. Therapeutic aptamers in clinical use or clinical development as of June 2009.

Compound ID	Therapeutic Target	Disease Indication	Route/Method Administration	Clinical Phase
Macugen^{TM}	VEDF	Macular degeneration	Intravitreal	Market
ARC1779	Won Willebrand factor	Thrombotic microangiopathy	Intravenous infusion	Phase 2
REG1	Factor IXa	Coronary artery bypass	Intravenous bolus	Phase 2
AS1411	Nucleolin	Acute myeologenous leukemia	Intravenous infusion	Phase 2
E10030	PDGF-b	Macular degeneration	Intravitreal	Phase 1
ARC1905	Complement factor 5	Macular degeneration	Intravitreal	Phase 1
NU172	Thrombin	Coronary artery bypass	Intravenous infusion	Phase 1

Since oligonucleotides usually reveal very limited cell membrane penetration due to electrostatic repulsion forces, most targets of therapeutic aptamers are located outside the cells such as plasma proteins or cell-surface proteins. Therefore, aptamer drugs normally need to spent sufficient time in the blood circulation and thus remain in the plasma compartment. However, most oligonucleotides generally encounter three major DMPK (distribution, metabolism and pharmacokinetics of a drug) hurdles: (1) rapid metabolic degradation, (2) rapid renal elimination and (3) rapid biodistribution from plasma into tissue[33]. Consequently, post SELEX modifications of the aptamer to increase plasma life time are normally required to generate aptamer drugs.

Post SELEX modifications of the initially selected aptamer displaying high binding affinity and therapeutic potential can improve drug like properties of the aptamer. The first major step which is important to limit manufacture costs and improve synthetic feasibility is to identify the functional moieties and minimize the aptamer sequence to the smallest possible size. Minimized aptamers generally have 15–45 oligonucleotides with molecular weights of ~5-15 kDa[33]. After minimization, further chemical modifications can be introduced systematically to improve metabolic stability or enhance binding affinity. For example, substitutions (e.g., 2’ -OMe, 2’ -F) at the 2’ position of the sugar ring or at the phosphate backbone [e.g. introduction of sulfur to replace non-bridging phosphodiester oxygen (P=S)] can enhance aptamer plasma life time[34]. Pegylation (conjugation with polyethylene(oxide), PEG) is another way to extends aptamer elimination half-life, which is a widely used strategy to extend drug circulating life time

for clinical use[35, 36]. PEG can be easily conjugated at one (5') or both ends (5' and 3') of the aptamer during chemical synthesis.

Very limited information of the toxicological properties of aptamers are available, except for the marketed aptamer product Macugen™[37]. The clinical trials accomplished for Macugen™ allowed a limited toxicological assessment due to the minimal systemic exposure from the low total dose administered (0.3 mg/eye), sporadic administration (every 6 weeks) and compartmental administration (intravitreal). However, in toxicology studies of therapeutic aptamers, the general toxicology profile of therapeutic aptamers has been reported[33] quite predictable and favorable. The previously reported oligonucleotide class effects such as immune stimulation, complement activation and anticoagulation are largely devoid in aptamers. NoAELs (no observed adverse effect levels) are generally in the 25–100 mg/kg/day range.

In short, therapeutic aptamers have been demonstrated as a new promising class of drugs for targeting plasma proteins or cell surface proteins. Chemical modifications of such aptamers are feasible at various positions to improve drug like properties. The pharmacokinetic and toxicological properties of aptamer drugs are favorable and they are devoid of the reported oligonucleotides side effects. However, still further data and more Aptamer drugs are required to intensively investigate the clinical properties of therapeutic aptamers.

1.4 Botulinum Neurotoxin infection and applications

Botulinum neurotoxins (BoNTs) produced by anaerobic bacteria *Clostridium botulinum* are the most potent toxins known with a mouse LD50 of roughly 1–5 ng/kg[38]. Seven botulinum neurotoxin serotypes (named BoNT/A to G) distinct biochemically and serologically have been characterized from anaerobic bacteria spores of the genus *Clostridium*. These bacterial spores are broadly present in the environment and are strongly resistant to a range of physical and chemical damages. Food contaminated by *Clostridium botulinum* spores, under suitably anaerobic conditions such as in cans, can generate bacterial cells producing one or more types of BoNTs. Therefore, botulinum toxin was also described as a "sausage poison" and "fatty poison"[39], as this bacterium often poisoned sausages or canned meat. Ingestion of BoNT-poisoned food potentially causes a fatal disease known as botulism which leads to paralysis starting with the muscles of the face and then spreading towards the limbs[38]. If left untreated, it leads to death because of the breathing muscles paralysis resulting respiratory failure. Infant botulism is also a common type of BoNTs infection caused by the colonization of the digestive tract of infants by *Clostridium botulinum*, which may cause sudden infant death. Botulism can also occur in wounds resulting from *Clostridium botulinum* growing in anaerobic wounds.

Despite its fatal toxic effects, BoNTs have also been used as a powerful therapeutic tool for treating a variety of neurological, ophthalmic and other muscle contraction disorders. For example, a popular botulinum toxin-containing drug, BOTOX, was approved by the

U.S. Food and Drug Administration (FDA) in December 1989 for the treatment of strabismus, blepharospasm, and hemifacial spasm in patients over 12 years old. And this product has also been used for cosmetic purposes to treat wrinkles.

1.5 Botulinum Neurotoxin structure and action mechanism

The seven serotypes of botulinum neurotoxins (BoNTs) share very similar structure and properties. In bacterial autolysis, they are produced as complexes composed of inactive single polypeptide toxin chains (M_r ~150 kDa) which are activated by proteolysis and other non-toxic accessory proteins[40]. The accessory proteins protect BoNTs through the stomach and dissociate when the complexes reach the small intestine where the toxins are absorbed into the bloodstream[41]. The active form of the toxin consists of a 150kDa-dichain protein composed of a 100kDa C-terminal heavy chain (HC) linked by a disulfide bridge to a 50kDa N-terminal light chain (LC) which is a zinc-protease (Figure 4). The HC can also be identified as two domains, the H_C domain (blue color) include a ganglioside binding site and a membrane receptor binding site which is responsible for neuroselective binding and the H_N domain (green color) functions as a chaperone for refolding of the LC domain after translocation[42].

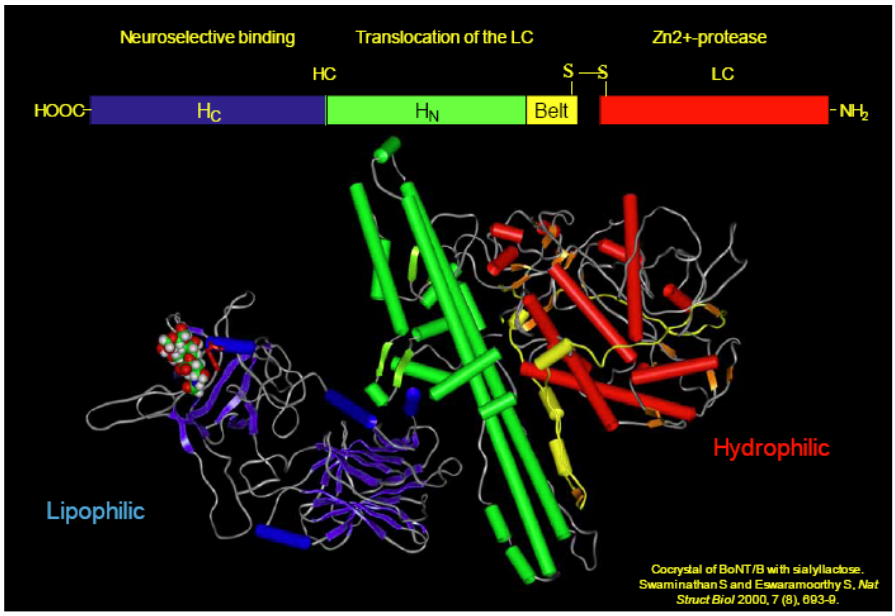


Figure 4. Crystal structure of BoNT/B illustrates the typical structural motifs of BoNTs[43].

BoNTs intoxication occurrs via a multistep process involving each of these functional domains (Figure 5): 1) The H_C domain interaction with Gangliosides at the neuromuscular junction resulting in neurospecific binding and diffusion along the motor neurons, 2) the membrane receptor binding site on the H_C domain is recognized by membrane receptors such as Synaptotagmins and internalization by receptor mediated endocytosis is initiated, 3) conformational changes of the H_N domain in the endosomes and release of the LC into the cytosol, and 4) cleavage of the SNARE protein by the LC domain, thereby preventing neurotransmitter release[44].

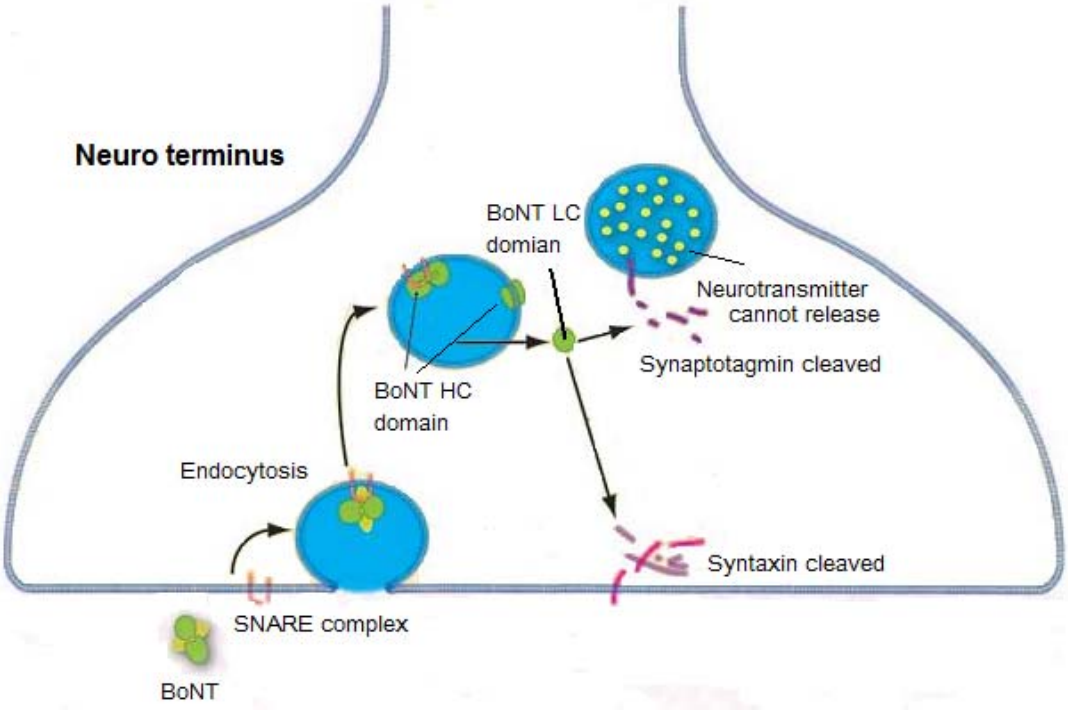


Figure 5. Demonstration of the intoxication mechanism of BoNTs.[41]

1.6 Treatment of botulism

The death by botulism is caused by respiratory failure due to paralysis of the respiratory muscles, which represents a secondary symptom of botulism. Therefore, if the symptoms of botulism are identified at an early stage, botulism could be treated by blocking any further step of the BoNTs intoxication pathway in order to prevent worsening of the paralysis symptoms. The only available drugs to treat botulinum intoxication currently are equine antitoxins which induce passive immunity to block the blood circulation of BoNTs [45]. Two primary Botulinum Antitoxins available are Trivalent (A,B,E) Botulinum Antitoxin derived from equine sources utilizing whole antibodies (Fab & Fc portions) and Heptavalent (A,B,C,D,E,F,G) Botulinum Antitoxin derived from

"despeciated" equine IgG antibodies with only Fab portions. Antitoxin treatment and modern clinical practice has reduced botulism fatality rates from 60% to about 10%[46]. However, the functional recovery of the nervous system could take several weeks to months or even longer. Currently, this antitoxin is not applied to treat infant botulism due to potential life-threatening side-effects such as anaphylaxis and serum sickness. Human botulinum immune globulin is still under investigation, which might have the potential to treat infants with fewer side effects since it is a homologous immunoglobulin[47]. Development of small molecular inhibitors for the treatment of BoNTs has also been strongly supported. Nearly all of the steps during BoNT pathogenesis have been exploited as potential therapeutic targets including toxin binding, translocation and catalytic activity. Several compounds with promising inhibiting ability or mitigating the paralysis have been identified. However, most of the compounds show unfavorable drug like properties such as very limited cell uptake or high cytotoxicity and therefore, they haven’t been considered for clinical studies[48].

1.7 Aim of project

In this project, the development of therapeutic aptamers which are able to block the ganglioside binding site and Synaptotagmin binding site on the H_C domain of BoNT/A represents a key concern. Due to the intoxication mechanism of BoNTs, these aptamers could in principle used as BoNTs inhibitors without cell membrane penetration. The mechanism of action would be similar as the antitoxins on the market, which were derived from equine antibodies (Section 1.6), but less immune side effects are expected

according to the clinical reports of several types of available therapeutic aptamers. Up to now, no rational approach for the identification of H_C inhibitors has been reported.

A preliminary study of BoNT H_C domain aptamer has been reported by Prof. Tok[28] using a single bead SELEX method. A short peptide sequence derived from the BoNT/A H_C domain was used in that study. However, the short peptide might not retain the active conformation of the neuroselective binding site and no binding affinity data of the generated aptamer for the whole BoNT protein was reported in that study.

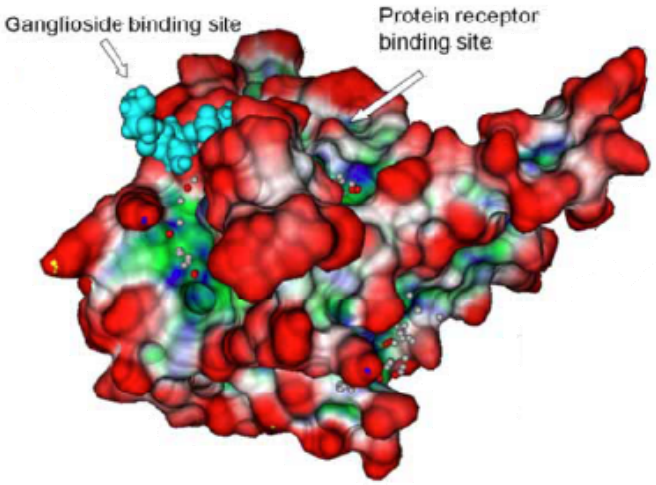


Figure 6. Crystal structure of the HC domain of BoNT/A where the ganglioside binding site and the protein receptor binding site are highlighted.

Co-crystallization of BoNT/B and gangliosides and part of the protein receptor peptide sequence as well as a combination of molecular modeling and mutation analyses facilitated the identification of the gangliosides binding site and protein receptor binding site on H_C domain of BoNT/A (Figure 6)[49]. Both of these two binding sites are exposed to the surface and located close, which should enable an interaction with aptamers and a

blockade of these binding events. In close cooperation with our collaborator, the ganglioside binding protein (*gsbp*) consisting both of the essential membrane binding sites and a retained active conformation has been expressed successfully. A His-tag is introduced just on the opposite side of these two binding sites to allow immobilization of this protein to a single Ni-NTA bead. Therefore, a similar single bead SELEX approach as reported by Prof. Tok's previously with some modifications on the procedures was designed and established herein to select aptamers with high binding affinities for *gsbp* and with potential to block the BoNT/A infection pathway.

Chapter 2. Experimental

2.1 Materials

Synthetic 29-mer ganglioside binding peptide with the following sequence HHHHHHGGGGDRVYINVVVKNKEYRLATN was ordered from Genescript (USA), and a purity of 98.1% was certified after HPLC analysis. The ganglioside binding protein (*gsbp*) was expressed and purified by Dr. Andreas Rummel from *Medizinische Hochschule Hannover*, Germany. Ni-NTA derivatized agarose beads and streptavidin derivatized agarose beads were purchased from Invitrogen (Carlsbad, CA). The 40nt random sequence containing the ssDNA library and both derivatized and underivatized primers were obtained from 1st-BASE (Singapore) with the following sequences: ssDNA Library: 5'-ATACCAGCTTATTCAATT-N40–AGATAGTAAGTGCAATCT-3'; Reverse Primer: 5'-(Biotin)-AGATTGCACTTACTATCT-3'; Forward Primer: 5'-(Fluorescein)-ATACCAGCTTATTCAATT-3'. M13 forward (–20): 5' GTAAAACGACGGCCAGT 3' and M13 reverse: 5' AACAGCTATGACCATG 3' are also synthesized by 1st-BASE (Singapore). GoTag DNA polymerase and other PCR reagents were all purchased from Promega (USA). PCR cloning kits were obtained from Fermentas Life Sceinces and Qiagen (USA). XL-blue competent cells were used for cloning. LB Agar plates were prepared from LB Agar (Lennox) Powder Growth Media, PTG and X-gal was obtained from Promega Pte Ltd. Fluorescein was purchased from Alfa Aesar.

2.2 Instruments

Sequencing was performed by Genewiz Inc. Beijing. Fluorescence polarization was measured by a Tecan Infinite F200 Microplate reader in PerkinElmer 384 Flat bottom Black microplates. Fluorescence images were recorded on an Olympus XTI-17 microscope.

2.3 Overview of SELEX process

The SELEX process to generate the *gsbp* aptamer has been modified from the originally reported single bead method [28]. (Figure 7) The single strand DNA (ssDNA) library was designed with 40nt random sequences in the middle region and two 18nt primer binding sites at each end. The target protein with His-tag was firstly coated on the Ni-NTA agarose bead. One bead coated with the peptides was incubated with 10uL of the ssDNA library. After incubation, free ssDNA was washed off and the bead with bound DNA binding was directly used for PCR. Reverse primers functionalized with biotin were used for PCR so that the antisense strand is labeled with biotin and can be removed via streptavidin beads. After separation, the sense strand was used as a new library for next cycle of the SELEX process. Since in each cycle, the relative amount of the peptide is limited, the competition during the binding process is much higher compared with a traditional column SELEX. Therefore, after less than 10 cycles aptamers displaying high binding affinities are achieved already. It is a key feature that only few pictograms of the peptide is needed for this kind of SELEX approach which is a big advantage considering the high costs of proteins such as the *gsbp*.

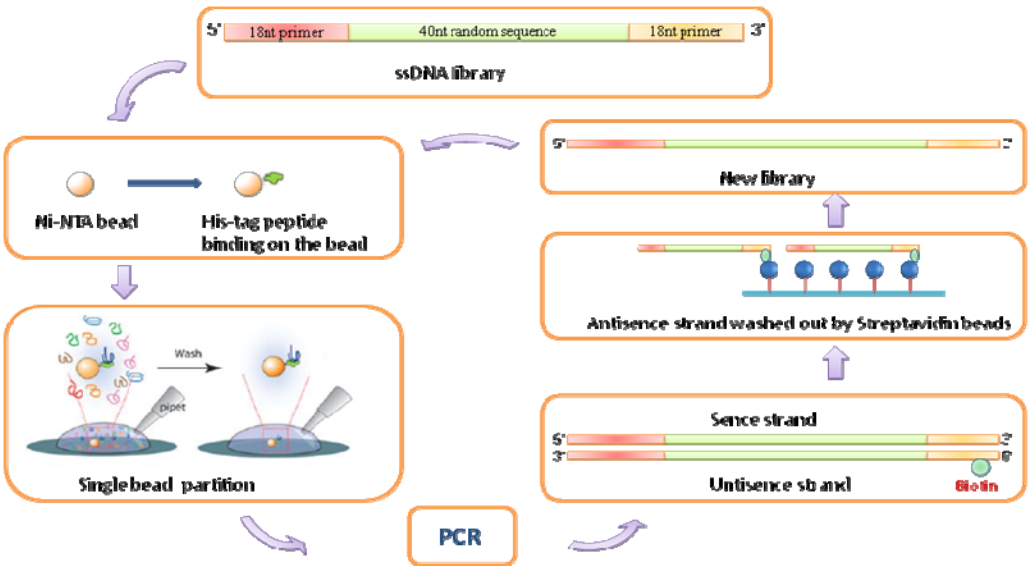


Figure 7. Representation of the single bead SELEX cycle.

The single bead SELEX method was first established using the reported ganglioside binding peptide [27] as target. Later on, the similar method was applied for *gsbp* native protein. A few but important modifications of the peptide SELEX procedure have been introduced in order to contribute to the higher sensitivity of the protein compared with the peptide at elevated temperature and under vigorous stirring. The general procedure of each SELEX step is described below and differences between the peptide SELEX and the protein SELEX are highlighted.

2.4. Loading of the His-tag peptide or protein onto a single Ni-NTA agarose bead

The sequence of the synthetic 19-mer ganglioside binding peptide consists of the amino acids 1177 to 1195, a short 4-mer glycine spacer and a 6-mer His-tag incorporated at the N-terminus. This peptide was dissolved in Milli-Q water with a final concentration of 1mM as stock solution. The *gsbp* native protein was also expressed with His-tag at the

opposite side of the essential membrane binding sites and it was obtained with a concentration of 15uM and used as obtained.

The Ni-NTA agarose beads in 20% ethanol stock solution were firstly washed 6 times with Milli-Q water and 6 times with binding buffer (20 mM HEPES, pH 7.4; 150 mM NaCl; 5 mM KCl; 2 mM $MgCl_2$; 2 mM $CaCl_2$). Subsequently, 5 to 10 beads with regular shape and similar average diameters were selected under the light microscope and transferred into a PCR tube. The beads were resuspended in 20μL of binding buffer, 0.08μL/bead of peptide stock solution or 0.5μL/bead of *gsbp* stock solution was then added. The solution was incubated at room temperature for 3hrs. After the first hour of incubation, the NaCl concentration was slowly adjusted to 1M. The beads were washed again 6 times with Milli-Q water and binding buffer after coating of the peptide. Both peptide and protein immobilized beads were stored in 100μL of binding buffer at 4^oC.

General procedure for washing a batch of beads: 1μL of Ni-NTA beads in ethanol were transferred to a 96-well plate, and diluted by 49μL of Milli-Q water. 5μL diluted beads were transferred to another well and further diluted by 45μL of Milli-Q water. About 50 beads in the well could be visualized under the microscope (10X magnification). The solution was well mixed by pipette, and then kept for 2 minutes until all beads had settled down. 40μL of water was carefully transferred from the upper layer with minimum movement of the beads on plates. Then 40μL of fresh Milli-Q water was added again to repeat this wash step.

2.5. Incubation and washing of the peptide or protein coated bead with the ssDNA library

One of the peptide or protein coated beads was transferred with 0.5μL of binding buffer via micropipette under the microscope to a Petri dish. The bead was washed 3 times with 20μL of Milli-Q water, and then transferred with 1μL of water to a 0.2mL PCR tube. 3μL of water, 5μL of 2X binding buffer and 1μL of ssDNA library was mixed with the bead in the tube (10μL of final volume). For peptide SELEX, the solution was heated at 95^{o}C for 7min and cooled down to 20^{o}C for 30min in the thermal cycler to minimize cross hybridization within the ssDNA library while this step was omitted for protein SELEX. The solution was then incubated at room temperature for 2hrs with gentle shaking.

After incubation, this 10μL solution was transferred to a Petri dish. The bead was washed 7 times with binding buffer and 3 times with water and finally transferred with 1μL water together to a new PCR tube and stored at 4^{o}C for PCR.

General procedure for washing one bead: One bead located on a Petri dish could be transferred in solution by a pipette. The bead was carefully transferred with 1μL of solution to a clean place under the microscope. 20μL of new binding buffer or water was added onto the bead. The drop was gently moved around by air derived from actuating a pipette. The bead should be circulated around in the drop, which can be monitored under

the microscope. After washing, the bead was transferred with 1μL of solution to another new place and the washing step was repeated.

2.6. Single Bead PCR optimization

In order to produce single stranded DNA after PCR, two PCR approaches have been examined. One is asymmetric PCR which is a PCR with one primer in very high excess compared with the other primer. The other approach is based on PCR with a biotin-labeled primer, which can be separated by streptavidin beads.

For asymmetric PCR, one bead was reacted in a 120μL of PCR solution. 6μL of forward primer (10μM) and 6μL of biotin-labeled reverse primer (0.1μM) were used. PCR conditions: preheat to 95^{o}C for 4min, 90 cycles of 94^{o}C 30s, 50^{o}C 45s, 72^{o}C 45s, final extension at 72^{o}C 10min.

For PCR with the biotin primer, one bead was also reacted in 120μL PCR solution. 6μL of 10μM of both forward and biotin-reverse primers were used. PCR conditions: preheat to 95^{o}C for 4min, 25 cycles of 94^{o}C and 30s, 49^{o}C and 45s, 72^{o}C and 45s, final extension at 72^{o}C for 10min.

2.7. Separation of sense strand ssDNA from double stranded DNA

To prepare streptavidin agarose beads for separation, 20μL streptavidin beads slurry were added into a 0.2mL PCR tube, centrifuged at 6.0X1000g for 1min. The supernatant was discarded carefully by a pipette. 90μL of autocleaved Milli-Q water was then added, the slurry was votexed and centrifuged at 6X1000g for 1min, supernatant was again carefully discarded. This wash step was repeated 5 times.

90μL of PCR sample was added into 10μL of streptavidin agarose beads. The solution was mixed by vortex and then incubated at room temperature for 1h. After incubation, the beads were centrifuged down at 6X1000g and the supernatant was carefully discarded. The beads were then washed with 90μL of water 3 times as described before. Subsequently, 50μL of sterilized NaOH solution (15mM) was added. For peptide SELEX, the resulting solution was first heated to 95^{o}C for 7 min and then cooled to 20^{o}C for 30min by a thermal cycler. For protein SELEX, 3hrs RT incubation with gently shaking was required. After incubation, the beads were centrifuged down and the supernatant was transferred into a new tube. ~0.5μL of 2.5% acetic acid was added to adjust the solution to neutral pH and the final pH was checked by pH indicator paper. The solution now containing clean ssDNA can be stored at 4^{o}C and used as a new library for next cycle of SELEX.

2.8. Cloning and sequencing

The clones of the selected aptamer sequences were generated using the InsTAclone PCR cloning kit (Fermentas) according to manufacturer's instructions. After overnight growth

on LB agar plates, 21 colonies were selected and amplified by PCR. The PCR reaction solution was directly sent to GeneWiz Inc. Beijing for further purification and sequencing.

2.9. Binding affinity test

The selected colonies were amplified by colony PCR with biotin-labeled reverse primer and fluorescein-labeled forward primer. The purification was performed by streptavidin agarose beads and the double strand DNA was separated into single strand DNA simultaneously (section 2.7). The whole process was carried out in the dark room to avoid photobleaching of fluorescein.

After purification and separation, the concentration of single strand aptamers was measured by agarose gel electrophoresis with a quantitative DNA ladder. Then, 5 μL of the aptamer solution was transferred into PerkinElmer 384 Flat bottom Black microplates and 10 μL of 2 x binding buffer (40 mM HEPES, pH 7.4; 300 mM NaCl; 10 mM KCl; 4 mM $MgCl_2$; 4 mM $CaCl_2$) was added. After which 5 μL of the desired concentration of the peptide or *gsbp* was added into the individual sample and incubated at room temperature in the dark for half an hour to allow binding. After incubation, the fluorescent polarization was obtained by using a Tecan Infinite F200 microplate reader. The excitation wavelength was set at 480 nm and the emission wavelength at 520 nm. The G-factor was corrected by 20nM fluorescein aqueous solution (1mP). At each peptide concentration, the polarization value (mP) was obtained by average of 5 independent measurements. The polarization (mP) data was then converted to anisotropy

data (r) by Equation 2, and the dissociation constant (Kd) was calculated by fitting the experimental data to Equation 3. The fitting curve was plotted by OriginPro 8 and the Kd values were also calculated.

Equation 2. $r = 2P/3-P$

Equation 3. $y = m1 + [m2/2 * ((m0 + m3 + m4) - ((m0 + m3 + m4)*(m0 + m3 + m4)-(4 * m0 * m3))0.5)]/m3$

where:

y = anisotropy in the presence of peptide

m1 = anisotropy in the absence of peptide(mP)

m2 = anisotropy changes in the presence of infinite amt of peptide(mP)

m0 = initial concentration of peptide (μM)

m3 = initial concentration of aptamer (μM)

m4 = dissociation constant (Kd)

Chapter 3. Results and Discussion

3.1 Establishment of the single bead SELEX method

3.1.1 Single bead PCR optimization

In order to get single stranded DNA after PCR, two PCR methods have been explored. One is asymmetric PCR approach where one primer is applied in very large excess compared with the other primer. The second approach includes PCR with a biotin-labeled primer and the separation by streptavidin beads. (Figure 8)

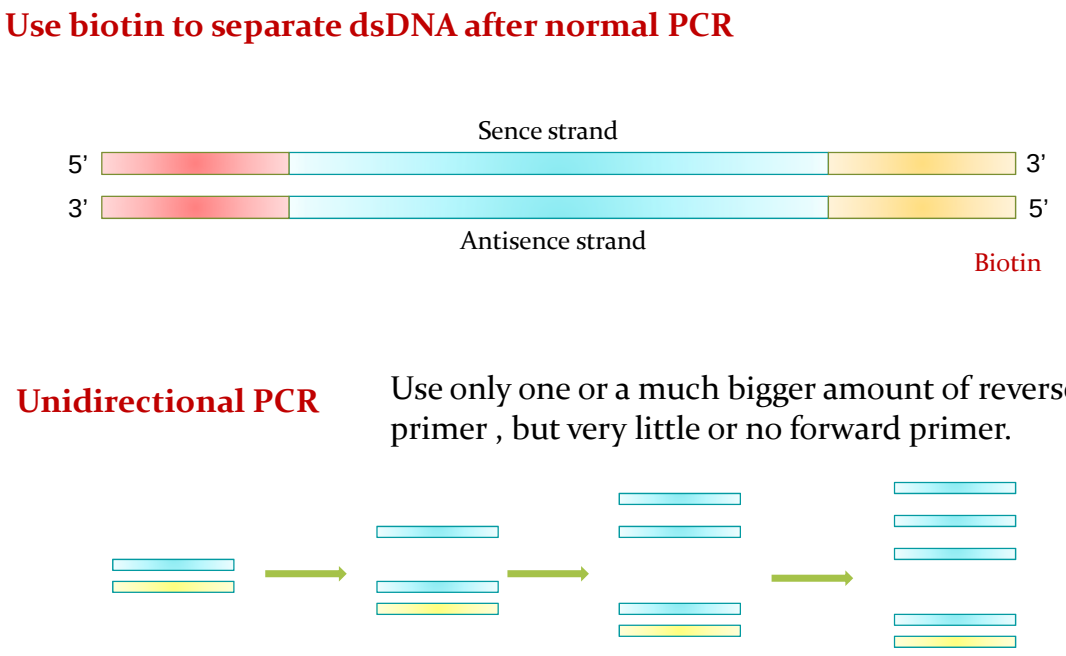


Figure 8. Illustration of the two PCR methods to receive ssDNA[50].

After the 1st SELEX cycle, PCR on the single bead coated with the ganglioside binding peptide was performed by these two methods and the outcome is shown in Figure 9. The

number of reaction cycles for PCR was also optimized. The biotin primer PCR achieves enough amount of product (76bp fragment) within 25 cycles (line 4), and with too much cycles, the product band become broad which indicating nonspecific reactions. The asymmetric PCR product is slightly lower than the double stranded PCR product, which indicated single strand product formation. The optimal number of cycles is 90 cycles (line 6), and nonspecific amplification abserved after 120 cycles (line 5). The best annealing temperature found was 50^{o}C. Both of these two PCR runs yield the correct product (line 4 and 6). However, as an important feature, separation of the biotin-labeled double strands with streptavidin agarose beads is much easier and can also achieve purification in one step. Therefore, this method was chosen for all following PCR cycles.

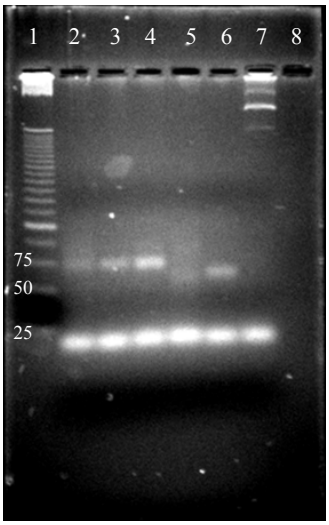


Figure 9. Comparison of the biotin primer single bead PCR and the asymmetric single bead PCR (4% agarose gel). Line 1: 25bp DNA ladder (Invitrogen); line 2: 9uL of PCR product from biotin primer PCR 35 cycles; line 3: 5uL of PCR product from biotin primer PCR 25 cycles; line 4: 9uL of PCR product from biotin primer PCR 25 cycles; line 5: 9uL of PCR product from asymmetric PCR 120 cycles; line 6: 9uL of PCR product from asymmetric PCR 90 cycles; line 7: negative control.

For biotin primer single bead PCR, the annealing temperature was further optimized to ensure the best PCR conditions. Results were shown in Figure 10. The best annealing temperature (49^{o}C) was using for all following SELEX cycles.

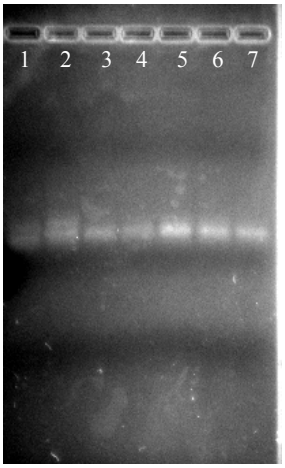


Figure 10. Annealing temperature optimization of biotin primer single bead PCR. Line 1 to 7: Annealing temperature 37^{o}C, 40 ^{o}C, 43 ^{o}C, 46 ^{o}C, 49 ^{o}C, 52 ^{o}C and 55 ^{o}C.

3.1.2 Protein coated beads monitored under the fluorescence microscope

In our initial design, the aptamer was labeled by a fluorescein dye that allows straight-forward detection via a fluorescence microscope. Before producing fluorescein-labeled aptamers, the fluorescein dye and the fluorescein-labeled primer were first incubated with the protein coated beads to record the background emission. However, we found that the fluorescein dye could be absorbed onto proteins most likely due to hydrophobic interactions, but these dyes are not absorbed by agarose beads (Figure 11 b, c). For the same reason, fluorescein-labeled primers could also interact non-specifically on protein-coated beads. This unspecific absorption leads to a high imaging background and reduces SELEX efficiency. Therefore, no fluorescein was further used in the SELEX cycles. However, since

specific (Figure 11d) and non-specific(Figure 11 b) interactions could be visualized, and the Ni-NTA agarose beads and protein coated agarose beads showed completed different interactions with fluorescein, this is a good choice to prove the success of protein immobilization.

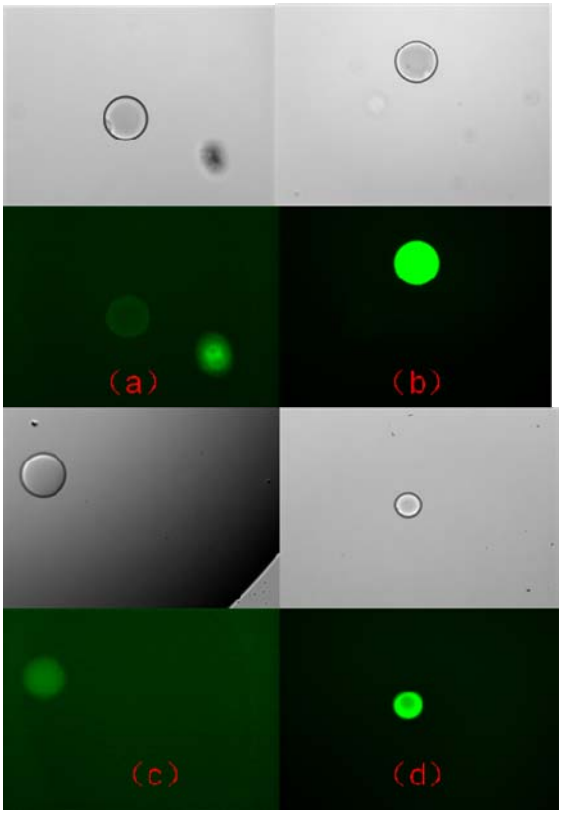


Figure 11. White light and fluorescence imaging of fluorescein incubated with peptide coated and non-coated beads. (a) Fluorescence background of peptide-coated bead (the bright dot is dust); (b) Fluorescein incubated with the peptide-coated bead and washed 3-times with water after incubation; (c) Fluorescein incubated with agarose beads and washed with water 3-times after incubation; (d) Fluorescein-labeled aptamer incubated with peptide-coated beads and washed 3-times with water.

3.1.3 Proof of washing efficiency.

During SELEX, a single protein-coated agarose bead was incubated with the ssDNA library. After incubation, several washing steps were used to completely remove unbound ssDNA, which is essential for achieving high affinity aptamers in a minimum number of SELEX cycles. In order to examine the washing efficiency, 1 μL of washing solution from the last washing step was subjected to PCR reaction and compared with the positive PCR of the single protein coated bead. The PCR products were analyzed by using agarose gel electrophoresis (Figure 12). The protein-coated bead with bound aptamers has a positive PCR-product shown at the 76 bp position on the gel, whereas no PCR product was detected in the PCR reaction of the washing solution.

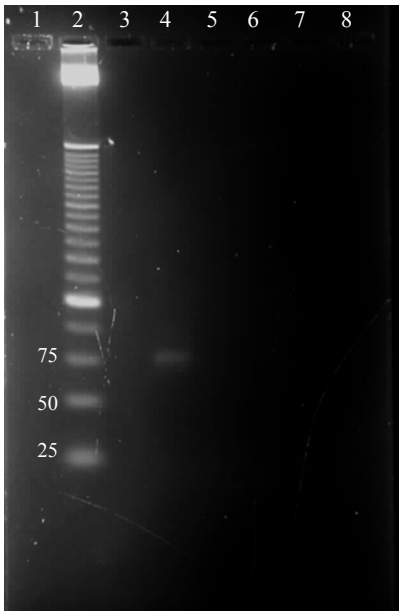


Figure 12. Proof of the washing efficiency (4% Agarose gel). Line2: DNA ladder; Line 4: PCR product from the single protein coated bead with bound aptamer; Line 6: PCR product from 1μL of the last washing solution.

3.1.4 Separation of "sense-strand" DNA from double-stranded PCR mixture

After biotin primer PCR reaction, double-stranded DNA (dsDNA) was separated by streptavidin agarose beads. The reaction mixture was firstly incubated with streptavidin-coated agarose beads to allow binding of the biotin-containing products. After incubation, the supernatant was kept in order to check whether the binding step was efficiently finalized (Figure 13 line 2 and 5). The beads were washed several times with Milli-Q water (Figure 13 line 3 and 6) and thereafter, 15mM NaOH was added to denature the double strands. After 1h denaturation, the supernatant was taken and neutralized by acetic acid (Figure 13 line 4 and 7), and used as a new library for the next SELEX cycle. The result of this separation step was investigated again via an agarose gel (Figure 13), which displayed a successful purification and separation of the DNA.

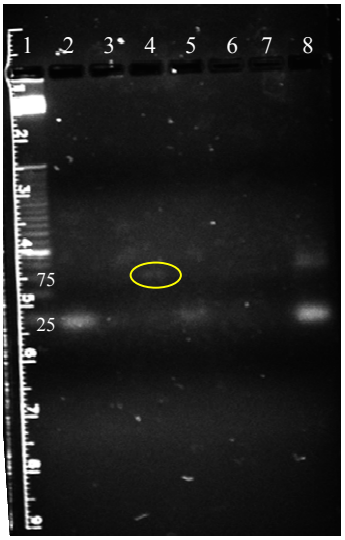
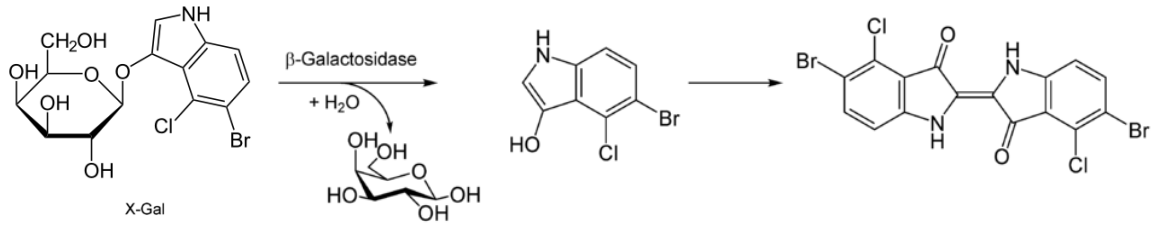


Figure 13. Double-stranded DNA separation (4% Agarose gel). Line1: DNA ladder; line 2: first wash solution after the PCR reaction mixture was incubated with streptavidin beads (primers were washed out while 76bp target PCR products were bound to streptavidin beads); line 3: second wash solution (nearly no DNAs inside); line 4: elution of sense-stranded ssDNA after denaturation with NaOH (yellow circle shows the ssDNA products

that are a little bit below compared with the dsDNA in line 8); line 5 to 7: same sample as line 2 to 4 but half loading amount; line 8: PCR reaction mixture before separation.

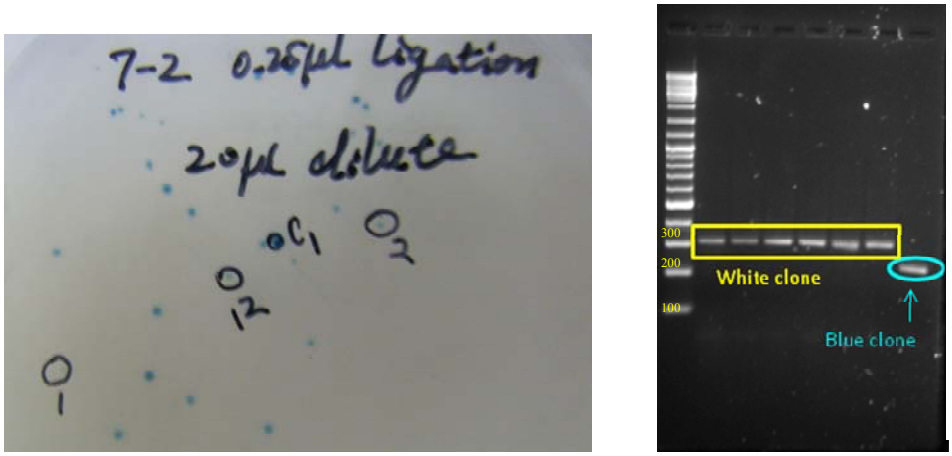
3.1.5 Cloning efficiency – blue and white screening

After the final cycle of the SELEX process, the PCR products were directly cloned into E.Coli cells and amplified by the Qiagen PCR cloning kit. Blue and white screening was perfomed to check ligation efficiency. The blue and white screening is a technique that allows for the quick and easy detection of successful ligation, without the need to individually test each colony. If the ligation was successful, the bacterial colony on Agar plate will be white; if not, the colony will be blue. The molecular mechanism is based on a genetic engineering of the lac operon in the E. coli strain. The chromosome of the host strain and the LacZ gene on the vector together encode a functional β-galactosidase enzyme. The foreign DNA can be inserted in vectors within the LacZ gene, thus disrupting the production of functional β-galactosidase. The competent cells with transformed vectors are grown in the presence of X-gal, which is colourless modified galactose sugar that is metabolized by β-galactosidase to form an blue product (5-bromo-4 chloroindole) (Scheme 1). The hydrolysis of colourless X-gal by the β-galactosidase causes the characteristic blue colour in the colonies containing vector without insert. White colonies indicate insertion of foreign DNA and loss of the cells' ability to hydrolyse the X-gal.



Scheme 1. β-galactosidase hydrolysis of X-gal to blue product 5-bromo-4 chloroindole.

From blue and white screening, 1.5µL of PCR product ligation resulted in an improved ligation efficiency compared with the 0.25µL of PCR product ligation. No obvious blue clone was found in the 1.5µL of PCR product ligation plate. However, since the inserted PCR product is too short, white clones also displayed a minor blue color. Subsequently, 20 white clones were randomly selected and amplified by the M13 primer clony PCR and a blue clone was also chosen as negative example (Figure 14). The PCR product with the aptamer inserted has 316bp in contrast to 238bp without ligation. Figure 14b reveals that the white clones all showed the 316bp target DNA products while in case of the blue clone no aptamer was inserted, which could be seen from the PCR products (238bp).The PCR solutions originating from the white clones were then send for sequencing.



(a) (b)

Figure 14. Blue and white screening of clone efficiency. (a) Blue and white E. Coli clones on LB Agar plate. (b) 2% Agarose gel electrophoresis showed the clony PCR sample products from blue and white clones. Line 1: 25bp DNA ladder; line 2 to 7: clony PCR samples from white clones; line 8: clony PCR sample from blue clone.

3.2 Generation of Aptamers for the Hc peptide (Apt-pep)

3.2.1 Selected sequencing of Apt-pep

After 3 cycles of SELEX, the aptamer-bound peptide-coated bead was subjected to a PCR reaction with non-labeled primers. The PCR solution was directly cloned into E. Coli cells using the InsTAclone PCR cloning kit (Fermentas) according to the manufacturer's instructions. After overnight growth on LB agar plates, 21 colonies were selected and amplified by M13 primer PCR. The PCR reaction solution was directly sent to GeneWiz Inc. Beijing for further purification and sequencing. Unfortunately, four of the sequencing results were not reliable which most likely due to insufficient amount of PCR products after purification. The remaining 17 aptamer sequences were summarized below. Three of these aptamers, which are highlighted in yellow color, have thereafter been investigated by a binding affinity assay.

>1

GGCGGTTCCTTGTGGTTCTTGAATGGTGCCTTGTGTGAT

>b2

CTGATGGGAGTAGACCAATCTGGGTTATGCCAAGGTGAC

>b6

GTTCGGGCCCAAAGTTGGTGTCTTTCAACTAACATGGCT

>b7

CCTATGGTGACCAAGGATGGATGCCCGTGCCATGGAGGC

>b8

ACACAGGGACCTTTTCCTATCGAACCTCGAGCGTATTCC

>b9

TGCCCCCTAATGTAGTTGCCGCAATTTGCCCAGTTCTTT

>b10

GCGTTATTGGGTTCGTGGGGCCGTTACACCTGGTACTCG

>b11

CCATACGCATGTTCCCCGGTTTTTGGATTGCTACCGTCC

>b12

GATCCCAGTCGACCTCTTCCTTTATCCGTAGGTGCCATC

>n1

CACCGAAAGTAGGTCAGCGAGTAACACCCGAGTTGATGA

>n7

AGGAGAATGCACGTCCGGGCGGCGGGATGAACGTGGTAT

>n9

CGAATATGTACAAGCTTTCGGTCGCTATGGTAAGCCCGT

>n11

GGCTGCAGACTTTCCGTGCAAACTGGTTTGGGTCGCTGA

>n12

CACCGGTACCATCCCACGTCCGTATGACGTTTTATTTCA

>n13

CCTTCCGCTTGTGTCCACCTATAGCACGCGCCATTGCAC

>n14

GACCGAAGGAGTAGCGGGAACGTCCCCATTGGCTCGGCT

>n16

TCCGGGCACACTGCGCTCCTGCCTGCTTGAGCTTTGTCT

>n17

CCGAGCCCTCAGCAGCGACGAGTTCCTACCGTTCCTCCG

>n18

GTCGGTAATCGCAACGGACCAGTAGCCAATTCCGCAGAT

3.2.2 Sequences alignment of the aptamers and similarity analysis

The obtained cloned sequences were subjected to homology analysis using the sequence alignment program ClustalW (available free-of charge from the website: www.ebi.ac.uk). The software allows to organize these 17 diverse input sequences into meaningful, homologous groups (Figure 15). Based on the above analysis, we have chosen 3 distinct sequences from the aptamer pool and subsequently examined their binding affinities toward the Hc-peptide. These three sequences were highlighted with red squares in Figure 15.

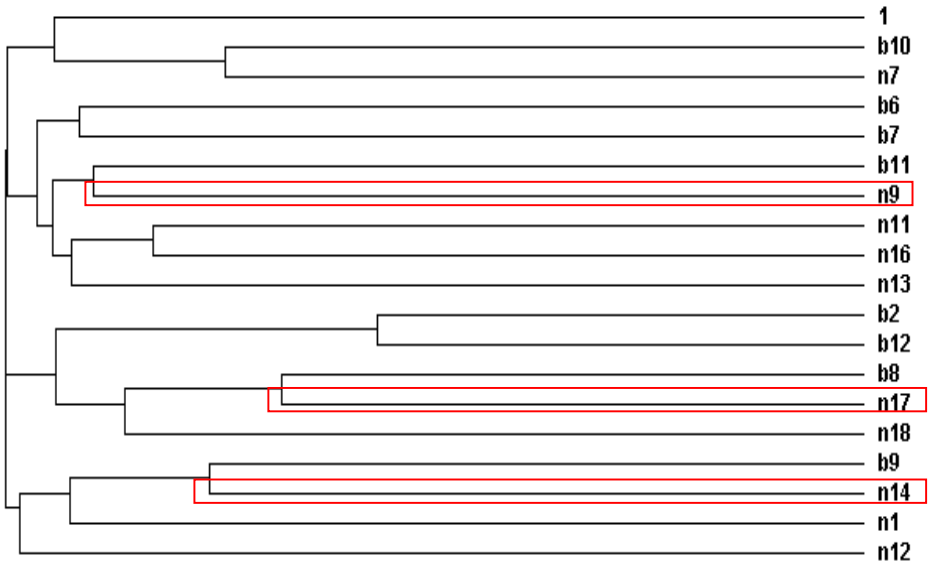


Figure 15. The *Cladogram Guide Tree* indicating the similarity score of the Aptamer sequences. The three selected sequences, which belong to different Aptamer families, were highlighted by red squares,

3.2.3 Investigation of the binding affinity of three representative aptamers

The binding affinity of aptamer n9, n17 and n14 were investigated by a fluorescence anisotropy binding affinity test. Fluorescence anisotropy is based on the detection of the depolarization of fluorescence emission after excitation of a fluorescent molecule by polarized light (Figure 16). Due to random character of diffusion, a linearly polarized excitation light will be translated into a less polarized emission light.[51] Thus, a high resultant of mP value (Fluorescence polarization) denotes the slow rotation of the labeled molecule, indicating that binding of large molecules probably did occur and vice-versa. It is suitable for aptamer binding studies as the tumbling motion of small aptamer molecules may be dramatically slowed down after binding to a larger protein molecule. Fluorescence anisotropy study offers numerous advantages over more conventional methods to study the binding of proteins to nucleic acids (particularly in that no hazardous radioactive waste is generated) and has a lower limit of detection in the sub-nanomolar range.

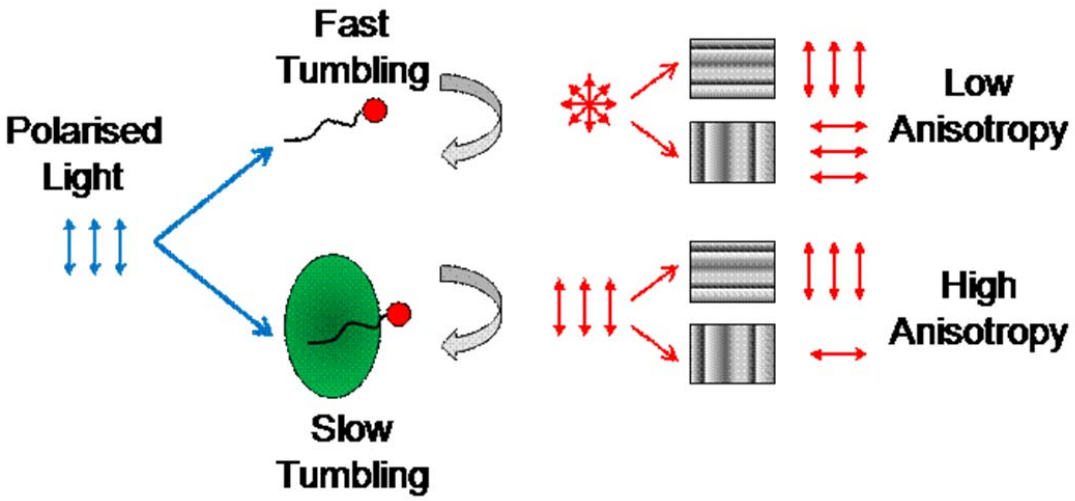
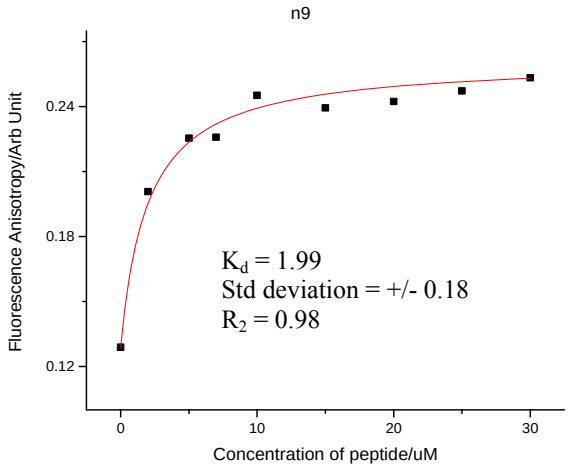


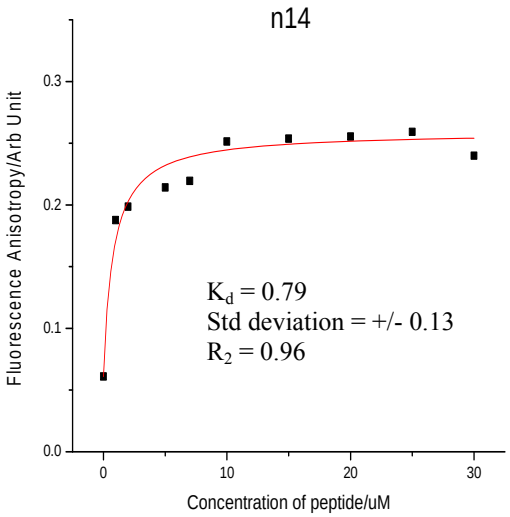
Figure 16. The principle of investigating binding events by fluorescent anisotropy.

Based on the fluorescence anisotropy study, the dissociation constants Kd of the formed complexes were determined and a considerable interaction between the peptide and the aptamer was found. In detail: the following Kd values were obtained: Kd of n9 = 1.99μM; Kd of n14 = 0.79μM and Kd of n17 = 0.72μM. The fluorescence anisotropy data and binding affinity fitting curve were shown in Figure 17.



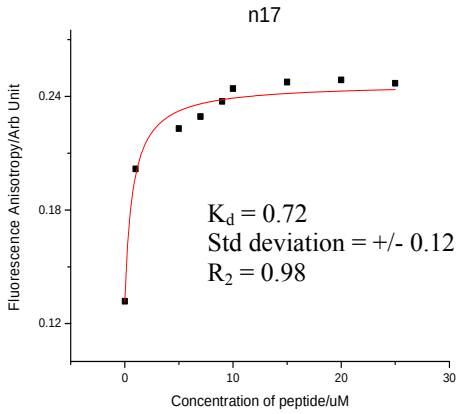
Data Table of Aptamer n9

[Hc peptide]	mP	r = 2P/3-P
0	181.6	0.129
1	197.2	0.133
2	273.6	0.201
5	303.8	0.225
7	304.4	0.226
9	255.2	0.186
10	327.6	0.245
15	320.6	0.239
20	324.2	0.242
25	330.0	0.247



Data Table of Aptamer n14

[Hc peptide]	mP	r = 2P/3-P
0	88.8	0.061
1	257.4	0.188
2	271.0	0.199
5	290.2	0.214
7	296.6	0.219
9	342.8	0.258
10	335.0	0.251
15	337.6	0.254
20	339.8	0.255
25	344.2	0.259



Data Table of Aptamer n17

[Hc peptide]	mP	r = 2P/3-P
0	185.4	0.132
1	274.8	0.202
2	312.2	0.232
5	301.0	0.223
7	308.6	0.229
9	318.2	0.237
10	326.4	0.244
15	330.4	0.248
20	331.8	0.249
25	329.6	0.247

Figure 17. Binding affinity data and fitting curve of Aptamers n7, n14, n17.

3.2.4 Analysis of aptamer structures

The three selected sequences were also subjected to the Rensselaer bioinformatics web server (http://mfold.bioinfo.rpi.edu) in order to visualize their potential secondary structures, which are summarized in Figure 18.

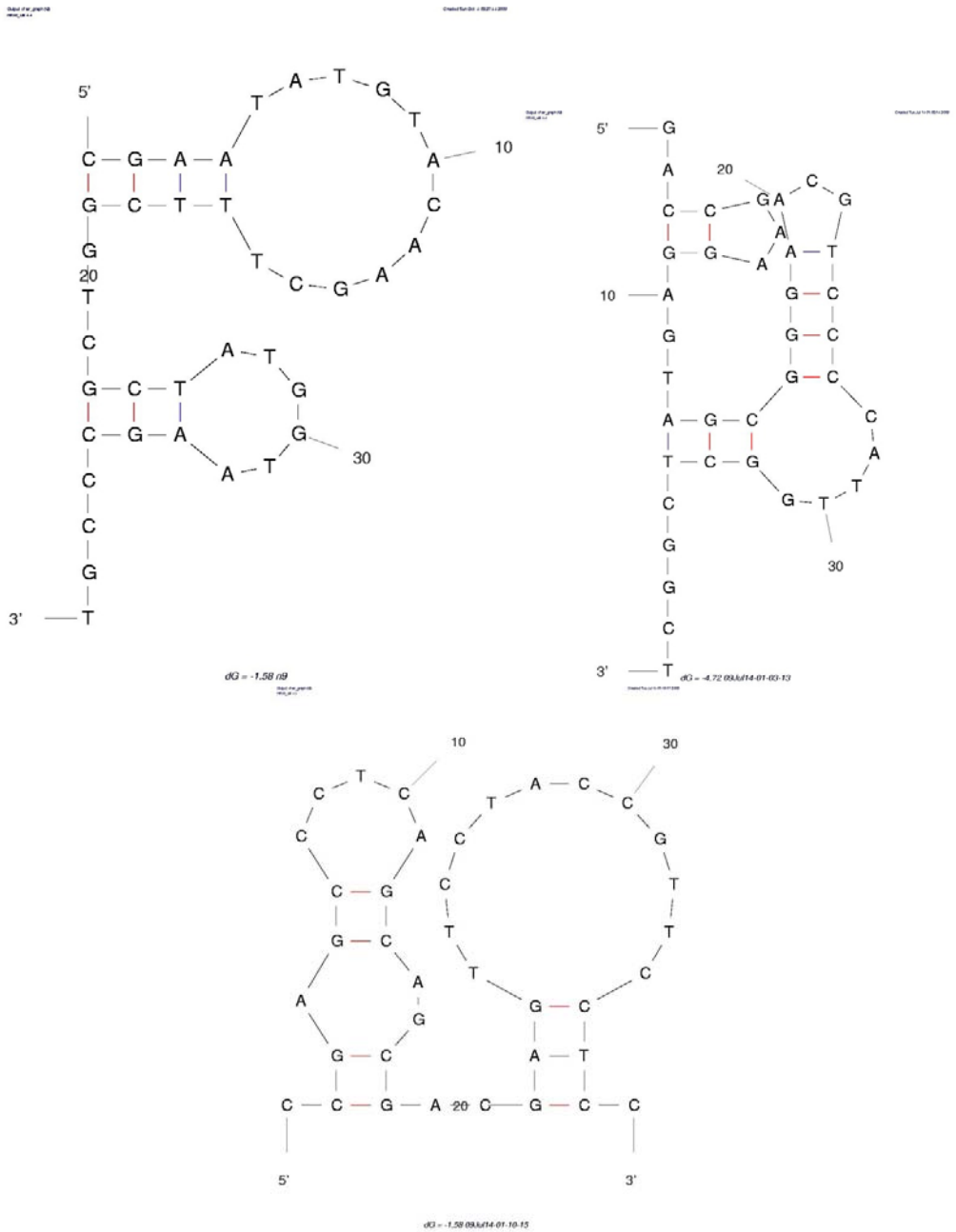


Figure 18. Predicted secondary structures for the three selected sequences based on Mfold software (http://mfold.bioinfo.rpi.edu) analysis.

3.2.5 Investigation of the binding affinity of Apt-pep to BoNT/A Hc domain protein

In order to highlight that it was crucial to use whole BoNT/A-Hc domain protein (*gsbp*), instead of 17mer Hc-peptide, as target for SELEX to get aptamers with potential to be BoNT/A inhibitor, we have tested aptamer n14 (its binding affinity to Hc-peptide is Kd=0.79μM) with BoNT/A-Hc domain protein in fluorescent polarization assay. No binding affinity was observed for ganglioside binding peptide aptamer toward native *gsbp* in 0~10μM range (Table 2).

Table 2. Fluorescence polarization assay for identifying the binding affinity between aptamer n14 and the BoNT/A-Hc domain protein.

BoNT-Hc concentration	0μM	1μM	2μM	4μM	7μM	10μM
mP	134.8	136.4	129.6	133.6	131.8	130.6

3.3 Aptamer for the *gsbp* (Apt-Hc)

3.3.1 Selected sequencing of Apt-Hc

After 6 SELEX cycles, the protein coated bead that contained bound aptamer was subjected to the PCR reaction with non-labeled primers. The PCR solution was directly cloned into E. Coli cells using the Qiagen PCR cloning kit according to the manufacturer's instructions. After overnight growth on LB agar plates, twenty colonies were selected and amplified by M13 primer PCR. The PCR reaction solution was directly sent to GeneWiz Inc. Beijing for further purification and sequencing. Four of the sequencing results were not reliable according to the chromatography data. The remaining 16 aptamer sequences were

summarized as follows. One of these aptamers has been investigated by a binding affinity assay which was highlighted with yellow color.

>LT5-2-1

CCAGTGGCGTATAGTATAGGGGGTCGCTTTGCAAGGTCA

> LT5-2-2

CAACGCCAACCCCCTTTGTAGCTCAGTCTTCCCGCCTCC

> LT5-2-3

ACCACCGTCATCCACTTTTCCCCTCGTGTCCGGACTTCA

> LT5-2-4

GAGATGAGCGCGCGTTAGGTTCAATGCGGACCCCGTGCG

> LT5-2-5

CCATGAGAACATAGCCTACCTGCCGACTATTCAGGTCCT

> LT5-2-6

GGGCACCGGACGATATAGGGAGAAAGGCCGTTAGCTGCC

> LT5-2-7

GGCCGACGCGAGACTTATGAGGTTTTACCCTTTCCCGTG

> LT5-2-8

CCATGAGGGCTAAGAATAAGATGAGCACACCCGTTCTCG

> LT5-2-9

CCCGCTATCCTCACTGCGCGATAATGTGAGTTTCGTGTT

> LT5-2-10

GCCCCGTATCAAGGTTTAATTACTACCCACAACCTACCA

> LT5-2-12

CCGACGATATCTAGCTCTGACACGCTACACGTAGCCCGT

> LT5-2-13

ACAACCGGGCGGCTCTCCTCAGTTATTGCTTATTCGTCG

> LT5-2-15

CCAGCTTATATATAATTTCCGGCCCGTTCACCCACCCCG

> LT5-2-17

CCCCGATAGACAAGCTGGGATCACACAACGTCGAGTTCT

> LT5-2-18

CCCACACCCTATCTTGTAATGCTTTCTCCACATGGCTTT

> LT5-2-19

CCACGCGACCTTTGACGCCGACCTGACTTATCGCTATCG

3.3.2 Sequences alignment and similarity analysis

The obtained clone sequences were subjected to homology analysis using the sequence alignment program ClustalW (available free-of charge via the website: www.ebi.ac.uk). The software is able to separate the greatly diverse 16 input sequences into meaningful groups (Figure 19).

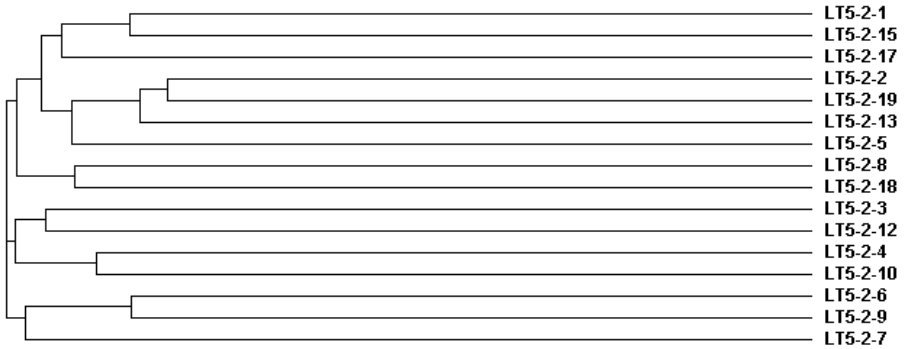
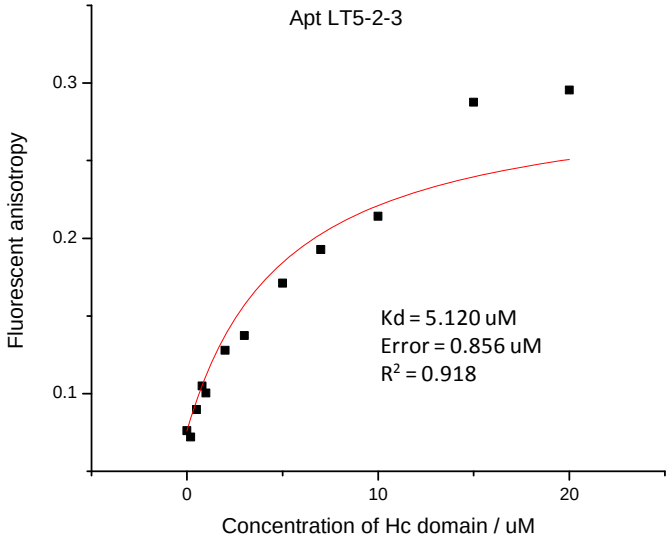


Figure 19. The *Cladogram Guide Tree* indicating the similarity score of the aptamer sequences.

According to the similarity score and *cladogram guide tree*, the identified aptamers pool is still too diverse and little similarity. This normally indicates that more SELEX cycles may be needed in order to get more potent aptamers.

3.3.3 Binding affinity of a representative aptamer

The binding affinity of aptamer LT5-2-3 for the *gsbp* protein was tested by fluorescent polarization spectroscopy. A binding affinity of 5μM with a standard deviation of +/- 0.86 was found and the fluorescence anisotropy data and the binding affinity fitting curve were shown in Figure 20.



Data Table of Aptamer LT5-2-3

[Hc peptide]	mP	r = 2P/3-P
0	90.6	0.062
0.2	92.4	0.064
0.5	114.6	0.079
0.8	130.6	0.091
1	124.2	0.086
2	160.2	0.113
3	169.6	0.120
5	198.6	0.142
7	240.4	0.174
10	257.8	0.188
15	356.8	0.270
20	357.0	0.270

Figure 20. Binding affinity correlation curve and anisotropy data summary of Aptamer LT5-2-3.

3.3.4 Analysis of aptamer structures

The sequences of LT5-2-3 were also subjected to the Rensselaer bioinformatics web server (http://mfold.bioinfo.rpi.edu) analysis for their secondary structures and shown in Figure 21. The real structure of this aptamer can only be derived from a crystal structure, which represents an additional, future goal.

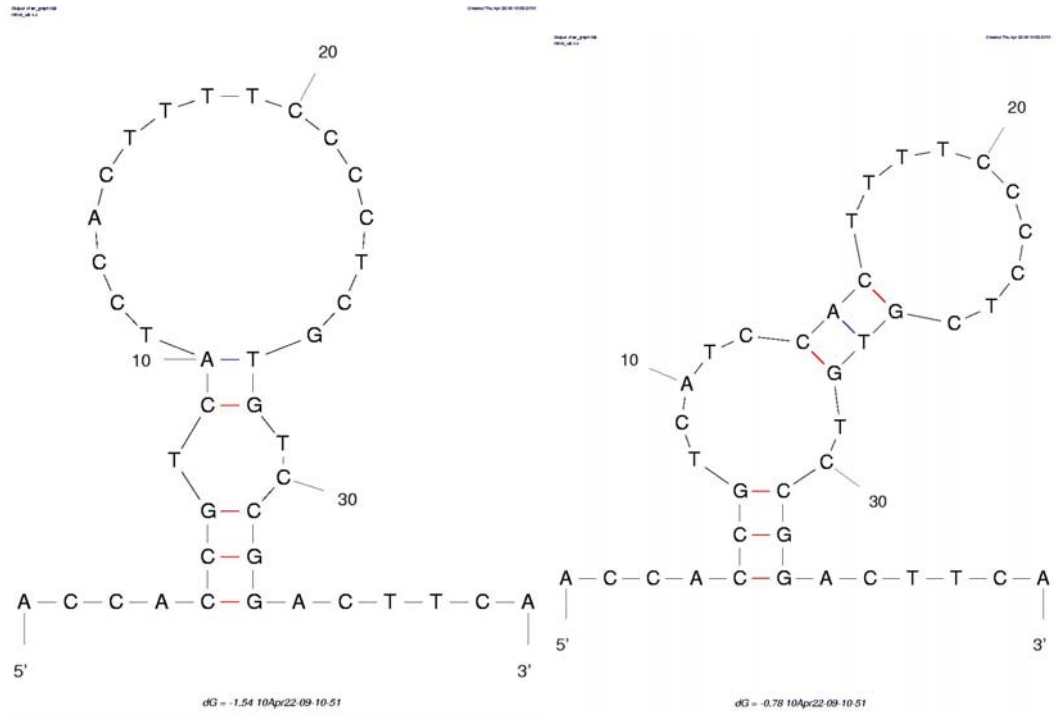


Figure 21. Predicted secondary structures for Apt LT5-2-3 based on Mfold software (http://mfold.bioinfo.rpi.edu) analysis.

Chapter 4. Conclusion

The H_C domain of the protein toxin BoNT/A contains both the ganglioside and the protein receptor binding site which are essential for the first step of the BoNT/A intoxication process. In this project aptamers as macromolecular inhibitors were designed and selected to interact with and block the H_C domain. Efficient blockade of the critical binding sites of the H_C domain would therefore prevent adhesion and endocytosis of BoNT by neuron cells. Cell membrane penetration of these aptamers is not required since the therapeutic targets are blood circulating BoNTs and therefore, the major drawback of aptamer drugs is less relevant here.

A single bead SELEX method has been established and described in detail. A commercially available, 19-mer peptide of the ganglioside binding peptide was used as the first target to set up the SELEX process. The protein immobilization conditions, PCR conditions, ssDNA library regeneration methods and PCR cloning conditions were all optimized in the course of this project. After 3 SELEX cycles, several moderately potent aptamers with binding affinities in the range of about 1μM were selected and analyzed. However, the chemically synthesized 19-mer peptide did most likely not maintain the correct, active conformation of native BoNTs and therefore, no significant binding affinity of these aptamers toward the native BoNT/A protein was observed.

Based on these important findings of the peptide aptamer (Apt-pep) SELEX, a similar SELEX procedure was applied for the *gsbp* called “small protein SELEX”. The target

protein used in this part reflects the neuroselective binding site of the H_C domain of BoNT/A (*gsbp*) and it was expressed with a His-tag provided by the collaborator. The original SELEX procedure has been modified with regard to the temperature and incubation time since protein SELEX requires several additional precautions due to the prerequisite to retain the active protein conformation in solution during the SELEX process, under these improved conditions, and after 6 cycles, aptamers with binding affinities of about 5μM for the native BoNT/A H_C domain were obtained.

More potent aptamers might be achieved after additional SELEX cycles or by post-SELEX modifications, which will explored in the future. All of these aptamer samples are currently being subjected to bioactivity tests by the collaboration partner.

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