DOI:10.14753/SE.2022.2604

SEMMELWEIS EGYETEM

DOKTORI ISKOLA

Ph.D. értekezések

2604.

PERCZE KRISZTINA

Patobiokémia
című program

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DOI:10.14753/SE.2022.2604

GENERATION OF RESPIRATORY SYNCYTIAL VIRUS SPECIFIC APTAMERS

PhD thesis

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Budapest
2021

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List of Abbreviations

ALPHA	Amplified Luminescent Proximity Homogenous Assay
APS	ammonium persulfate
BME	MTA-BME "Lendület" Chemical Nanosensors Research Group, Department of Inorganic and Analytical Chemistry, Budapest University of Technology and Economics
BSA	bovine serum albumin
CCL2 / MCP-1	monocyte chemoattractant protein 1
CX3C	fractalkine receptor
CXCL12	stromal cell-derived factor 1
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
EMA	European Medicines Agency
F protein	fusion glycoprotein
FAM	fluorescein amidite
FDA	US Food and Drug Administration
FP	fluorescence polarization
G protein	attachment glycoprotein
HRV	human Rhinovirus
HTS	high-throughput sequencing
K_D	dissociation constant
kDa	kilodalton
LRTI	lower respiratory tract infection
MES	2-(N-morpholino)ethanesulfonic acid
MST	Microscale Thermophoresis
NIAID	National Institute of Allergy and Infectious Diseases

NIH	National Institutes of Health
O/N	overnight
OTA	ochratoxin A
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
PFU	plaque forming unit
Poly(dI-dC)	poly(deoxyinosinic-deoxycytidylic) acid
qPCR	quantitative polymerase chain reaction
RADT	rapid antigen detection test
RNA	ribonucleic acid
RSV	Respiratory syncytial virus
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELEX	Systematic Evolution of Ligands by Exponential enrichment
SH protein	small hydrophobic protein
SOMAmer	slow off-rate modified aptamer
ssDNA	single stranded deoxyribonucleic acid
TAdUTP	5-[(3-indolyl)propionamide-N-allyl]-2’-deoxyuridine-5’-triphosphate, 5-indolyl-AA-dUTP
TBE	Tris-Borate-EDTA buffer solution
TE	Tris-EDTA buffer solution
TEMED	tetramethyl ethylenediamine
T_m	melting temperature
U	unit
VEGF	vascular endothelial growth factor
$VEGF_{165}$	vascular endothelial growth factor 165
VLP	virus-like particle
vWF	von Willebrand factor

1. Introduction

Reliance on antibodies both for the prompt detection of proteins and targeted treatment of diseases is rapidly emerging in the medical field. Consequently, the recent upsurge in demand for highly specific antibodies is unprecedented and can hardly be met due to the complexity of antibody production and purification. This unmet demand inevitably leads to the development and discovery of alternative receptors. Owing to the advantageous characteristics and ease of production, aptamers have been proven to be suitable alternatives to antibodies in a wide range of therapeutic and diagnostic applications. Aptamers are single-stranded oligonucleotides exhibiting high specificity and selectivity towards their target molecule by folding into unique three-dimensional structures. They offer a variety of benefits over antibodies, e.g., their *in vitro* selection which can theoretically target any molecule, even toxic or non-immunogenic compounds as opposed to the production of antibodies using animals or the hybridoma technology. The time consumption and high cost of latter methods are unparalleled to the selection of aptamers. Due to the inherent properties of aptamers, there is low batch-to-batch variation, high stability in various environmental conditions, and low immunogenicity that makes them a noteworthy drug candidate. Synthesis and modifications of aptamers are easier and more controllable than that of antibodies. Even though there is a myriad of advantages of aptamers in comparison to antibodies, aptamers have yet to reach their translational potential for therapeutics or diagnostics due to several reasons. On one hand, the practical applicability of the published aptamers is often not thoroughly assessed in a clinically relevant setting. On the other hand, challenging the well-established antibody-based methods requires a great capital investment and novel know-how that delays the commercialization of aptamer-based products.

1.1. The aptamers

Aptamers are short, single stranded oligonucleotides of unique 3D conformations that selectively bind their target molecules with high affinity and specificity.[1,2] Two independent research groups discovered and published the first aptamers in 1990. The term “aptamer”, coined by Ellington and Szostak, is derived from the Latin “aptus”, meaning “fitting” and the Greek “meros”, meaning “particle”. Their research group obtained selective aptamers for organic dyes.[3] Tuerk and Gold generated aptamers for

bacteriophage T4 DNA polymerase and named their method "SELEX" (*Systematic Evolution of Ligands by EXponential enrichment*, Figure 1).[4] Both groups exploited random RNA libraries as it was believed that the conformational flexibility of RNA oligonucleotides is higher than those of DNAs.[5] Currently, most laboratories utilize DNA oligonucleotide libraries for aptamer selection as the possibility of selecting high affinity aptamers from both DNA and RNA oligonucleotide libraries became apparent.[6]

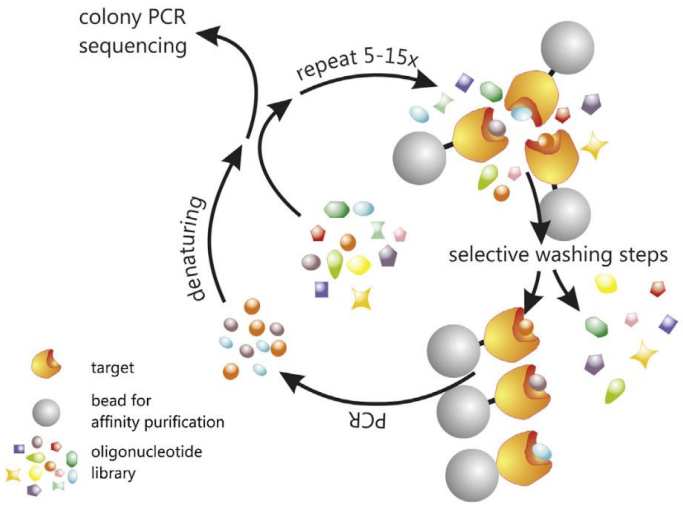

Figure 1 Principle of SELEX.[7] A diverse single stranded oligonucleotide library has a random region flanked by two constant primer-binding sites, which are used for PCR amplification of the library. This library, containing 10^{14}-10^{16} different sequences, is incubated with the target molecule. Sequences in complex with the target are separated from the unbound sequences and amplified by PCR, then this enriched library is regenerated and applied in the next round of selection. Multiple rounds (typically 5-15 rounds) of partitioning and amplification are performed until the library is enriched for a collection of oligonucleotides that possess high affinity towards the target molecule. Eventually, the nucleic acid sequences in the enriched oligonucleotide pool are identified by sequencing.

In terms of target selectivity and binding affinity, aptamers rival antibodies since both receptors exhibit dissociation constants (K_D) at nM-pM range.[8] The advantages of aptamers over antibodies derive from their chemical properties and *in vitro* synthesis. Theoretically, any molecule can be targeted by aptamers as opposed to the *in vivo* generation of antibodies where only immunogenic and non-toxic molecules can be applied. SELEX can be engineered so that aptamers recognize specific regions of the target molecule under a range of conditions tailored to the future application of the aptamer.[8] High affinity aptamers have been generated for proteins and peptides[9–11], small organic molecules[12], ions[13], viruses[14], whole cells and even for tumours *in*

vivo.[15,16] Chemical modifications and conjugations are easier to perform than that of antibodies. Due to the small size of aptamers (30-100 nucleotides), large amounts can be synthetized via the cost-effective phosphoramidite chemistry with low batch-to-batch variation.[17] Additionally, oligonucleotides possess greater temperature stability than proteins. These molecules have been shown to be able to replace antibodies in a battery of applications, such as biosensors[18,19] or molecular imaging probes[20,21] (Table 1).

Table 1 Currently available aptamer-based diagnostic products, their mode of detection, applications and limitations.[22]

Product	**Mode of detection**	**Application**	**Limitation**
OTA-Sense and AflaSense	Fluorescence-based assay	Detection of Mycotoxins in food	Requires extraction of toxin from the sample
AptoCyto	Aptamer-based flow cytometry	Aptamer- and magnetic bead-based isolation of biomarker positive cells	Dependence on expensive instrument (flow cytometer)
AptoPrep	Fluorescence-based assay and mass spectrometry	Aptamer-based pulldown of biomarker-positive cells and protein isolation	4°C storage is recommended for long term storage
SOMAscan	SOMAmer-based detection and quantification of biomarkers	A highly efficient platform for biomarker discovery and diagnostics	Multistep process, the platform is only available through the company
CibusDx	Electrochemical sensing	Detection of foodborne pathogens	Special instrument-based technology
OLIGOBIND	Fluorogenic activity assay	Detection of active thrombin	Platelet contamination in plasma sample may interfere with the assay

The low immunogenicity and toxicity of aptamers makes them excellent therapeutic agents (agonist, antagonist, or inhibitor) or drug delivery candidates.[23–26] Several aptamers targeting proteins associated with pathogenesis were published. Among them were numerous thrombin aptamers[27,28] and ones targeting cell surface proteins, e.g., vascular endothelial growth factor (VEGF). The latter provided the foundation for Macugen (pegaptanib), the first-in-class aptamer-based drug approved by the US Food and Drug Administration (FDA) for the treatment of macular degeneration (Table 2).[25,29]

Table 2 Therapeutic aptamers currently in clinical trials.[22,24]

Aptamer	Target	K_D	Condition	Clinical status
Pegaptanib / Macugen	$VEGF_{165}$	200 pM	Ocular disease	FDA-/EMA-approved, phase IV
E10030	PDGF	0.1 nM	Ocular disease	Phase III
ARC1905	Human complement C5	2-5 nM	Ocular disease	Phase II/III
AS1411 (AGRO100)	Nucleolin	54.8 ± 7.3 nM	Oncology	Phase II
NOX-A12	CXCL12	200 pM	Oncology	Phase II
NOX-E36	CCL2 / MCP-1	1.32 nM	Inflammatory disease	Phase II
NOX-H94	Hepcidin	0.65 ± 0.06 nM	Anemia	Phase II
ARC1779	A1 domain of vWF	2 nM	Anti-coagulation	Phase II
NU172	Thrombin	Not published	Anti-coagulation	Phase II
REG1 system	Coagulation factor IXa	2.83 ± 0.4 nM	Anti-coagulation	Phase III

PDGF: Platelet-derived growth factor; $VEGF_{165}$: Vascular endothelial growth factor 165; CXCL12: stromal cell-derived factor 1; CCL2 / MCP-1: monocyte chemoattractant protein 1; vWF: v on Willebrand factor; EMA: European Medicines Agency

1.1.1. SELEX

Traditionally, SELEX requires the target molecule to be immobilized on a solid support so the partition of bound and unbound sequences can be accomplished. Using magnetic beads (FluMag-SELEX) for immobilisation ensures rapid and efficient separation of these molecules and facilitates stringent washing steps, besides, it enables the use of small amounts of target.[30] Other options for the partitioning during SELEX include, for example, the exploitation of the change in electrophoretic mobility between bound and unbound nucleic acids in capillary electrophoresis (CE-SELEX).[31]

The purity of the target ligand is vital to guarantee the selectivity of generated aptamers. In case of protein targets, this condition can be realised using recombinant proteins with fusion tags that also aid the oriented immobilization of proteins, consequently, the exposition of the desired epitope during the aptamer selection. However, oligonucleotides will also inevitably bind to the support, the cross-linker used for the immobilization and all other matrix components. Hence, negative selection steps must be introduced; sequences that interact with these interferents are eliminated by incubating the aptamer library with the immobilization matrix, then using the oligonucleotides in the supernatant for the next positive selection step.[6] To further

increase the selectivity of aptamers, counter-selection steps may be introduced where the oligonucleotide library is incubated with molecules showing structural homology to the target molecule. Using this approach, it is possible to select aptamers capable of distinguishing between D and L-enantiomers of small molecules, or molecules differing in a single methyl group.[32–34] Generally, in each SELEX selection cycle, incubation time with the target molecule decreases, meanwhile the stringency of washing steps increases.[7] The introduction of competitors into the selection buffer, e.g. BSA, Poly(dI-dC) or dextran-sulphate aids the elimination of aspecific and low affinity oligonucleotides from the aptamer pool.[35,36]

In 1992, the first RNA aptamer with therapeutic potential for viral infections was described by Tuerk and Gold that specifically inhibited the reverse transcriptase activity of human immunodeficiency virus type 1 (HIV-1).[11] The aptamer was generated by the employment of the traditional SELEX method using immobilized protein. Currently, the so-called cell-SELEX, where selection is performed on infected whole cells or cells expressing virus proteins on their surface is a likewise widespread method.[37,38] A variation of cell-SELEX also emerged, in which the selection target is either intact or inactivated virus or virus-like particle (VLP) that is immobilized by affinity interaction or by covalently linking to a solid support.[39–41] All approaches have their advantages and shortcomings. While the protein demand of traditional SELEX can be met using protein overexpressing systems, most of the diagnostically significant virus proteins are modified posttranslationally, which might not be fully represented when expressed by cell systems.[42] Selection of aptamers for these improperly modified proteins might result in aptamers with no practical applicability since they do not recognize the virus proteins in their native state. Even though cell-SELEX and its variations similarly require the purification of the whole virus, which is nonetheless an intricate process, these methods are more straightforward as opposed to the complexity of reproduction of the adequate glycosylation patterns of native virus proteins. Furthermore, it is possible to perform cell-SELEX without prior knowledge of the structures of the viral proteins.[43] Affinity immobilization of viruses ensures the purity of the target as well as provides an opportunity to perform stringent counterselection steps against critical interferents, which may further improve the selection process.[44]

Nevertheless, the overall success rate of generating highly specific, functional aptamers using SELEX is only approximately 30% for protein targets, and their routine use is hindered by their degradation due to the pervasive nucleases.[5] Additionally, the commercialization of an aptamer-based therapeutic or diagnostic tool needs a greater investment than that of antibodies, where well-established methods shorten the time to reach their translational potential.[45]

1.1.2. Modified aptamers

The possible aptamer-target interactions can include a mixture of hydrogen bonding, electrostatic interactions between charged groups, π-π stacking between aromatic compounds and the nucleobases of aptamers, van der Waals forces and the hydrophobic effect.[46] The latter is less pronounced in the case of aptamers consisting of natural nucleotides; the possible interactions between proteins and natural oligonucleotides are constrained due the limited chemical diversity of the nucleobases as opposed to that of amino acids.[47] Therefore, a series of unnatural modifications have been incorporated into aptamers in order to improve the efficiency of SELEX and increase the durability of aptamers in the prevailing environmental and physiological conditions.[48]

The first modified aptamers were produced by the post-SELEX approach, that is, aptamers were first isolated by the traditional SELEX using natural RNA or DNA libraries, then the unnatural nucleotides were incorporated at given positions during the chemical synthesis of oligonucleotides.[49] The shortcoming of this method is well-illustrated by the history of the first commercialised aptamer-based drug. The lead molecule of pegaptanib was isolated from a 2'-F ribose-modified RNA library; in order to increase the half-life of the aptamer, SELEX was followed by the insertion of further modified nucleotides in various locations and the laborious and cost-ineffective individual characterization of each aptamer candidate.[29] Mod-SELEX has been utilized to evade post-SELEX: oligonucleotide libraries of nonstandard nucleotides have been created either by directly introducing the modified nucleotide during the synthesis or by the addition of "clickable" nucleotides and the post-synthetic functionalization by click-chemistry.[48,50] Three broad categories in modifications can be distinguished according to the involved component of oligonucleotides, that is, sugar, phosphodiester backbone, and nucleobase modifications (Figure 2).[51] Numerous unnatural oligonucleotides have been applied in SELEX with a diverse success rate, e.g., Spiegelmers (highly nuclease

resistant L-stereoisomer oligonucleotides[52]), Locked Nucleic Acids (oligonucleotides possessing a 2'-O, 4'-C methylene bridge, conferring nuclease resistance and structural stability to the oligonucleotide[53]) and the most promising ones, the so-called SOMAmers (slow off-rate modified aptamers, base modified oligonucleotides at the 5-position of deoxyuridine[5]). SOMAmers were successfully selected even for proteins that failed SELEX using an unmodified DNA library, moreover, most of them exhibited strongly improved, sub-nanomolar K_D values.[5]

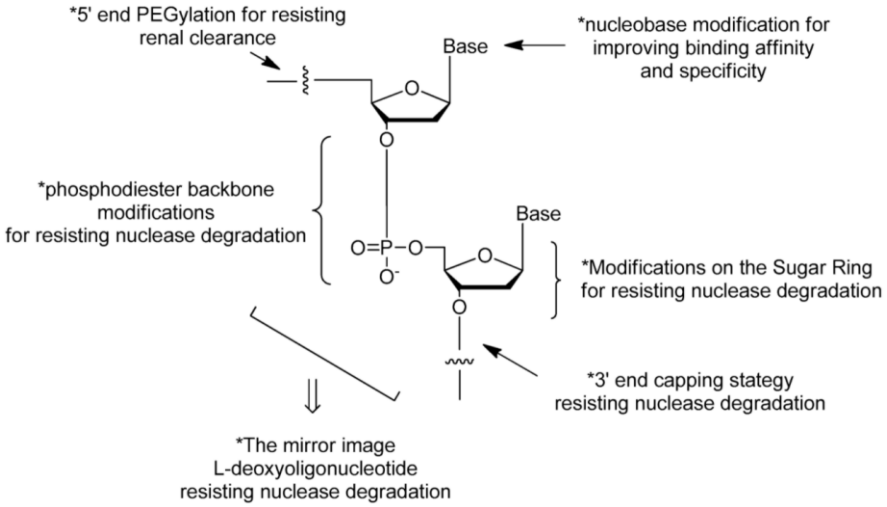

Figure 2 Common strategies for the chemical modifications of nucleotides in nucleic acid aptamers and their functions.[54]

Concisely, introduction of hydrophobic and aromatic functional groups at positions oriented away from the hydrogen bonding sites of the bases, that is, the 5-position of pyrimidines and the 8-position of purines seemed to have the most substantial effect on the outcome of aptamer selections.[55,56] It was also demonstrated by solving the crystal structures of SOMAmer-target complexes that these hydrophobic modifications exhibit a hydrophobic binding surface for the target molecule by forming a cluster and are stabilized in a highly compact conformation.[57–59]

While the initial modified oligonucleotide library could be generated by solid-phase synthesis and by click chemistry, the amplification of target selective oligonucleotides in SELEX requires specific polymerases that are capable of incorporating the unnatural nucleotide.[60] Several different commercial DNA polymerases have been studied to evaluate their ability of accepting modified nucleotides as substrates of DNA polymerisation. These studies proved that family B polymerases are superior to the members of family A, both in terms of effectivity and accepting a more extended

repertoire of unnatural nucleotides.[61–63] Some of these enzymes were successfully applied to amplify base-modified aptamers[50,64–66], although very little have been published illustrating the distortion of oligonucleotide libraries upon incorporation of non-natural nucleotides by PCR yet.

Following SELEX, the enriched oligonucleotide pool consists of a myriad of aptamer candidates and they possess distinct affinity and specificity towards the target molecule. Sequencing either using Sanger's method or high-throughput sequencing (HTS) and applying *in silico* analysis provides help for the lead aptamer identification. There are various online tools for *in silico* analysis of aptamers: primary sequence analysis (e.g. FASTAptamer[67]), consensus motif discovery (e.g. MEME[68]), secondary and tertiary structure prediction (e.g. Mfold[69] and 3DNA[70]) and docking simulations (NPDock[71]).[72] The latter two methods are mostly suited for aptamers consisting of natural nucleotides and prove to be inapplicable if a modified nucleotide is introduced into the aptamer. Due to PCR bias[73], the most abundant sequence or motif holding aptamer in the final oligonucleotide pool is not necessarily the best binder; therefore, a combination of *in silico* and functional analysis of aptamers is a superior approach to pinpoint the lead aptamer.

1.2. The Respiratory Syncytial Virus (RSV)

Viral or bacterial infections of the respiratory system occur throughout one's lifetime. The severity of infections varies from a mild upper respiratory tract infection to a severe lower respiratory tract infection (LRTI), often leading to bronchitis, bronchiolitis and pneumonia. *Streptococcus pneumoniae*, *Haemophilus influenzae* and Respiratory syncytial virus (RSV) are the major infectious agents responsible for significant morbidity and mortality, especially in childhood.[74,75]

Almost all children in their first couple of years of life undergo RSV infection at least once and in the majority of cases the disease resembles the symptoms of the common cold. Secondary bacterial infections occur less than 1% of RSV cases, on the other hand, co-infections with other respiratory viruses are more frequent and increase the risk of severe LRTIs leading to hospitalization, e.g. co-infection with Rhinovirus, parainfluenza virus-3, human metapneumovirus, or adenovirus.[76–79] The highest risk factors for the development of a severe RSV infection are prematurity, low birth weight and chronic lung disease.[80,81] The subsequent development of asthma among children

who are hospitalized due to severe RSV infection is significantly higher.[81] Additionally, infection of both the elderly and immunosuppressed patients are of great concern as RSV is most common viral pathogen that is transmitted nosocomially in both paediatric wards and nursing homes.[75,82–84] Especially in the case of the neonates and the immunosuppressed, the symptoms of RSV infections may appear to be atypical, thus hindering the diagnosis and delaying crucial treatment.[83]

RSV spreads by close contact with infectious secretions, i.e., aerosols of large particles or self-inoculation after touching a contaminated surface.[83] The infection takes place first in the epithelial lining of the nasopharynx, then it spreads to the alveoli of the lower respiratory tract.[85] *In vivo*, ciliated epithelial cells and type I alveolar pneumocytes are targeted by RSV.[86] Reinfection can occur multiple times during one's lifetime due to the capacity of RSV to evade innate immunity and the failure of adaptive immunity to prevent reinfection.[87] It is estimated that 33 million RSV-associated LRTI results in 3.2 million hospital admissions annually and between 94 600 and 149 400 deaths worldwide, making RSV a disease burden comparable to influenza.[88,89] Despite the significance of RSV, the development of a safe and clinically effective vaccine against RSV is hampered by several factors including the early age of infection and the lack of adequate animal models for the pathogenesis of human RSV infection.[90] Management of RSV infection is highly supportive, currently there is only one commercially available humanized monoclonal antibody treatment (Palivizumab) for the prophylactic therapy of high-risk infants.[91]

RSV belongs to the *Orthopneumovirus* genus within the *Pneumoviridae* family, in the order of *Mononegavirales*.[86] It is an enveloped RNA virus with non-segmented, single-stranded, negative-sense genome.[92] Its name derives from the characteristic cytopathic effect that occurs during infection, that is, the formation of syncytia: membranes of the neighbouring cells fuse together forming a multiple nuclei harbouring cell.[92]

The RSV genome codes for 5 structural, 2 non-structural and 3 envelope proteins. These are the following: large (L) protein, nucleocapsid (N), phosphoprotein (P), matrix (M) that translates into M2-1 and M2-2 proteins due to overlapping reading frames, and two non-structural proteins (NS1 and NS2). To form the envelope, 3 transmembrane proteins are expressed, namely the fusion (F) glycoprotein, the attachment (G) glycoprotein and the small hydrophobic (SH) protein.[86]

The SH protein acts as a viroporin, and there is evidence for its role in viral fusion; however, SH gene lacking RSV is also infectious and triggers syncytia formation.[86,93] The membrane-bound form of G protein is responsible for the attachment of RSV to the host cell membrane by interaction with host cell surface molecules, meanwhile its secreted isoform plays a role in immune evasion.[94] The attachment is likely to occur through the interaction between a basic heparin-binding region present on G protein and the negative charged regions of heparan sulphate on the cell surface or possibly by the interaction of G protein with CX3C-chemokine receptor 1.[95] The function of the F protein is the mediation of fusion of the host cell and viral membrane and cell entry.[81,86] It has 5-6 glycosylation sites depending on the strain. In order to be fusogenic, it must be proteolytically cleaved into F1 and F2 subunits (15 and 55 kDa, respectively) and assemble into a heterodimer structure covalently linked by two disulphide bonds, which then associates into the trimeric F protein. This conformation is unstable and either spontaneously or upon a trigger folds into the postfusion form. During this reassembly, the F1 subunit refolds into a long α-helix, meanwhile the fusion peptide is repositioned towards the host cell membrane. This process is irreversible and the postfusion F protein is very stable. The fusion peptide could insert into the membrane of the host cell thus F protein will span both membranes. This facilitates the injection of the ribonucleoprotein complex into the host cell cytoplasm where the replication and transcription takes place. [86,92]

There is a single RSV serotype, which can be divided into two major antigenic subgroups: A and B. These groups have 81% nucleotide identity, amino acid sequences of F protein ectodomain vary only by approx. 5% between RSV A and B. On the other hand, there is great diversity in G protein among the two subgroups.[81,86,92] During epidemics, these subtypes co-circulate and usually RSV-A is the more dominant.[96]

Neutralizing antibodies target both the F and the G protein upon natural infection by RSV. The variability both in the sequence and the mucin-like glycosylation pattern of G protein in different subtypes of RSV might be advantageous for the virus considering the viral attachment and steric masking of the F protein from neutralizing antibodies.[86,97] Antigenic drift of F protein is less significant compared to that of G protein, and F protein induces higher levels of neutralizing antibodies *in vivo*. Consequently, F protein is a more favourable target for antiviral development.[86,91,98,99]

1.2.1. Current treatment for RSV infection and vaccine development

In all age groups, the treatment of RSV infection is primarily based on supportive care. Ribavirin, a small molecule that inhibits the replication of RNA and DNA viruses may be applied. However, solely high-risk patients are treated with ribavirin due to the complexity of ribavirin administration, high cost and possible adverse effects.[76,100,101] A humanized murine monoclonal antibody, Palivizumab, is currently the only prophylactic treatment option also for high-risk infants to prevent serious RSV infection. Palivizumab targets an epitope on the antigenic site II of F protein which is present on both the prefusion and postfusion conformation of F protein (Figure 3).[86]

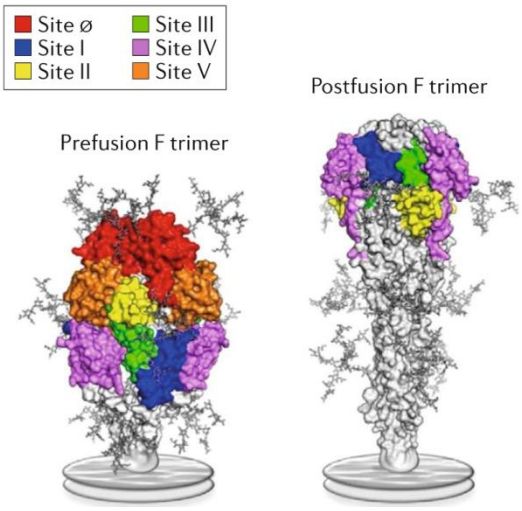

Figure 3 Location of the major antigenic sites on the prefusion and postfusion conformations of the respiratory syncytial virus (RSV) fusion (F) protein.[86]

A multitude of other human antibodies were raised against F protein and many of them exhibit greater neutralization capacity than Palivizumab. One example is the D25 antibody, which targets the prefusion F specific antigenic site Ø. It is suggested that D25 renders stronger neutralizing capability by stabilising the prefusion conformation of F protein via binding to antigenic site Ø. [102,103]

As a potent vaccine candidate, the prefusion form of F protein is a reasonable choice. However, preserving the F protein in its prefusion form without the help of an antibody poses a challenge. McLellan *et al.* provided one of the most stable mutants of F protein (RSV-A) using structure-guided stabilization, the so-called the DS-Cav1. DS-Cav1 is a subunit vaccine candidate for pregnant women.[89] It has been stabilized by an intrasubunit disulfide bond as two serines were substituted by cysteines (“DS”) and two hydrophobic cavity filling mutations (“Cav”) in the F1 subunit.[104] DS-Cav1 elicits

systemic immune response in healthy adults after a single dose and provides 10-fold increase in neutralizing activity of serum.[105,106]

Small molecule fusion inhibitors may also stabilize the conformation of the prefusion form by binding its central cavity and interacting with the fusion peptides. This event prevents the insertion of the fusion peptides into the host cell membrane thus inhibits the infection.[86] Such an antagonist, called GS-5806, has shown to reduce viral load and disease severity during the treatment of healthy adults who were challenged with a clinical isolate of RSV in phase II trials.[107]

1.2.2. Current methods for the detection of viral infections

Rapid diagnosis of viruses is crucial for creating the most appropriate treatment plan. However, the traditionally applied methods, such as viral culturing require not only highly skilled staff but are also time-consuming. Therefore, qPCR of the viral genome has become the golden standard for detection of viral infections, alongside with the use of surface protein selective antibodies for immunoassays. The latter approach can be applied using direct immunofluorescence staining or antibodies can be used in various rapid antigen detection tests (RADTs), which are widely commercialized in kit format.[108] Detection of viral proteins are the most suitable for rapid tests, which is highlighted by the fact that the majority of the results reported to an RSV surveillance program were produced using RADTs.[109] These rapid assays are applied for the detection of either RSV F protein or nucleocapsid proteins, and commonly available as enzyme immunoassays (EIAs), immunochromatographic tests, or optical immunoassays.[110] In case of the elderly, who presumably shed a low amount of virus, viral culture and antigen tests are impractical. A combination of serology by EIAs and qPCR provide the best sensitivity and specificity in the adult population.[100] Especially in resource-limited settings, antibody-based antigen detection tests are less suitable due to loss of reliability caused by, e.g., thermal inactivation. A solution for the latter problem could be the substitution of antibodies with different types of receptor molecules, for example, aptamers.[111,112]

DOI:10.14753/SE.2022.2604

2. Objectives

The respiratory syncytial virus (RSV) is the most common cause of acute lower respiratory tract infection during infancy and late adulthood with a disease burden comparable to that of influenza. Appropriate clinical management and diagnostics of RSV is hindered by the fragility, immunogenicity and high cost of antibodies. The aim of the thesis work is to exploit the unique attributes of aptamers to provide RSV specific oligonucleotides of diagnostic and therapeutic potential.

In order to realize our goals, the following tasks were carried out:

1. Design of a SELEX procedure that circumvents the time-consuming target protein purification and may be generally applied for selection of virus specific aptamers;
2. Selection of highly specific and selective aptamers that are suitable for the determination and distinction of RSV from its most common co-infecting agent, HRV;
3. Characterization of aptamers using several approaches to determine target specificity and selectivity in complex biological matrices at clinically relevant virus titres;
4. Development of a suitable method for the generation of oligonucleotide libraries of modified nucleotides by PCR;
5. Devising a SELEX protocol for exploiting the modified oligonucleotide library to provide aptamers of therapeutic potential for RSV infection;
6. Identification of a lead aptamer candidate for virus infection inhibition assay.

3. Methods

Table 3 Solutions and their composition.

Name	Composition
10x PBS	1.37 M NaCl, 27 mM KCl, 20 mM KH_2PO_4, 100 mM $Na_2HPO_4 \cdot 2H_2O$, pH 7.5
TE	10 mM Tris, 0.1 mM EDTA, pH 8.0
5x Laemmli sample buffer	0.312 M Tris, 10% SDS, 250 mM DTT, 50% glycerol, 0,01% bromophenol blue, pH 6.8
5X TBE	1.1M Tris; 900mM borate; 25mM EDTA; pH 8.3
Coomassie-blue Destain solution	10% ethanol, 2% ortho-phosphoric acid in distilled water
Coomassie-blue solution	0.02% Coomassie Brillant Blue G-250, 5% $Al_2(SO_4)_3.18xH_2O$, 10% ethanol, 2% ortho-phosphoric acid in distilled water
Dynabeads 2x Binding&Washing buffer	20 mM Tris-HCl (pH 7,5), 2 mM EDTA, 2 M NaCl
Laemmli separation gel buffer	1.5 M Tris, 0.4% SDS, pH 8.8
Laemmli stacking gel buffer	1 M Tris, 0.8% SDS, pH 6.8
MES buffer	50mM MES, pH 6.0

Table 4 Sequences, modifications and manufacturer of DNA libraries and primers.

Name of oligo	Sequence (5'-3')	Modification	Manufacturer
Library no. 8 (L8)	GAT CGA GCC AGC TAC GTC -(N40)- GAC ATG CCT CAG CGA CTA	Ø	IDT
Forward primer for L8	GAT CGA GCC AGC TAC GTC	Ø / 5'-Biotin / 5'-Cy5	IDT
Reverse primer for L8	TAG TCG CTG AGG CAT GTC	Ø / 5'-Biotin / 3'-Phos	IDT
Complement of reverse primer for L8	GAC ATG CCT CAG CGA CTA	Ø	Eurofins
M13 forward	GTA AAA CGA CGG CCA G	Ø	Sigma Aldrich
M13 reverse	CAG GAA ACA GCT ATG AC	Ø	Sigma Aldrich

3.1.1. Immobilization of RSV F to paramagentic beads

The stabilised form of prefusion ("DS-Cav1") and postfusion F protein was used as targets in SELEX; these purified proteins were kindly provided by Peter D. Kwong's research group (Vaccine Research Center, NIAID, NIH, USA). 5.7 mg of beads from MACSflex™ MicroBead Starting Kit (Miltenyi Biotec) was reconstituted in 257 µl of Reconstitution Buffer. This bead was modified using 15 µg of F protein in 25 µl of MES buffer by O/N incubation at 4°C. Success of immobilization was determined using SDS-PAGE (Table 5) by Coommassie-blue staining (Table 3).

Table 5 Composition of acrylamide gel for analysis of proteins.

Acrylamide gel for analysis of proteins		12%	15%
Separation gel	distilled water	2,19 ml	1,8 ml
	Laemmli separation gel buffer	1,3 ml	1,3 ml
	40% acrylamide (37.5:1)	1,56 ml	1,95 ml
	10% APS	50 µl	50 µl
	TEMED	2 µl	2 µl
Stacking gel	distilled water	1,44 ml	
	Laemmli stacking gel buffer	280 µl	
	40% acrylamide (37.5:1)	260 µl	
	10% APS	20 µl	
	TEMED	2 µl	

3.1.2. Selection of modified aptamers for RSV

An aptamer selection was performed to generate RSV F protein selective aptamers using a tryptophan-like side chain holding aptamer library. The SELEX of RSV F specific modified aptamers were obtained in 7 iterative cycles with increasing selection pressure. The modified oligonucleotide library was heated to 95 °C for 5 min and immediately cooled on ice prior to each selection cycle. First, approx. 1 nmole of the oligonucleotide library was incubated with non-modified Miltenyi beads in selection buffer (10 µg/ml BSA, 0.02% Tween 20, 1 µg/ml Salmon sperm DNA, 10 µg/ml mucin in PBS) for 60 minutes using mild shaking to exclude the bead binding oligonucleotides. The F protein modified beads were incubated with the supernatant of the previous step for 30 minutes, then washed with 2 × 1000 µl PBS, followed by isolation of DNA pool using alkali elution and neutralization of F protein-bound oligonucleotides. Water-in-oil emulsion PCR was carried out to amplify the bound sequences using Micellula DNA Emulsion & Purification Kit (Roboklon). The PCR mixture contained KOD XL 1x reaction buffer, 2 U of KOD XL polymerase (Toyobo) 0.4–0.4 µM of untagged forward and biotinylated reverse primers of L8 library (Table 4), and 0.2 mM each CleanAmp dATP, dGTP, dCTP (Trilink) mixed with TAdUTP (5-[(3-indolyl)propionamide-N-allyl]-2’-deoxyuridine-5’-triphosphate, Trilink).

Amplification conditions were: 3 min at 94 °C, 25 cycles of 94 °C for 30 s, 60 °C for 5 s, 74 °C for 30 s and 74 °C for 5 min after the last cycle. The success of amplification was monitored by 10% polyacrylamide gel electrophoresis (Table 6) and visualized using GelGreen DNA dye (Biotium).

For the regeneration of ssDNA by alkali denaturation, the PCR product was coupled to 25 µl streptavidin-coated paramagnetic beads (Dynabeads Streptavidin, M-280, Thermo

Scientific) for 15 min and washed with 3 × 1000 µl of PBS. The non-biotinylated strands were separated from the immobilized complementary strand by 10 min incubation with 50 µl of fresh 20 mM NaOH. The eluted ssDNA was immediately neutralized by addition of 7.5 µl of 200 mM NaH_2PO_4. In the following rounds of selection, the postfusion form of F protein was used either coupled to paramagnetic beads as a counter-target molecule (in rounds no. 4, 6, and 7) or the selection buffer was complemented with an excess of postfusion F (5-10 times in excess to the prefusion form in round 2, 3, 5, and 7). To further increase the selection pressure, the incubation conditions and washing steps were changed. In round 4, 5, 6, 7 the incubation time was reduced to 25, 20, and 15 min, respectively. In the third round an additional washing step with 1 ml of 0.15 mM dextran-sulphate in PBS (pH 7.4) was also introduced, in the fourth round, the bound sequences were challenged by washing the beads with 1 ml of 18 µM L-tryptophan solution as well. In the final selection round, the incubation time was 15 min and the beads were washed twice with 1 ml of 0.3 mM dextran-sulphate solution, twice with 1 ml of 18 µM L-tryptophan solution and twice with PBS solution. The PCR product of the last selection step was inserted into a cloning vector (Zero Blunt TOPO PCR Cloning Kit, Thermo Fischer Scientific) and transformed into α Select Gold Efficiency chemically competent *E. coli* cells (Bioline). 130 of the colonies were analysed by colony PCR (using an M13 primer set, Table 4) and capillary electrophoresis (LabChip GX, PerkinElmer) using the DNA 1K Reagent Kit with DNA HT 5K LabChip single sipper chip. For the latter, the colony PCR products were diluted 40x in TE buffer. The sequences of colony PCR products of correct size were determined by Sanger sequencing.

Table 6 Composition of acrylamide gel for analysis of DNA.

Acrylamide gel for analysis of DNA	**10%**
distilled water	3,2 ml
5x TBE buffer	1,2 ml
30% acrylamide (29:1)	1,6 ml
10% APS	100 µl
TEMED	5 µl

3.1.3. Production of modified aptamers for AlphaScreen and MST

AlphaScreen requires biotinylated aptamers which were generated by PBA-PCR (primer blocked asymmetric PCR). The 25 µl PCR mixture contained KOD XL 10x reaction buffer, 2 U of KOD XL polymerase, 0.5 µM of biotinylated forward primer, 25

nM untagged reverse primer, 475 nM 3'-P reverse primer, 0.2 mM each CleanAmp dATP, dGTP, dCTP mixed with TAdUTP, 0.5 µl of 40x diluted colony PCR template. Amplification conditions were: 3 min at 94 °C, 45 cycles of 94 °C for 30 s, 60 °C for 5 s, 74 °C for 30 s and 74 °C for 5 min after the last cycle. Following PCR, the complement of the reverse primer was added at 5 µM concentration, the mixture was heated to 95 °C for 10 minutes. The success of amplification was monitored by 10% polyacrylamide gel electrophoresis (Table 6). Cy5 labelled aptamers were prepared for MST as described in 3.1.2 with the distinction of using Cy5 labelled forward primers instead of the untagged primers.

3.1.4. Screening of modified aptamers

The interaction assays were performed using white 384-well Optiplates (PerkinElmer) in total volume of 18 µl using Protein A acceptor and Streptavidin donor beads (PerkinElmer). Varying amount of the prefusion and postfusion form of RSV F protein in PBS supplemented with mucin (10 ug/ml), BSA (1 mg/ml), Salmon sperm (1 ug/ml) and were incubated with 10 nM final concentration modified biotinylated aptamer and F protein selective antibody. Following 20 min incubation at RT, the acceptor and donor beads were added at 20 µg/ml final concentrations in two steps. First, the Protein A acceptor beads were added and incubated for 30 minutes at RT and that was followed by the addition of Streptavidin donor beads and further 30 min incubation. Fluorescent signal was detected by using an EnSpire multilabel plate reader (PerkinElmer).

3.1.5. Determination of K_D using MST

10, 15 or 20 nM of Cy5-labelled aptamers were mixed with a 16-fold, 1:1 serial dilution of either the prefusion form or the postfusion form of F protein. All mixtures were prepared in 0.05% Tween 20 in PBS and the measurement was carried out in Monolith NT™ Standard capillaries (NanoTemper Technologies). Excitation power and MST power of Microscale Thermophoresis instrument Monolith NT.115 (NanoTemper Technologies) was set to 50% and 20%, respectively. The temperature was set to 25°C during the measurements. All obtained data was analysed using the MO.Affinity Analysis v2.3 software (NanoTemper Technologies), where dissociation constants were calculated from the fitted curve using Michaelis-Menten kinetics.

DOI:10.14753/SE.2022.2604

4. Results

4.1. Selection of aptamers for RSV

4.1.1. SELEX procedure

The choice of target molecule is a critical decision when designing an aptamer selection procedure. In this case, SELEX was performed using inactivated whole RSV and HRV, as selection targets and counter targets, respectively. Prior to each selection step, RSV was *in situ* captured by Palivizumab-coated paramagnetic beads to ensure proper separation of the RSV-bound and unbound aptamer fractions. Negative selection steps were also performed to ensure the exclusion of aptamers binding to either the protein A paramagnetic beads or the antibody used during the selection (Figure 4).

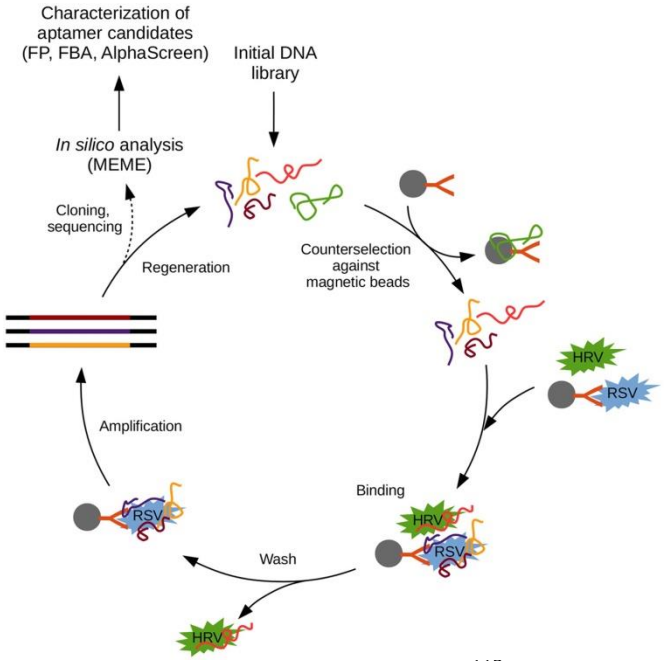

Figure 4 Schematics of the applied SELEX procedure.[113]

Five selection steps with increasing selection pressure were applied to ensure the specificity of aptamers. PCR products from the last selection cycle were inserted into a cloning vector and the sequences of 96 colonies were determined by Sanger sequencing. *In silico* analysis was carried out using Microsoft Excel and MEME motif search, the former revealed two aptamers in duplicated copies (B5, F6 and D10, D12), the latter showed three pairs holding a 9-nucleotide long consensus sequence motif (H8, E6; E11, E10; B10, F10) (Figure 5). Another single copy aptamer, H5, was also chosen randomly. Further characterization of aptamers was restricted to these candidates.

B5	AAGCCCCGTACCAAAGAAGGTCAGGTGTCT		
F6	AAGCCCCGTACCAAAGAAGGTCAGGTGTCT		
D10	TAAGAGGCAGACAGTAAGAACAACTACCTT		
D12	TAAGAGGCAGACAGTAAGAACAACTACCTT		
H8	AGTGCGGTGAG	**CCGTCGGAC**	ATACAAATAC
E6	AC	**CCGTCGGAC**	TCGGCCATAAATTAAAGGC
E11	TCATTAGGTGA	**GTGTCCGTTCT**	ACACTATA
E10		**GTGTCCGTTCT**	TATTGGCGGCTCCCAATGT
B10	TCCATATCG	**TTTAGCGTAC**GG	TGGCAGTCT
F10	A	**TTTAGCGTAC**TG	CCAACCATCAGGCGCCA
H5	TCGCCGTCCTGATCAGTAGCAGCCAGAACG		

Figure 5 The sequence alignment of selected oligonucleotides. Two aptamers in duplicated copies (B5, F6 and D10, D12); three pairs of aptamers holding an identical nucleotide sequence motif (H8, E6; E11, E10; B10, F10) and a randomly chosen aptamer (H5) from the rest of the oligonucleotides that possessed unique sequences.[113]

4.1.2. Analysis of selected aptamers

The practical applicability of aptamers must be determined by their characterization with various methods and in both ideal buffers and complex biological matrices. Therefore, we aimed at studying the virus binding capacity of aptamers using different approaches. First, the consensus motif holding aptamers were studied in selection buffer with fluorescence polarization (FP) by our collaborator at the MTA-BME "Lendület" Chemical Nanosensors Research Group, Department of Inorganic and Analytical Chemistry, Budapest University of Technology and Economics (BME). The FAM-labelled aptamers were synthesized in our laboratory.

FP relies on the fact that if a fluorescently labelled molecule is excited by polarized light, the degree of polarization of the emitted light will be inversely proportional to the rate of molecular rotation.[114] This phenomenon makes the real-time measurement of selective interactions between small, labelled ligands and larger biomolecules possible in biological matrices, without the need of immobilization of either biomolecule.[115] Direct binding studies and competition assays are both feasible using FP.[114,116]

FP analysis determined that all aptamers bound to RSV and HRV in a concentration dependent manner (Figure 6). The changes in FP value are influenced by two factors, i.e., the binding of the aptamer to the target molecule and the conformation of the aptamer that could constrain the free rotation of the FAM label. As these factors vary from aptamer to aptamer, classification of aptamers solely based on the FP measurements must be handled cautiously. Notwithstanding, three aptamers, E10, B10

and H8 seem to possess higher RSV binding capacity and H8 is clearly superior in discriminating between RSV and HRV.

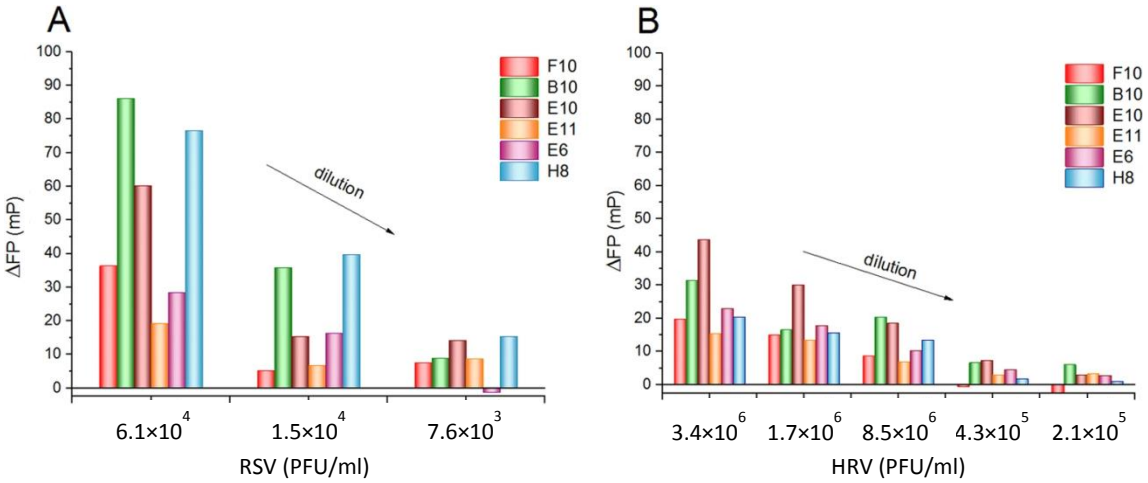

Figure 6 Fluorescence polarization values of mixtures containing 0.5 nM FAM-labelled aptamers and various amounts of RSV (**A**) and HRV (**B**) virus preparations. Concentration in PFU equivalents of HRV is approx. two orders of magnitude higher than that of the RSV.[113]

Next, we set out to analyse the aptamer candidates in selection buffer and a biologically relevant matrix, throat swab. One of the most traditional biomolecule interaction assays, the filter binding assay, was utilized for this purpose. This technique relies on aptamer-protein complexes that are spotted directly onto a pre-treated nitrocellulose membrane. Stringent washing steps allows the non-binder aptamers to pass through the filter, while aptamer-protein complexes are retained and can be detected.[117,118] Fluorescently labelled aptamers in complex with their target protein may be visualized using a fluorescence scanner.

FAM-labelled fluorescent copies of aptamers were synthesized using the oligonucleotides which were found in duplicates (B5 and D10), the randomly chosen sequence (H5) and the most promising consensus sequence holding oligonucleotide (H8). A constant amount of FAM-labelled aptamers was mixed with decreasing amount of inactivated RSV or HRV particles (3.4×10^6 PFU/ml to 8.5×10^5 PFU/ml), in selection buffer and throat swab matrix. Throat swab was obtained from the nasopharynx and oropharynx of healthy volunteers. Samples were spiked with RSV and HRV at clinically relevant concentrations. After incubation, the mixtures were blotted onto nitrocellulose membrane using vacuum, and stringent washing steps were carried out before fluorescence analysis.

All aptamers were selective to RSV in selection buffer and seemed to remain selective even when challenged with throat swab matrix, since they produced a higher fluorescence signal with the RSV than HRV containing mixtures (Figure 7). The consensus motif holding aptamer, H8, provided the highest signal, while the lowest signals were produced by the sequences present in duplicates (B5 and D10).

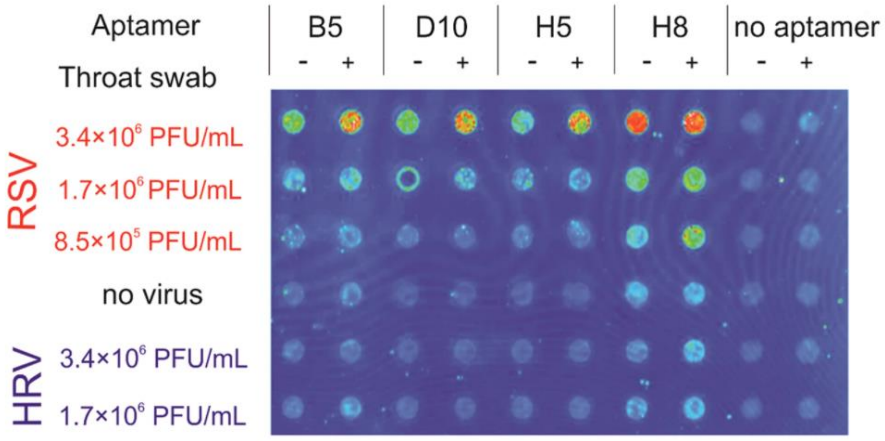

Figure 7 FAM-labelled aptamers were incubated with preparations of RSV or HRV, either in selection buffer or selection buffer complemented with throat swab matrix, then transferred onto nitrocellulose membrane using vacuum and extensively washed before fluorescence imaging. A high fluorescent signal in case of RSV containing samples indicates the selectivity of aptamers.[113]

Another approach, Amplified Luminescent Proximity Homogenous Assay (ALPHA) was also implemented to further assess the binding of aptamers to RSV. AlphaScreen is versatile no-wash assay developed to measure the interaction of biomolecules in a homogenous assay. In ALPHA, donor and acceptor beads are coated with biomolecules. Upon excitation of the donor bead, a singlet oxygen is generated that can travel approx. 200 nm in solution. If there is an acceptor bead in this proximity due to the interactions between the immobilized partners, energy transfer occurs that results in the generation of chemiluminescence.[119]

The RSV selective commercial antibody, Palivizumab, and biotinylated oligonucleotides were immobilized on Protein A and Streptavidin coated beads, respectively. The measured fluorescence indicates the RSV binding of aptamers (Figure 8). The results of ALPHA measurement confirmed that all aptamers recognized RSV and the most promising aptamer candidate is the consensus motif holding oligonucleotide, H8 (Figure 8). Of note, none of the aptamers showed an increased fluorescence signal when incubated with HRV.

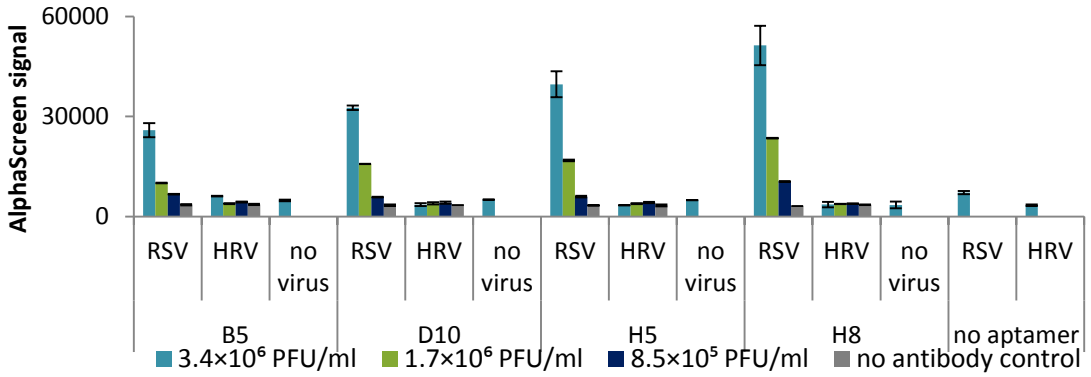

Figure 8 Aptamer-virus complexes were formed using a constant concentration of biotin labelled aptamers and varying amounts of RSV or HRV formulated in selection buffer. Then Streptavidin coated donor beads and F protein selective antibody modified Protein A acceptor beads were added and the fluorescence intensities were measured.

ALPHA is a highly versatile method, in which a panel of acceptor and donor beads can be combined for the assays. Therefore, it was possible to perform both an antibody-aptamer and an aptamer-aptamer sandwich assay by using the highest affinity aptamer, H8 in throat swab matrix. In theory, aptamer-based sandwich assays for virus detection can be implemented by applying the same aptamer as capture and recognition element of the sandwich assay since many copies of the envelope proteins are present on the virus surface.

The experimental setup for the antibody-aptamer sandwich was as follows. The antibody and the aptamer were mixed with varying amounts of inactivated RSV and HRV in throat swab containing buffer. Next, the Protein A acceptor bead was added and incubated with the mixture. Finally, the solution was complemented with Streptavidin donor beads and following the incubation, the chemiluminescent signal was measured. The aptamer-aptamer sandwich assay differed from this setup since the biotinylated aptamers were separately immobilized onto the Streptavidin acceptor and donor beads and then mixed with the virus containing throat swab samples.

Both experimental setups succeeded in detecting clinically relevant titres of RSV, producing high fluorescence signals and showed no signal increase when combined with HRV (Figure 9). Notably, the aptamer-based sandwich detection showed a greater sensitivity than that of antibody-aptamer assay for RSV detection.

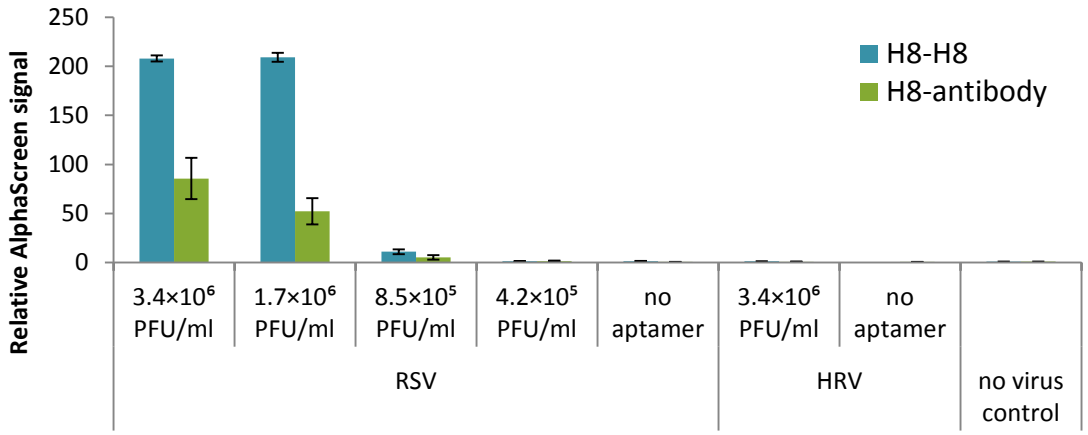

Figure 9 Biotin-labelled aptamer and F protein selective antibody was added to RSV-spiked throat swab mixtures. After the addition of the streptavidin coated donor and protein A coated acceptor beads, the fluorescence intensity was measured. In case of the aptamer-based sandwich assay, the streptavidin coated beads were modified with the biotin-labelled aptamers prior to mixing them with RSV-spiked throat swab mixtures, then the fluorescence intensities were measured. Relative AlphaScreen signal was calculated by forming a ratio of the sample fluorescence and the virus-free background fluorescence.

4.1.3. Determination of the virus protein specificity of the most promising aptamer candidate by FP

RSV has three surface proteins, namely the attachment (G) protein, the fusion (F) protein and the small hydrophobic (SH) protein. During the selection and characterization of aptamers, an anti-RSV antibody was utilized that recognizes a conformational epitope of F protein. As many copies of the surface proteins of RSV are present, in theory, all of them are possible targets for aptamers during SELEX. To determine if aptamer H8 and the antibody used during selection compete for the same epitope, a fluorescence polarization based competitive assay was set up by our collaboration partner at BME. Alexa-488 labelled commercially synthesized H8 aptamer was mixed with inactivated RSV, both at constant concentrations, and their fluorescence polarization was measured in 5-minute increments and the results demonstrated the formation of virus-aptamer complex after 20 minutes of incubation (Figure 10).

In the next experiment, the H8 aptamer was mixed with two orders of magnitude higher concentration of anti-RSV antibody (Palivizumab) and incubated with the inactivated RSV sample. There was no evident difference in the fluorescence polarization between the H8 aptamer only samples and samples containing both antibody and aptamer

(Figure 10 A). These data prove that the H8 aptamer and the Palivizumab antibody did not compete for the same recognition site.

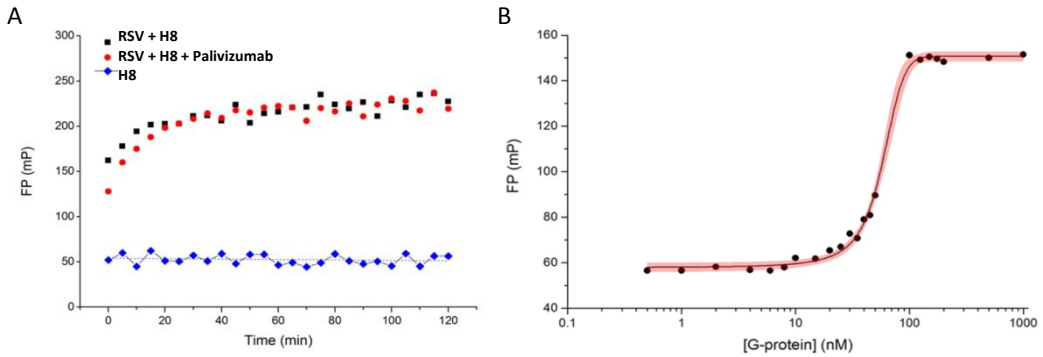

Figure 10 A The fluorescence polarization value of Alexa-488 labelled H8 aptamer greatly increases with addition of RSV indicating their interaction. Upon addition of an F protein selective antibody in a hundred times excess to this mixture, no change in fluorescence polarization value is detected illustrating that the aptamer and antibody do not compete for the same epitope. **B** A constant concentration of Alexa-488 labelled H8 aptamer was incubated with various amounts of G protein and fluorescence polarization was measured. The data points obtained were fitted with a 1:1 stoichiometry dose-response curve; the 95% confidence band of the fit is highlighted with red.[113]

In search for the target protein of H8, the attachment G protein of RSV was tested as a possible interaction partner by florescence polarization. To measure the putative interaction, a constant concentration of Alexa-488 labelled H8 was mixed with various concentrations of recombinant G protein and changes in fluorescence polarization was measured. There was clear FP change upon addition of the protein and a 30 nM K_D value was calculated for H8 aptamer and G protein complex by using a 1:1 binding model (Figure 10 B).

4.1.4. Stability of the most promising aptamer in throat swab matrix

The lack of modification that could protect the H8 aptamer from naturally occurring endo- and exonucleases of throat-swab sample could result in rapid degradation of the aptamer. To determine the stability of our aptamer, we measured the concentration change of H8 upon infusion with throat swab matrix by qPCR. Samples were taken from the throat swab mixture containing 100 pM H8 aptamer at various time points up to 24 hours.

A slight change in concentration was detected during the first 2 hours of incubation, the concentration of the H8 aptamers was approx. 90 pM. Following one day incubation, the concentration of H8 aptamer was calculated to be ~30 pM (Figure 11).

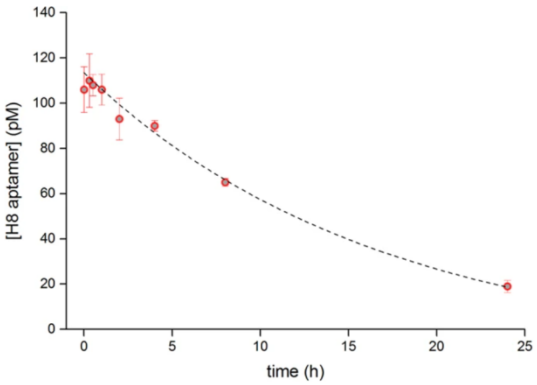

Figure 11 Stability of aptamer H8 in throat swab matrix. 100 pM of H8 aptamer was complemented with throat swab sample and the decrease of aptamer concentration over time was measured by qPCR. [113]

4.2. Selection of modified aptamers for RSV F protein

4.2.1. Choice of modified nucleotide

Amino-acid side chain holding nucleotides are reported to increase the success rate of SELEX.[54] 5-indolyl-AA-dUTP (TAdUTP, Figure 12) was chosen to be incorporated into an oligonucleotide library as it not only contains a tryptophan-like side chain, but also possesses a peptide bond. These modifications might be beneficial to target the hydrophobic cavities on site Ø (Figure 3) using TAdUTP holding aptamers as the tryptophan-like group may confer high affinity interactions with hydrophobic amino acids and the peptide bond provides flexibility to the sidechain.

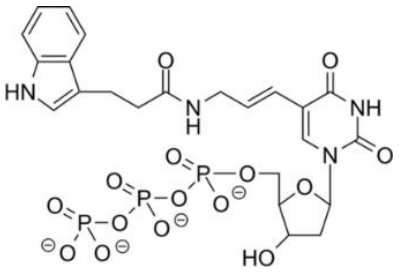

Figure 12 Structure of 5-Indolyl-AA-dUTP (TAdUTP). The incorporation of TAdUTP provides tryptophan-like physiochemical properties to oligonucleotides.[120]

4.2.2. Incorporation of TAdUTP into oligonucleotides

Incorporation of a modified nucleotide into oligonucleotides poses a challenge to the routinely used DNA polymerases of PCR. First, we set out to analyse eight thermostable polymerases that were previously reported to accept modified nucleotides as substrates to see if they could incorporate TAdUTP instead of thymine into the amplicons (Table 7).

Table 7 Properties and TAdUTP incorporating capacity of the studied thermophilic DNA polymerases.[120]

Polymerase	3'-5' exonuclease activity	TAdUTP incorporation	Specifications	Family
iProof	yes	no	*Pyrococcus*-like enzyme with a processivity-enhancing Sso7d domain	B
OneTaq	yes	no	Mixture of family A and B Taq and Deep Vent	A & B
Vent(exo-)	no	yes	Isolated from *Thermococcus litoralis*	B
PWO Superyield	yes	no	Isolated from *Pyrococcus woesei*	B
Q5U	yes	no	High-fidelity DNA polymerase fused to a processivity-enhancing Sso7d domain	B
KOD XL	yes/ no	yes	Mixture of KOD and KOD(exo$^-$)	B
Pfu	yes	no	Isolated from *Pyrococcus furiosus*	B
Therminator	no	no	9°N exo- variant	B

Three different oligonucleotides were applied as templates of different base composition (Figure 13 A). None of the DNA polymerases with 3'-5' exonuclease activity produced amplicons when dTTP was substituted with TAdUTP in the PCR mixture. Amplification reactions were only successful in generating PCR products of expected size in two cases, that is, with application of Vent(exo-) and KOD XL polymerases (Figure 13 B, C), which both lack 3'-5' exonuclease activity. A somewhat lower yield was observed when Vent(exo-) was used, while the substitution of dTTP with TAdUTP did not seem to affect the efficiency of KOD XL (Figure 13 C). Interestingly, no amplicons were generated when the third proofreading activity lacking enzyme, Therminator, catalysed the reaction.

DOI:10.14753/SE.2022.2604

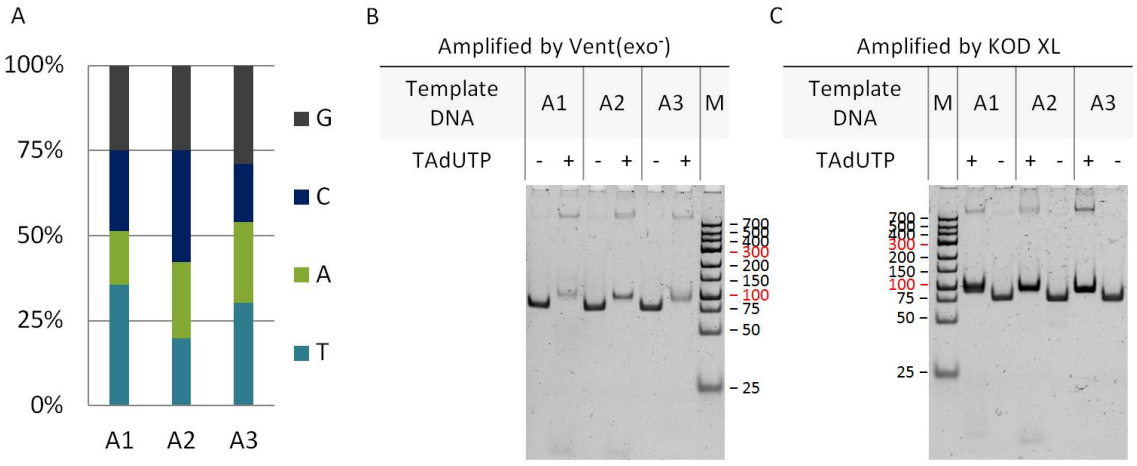

Figure 13 Amplicons generated by Vent(exo-) and KOD XL polymerases using dTTP or TAdUTP. **A** Oligonucleotide templates with different nucleotide ratios. **B, C** 5 µL of reaction mixtures from PCR, which was catalysed by either Vent(exo-) (**B**) or KOD XL (**C**), were separated by PAGE and visualised with GelGreen dye.[120]

4.2.3. Effect of TAdUTP on the physicochemical characteristics of oligonucleotides

The modified nucleotide containing amplicons showed a slower migration in PAGE analysis implying the successful incorporation of TAdUTP. It has been described that alteration of the nucleotide structure can cause difference in physiochemical properties, which can be inspected using melting curve analysis. The amplicons either containing the modified nucleotide or natural nucleotides were analysed by gradually decreasing the temperature and measuring fluorescence intensity. The obtained data suggested that the insertion of TAdUTP into oligonucleotides resulted in about 5–10 °C lower melting temperature (T_m) compared to that of dTTP containing amplicons (Table 8).

Table 8 Melting temperature analysis of generated PCR products. Incorporation of TAdUTP results in a decreased T_m of PCR products.[120]

	A1		A2		A3	
TAdUTP	-	+	-	+	-	+
T_m ± SD [°C]	88.64 ± 0.03	78.74 ± 0.07	89.60 ± 0.03	82.22 ± 0.05	89.00 ± 0.03	83.55 ± 0.05

4.2.4. Amplification of modified aptamer library

Generation of a random oligonucleotide library by PCR poses a challenge even if no modified nucleotides are applied in the process due to the large sequence space of library. Amplification of such libraries with open PCR usually results in by-product formation (Figure 14 A), as the random region of the library potentially binds to others via complementary bases and serves as a primer, then the 3'OH can be extended by the

DNA polymerase. To evade this issue, water-in-oil emulsion PCR was exploited to amplify an aptamer library containing up to 10^{14} different sequences. PAGE analysis revealed that the by-product formation greatly decreased when the micro-reactor forming emulsion PCR was applied to generate oligonucleotide libraries in reactions catalysed by Vent(exo-) or KOD XL (Figure 14 B). A decreased migration is also observed in TAdUTP containing reaction mixtures in library generation indicating that both polymerases readily incorporate TAdUTP into random oligonucleotide libraries. Interestingly, KOD XL seems to produce a higher yield of library when dTTP is substituted with TAdUTP. The downstream process of emulsion PCR consists of discarding the oil phase of the emulsion and purification of the DNA on a silica membrane or bead-based DNA purification column. The commercially available kits are designed to purify non-modified DNA, thus identifying the one with the highest efficacy for purification of TAdUTP containing DNA is crucial. Out of the tested three kits, Zymo's Oligo Clean and Concentrator provided the highest recovery of modified DNA (Figure 14 C).

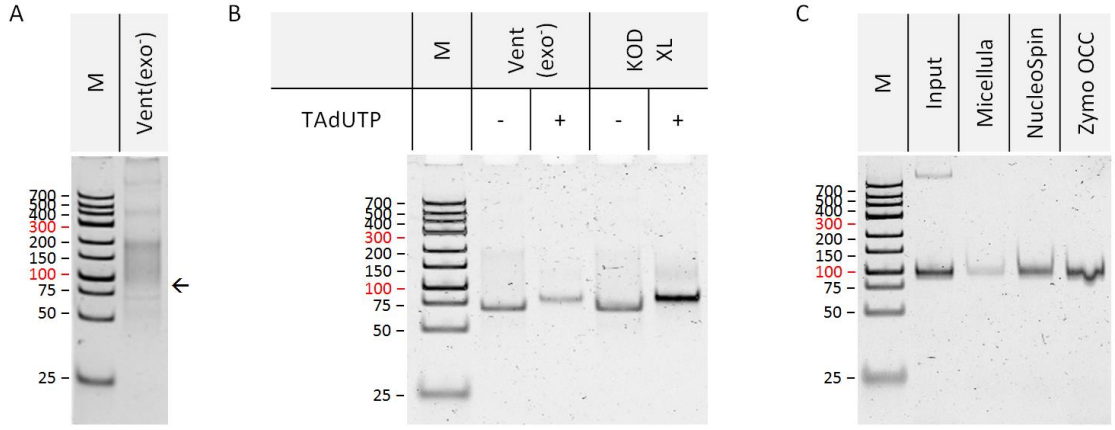

Figure 14 Generation of oligonucleotide library by PCR. **A** Amplification of a library by Vent(exo-) in a conventional, open PCR. The arrow marks the expected size of aptamer library. **B** Amplification of a library by Vent(exo-) and KOD XL polymerases in water-in-oil emulsion PCR. **C** Purification of modified oligonucleotides by three commercially available DNA purification kits. 5 µL of PCR mixtures or eluted fractions were separated by PAGE and visualised using GelGreen dye.[120]

4.2.5. High-throughput sequencing analysis of generated libraries

Nor KOD XL, neither Vent(exo-) has proofreading activity, which might alter the composition of generated DNA libraries. To study if TAdUTP has any effect on the fidelity of these DNA polymerases, high-throughput sequencing of the above-mentioned amplicons (A1-A3, Figure 13) was carried out. Analysis of the obtained data

indicated that the substitution of dTTP with TAdUTP had a minor effect on the error rate of DNA polymerisation and the fidelity of both enzymes was in concert with the previously published values (Table 9).

Table 9 The published and observed error rate of Vent(exo-) and KOD XL, and the ratio of unique sequences in the amplified library in presence of dTTP and TAdUTP, respectively.[120]

	Vent(exo-)		KOD XL	
TAdUTP	-	+	-	+
Error rate observed ± SD [$\times 10^{-6}$]	555 ± 398	418 ± 343	256 ± 153	336 ± 192
Published error rate [$\times 10^{-6}$]	200[121]		3-4<Taq[60]	
Ratio of unique sequences to all filtered reads [%]	98.93	99.08	98.96	99.00

The ratio between the unique sequences and all filtered reads indicates that the sequence space of the generated oligonucleotide library is near the theoretical maximum and is quite similar in the natural nucleotide and the TAdUTP containing library. However, the nucleotide composition of these libraries differed as the amplification of libraries holding the modified nucleotide resulted in a slightly lower percentage of A and T (Table 10).

Table 10 The nucleotide composition of the libraries generated in the presence of TAdUTP or dTTP by either Vent(exo-) or KOD XL.[120]

	Vent(exo-)		KOD XL	
TAdUTP	-	+	-	+
A [%]	27.10	22.50	25.00	22.30
T [%]	27.20	22.10	25.30	22.20
G [%]	22.70	27.80	24.50	27.90
C [%]	23.00	27.60	25.10	27.70

4.2.6. Selection of modified aptamers for RSV F protein

Based on the evidence that RSV infection can be prevented by stabilizing the F protein in its prefusion form[106], we hypothesise that an equal effect can be achieved by a modified aptamer. Hence, we set out to produce modified aptamers of therapeutic potential for RSV. Mod-SELEX was carried out using both the immobilized prefusion and postfusion form of RSV F protein by using the former as the target for the selection procedure and the latter as a counter-target molecule.

An aptamer selection targeting the prefusion form of F protein was successfully performed using the tryptophan-like side chain containing aptamer library. Seven selection steps with increasing selection pressure were applied to ensure the specificity

of prefusion F selective aptamers. As a counter-selection target, the postfusion form was used in 6 out of 7 rounds. The selection pressure was further increased by applying competitor molecules, e.g., L-tryptophan, mucin, BSA, Salmon DNA. After cloning and Sanger sequencing the final selection step, the obtained sequences were analysed with BioEdit software. In the 96 sequenced aptamers, the primary sequence analysis revealed that 10 aptamers were present in multiple copies, showing a definite enrichment of potential RSV selective modified aptamers. MEME Motif search did not discover a consensus sequence.

4.2.7. Screening of modified aptamers using AlphaScreen

Aptamers being present in multiple copies do not necessarily possess a higher affinity towards the target molecule, as our previous studies have demonstrated. Therefore, all aptamers were screened by AlphaScreen for their prefusion F protein binding capacity.

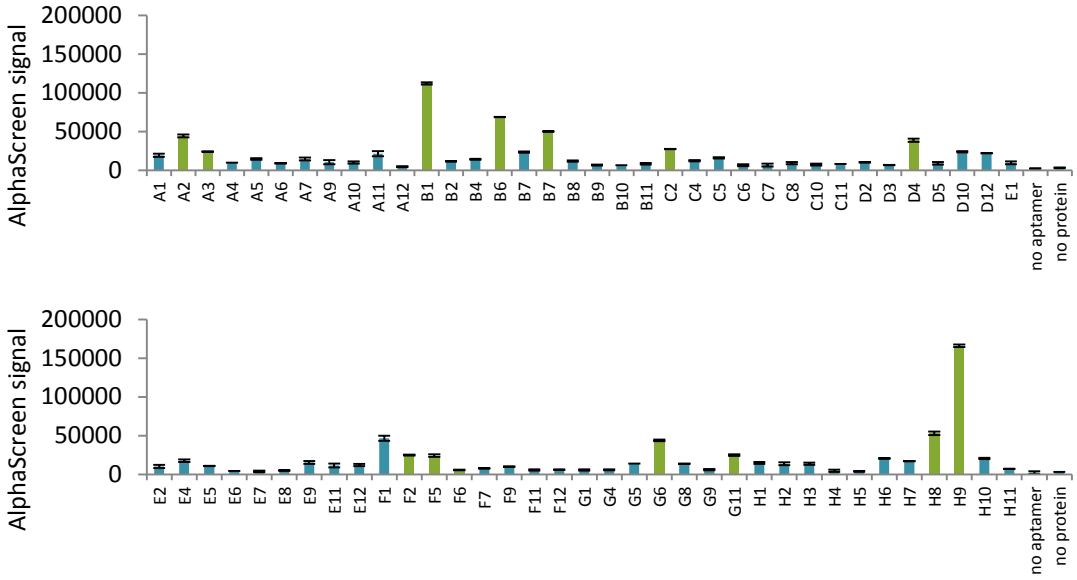

Figure 15 Screening of modified aptamers using AlphaScreen. Biotin-labelled aptamers were mixed with the prefusion form of F protein (at a concentration of 30 nM) and Palivizumab. An increased fluorescence signal is detected upon binding of aptamers to prefusion F protein. Aptamers chosen for further studies are indicated as green bars.

To this end, the modified aptamer candidates, prefusion form of F protein, and Palivizumab antibody was mixed. Palivizumab binds both the prefusion and the postfusion form of F protein. This experimental setup was chosen to evade aptamers that bind the same epitopes as Palivizumab. The antibody and the biotinylated aptamers were immobilized on Protein A and Streptavidin coated beads. The measured fluorescence indicates the binding of aptamers to the prefusion F protein. Among the 70

aptamers, almost all aptamers showed binding to the target molecule and 14 aptamers produced 10-50 times higher fluorescence signal upon binding the target protein in comparison to the no F protein or no antibody containing samples (Figure 15).

Next, the above 14 aptamers were mixed with either the prefusion or the postfusion form at different concentrations in the above-described experimental setting to determine the F protein discriminating capacity of aptamers. All analysed aptamers produced an approx. 2 times higher relative fluorescence signal when mixed with the prefusion form (Figure 16) than upon mixing with the postfusion form.

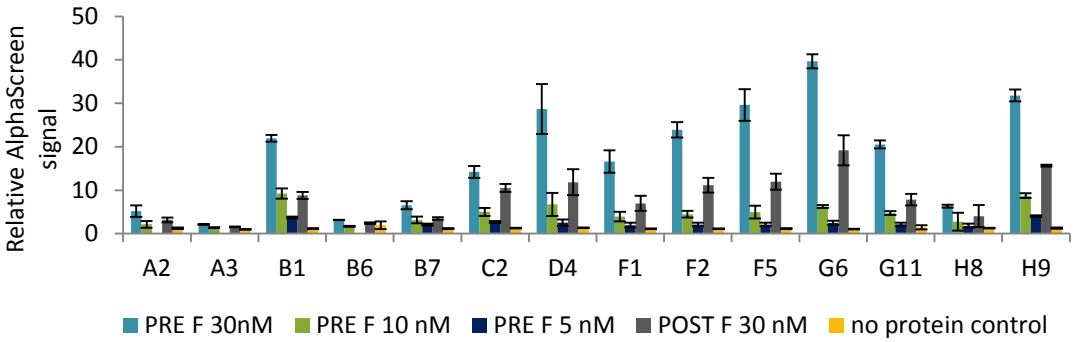

Figure 16 Determining the ability of modified aptamers to distinguish between the prefusion and postfusion form of F protein by AlphaScreen. Biotin labelled aptamers were mixed with either the prefusion or postfusion form of F protein (at various concentrations) and Palivizumab. Relative AlphaScreen signal was calculated by forming a ratio of the sample fluorescence and the aptamer-free background fluorescence. Increase in this value indicates selective binding of aptamers.

4.2.8. Determination of K_D of the modified aptamers by MST

Microscale Thermophoresis (MST), a widely exploited method for the analysis of interactions between biomolecules in solution, was applied in our studies to determine the K_D of the most promising aptamer candidates. It is based on thermophoresis, that is, the movement of molecules according to the temperature gradient. Thermophoresis is affected by a wide range of molecular attributes, such as molecular weight, conformation, charge, and solvation shell. MST allows the detection of minuscule changes of these previous properties, which are altered upon binding of an interacting partner.[122,123] In an MST experiment, the concentration of the smaller, fluorescently labelled molecule is kept constant when mixed with a serial dilution of its unlabelled interaction partner that is larger in size.

Aptamers producing the highest fluorescence signals in AlphaScreen measurements, e.g., F2, F5, and G6, did not prove to possess high affinity towards the prefusion F

according to MST (Table 11). The lowest K_D values were acquired in case of A2, B7 D4 and H9.

A possible reason for this contradiction might be the inherent differences between the two applied aptamer characterising methods. AlphaScreen requires the immobilization of the interaction partners, meanwhile MST determines the interaction between two partners in free solution. These results highlight the importance of applying different methods for identification of the prime aptamer candidates. Even though the two approaches produced non-comparable results in this case, both can be viewed as valuable inputs for further experiments in terms of the applicability of these aptamers.

Table 11 K_D of the 14 outstanding aptamers was determined by MST. A 16-fold serial 1:1 dilution was made of F proteins (prefusion F and postfusion F, labelled as pre F and post F, respectively) and mixed with a constant concentration of Cy5-labelled modified aptamers, and then the IR induced thermophoresis was measured. NA means that the Affinity MO program was unable to fit a model to the obtained data points using Michaelis-Menten kinetics.

Aptamer name	Target molecule	K_D (nM)	± SD (nM)
A2	pre F	181.9	29.4
	post F	1751.1	607.7
A3	pre F	450.9	87.4
	post F	3845.7	3824.7
B1	pre F	1773.1	577.6
	post F	4803.2	1549.1
B6	pre F	11559.8	7767.0
	post F	NA	
B7	pre F	449.7	125.0
	post F	1362.9	876.7
C2	pre F	1309.5	433.1
	post F	23405.3	26417.1
D4	pre F	372.9	111.6
	post F	2742.9	1049.0
F1	pre F	863.7	207.4
	post F	14487.5	32767.8
F2	pre F	791.6	180.7
	post F	59346.4	317154.1
F5	pre F	1725.9	956.7
	post F	10959.1	10913.9
G6	pre F	1432.0	577.5
	post F	NA	
G11	pre F	582.7	256.7
	post F	NA	
H8	pre F	1690.0	674.5
	post F	NA	
H9	pre F	150.6	41.7
	post F	928.2	469.8

5. Discussion

5.1. Aptamers for diagnostic purposes of RSV infection

Most of the currently applied viral diagnostics relies on the specific detection of the viral genome by PCR-based methods. These approaches are sensitive, highly specific and fast; however, they usually require well-trained personnel to perform these expensive tests. Detection of viral proteins are more suitable for rapid tests, presently, all the commercially available antigen-detection based virus diagnostic tools depend on highly selective antibodies. While numerous aptamers have been selected for virus identification, their application for viral diagnostics in clinical setting has not been realized, yet.

To evade the difficult virus purification steps, our rationally designed SELEX method relies on immobilizing the whole virus on an RSV selective antibody-conjugated paramagnetic bead. The immobilized RSV was used as the target during the aptamer selection and each selection step was performed with an increasing selection pressure. To further intensify the selection pressure, a common co-infecting agent of RSV, HRV was introduced during the counter-selection steps.

Even a successful SELEX results in a panel of aptamer candidates; therefore, pinpointing the most promising oligonucleotide requires diverse approach. We applied three different methods to identify high-affinity aptamers that selectively bind RSV and applicable in complex biological matrices. Using *in silico* methods, we identified consensus motif holding oligonucleotides and duplicate copies of aptamers. First, we applied fluorescence polarization to analyse the binding affinity of the consensus motif holding aptamers to both RSV and HRV. Affinities of these aptamers to their target differed greatly illustrating that the affinity of aptamers cannot be predicted solely by *in silico* methods even if they hold consensus motifs. Next, to assess the applicability of the aptamers in the biologically relevant matrix of respiratory diseases, throat swab samples were spiked with the viruses and the binding of aptamers were determined by filter binding assay and AlphaScreen. Both approaches demonstrated selective binding of the aptamers.

Our approach to study selectivity, affinity and practical applicability of the aptamers using various methods ensured that the most promising candidate is chosen for further studies. Importantly, the applied methods needed only a small amount of labelled

oligonucleotides which could be synthetized using PCR; thus, these methods are feasible in general molecular biology laboratories.

Many factors influence the success of SELEX, amongst them are the oligonucleotide library design, the applied DNA polymerase, and arrangement of PCR. Furthermore, the polymerase reaction generally does not amplify all the template sequences equimolarly, thus a bias is introduced into the library. Our data also confirmed that this effect takes place during SELEX since the aptamer with the highest affinity was present only in one copy in the sequenced oligonucleotide pool, and the aptamers which were present in duplicates showed the lowest affinity towards the target molecule. We studied if the superior aptamer is applicable to detect clinically relevant concentrations of RSV in throat swab sample. Two sandwich type assay setups were applied using AlphaScreen: H8 was used as both capture and recognition elements or H8 was combined with F protein selective antibody. Both setups were suitable for detection of RSV at 5 log PFUe in throat swab sample. Our most promising aptamer candidate of diagnostic potential, H8, was further characterized by FP and the obtained results demonstrated that H8 does not compete with the F protein selective antibody for the same epitope. By the next set of experiments, it was shown that H8 binds the G protein of RSV with a low nanomolar dissociation constant. This binding affinity is superior to many virus-selective aptamers reported so far. Additionally, the practical applicability of H8 was demonstrated by the development of a fluorescent aptamer-based nanoparticle tracking analysis method for virus counting.[124]

It is a common belief that non-modified aptamers have short half-life in biological matrices due their sensitivity to nucleases. On the contrary, it has been described in several cases that non-modified aptamers kept their integrity even in such complex matrices as blood serum.[125] Degradation of our aptamer in throat swab sample was monitored using qPCR and the obtained data exhibited that it remained intact for hours. This is within the timeframe of viral diagnostic tests; therefore, degradation of the aptamer would not limit its applicability in diagnostic devices.

5.2. Modified aptamers with therapeutic potential of RSV infection

A continuous effort in aptamer-related research is made to increase the stability and the practical applicability of selected aptamers. One of the emerging trends over the last decade is the introduction of protein-like side chain holding nucleotides into the

oligonucleotide library that may confer not only protection against the omnipresent nucleases but also higher affinity than that of non-modified aptamers. The introduction of these modifications might alter the hydrogen bond formation of base pairs; therefore, their enzymatic amplification may be affected. These difficulties could dramatically increase the error rate of DNA amplification and the efficacy of polymerisation might also decline. The enrichment of target-specific oligonucleotides by PCR is the one of the essential steps of SELEX; thus, minimization of these shortcomings is crucial to improve the success rate of modified aptamer selection. A comprehensive analysis of the PCR distorting effect of a commercially available amino acid-like side chain holding nucleotide, 5-indolyl-AA-dUTP (TAdUTP), was performed in order to identify the most promising thermostable DNA polymerase and reaction conditions for the generation of base modified oligonucleotides and reveal the alleged undesirable consequences stemming from its usage.

Our data verifies that only 3'–5' exonuclease deficient DNA polymerases accept nucleotides with amino acid-like side chains as substrates in PCR. Although the 3'–5' exonuclease activity deficient Vent(exo-) and KOD XL enzymes incorporated TAdUTP into the PCR product, the lack of this activity does not certainly warrant the capability to incorporate the modified nucleotide as the third studied 3'–5' exonuclease domain lacking polymerase, Therminator, failed to accept TAdUTP. Alterations of the physicochemical properties of the modified nucleotide holding PCR products were verified by gel electrophoresis and melting curve analysis. The lowered melting temperature of TAdUTP containing products indicated that the stability of the DNA duplex is weakened if TAdUTP was incorporated. Considering the latter data, Vent(exo-) and especially KOD XL seem to be insensitive for the improperly formed hydrogen bonds as both enzymes catalysed reactions using TAdUTP, and the enzymatic activity of KOD XL was not decreased by the replacement of dTTP with TAdUTP.

The oligonucleotide library production and amplification during SELEX is generally hampered by excessive by-product formation that could be alleviated by the application of water-in-oil emulsion PCR that could also be exploited to generate modified nucleotide libraries with a negligible amount of by-product formation. Furthermore, the high-throughput sequencing of PCR products acquired either by amplification of single oligonucleotides or random oligonucleotide libraries demonstrated that the error rates of

the enzymes and sequence space of the library remained unaltered when using TAdUTP. Collectively, KOD XL and TAdUTP can be effectively employed for aptamer selection without distorting the sequence space of random oligonucleotide libraries.

Having established the conditions of modified nucleotide holding aptamer generation, we set out to select aptamers for the F protein of RSV. The immobilized prefusion form of F protein of RSV was targeted in a mod-SELEX process by using the protein-like side chain possessing aptamer library produced by KOD XL. Each selection step was preceded by a negative or counter selection step, either for the immobilized postfusion form of F protein or the immobilization matrix. To further increase the selection pressure, the selection buffer was complemented with the postfusion form of F protein, mucin, L-tryptophan, BSA, Salmon sperm DNA; thus, the competition for binding sites occurred in the presence of critical interferents.

Based on our previous findings, screening of aptamers to identify the most promising candidate is inevitable. Hence, we applied a sandwich-type interaction assay, the AlphaScreen, to pinpoint the lead aptamers that selectively bind the prefusion form of F protein. Fourteen of the 70 tested aptamers showed high affinity towards prefusion F, selectivity of these aptamers was also assessed by using the postfusion F. All 14 aptamers bound for both forms of F protein; however, at least two times higher relative AlphaScreen signals were generated when aptamers were complexed with the prefusion F. Next, the binding affinity of the aptamers to the prefusion and postfusion form of F protein was determined by microscale thermophoresis (MST). The obtained data indicated ten times higher affinity for the pre-fusion F protein compared to the post-fusion form of the protein; yet, some of the outstanding aptamers in AlphaScreen seem to have mediocre K_D values according to MST. This controversy might have occurred due to the fundamental differences between the two interaction assays; meanwhile AlphaScreen requires the immobilization of the interaction partners, MST determines the interaction between two partners in free solution. Even though the two approaches produced inconsistent results in this case, utilizing different methods for identification of the prime aptamer candidates is nevertheless essential in terms of the practical applicability of aptamers.

6. Conclusions

The rationally designed SELEX method that applied monoclonal antibody immobilized whole inactivated RSV particles as targets of selection and counter-selection steps using a common co-infecting virus, HRV, resulted in selective aptamers with potentially diagnostic value.

Our approach generated an aptamer, which remains to be capable of detecting RSV and distinguishing it from HRV at clinically relevant concentrations even when challenged with complex biological matrices.

Our variation of cell-SELEX could be generally applied for other viruses and may accelerate the process of aptamer selection since there is no need to use purified viral protein and the target virus could be directly immobilized using the antibody.

To generate chemically modified oligonucleotide libraries containing 5-indolyl-AA-dUTP (TAdUTP), two DNA polymerases, Vent(exo-) and KOD XL can be applied. Substitution of dTTP with TAdUTP has no significant effect on their error rate of these polymerase and do not distort the sequence space of the aptamer library.

Amalgamation of water-in-oil emulsion PCR with the application of KOD XL thermostable polymerase could be the method of choice for the selection of chemically modified aptamers with amino acid-like properties since it evades excessive by-product formation during SELEX.

The presented data also highlights that the most abundant sequences do not necessarily possess high affinity towards the target molecule; therefore, the screening of aptamers is inevitable and further functional analysis of aptamer candidates by diverse approaches is required to pinpoint the most appropriate aptamers.

Devising and implementing a mod-SELEX protocol using a modified library possessing tryptophan-like side chains to generate F protein selective oligonucleotides results in high-affinity aptamers that may stabilize the F protein in its prefusion form, thereby inhibiting RSV infection.

DOI:10.14753/SE.2022.2604

7. Summary

The rapid detection of the unceasingly emerging new virus strains and the development of novel diagnostic and therapeutic solutions are constant challenges for both the medical field and the pharmaceutical industry. This unmet clinical need is especially highlighted in the case of respiratory syncytial virus (RSV) as there is no preventive medicines approved yet and the shortcomings of conventional virus detection methods hinder its prompt and accurate diagnosis. Aptamers, the single stranded oligonucleotides with antibody-like specificity and affinity, are promising tools for detecting viruses and treating viral infections. Therefore, we aimed at providing both diagnostic aptamers and modified nucleotide holding aptamers of therapeutic potential.

Our approach for generation of aptamers with diagnostic potential circumvents the drawbacks of using virus proteins as SELEX targets by immobilization of target virus onto magnetic beads using a virus-selective antibody. Intensifying the selection pressure by counter-selection steps with a common co-infecting virus resulted in the isolation of highly selective aptamers. This protocol generated an aptamer that selectively detects RSV in complex matrices at clinically relevant titres; thus, our work provides a proof of our rationally designed procedure for the generation of virus selective aptamers with potential clinical application.

To identify modified nucleotide holding aptamers of antiviral effect, first, an oligonucleotide library bearing an amino acid-like side chain was created. Incorporation of the modified nucleotide, TAdUTP, by Vent(exo-) and KOD XL was studied extensively. These thermostable DNA polymerases are capable of substituting dTTP with TAdUTP. The high-throughput sequencing analysis showed that neither the error rate nor the PCR bias was significantly elevated by the replacement of a natural nucleotide. Our method relies on that water-in-oil emulsion PCR and is adequate for the generation of TAdUTP holding aptamer libraries. A mod-SELEX protocol to evolve aptamers that selectively bind the prefusion form of F protein was implemented and the isolated aptamers were characterized by different approaches. This protocol yielded aptamers that possess dissociation constants in the nanomolar range, thereby potentially exhibiting virus neutralizing properties.

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9. Bibliography of the candidate's publications

Publications related to the thesis:

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IF:4.122

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IF:3.164

Publication not directly related to the thesis:

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IF:5.977

DOI:10.14753/SE.2022.2604

10. Acknowledgements

I would like to thank my supervisor, Dr. Tamás Mészáros, for his unceasing personal and professional help and support even in the most difficult times and for the opportunity to participate in such a captivating and fruitful research.

I am grateful to all the past and current members of our work group that we shared not only great scientific achievements but many laughs together: Anna Gyurkovics, Dr. Zsuzsanna Szeitner, Dr. Szilvia Nagy, Dr. Éva Nagyné Scholz, Judit András, Zoltán János Tolnai, Brigitta Kállai, Ákos Harkai and Júlia Sziládi.

I would like to express my appreciation to the former and present directors of the department, Prof. Dr. Gábor Bánhegyi, Dr. Gergely Keszler and Prof. Dr. Miklós Csala for providing me with the opportunity to pursue my PhD and work at the Department of Molecular Biology at the Institute of Biochemistry and Molecular Biology, Semmelweis University. I also thank our colleagues who never failed to help me with administrative issues and ensured the technical background for my PhD work: Valéria Béláné Szénási, Mária Gránicz, Józsefné Bombicz and Kinga Sonnevend.

My family has my eternal gratitude for their above and beyond care and support that let me choose this path. No words can describe how much I appreciate my friends for their continuous encouragement and inspiration.