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In vitro Selection and Characterization of single stranded DNA Aptamers Inhibiting the Hepatitis B Virus Capsid-Envelope Interaction

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My Family

Contents

1 INTRODUCTION 1

2 REVIEW OF THE LITERATURE 2

2.1 HEPATITIS B VIRUS (HBV) 2
2.1.1 HISTORY AND TAXONOMY 2
2.1.2 EPIDEMIOLOGY AND PATHOGENESIS 3
2.1.3 VIRION STRUCTURE 4
2.1.3.1 ROTEIN COMPOSITION OF HBV PARTICLES 4
2.1.3.1.1 SURFACE PROTEINS (HBS) 4
2.1.3.1.2 TRANSMEMBRANE TOPOLOGY OF SURFACE PROTEINS (HBS) 6
2.1.3.1.3 CORE PROTEIN (HBC) 7
2.1.3.1.4 HB_E PROTEIN 9
2.1.3.1.5 HEPTATITIS B POLYMERASE (P) PROTEIN 9
2.1.3.1.6 HEPTATITIS B X PROTEIN (HBX) 9
2.1.3.2 HBV GENOME 10
2.1.4 HBV LIFE CYCLE 11
2.1.5 ENVELOPMENT OF CORE PARTICLES 12

2.2 APTAMERS 14
2.2.1 NATURE AND THEORY 14
2.2.2 TECHNOLOGY 14
2.2.2.1 OLIGONUCLEOTIDE LIBRARY 14
2.2.2.2 STANDARD SELECTION PROCESS (SELEX) 16
2.2.2.3 SITE-DIRECTED SELECTION OF APTAMERS 18
2.2.2.4 AUTOMATED APTAMER SELECTION 19
2.2.3 APTAMERS AND ANTIBODIES 19
2.2.4 APTAMERS IN DIAGNOSTICS 20
2.2.5 APTAMERS IN THERAPEUTICS 21
2.2.6 APTAMERS AGAINST HEPATITIS VIRUSES 21

3 OBJECTIVES 22

4 MATERIAL AND METHODS 23

4.1 MATERIAL 23
4.1.1 ANTIBODIES 23
4.1.2 APTAMERS 23
4.1.3 BACTERIAL STRAINS 23
4.1.4 BACTERIAL MEDIA AND ANTIBIOTICS 24
4.1.5 CAPSIDS OF HBV 24
4.1.6 CELL LINE 24
4.1.7 CELL CULTURE MEDIA 24
4.1.8 CHEMICALS AND REAGENTS 24
4.1.9 ENZYMES 26
4.1.9.1 RESTRICTION ENZYMES 26
4.1.9.2 OTHER ENZYMES 26

4.1.10 DEVICES 26
4.1.11 KIT SYSTEMS 28
4.1.12 LABORATORY CONSUMABLES 28
4.1.13 DNA AND PROTEIN MARKERS 28
4.1.14 PLASMIDS 29
4.1.15 SOLUTIONS AND BUFFER SYSTEMS 32
4.1.16 PRIMERS 33
4.1.17 SOFTWARE 34

4.2 METHODS 35

4.2.1 DNA TECHNOLOGY 35
4.2.1.1 CONVENTIONAL POLYMERASE CAHIN REACTION (PCR) 35
4.2.1.1.1 PCR DURING HBV WT AND MUTANT CAPSID EXPRESSION 35
4.2.1.1.2 PCR DURING APTAMER SELECTION AND SEQUENCING 36
4.2.1.2 PURIFICATION AND CONCENTRATION OF DNA 36
4.2.1.2.1 PHEENOL CHLOROFORM EXTRACTION 36
4.2.1.2.2 ETHANOL PRECIPITATION OF DNA 37
4.2.1.2.3 PURIFICATION OF DNA SOLUTIONS AND PCR PRODUCTS 37
4.2.1.3 GEL ELECTROPHORESIS 37
4.2.1.3.1 AGAROSE GEL ELECTROPHORESIS 37
4.2.1.3.2 DENATURING-UREA POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) 37
4.2.1.4 EXTRACTION OF DNA FROM GELS 38
4.2.1.4.1 EXTRACTION FROM AGAROSE GEL 38
4.2.1.4.2 EXTRACTION FROM POLYACRYLAMIDE GEL 38
4.2.1.5 STREPTAVIDIN INDUCED ELECTROPHORETIC MOBILITY SHIFT FOR SSDNA PREPARATION 39
4.2.1.6 DETERMINATION OF DNA CONCENTRATIONS 39
4.2.1.7 DNA CLONING 40
4.2.1.7.1 DNA RESTRICTION 40
4.2.1.7.2 DNA LIGATION 41
4.2.1.7.3 TRANSFORMATION OF BACTERIA WITH LIGATED DNA 42
4.2.1.7.4 PLASMID PREPARATION 43
4.2.1.8 DNA SEQUENCING 44
4.2.1.9 DNA QUANTIFICATION BY SPECIFIC REAL-TIME PCR 44

4.2.2 PROTEIN TECHNOLOGY 46
4.2.2.1 PROTEIN EXPRESSION 46
4.2.2.2 PROTEIN PURIFICATION 46
4.2.2.2.1 CELL LYSIS 46
4.2.2.2.2 PROTEIN PRECIPITATION 46
4.2.2.2.3 PROTEIN CONCENTRATION 47
4.2.2.2.4 CHROMATOGRAPHY 47
4.2.2.2.5 SUCROSE GRADIENT ULTRACENTRIFUGATION 48
4.2.2.3 PROTEIN DETECTION 48
4.2.2.3.1 SDS-PAGE 48
4.2.2.3.2 AGAROSE GEL ELECTROPHORESIS 49
4.2.2.3.3 CAPILLARY BLOTTING 49
4.2.2.3.4 ELECTRO BLOTTING 49
4.2.2.3.5 IMMUNOSTAINING 50
4.2.2.3.6 COOMASSIE STAINING 50
4.2.2.4 DETERMINATION OF PROTEIN CONCENTRATION 51

4.2.3 IN VITRO SELECTION OF APTAMERS 51
4.2.3.1 FILTERATION PARTITION METHOD 51
4.2.3.2 SELECTION PROCEDURE 52
4.2.3.2.1 SNAP COOLING AND PRE-SELECTION OF APTAMERS 52

4.2.3.2.2 POSITIVE SELECTION OF APTAMERS 52
4.2.3.2.3 NEGATIVE SELECTION OF APTAMERS 53
4.2.3.2.4 APTAMER SEQUENCING 54
4.2.3.3 APTAMER SECONDARY STRUCTURE PREDICTION 54
4.2.4 IMMUNOLOGICAL & BIOPHYSICAL ASSAYS 54
4.2.4.1 IMMUNOPRECIPITATION ASSAY 54
4.2.4.1.1 PREINCUBATION OF AGAROSE BEADS AND ANTIBODY 54
4.2.4.1.2 INCUBATION OF APTAMERS AND HBV CAPSIDS 55
4.2.4.1.3 IMMUNOPRECIPITATION 55
4.2.4.1.4 EXTRACTION OF BOUNDED APTAMERS 55
4.2.4.1.5 APTAMERS QUANTIFICATION 56
4.2.4.2 HBV VIRION IMMUNOPRECIPITATION 56
4.2.4.3 FILTERAION ASSAY 56
4.2.4.4 DETERMINATION OF DISSOCIATION CONSTANT FOR BINDING OF APTAMERS TO CAPSIDS 56
4.2.5 CELL CULTURE TECHNIQUES 57
4.2.5.1 CULTIVATION OF HUH7 57
4.2.5.2 FREEZING OF CELLS 57
4.2.5.3 THAWING OF CELLS 57
4.2.5.4 TRANSFECTION OF HUH7 58
4.2.5.5 HARVEST OF SUPERNATANT 58

5 RESULTS 59

5.1 PRODUCTION AND PURIFICATION OF HBV RECOMBINANT CAPSIDS 59
5.1.1 CONSTRUCTION OF EXPRESSION VECTORS 59
5.1.2 CAPSID OVEREXPRESSION 60
5.1.3 CAPSID PURIFICATION 62
5.1.3.1 PRECIPITATION OF PROTEINS 62
5.1.3.2 SIZE EXCLUSION CHROMATOGRAPHY 63
5.1.3.3 SUCROSE GRADIENT ULTRACENTRIFUGATION 65
5.1.4 DETERMINATION OF THE CONCENTRATION OF PURIFIED CAPSIDS 66
5.2 *IN VITRO* SELECTION OF APTAMERS 67
5.2.1 APTAMERS QUANTIFICATION BY REAL-TIME PCR 67
5.2.2 EFFICACY OF ALKALINE PRETREATED FILTERS 68
5.2.3 OPTIMIZATION OF APTAMERS PCR AMPLIFICATION 69
5.2.4 SELEX WITH COUNTER SELECTION 70
5.3 CHARACTERIZATION OF SELECTED APTAMERS 73
5.3.1 APTAMER SEQUENCING 73
5.3.2 SECONDARY STRUCTURE PREDICTION 74
5.3.3 BINDING CHARACTERISTICS 75
5.3.3.1 BINDING AFFINITY AND SPECIFICITY 75
5.3.3.2 K_D DETERMINATION 77
5.4 *IN VITRO* INHIBITION OF NUCLEOCAPSID ENVELOPMENT 80

6 DISCUSSION 82

6.1 *E.COLI* HBV EXPRESSED CAPSIDS 82

6.2 PURIFICATION OF THE EXPRESSED CAPSIDS 83

6.3 OPTIMIZATION OF THE *IN VITRO* SELECTION 84

6.4 STRUCTURAL FEATURES OF THE APTAMERS 87

6.5 BINDING AFFINITY AND SPECIFICITY OF THE APTAMERS 87

6.6 INHIBITION OF HBV NUCLEOCAPSID ENVELOPMENT IN HUH 7 CELLS 88

7 SUMMARY 90

8 ZUSSAMENFASSUNG 91

9 REFERENCES 93

10 LIST OF FIGURES 111

11 LIST OF TABLES 113

12 APPENDIX 114

13 ACKNOWLEDGEMENT 115

Abbreviations

µ	micro
aa	Amino acid
ATP	Adenosintriphosphate
bp	Base pair
BSA	Bovine Serum Albumin
CE	Capillary Electrophoresis
C-Protein	Core Protein
ccc	circular covalent closed
Da	Dalton
DHBV	Duck Hepatitis B Virus
DNA	Deoxyribonucleic acid
ds	double strand
DTT	Dithiothreitol
E.*coli*	Escherichia coli
EDTA	Ethylendiamine tetra-acetic acid
EMSA	Electrophoretic Mobility Shift Assay
ER	endoplasmic reticulum
EtBr	Ethidium bromide
EtOH	Ethanol
FC	Flow cytometry
FDA	Food and Drug Administration
g	gramm
G	Guanosin
h	hour
HBeAG	Hepatitis B E Antigen
HBsAG	Hepatitis S Antigen
HBV	Hepatitis B Virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
IFN	Interferone
IP	Immunoprecipitation
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRES	Internal Ribosomal Entry Site
kb	kilo base
K_d	Dissociation constant
kDa	kilo dalton
l	Liter
Lac	Lactose
LB	Liquid broth
m	milli
mA	milli Amper
MBD	Matrix Binding Domain
MD	Matrix Domain
MDa	Mega Dalton
mRNA	messenger Ribonucleic acid
nm	nanometer
nt	nucleotide
No.	number
OD	Optical density
ORF	Open Reading Frame
P	Polymerase
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
pg	pregenomic
PK	Protein kinase
Pr	Protein
PRE	post transcription regulatory element
RBS	Ribosomal Binding Site
rc	relaxed circular
RNA	Ribonucleic acide
rpm	round per minute
RT	Room Temperature
rT	reverse Transcriptase
SDS	Sodium Dodycyl Sulfate
s	second
SELEX	Systematic Evolution of Ligand by Exponential enrichment
SPR	Surface Plasmon Resonance
ss	single strand
SVP	subviral particle
T=	triangular
TAE	Tris Acetate EDTA buffer,
TB	Terrific Broth
TBE	Tris Borate EDTA buffer
TBS	Tris buffered saline
TE	Tris-EDTA Puffer
TEMED	Tetramethylethylendiamine
TM	Trans-membrane
TNE	Tris Borate EDTA buffer
Tris	Tris (hydroxymethyl) - aminomethane
U	Units
UV	Ultraviolett
V	Volt
WHO	World Health Organization
WHV	Woodchuck Hepatitis Virus
WT	wild type
3D	Three dimensional

1 Introduction

The hepatitis B virus (HBV) causes acute and chronic human liver infections. HBV infections are globally distributed as more than 350 million people are chronically infected with a mortality rate of approximately 1 million people per year. To date only interferone and nucleoside/nucleotide derivatives as reverse transcriptase inhibitors are approved by FDA for treatment of chronic hepatitis B virus infections (Conjeeveram and Lok, 2003). These agents can rarely achieve a sustained suppression of HBV replication and in many cases this approach leads to remission of liver disease. One major problem is that all available antiviral substances specific against HBV are against the same target, the viral reverse transcriptase. Therefore, new antiviral therapeutic agents directed against novel targets are required.

HBV is the most prominent member of family *Hepadnaviridae*. The mature virus particle composed of a unique, incomplete, double stranded DNA genome packged into an icosahedral capsid which is surrounded by an envelope. The viral envelope carries three surface proteins which termed according to their size as large (L), middle (M) and small (S) surface proteins (Seeger and Mason, 2000). During HBV capsid envelopment, a specific, highly conserved domain (matrix binding domain, MBD) on the capsid surface binds to the matrix domain (MD) in the L surface protein. These two domains (MBD and MD) interact with each other specifically during virus budding (Bruss, 1997; Pairan and Bruss, 2009). Interfering with this interaction e.g. by a molecule binding to MBD on the capsid surface is a possible strategy for antiviral intervention.

Aptamers are low molecular weight molecules, selected from a random library of nucleic acids (RNA or ssDNA). They can bind to target molecules e.g. proteins by a three dimensional (3D) recognition. The aim of this work was to select an aptamer with a high binding affinity to the MBD on HBV capsid surface. Such a molecule can potentially inhibit the specific MBD-MD interaction and consequently, abolishing the capsid envelopment. Thus, an aptamer with high binding affinity to MBD can be used as a starting point to develop a new antiviral agent against the HBV infection.

In this study, a ssDNA aptamer with high binding affinity to the MBD on HBV capsid surface was selected showing inhibition of HBV secretion in cell culture.

2 Review of the literature

2.1 *Hepatitis B Virus*

2.1.1 History and taxonomy

The hepatitis B virus (HBV) was the first virus among human hepatitis viruses from which the proteins and the genome were detected and characterized. By epidemiological observations, two types of hepatitis transmission were identified: type A which was transmitted by the faecal-oral route and type B which was transmitted parenterally (Findlay et al., 1938). An unknown antigen in the blood of an Australian aborigine (Australia antigen) was discovered by Blumberg and his colleagues and it was realized that the appearance of this antigen was tightly related to type B hepatitis (Blumberg et al., 1967). Three years later, Dane discovered in the serum of hepatitis B patients 42 nm large virus-like particles (Dane particles) that carried this antigen on their surface, and these particles were considered to be the hepatitis B virus. In addition, 22 nm small spherical and filamentous particles were discovered and were then shown to be subviral particles (Dane et al., 1970). HBV infection was known to induce liver inflammation (Findlay et al., 1938). In 1970, it was assumed from epidemiological data that HBV may induce liver cancer and this was augmented by the discovery of an HBV-like agent in woodchucks (marmot-like animals from North America), which had been observed to develop liver cancer (Summer et al., 1978).

HBV is a member of the family *Hepadnaviridae*, its name derived from the hepatotropism and DNA genome (Howard, 1995). The family *Hepadnaviridae* comprises two main genera: genus *Orthohepadnavirus* (viruses infecting mammals) and genus *Avihepadnavirus* (viruses infecting birds) which are divided into species. The most prominent member of the genus *Orthohepadnavirus* is HBV while that of the genus *Avihepadnavirus* is the duck hepatitis B virus (DHBV). Variants of HBV can be currently classified into eight genotypes which involve 24 subgenotypes in-between. The genotypes were designed in an alphabetic manner A–H. The genetic divergence among A-E and G genotypes is around 8-9% while genotype F and its related genotype H are of higher sequence divergence (approximately 13%) (Norder et al., 2004; Schaefer, 2005; Arauz-Ruiz et al., 1997). New variable hybrid hepatitis B viruses can evolve by recombination between different parental genotypes (Norder et al., 1996).

2.1.2 Epidemiology and pathogenesis

HBV has a global high incidence rate. Out of more than 2 billion world-wide HBV infected people, 360 million individuals are chronically infected and act as carriers (Hollinger and Liang, 2001). Annually, more than 4 million individuals are newly infected by HBV, and nearly one million people die from chronic active hepatitis, cirrhosis or liver cancer (WHO, 2001). HBV genotypes show some sort of specific geographical distributions: Genotype D appears to be globally distributed (Europe, Africa, and Asia). Genotype A is mainly present in central Africa, genotypes B and C in east and south-eastern Asia, genotype E in west, sub-saharan Africa and genotypes H and F are largely confined to aboriginal Indian populations in central and south America. Genotype G is mostly detected in co-infection with other HBV genotypes. Many epidemiological studies showed high incidences of hepatocellular carcinoma (HCC) in the HBV endemic regions. In addition, differences in the potential to cause HCC have been reported among HBV genotypes (Beasley, 1988; Tanaka et al., 2008).

The incubation period of HBV is 120 days on average. HBV infection takes place either horizontally or vertically. The horizontal transmission occurs by direct contact with infectious blood or other body fluids. The prenatal HBV transmission showed the greatest risk for infants born to women who are HBeAg-positive and ranges from 70% to 90% at 6 months of age. The iatrogenic transmission of HBV can also happen because the virus is stable on environmental surfaces for more than one week (Hoofnagle et al., 1978; Stevens et al., 1979; Bond et al., 1981).

Most HBV infected persons develop a clinical or sub-clinical self-limiting acute hepatitis and within a few weeks post-infection spontaneous clearance of HBV-infected hepatocytes or suppression of viral expression takes place. However, some infected individuals develop chronic infection. Only 25-50% of cases of acute HBV infection are symptomatic; the remainder are asymptomatic. Following the incubation period, symptoms of the pre-icteric phase begin to appear. These symptoms include anorexia, nausea, vomiting, weakness and pain in the right upper body quarter. In addition, the hepatic transaminases reach a peak. Once the icteric phase (lasts nearly 3 weeks) starts these symptoms and the high levels of transaminases begin to decline. Following the symptomatic phase the convalescent phase starts and last for up to six months with nearly complete disappearance of symptoms (Alter, 2003).

An HBV specific T cell response is considered the main factor affecting and modulating the virus pathogenesis. HBV variants may influence the course of disease and on the other hand they may counter act the efficacy of antiviral therapy (Rabe et al., 2003; Baumert et al., 2007).

Although there is no definite treatment for acute hepatitis B, some studies showed that high doses of lamivudine are recommended in patients with severe acute HBV infections to reduce the risk of progression to fulminant hepatitis (Lisotti et al., 2008). Some nucleoside or nucleotide analogues were approved by FDA for the treatment of chronic hepatitis B e.g. lamivudine, adefovir, entecavir and emtricitabine. These analogues are used alone or in combination with the immune modulator IFN-α (Conjeeveram and Lok, 2003). Strategies for immunization using hepatitis B vaccines were followed in most countries all over the world to prevent hepatitis B virus infection (Van Damme and Vorsters, 2002).

2.1.3 Virion structure

The blood of HBV-infected persons carries three types of virus-associated particles: mature virus particles and subviral particles (SVP) which involve HBsAg spheres and HBsAg filaments (Fig. 1). The virus appears under the electron microscope as a spherical double-shelled structure with a diameter of 42–45 nm. The outer shell resembles the viral envelope and is formed by the envelope proteins together with the classical host lipid bilayers while the inner one is referred to as the core particle or capsid and is composed of capsid- or core protein (HBc protein). The viral capsid consist of 180 or 240 identical core proteins, which form capsids of 32 and 36 nm in diameters with a T=3 and T=4 symmetry, respectively. The virus capsid encloses ds/ss positive DNA genome linked covalently at its 5′ end with the viral polymerase (Crowther et al., 1994; Kenney et al., 1995).

2.1.3.1 Protein composition of HBV particles

The hepatitis B virus minus DNA strand contains 4 open reading frames (ORFs) which encode for seven proteins, 3 surface proteins (SHBs, MHBs and LHBs), the core protein (HBc), a secretory protein (HBe), the viral polymerase (P) and the X proteins.

2.1.3.1.1 Surface proteins (HBs)

The surface proteins (envelope proteins) are classified into 3 different proteins, small (S), middle (M) and large (L) protein and they are encoded by a single open reading frame (ORF

S) which is divided into the S gene, pres1 region and pres2 region (Heermann et al., 1984). The different HBV genotypes showed S gene and pres2 region of constant length, however the pres1 and pres2 regions show higher amino acid divergence than the S gene.

The small hepatitis B surface protein (SHBs), encoded by the S gene is 226 amino acids long. This protein is one of the main constituents of all forms of HBV particles; it is synthesized by the virus in high quantities. SHBs has a conformational, highly antigenic epitope (HBsAg, Hepatitis B surface antigen) which is composed of the "*a*" determinant flanked by two mutual *d*/*y* determinants at the amino acid position 122 and *w*/*r* determinants at the position 160 (Bancroft et al., 1972; Torre and Naoumov, 1998). HBsAg "*a*" determinant has two-loop structure which protruded on the surface of the viral particles and its residues, aa 124-147, are relatively conserved in all HBV genotypes. The "*a*" determinant is directly involved in inducing neutralising antibodies. It forms the base of current HB vaccines (Chen and Oon, 1999; Bartholomeusz and Schaefer, 2004). At asparagine 146 of the S protein, there is a signal for the addition of an N-linked glycan which is present in approximately half of the SHBs molecules. Therefore, SHBs shows two different forms, an unglycosylated form of 24 KDa and a glycosylated form of 27 KDa molecular weight (Peterson, 1981).

The middle hepatitis B surface protein (MHBs), encoded by the S gene and the pres2 region (an additional 5′ open reading frame with 55 codons). The N terminal 55 amino acids domain is mostly hydrophilic and contains a dominant epitope located at the surface of the envelope (Tiollais et al., 1985). In addition to the first glycosylation site of the S protein at as asparagine 146, there is a second glycosylation site at asparagine 4 of the preS2 domain. Therefore, MHBs can be synthesized into 3 different forms: as an unglycosylated protein of 30 KDa, a single glycosylated protein with a glycan residue at asparagine 4 of 33 KDa and double glycosylated protein of 36 KDa (Heermann et al., 1987; Mehta et al., 1997).

The large hepatitis B surface protein (LHBs) is encoded by the pres1 domain in addition to the pres2 and S domains. The preS1 domain encodes for 108 or 119 aa. Overexpression of LHBs alone results in the retention of the protein in the ER, which may lead to the development of hepatocellular carcinoma (Chen and Oon, 1999). LHBs is monoglycosylated although it contains a second glycosylation site at aa 4 of the PreS2 region so; LHBs can be synthesized into two different forms: an unglycosylated protein of 39 KDa and a glycosylated protein of 42 KDa (Heermann et al., 1987; Hildt et al., 1996).

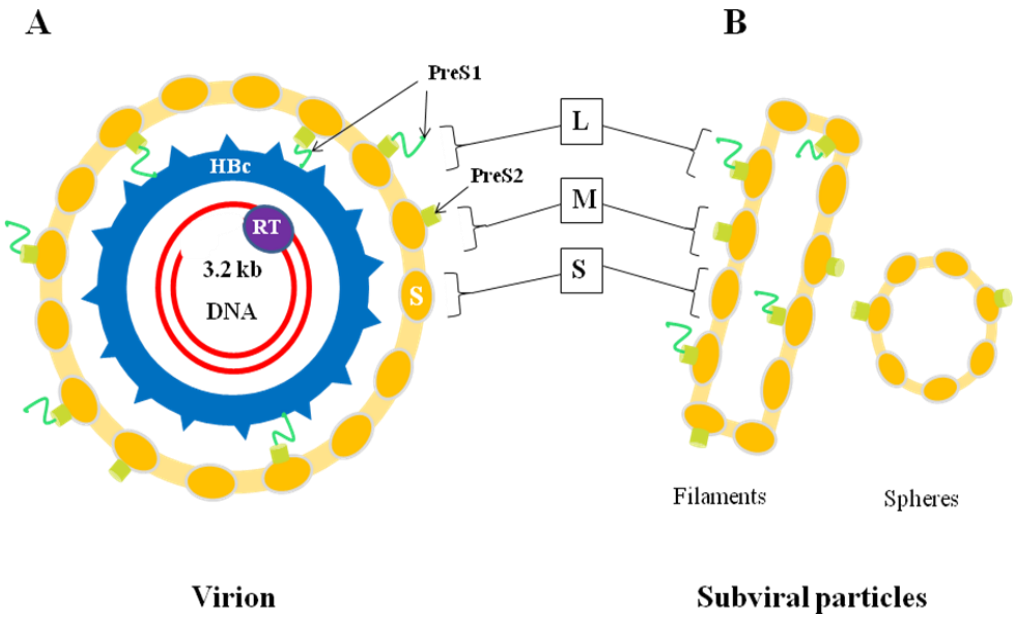


Fig. 1. Morphology and structure of HB associated particles. (A) Schematic diagram of HBV particle. The HBV particle is composed of a 3.2 kb partially ds DNA which is covalently linked by its 5' end to the DNA polymerase and encapsidated by a capsid composed of 180-240 core proteins. This capsid is surrounded by an envelope carrying small, middle and large surface proteins (HBs). The three envelope proteins contain an identical S domain. MHBs contains the additional pre-S2 domain while LHBs contains the additional pre-S1 domain together with the pre-S2 domain. **(B)** Schematic diagram of subviral particles. The subviral particles consist of the same proteins as the virion envelope but the spheres contain fewer LHBs.

2.1.3.1.2 Transmembrane topology of the surface proteins

The surface proteins are synthesized at the ER and show a complex transmembrane topology (Bruss, 2004) (Fig. 2). The N and C termini of S protein are disposed externally in the mature particles (luminal disposition) of both. Therefore, the protein traverses the ER membrane at least twice. The ER membrane insertion takes place by two N terminal domains, aa 11-28 (TM1, transmembrane domain 1) and aa 80-98 (TM2, transmembrane domain 2), which are spaced by a hydrophilic region exposed internally in the mature particle (cytoplasmic disposition) (Eble et al., 1987; Bruss and Ganem, 199lb). There is a second hydrophilic loop, aa 99-168 aa, exposed on the luminal side which carries the major epitope and the glycosylation site (Stirk et al., 1992). The C terminal region of the S protein is hydrophobic and may contain another two transmembrane domains (TM3and TM4) (Eble et al., 1986).

The M and S proteins have an identical topology as the hydrophilic preS region of M and the preS-specific epitopes protrude on the surface of the mature particles (Kuroki et al., 1990;

Heermann and Gerlich, 1991). The preS2 region of M protein shows luminal disposition and this was augmented by its glycosylation (Heermann et al., 1984).

The topology of L protein shows some alterations relative to the S and M proteins. The preS1 and preS2 domains of the L protein are initially disposed on the cytosolic side of the ER (i-preS; internal preS). This explains the partial glycosylation of asparagine 146 in the S domain but not of asparagine 4 located in preS2. However, around 50% of L protein in mature particles shows a contrary topology with the preS1 and preS2 domains which protruding on the surface of the mature particles (e-preS; external preS) (Bruss et al., 1994; Bruss and Vieluf, 1995; Prange and Streek, 1995). The L protein shows myristoylation at glycine 2 which seems to be essential for viral infectivity (Persing et al., 1987; Gripon et al., 1995; Bruss et al., 1996).

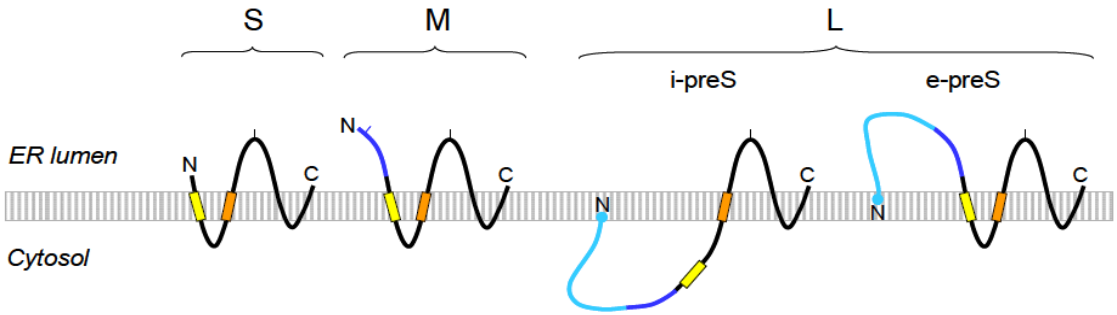


Fig. 2. Transmembrane topology of the HBV surface proteins. The S protein (black line) containing TM1 (yellow barrel) and TM2 (orange barrel). The M protein consisting of S and the preS2 domain (dark blue line). The L protein carries the additional preS1 domain (cyan line). Black and dark blue bars indicate glycosylation sites while the cyan dot resembles the myristoylation of L (Schittl, 2012).

2.1.3.1.3 Core protein (HBc)

The HBc protein is the major component of the nucleocapsid shell. HBc is either 183 or 185 amino acids long depending on the genotype of the virus and its molecular weight is 21 KDa. It is expressed in the cytosol of the infected hepatocytes. It packages its own mRNA and the viral polymerase after formation of the RNA-polymerase complex and assembles into core particles (Ou et al., 1986; Nassal et al., 1992).

The core protein involves two different domains, the N-terminal 144 aa domain and the C-terminal arginine rich domain. The N-terminal 144 aa domain is essential for capsid formation. Cryo-electron microscopy and crystallization reveals that the N-terminal domain builds up five α−helices arranged in an anti-parallel orientation forming a spike between α3 and α4 (Conway et al., 1997; Wynee et al., 1999). The C-terminal arginine rich domain is a

multifunctional domain. It is essential for RNA packaging and it syntheses the viral positive DNA strand (Hatton et al., 1992).

The HBV capsid is constructed from 180 or 240 copies of the core protein. Assembly of a core particle is initiated by formation of a core protein dimer (Fig. 3A) which rapidly assemble to the icosahedral capsids with a T=3 or – more frequently – T=4 symmetry (Fig. 3B) (Endres and Zlotnick, 2002; Roseman et al., 2005). The HBV capsid is not a completely closed protein shell; it shows holes of nearly 2 nm diameter. These capsid holes allow the nucleotides which are required for DNA synthesis of a mature viral genome to enter the lumen. During the maturation of the viral genome, these holes were thought to undergo conformational changes thereby exposing the C terminus of the C protein on the capsid surface facilitating the transport of the viral DNA into the nucleus (Kenney et al., 1995; Kann et al., 1999).

The expression of HBV core protein and its mutants can be carried out in heterologous systems, e.g. *Escherichia coli*. The expressed core proteins assemble into capsids even in the absence of the viral genome and it has been shown that the first 144 amino acids of the core protein are sufficient for assembly (Birnbaum and Nassal, 1990). It was assumed that HBV capsids that are assembled in *E. coli* have the same morphology as authentic capsids from virions of infected liver (Kenney et al., 1995).

In chronic hepatitis B, the HBc protein considered the major target of the host immune response and it contains several immunodominant epitopes which assist in the evolution of escape mutants (Kao, 2002).

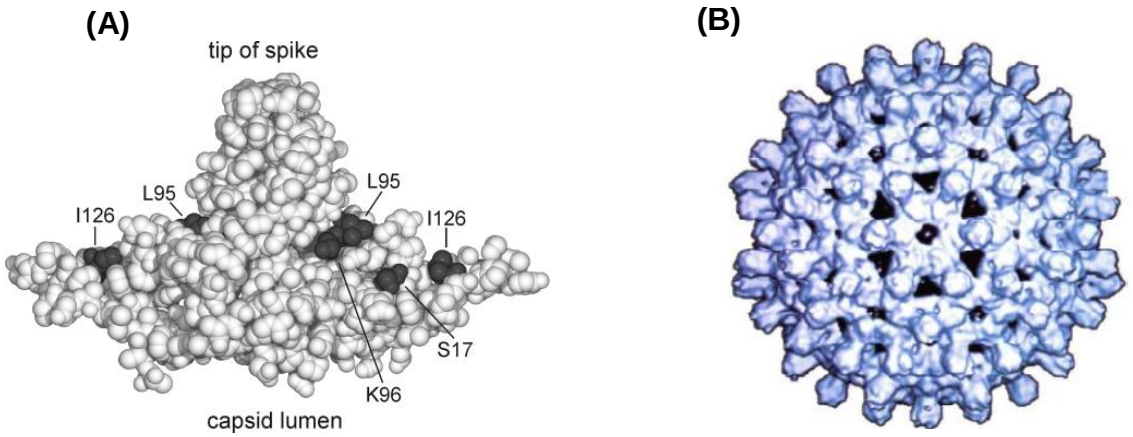


Fig. 3. Morphology of HBV capsid and the core homodimer. (A) Sphere model of a HBV core homodimer showing the amino acid residues important for capsid envelopment (black spheres) (from Pairan and Bruss, 2009). **(B)** External cryo-electron micrograph of T=4 symmetrically assembled HBV capsid (from Conway et al., 1997).

2.1.3.1.4 HBe protein

The HBe protein is a secretory form of the HBc protein. It is a non-structural protein that shares about 90% of its amino acids with the HBc protein. The pre-C sequence at the 5′ terminal part of ORF C encodes for a hydrophobic α-helix, which is a secretion signal cleaved off by a signal peptidase, and prevents the folding of the HBe protein similar to HBc protein. It is essential for the translation/translocation of HBe into the lumen of the endoplasmic reticulumn and the release of HBe into the circulation of the infected patient. Although HBe and HBc proteins have nearly identical amino acid sequences but there is little antigenic homology between them because of the differences in their folding (Bruss and Gerlich, 1988; Bruss and Ganem, 1991; Wasenauer et al., 1992).

2.1.3.1.5 Hepatitis B polymerase (P) protein

The P protein is a 90-kDa protein, the largest protein among the HBV proteins, is encoded by the ORF P (Bartenschlager et al., 1992). The ORF P has several functions in HBV replication, such as RNA pregenome (pgRNA) encapsidation, priming of DNA synthesis, reverse transcription, and (+) strand DNA-polymerisation (Burda et al., 2001). The HBV polymerase is composed of four distinct domains: an N-terminal domain (TP) that serves as the primer for the reverse transcription, a spacer region of unknown function, reverse transcriptase (RT)/polymerase domain and ribonuclease H (RNase H). The TP domain linked to the 5′ end of the minus-strand of the genome is also termed primase as it is essential for the priming of minus-strand synthesis (Bartenschlager and Schaller, 1988). The reverse transcriptase (RT)/polymerase domain is multifunctional, it is responsible for the synthesis of minus strand DNA from pgRNA by reverse transcription, builds the plus DNA strand by its polymerase function (Köck et al., 2003). The RT/polymerase domain is assumed to have a structure similar to the RT of retroviruses with fingers of a palm (Beck et al., 2002; Torresi et al., 2002). The RNase H domain cleaves and degrades the RNA if it is present in hybrids of RNA and DNA.

2.1.3.1.6 Hepatitis B x protein (HBx)

The X protein is encoded by the ORF X, the smallest ORF in the HBV genome. It is composed of 154 amino acids with a molecular weight of 17 KDa. HBx protein is present only in orthohepadnaviruses and not in avihepadnaviruses. The HBx protein function is still not fully understood, but it is assumed that it inhibits the viral protein degradation inside the host cell (Chen and Oon, 1999) and plays a role in hepatocarcinogenesis (Kew, 2011).

2.1.3.2 HBV genome

The DNA of HBV appears under the electron microscope, compact, circular and partially double stranded and it is of 3.2 kb long (Robinson et al., 1974). In HBV virions the genome has an incomplete plus-strand with defined 5′ end but a variable 3′ end and a complete minus-strand, the coding strand, which has defined 5′ and 3′ ends. The viral polymerase is covalently bound to the 5′ end of the minus strand while the 5′ end of the plus-DNA strand is capped by an 18 base long oligoribonucleotide, which serves as a primer. The minus-strand has terminal redundant sequences of 8–9 bases in its both ends, resulting in a region in which the genome is triple-stranded (Will et al., 1987). There are two short direct repeats of 11 nucleotides length (DR1 and DR2) present at the 3’ end of the negative and the 5′ end of the plus strand, respectively (Fig. 4).

All genomes of mammalian hepadnaviruses contain four partially-overlapping ORFs, which are encoded by the same minus-DNA strand. These four ORFs (ORF S, ORF P, ORF C and ORF X) code for in total 7 proteins, ORF S which encodes the large, middle and small envelope proteins is completely located within the ORF P which encodes the DNA polymerase, ORF C which encodes the HBc and HBe proteins and ORF X which encodes the HBx protein overlap partially with ORF P (Fig. 4).

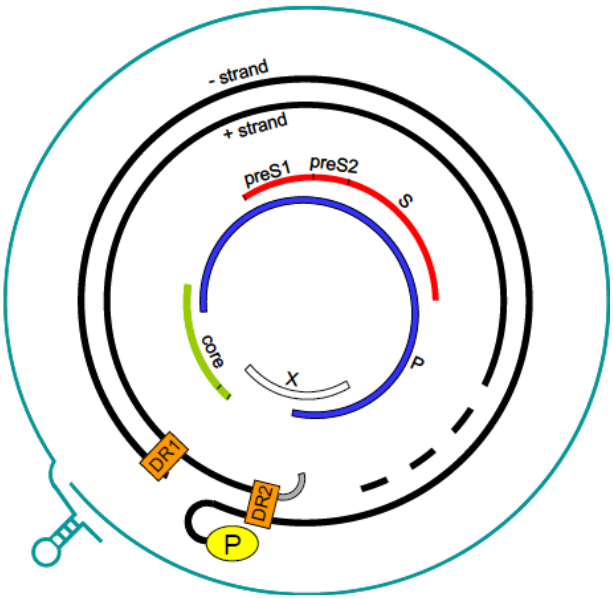


Fig. 4. HBV DNA genome showing the circular arrangement of the four overlapping but frame-shifted open reading frames. The partially double stranded DNA genome (thick black lines) contains four overlapping open reading frames (ORF C: green; ORF S: red; ORF P: blue and ORF X: white). The minus strand bound covalently to the endogenous polymerase (yellow oval) at the 5′ end. A small RNA primer (grey line) located at the 5′ end of the positive strand. The orange boxes refer to the direct repeats DR1 and DR2. The outer cyan circle refers to the posttranscriptional pregenomic RNA which involves the epsilon signal at its 5′ end (Schittl, 2012).

2.1.4 HBV life cycle

The HBV life cycle (Fig. 5A) starts with the attachment of the virion by its envelope to a hepatocytes surface receptor. Recently, it was identified that sodium taurocholate cotransporting polypeptide (NTCP) which is mainly expressed in the liver, is a functional receptor for HBV and HDV (Yan et al., 2012). Primary hepatocytes are the first cells used as infectivity systems for HBV. Although many cell lines, e.g. HepG2 and HuH 7 cell lines are permissive for HBV replication after transfection but they are not susceptible to HBV infection and this was owed to this phenomenon is caused by an ongoing de-differention process which blocks the virus uptake by cells and this was augmented by the loss of susceptibility of all primary hepatocytes to their corresponding virus within a few days after they are taken into culture. Gripon and his colleagues had established a new cell line called HepaRG supported HBV infection comparable to PHH (Gripon et al., 2002).

Once the capsid enters the hepatocyte, it is transported to the nucleus by the assistance of the nuclear localization signal at the C terminus of the core protein. Inside the nucleus, the remaining gap of HBV plus-DNA strand is filled by the celluar polymerase (Summers et al., 1975; Landers et al., 1977) and then the viral genome is converted to a covalently closed circular DNA (cccDNA) which undergoes transcription to continue the viral replication. The cccDNA serves as the template for synthesis of five viral transcripts (mRNAs) by the action of cellular RNA polymerase II. There are two mRNAs of approximately 3.5 kb long, one serves the translation of the precore secretory protein (HBe) and the other is the pgRNA which encodes for the nucleocapsid protein and the polymerase/RT protein. There are 2.4 and 2.1 kb mRNAs encoding the surface proteins (HBs, MHBs and LHBs) and a 0.9 kb mRNA encoding the X protein. The pgRNA is packaged with the polymerase/RT protein by its encapsidation signal at the 5′ ε-stem loop, into core particles and then reverse transcribed by the polymerase into progeny HBV DNA (Tavis and Ganem, 1996; Günther et al., 1997; Kann et al., 1999).

During the reverse transcription of the pgRNA into the (-) DNA strand by the endogenous polymerase, the pgRNA template is degraded by the HBV polymerase RNase H activity leaving a small segment. This segment of RNA is composed of the 5′ Direct Repeat 1 region (DR1) which is translocated and anneales to the 3' direct repeat 2 region (DR2). This RNA oligomer is used as a primer for the synthesis of (+) DNA. The (+) DNA synthesis is then continued by the polymerase. The short terminal redundancy (r) on the negative strand is also copied forming the 5′ r. The new mature viral nucleocapsids transfer to the ER, where they are associated with the envelope proteins that have previously been inserted as integral

membrane proteins into the lipid membrane of the ER and finally the newly formed virions bud into the lumen of the ER, from which they are secreted via the Golgi apparatus out of the cell (Lien et al., 1986; Mahoney and Kane, 1999).

The sera of highly viraemic HBV carriers contain huge amounts of non-infectious subviral particles (SVP) composed of excessive HBs protein (HBsAg). Most of these are spherical particles of 17–25 nm, which are secreted in 100–10000-fold excess over virions. The subviral particles have neither capsid nor HBV DNA and thus they are non infectious. The different subviral particles conformations contain different ratios of S/L HBs. The filamentous form is correlated with a higher concentration of the L protein (Heermann et al., 1984). The formation and assembly of subviral particles take place in a post-ER pre-Golgi compartment (Simon et al., 1988).

2.1.5 Envelopment of core particles

Only mature capsid can undergo envelopment while immature ones containing pgRNA can not be enveloped. It is assumed that the synthesis of the minus DNA changes the conformation of the nucleocapsid exposing a specific signal which is essential for capsid envelopment. The matrix binding domain (MBD) is a specific domain located at the base of the spike and in the groove between capsid spikes having an important role in HBV assembly. The introduction of point mutations in this domain e.g. I126, K96, L95 and S17 (Fig. 3A) allows capsid assembly but results in the inhibition of capsid envelopment. Mutations at the tip or stem of the capsid spike had no impact on the envelopement process (Ponsel and Bruss, 2003; Parian and Bruss, 2009).

The viral envelope proteins, especially L protein, are considered to play a key role in the envelopment of HBV capsids (Bruss and Ganem, 1991). A smaller domain between aa 103 and 124 in the cytosolic portion of L protein plays an essential role in HBV nucleocapsid envelopment so, it was termed as the matrix domain (MD) (Bruss, 1997). Furthermore, the minimal distance between this domain and TM1 was determined to be 26 amino acids which would fit well to the length of the capsid spike (Le Seyec et al., 1998; Kluge et al., 2005). *In vitro* binding assays using peptides corresponding to the MD of L protein revealed also a direct interaction between MD of L protein and the MBD on the capsid surface (Fig. 5B) (Poisson et al., 1997). Both, HBV capsids and L protein have the ability to bind to γ2-adaptin, a protein important in the ESCRT-mediated multivesicular body (MVB) / lysosome sorting pathway. The core protein amino acid residue K96 was also shown to be essential for the recognition of γ2-adaptin (Hartmann-Stühler and Prange, 2001; Rost et al., 2006;

Döring et al., 2010). The budding site of HBV virions is still not clear but it was observed that the induction of mutations in the endosomal sorting complex (ESCRT-complex) inhibits virion release (Lambert and Prange, 2007).

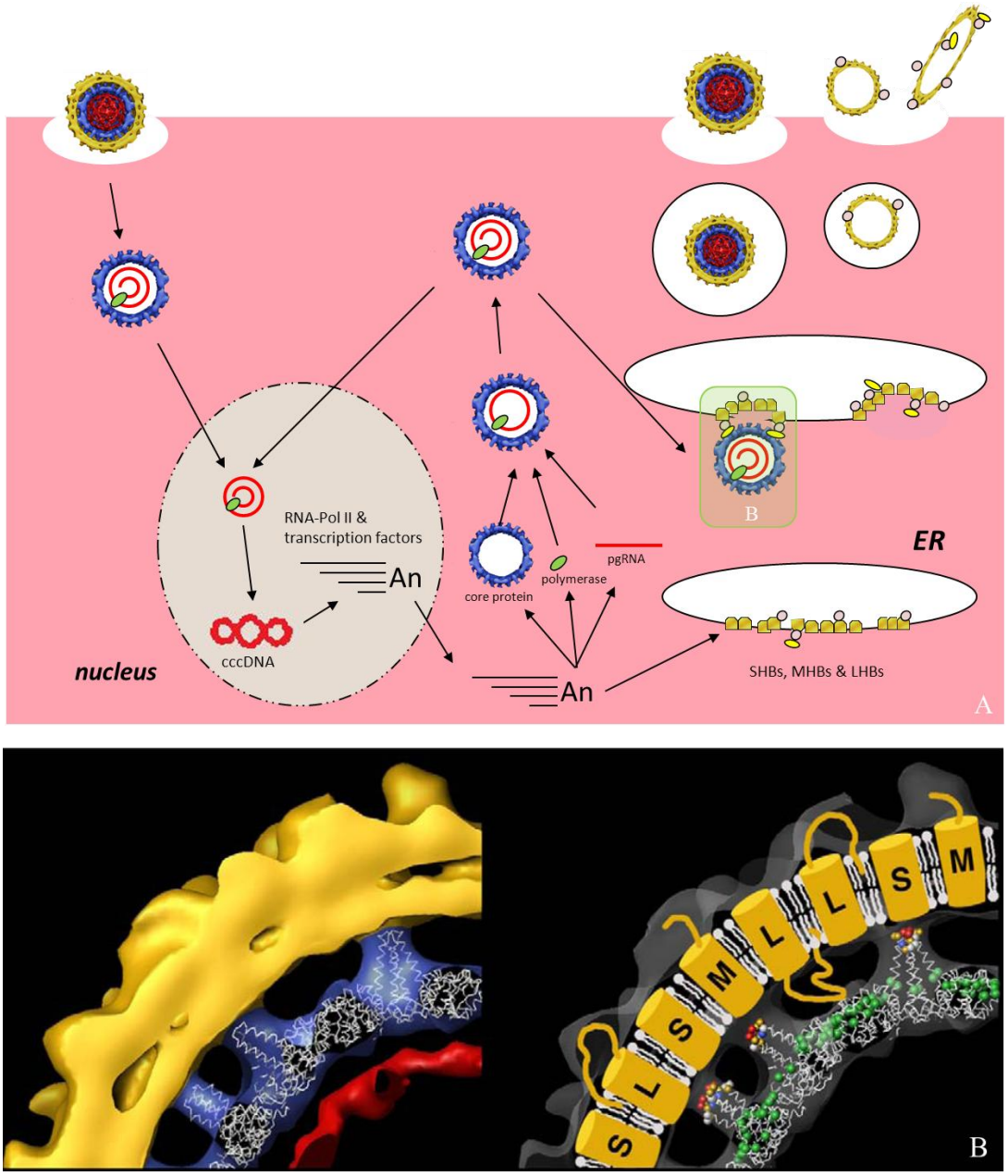


Fig. 5. Life cycle and envelopment process of HBV. (A) HBV binds to the surface of hepatocytes and enters the cells with the help of its envelope proteins (receptor mediated endocytosis). Inside the cell, the capsid is transported to the nucleus where the partially circular DNA is converted to covalently close circular DNA (cccDNA). HBV cccDNA serves as a template for transcription of mRNAs and the pgRNA. The pgRNA is then encapsidated into core proteins and reverse-transcribed. The core particles with the newly synthesised partially-circular genomes are finally packaged into viral envelopes in the ER, and then exocytosed with the synthesized subviral particles out of the cell. **(B)** X-ray crystal model of HBV virion showing an interaction between specific core residues (green spheres) with the interior loop of the L protein but without penetration of the capsid spike into the envelope (Dryden et al., 2006).

2.2 *Aptamers*

2.2.1 Nature and theory

The term aptamer is derived from the Latin word “aptus”– which means fitting and the Greak word “meros” – which means particle. Aptamers are short nucleic acids or peptides with a specific and complex three–dimensional (3D) shape characterized by stems, loops, bulges, hairpins, pseudoknots, triplexes, and/or quadruplexes. Based on their 3D structure, aptamers can bind to a wide variety of targets. Binding of the aptamer to the target molecule results from structure compatibility: stacking of aromatic rings, electrostatic and van der Waals interactions, hydrogen bindings, or from a combination of these effects (Ellington and Szostak, 1990; Hermann and Patel, 2000, Feng and Hu, 2008).

In 1990, screening and selection of RNAs libraries against T4 DNA polymerase and many organic dyes were achieved. Ellington and Szostak called the selected RNA ligands as aptamers while the selection process was termed by Tuerk and Gold as SELEX (Systematic Evolution of Ligands by EXponential enrichment) (Ellington and Szostak, 1990; Tuerk and Gold, 1990).

Aptamers are high-affinity and high-specificity ligands and they are mostly acting as inhibitors as they often bind to the functionally important parts of their targets (Eaton et al., 1995; Proske et al., 2005)

SELEX is a process involving the progressive purification from a random library of nucleic acid molecules or peptides (aptamers) with a high affinity for a particular target by repeated rounds of partitioning and amplification (Gopinath, 2007). Briefly, randomized pools of RNA, ssDNA or peptides are incubated with target molecules under specific selection conditions. The bounded aptamers are partitioned away from non-binders, amplified to generate a new pool, and the process is repeated until sequences with suitable phenotypes are obtained or until sequence diversity is greatly reduced (Hermann and Patel, 2000).

2.2.2 Technology

2.2.2.1 Oligonucleotide library

The starting point of a SELEX process is a chemically synthesized random oligonucleotide library. Libraries containing a random region of maximal 20-60 nt in length and flanked at

its both ends by two fixed sequences for PCR fragments amplification are used (Conrad et al., 1996).

Both RNA and ssDNA libraries are used in SELEX procedures. In principal, the affinity or specificity of ssDNA aptamers and RNA ligands is not different. The advantage of RNA aptamers is that they can be expressed inside of cells, which may be of great importance in experiments *in vivo*. On the other hand, DNA aptamers show higher stability and their selection is simpler and faster. Owing to this, during recent years DNA aptamers have become more and more widespread (Breaker, 1997).

For the synthesis of the random region in ssDNA library, a mixture of all four deoxyribonucleotide derivatives is added to the reaction mixture allowing the random incorporation of a nucleotide into the growing molecule. To obtain an RNA library, the promoter sequence for the RNA polymerase of bacteriophage T7 is introduced into the 5′ terminal region of the ssDNA library, dsDNA is obtained by a polymerase chain reaction (PCR), and then an *in vitro* transcription is carried out. The synthesis of random sequences is relatively cheap. The obtained sequences depend on the ratio of the four nucleotides used which differs according to the manufacture process (Famulok and Mayer, 1999; Kulbachinskiy, 2007).

Concerning the arrangement and type of randomization, different types of nucleic acid libraries can be used in SELEX, classical libraries (Tuerk et al., 1992; Burke et al., 1996), structurally constrained libraries (Biroccio et al., 2002; Hamm et al., 2002), libraries on the basis of a known sequence (Hirao et al., 2004), libraries free of fixed sequences (Vater and Klussmann, 2003), and libraries on the basis of genomic sequences (Shtatland et al., 2000).

The complexity of the library can be determined easily as 4^n (n is the number of positions in the random sequence). For example, the complexity of a library with twenty five randomized nucleotides is 4^{25} or approximately 10^{15}.

Aptamers with chemically modified nucleotides can be used for achieving special purposes, to magnify the potential variety of oligonucleotides, to introduce new features e.g. functional groups providing new possibilities for the interaction with target molecules, to improve the stability of the aptamers or to increase their resistance to nucleases (Eaton et al., 1995; Kusser, 2000). There are two standard approaches for obtaining chemically modified aptamers: the first approach is by using modified oligonucleotides directly during the selection. However, a problem might be that the ability of the nucleotide to serve as a substrate for RNA or DNA

polymerase is influenced. In the second approach, the already selected aptamers are modified but these post-selection modifications may result in lower affinity to their targets (Kulbachinskiy, 2007). There are different techniques for obtaining modified aptamers: (1) To improve the aptamer stability and their nuclease resistance, F or NH2 group can be introduced in the 2´-position of ribose (Jayasena, 1999; Nimjee, 2005) or by creating spiegelmers using aptamers composed of natural D–oligonucleotides which can be selected against mirror image targets, such as D–amino acid peptides, rather than natural L–amino acid peptides. After the isolation of the aptamer they can be chemically synthesized as L–oligonucleotide (Spiegelmer) and will bind to the natural L–amino acid peptide targets (Klussmann et al., 1996; Nolte et al., 1996). (2) To improve the affinity and the specificity of the aptamers to their protein target, modified oligonucleotides (photoaptamers) containing functional groups that can be activated upon irradiation (such as 5-iodo-, 5-bromo-, and 4-thiouridine) forming covalent cross-links with their protein target can be used (Jensen et al., 1995). (3) To analyze the binding of the aptamers to their target protein, modified aptamers containing fluorescent groups can be used (Nutiu, 2005).

2.2.2.2 Standard Selection Process (SELEX)

The scheme of the standard SELEX procedure (Fig. 6) starts with the incubation of the oligonucleotide library with the target molecule. Then the selection step is carried out by the separation of bound oligonucleotides from those that are not bound. Selected oligonucleotides are then amplified. The amplification is performed by PCR in the case of DNA, and by RT-PCR followed by *in vitro* transcription in the case of RNA. One cycle of target binding, selection and amplification is called a SELEX round. The SELEX rounds are repeated several times, and some of the oligonucleotides selected in the final round of the experiment are sequenced and evaluated.

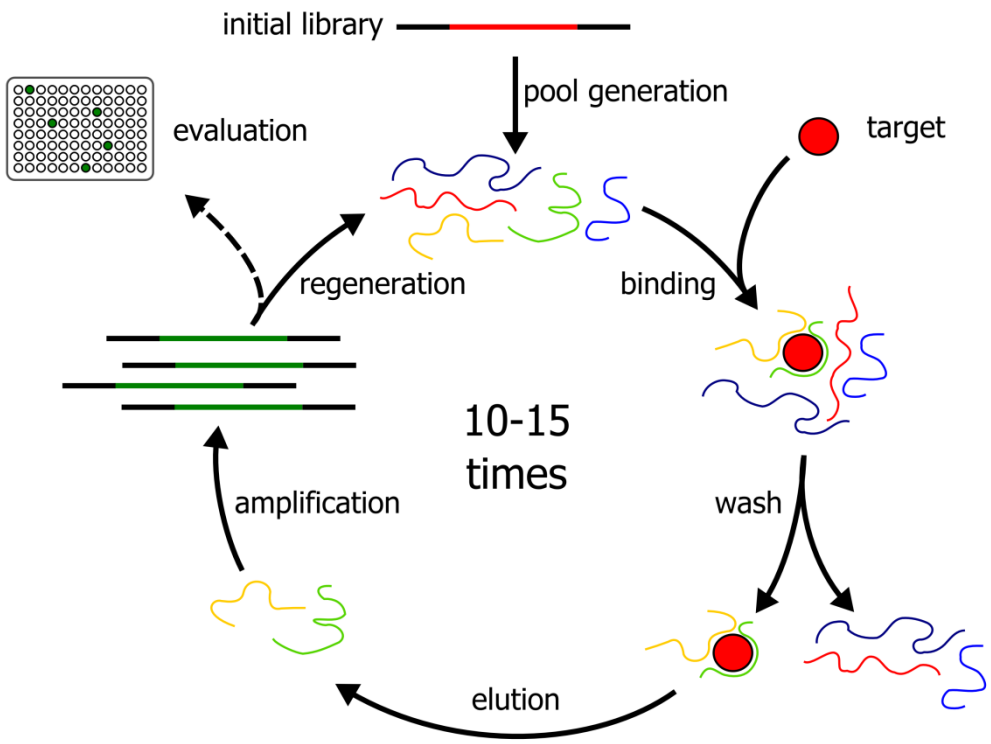


Fig. 6. General scheme of the standard SELEX procedure. A library of DNA or RNA molecules is incubated with the target molecule, and the bound ones are separated from the rest. The sequences with affinity for the target are subsequently amplified to generate a pool of molecules that bind to the protein of interest. After several rounds, aptamers with high affinity and specificity can be selected. *http://www.cd-genomics.com/Aptamers/SELEX.htm.*

The partitioning of the aptamer–target complex from non specific molecules can be achieved by various techniques. The most commonly used method for protein targets partitioning is filteration through nitrocellulose filters (Tracy and Kowalczykowshi, 1996; Bianchini et al., 2001). The selection processes using nitrocellulose membranes usually require up to 12-15 selection cycles. Alternatively, the use of functionalized magnetic adsorbent particles with a magnetic separation system has also been considered to be a useful tool for the separation of protein and nucleic acids (Gopinath, 2007). Also, using affinity tags like glutathione S-transferase and streptavidin-derivitized surfaces (Dobbelstein and Shenk, 1995; Cox and Ellington, 2001) or column matrices like sepharose (Ciesiolka et al., 1995) can be used to reduce the number of required selection cycles. A counter–selection against the partitioning matrix is a very important step to avoid the isolation of sequences that have affinity to the matrix (Gold, 1995). During recent years, more effective separation methods are reported, e.g. Capillary Electrophoresis (CE), Flow Cytometry (FC) (Davis et al., 1997), Electrophoretic Mobility Shift Assay (EMSA) (Tsai and Reed, 1998), Surface Plasmon Resonance (SPR) (Misono and Kumar, 2005) or centrifugation (Rhie et al., 2003).

For the selection of RNA aptamers, the random DNA oligonucleotide library has to be transformed into a RNA library before starting the first round of a RNA SELEX process. A sense primer with an extension at the 5′ end containing T7 promoter sequence and an antisense primer are used to convert the ssDNA library into a double–stranded (dsDNA) library by PCR. The dsDNA is then *in vitro* transcribed by T7 RNA polymerase resulting in a randomized RNA library which can be used in SELEX. For further rounds of selection, the same procedures should be carried out after each round (Homann and Göringer, 1999).

For the selection of DNA aptamers, the process is simpler than RNA SELEX as the library can be used directly in the first round of selection. The primer set derived from the fixed sequences at the 5′ and 3'end enable the amplification of the selected oligonucleotides in each SELEX round. After PCR amplification, a ssDNA preparation must be performed to generate a ssDNA pool for the next round. Many methods are used for ssDNA preparation e.g. (1) A biotin residue is introduced into one of the primers used for amplification and both DNA strands are separated under denaturing conditions either in a polyacrylamide gel after a pre-incubation step with streptavidin or directly into a column containing streptavidin (Agratis, 1996; Murphy et al., 2003). (2) An a symmetric PCR, in which one primer initiates DNA synthesis much more efficient than the other primer which is relatively unproductive, leading to the accumulation of ssDNA synthesized from the efficient primer (Ellington and Szostak, 1992). (3) A hexaethyleneglycol (HEGL) spacer, a terminator for *Taq* polymerase, and a polyA tail are added at the 5′ end of the reverse primer. This leads to elongation of only one strand (–strand). Afterwards, the two strands can be separated according to their size using electrophoresis under denaturing conditions (Williams and Bartel, 1995). (4) A phosphate group is introduced into the 5′ end of one primer. Then the PCR amplified product is treated with the phage lambda exonuclease that digests the phosphorylated strand of DNA (Fitter and James, 2005).

2.2.2.3 Site-directed selection of aptamers

Complex target SELEX is a SELEX used for selection of aptamers against many heterogeneous targets, e.g. whole cells. It is used mostly to generate new biomarkers especially when biomarkers are not known in advance (Shamah et al., 2008). Aptamers against whole trypanosomes were successfully selected by this approach (Homann and Göringer, 1999).

There are many different methods can be used to avoid selection of aptamers to an undesirable epitope or to obtain ligands to a particular epitope of a protein target: (1) By the method of counter selection, aptamers are selected which interact with the full-size protein but do not bind to the mutant protein devoid of this epitope. The oligonucleotide library is firstly incubated with the whole protein target, and then oligonucleotides that do not interact with the mutant protein lacking the site of interest are selected (Andreola et al., 2001). (2) The method of competitive elution of aptamers using another ligand binding in the same site of the protein (Hale and Schimmel, 1996; Bridonneau et al., 1999). (3) Method of blended selection, uses oligonucleotides carry a known ligand specific for this protein. So, the selected aptamers can interact with a site near the binding site of this ligand (Charlton et al., 1997). (4) Aptamers can be selected against a peptide corresponding to any epitope of protein target. The selected aptamers can consequently recognize this epitope within the full-size protein (Bianchini et al., 2001). (5) For the selection of aptamers using the anti-idiotypic approach, the first stage antibodies specific for a protein partner of the target protein are generated while in the second stage, aptamers interacting with the obtained antibodies are selected. Consequently, the selected aptamers will have affinity to the target protein (Hamm et al., 2002).

2.2.2.4 Automated aptamer selection

The traditional methods of aptamers selection are time consuming and laborious. Many attempts for automating *in vitro* selection of aptamers have been done successfully. E.g. Cox and colleagues used a system based on an augmented Beckmann Biomek 2000 Pipetting robot which was adapted to select aptamers against a protein by some modification and generated aptamers to hen egg white lysozyme. This robotic work station can carry out eight selections in parallel and will complete 12 rounds of selection in two days (Cox and Ellington, 2001).

2.2.3 Aptamers and antibodies

Aptamers have several properties which make them mostly override antibodies and in addition, potential attractive therapeutic agents (Rusconi et al., 2002). Aptamers, like antibodies, bind to their targets by three dimensional (3D) recognition. Aptamers characterized by their high specificity and high affinity to their targets as antibodies with K_ds in the low picomolar to low nanomolar range. Aptamers are more stable, especially DNA aptamer, than antibodies and display lower or no immunogenicity (Eyetech Study Group, 2003). In comparison with the antibody technology, aptamer research is still new but promising and its progress is fast.

2.2.4 Aptamers in Diagnostics

2.2.4.1 Aptasensors

Aptasensors are recognitive biosensor elements. Their main structural component is the aptamer (O'Sullivan, 2002). Aptamers can be chemically modified without influencing their affinity to incorporate particular reporters and also they can easily be labelled to be used in diagnostics (Balamurugan et al., 2008; Ulrich and Wrenger, 2009). Aptasensors can be classified into two main types, optical and electrochemical aptasensors, (1) Optical aptasensors include aptamers labelled with fluorescence, luminophore, enzyme, nanoparticles or aptamer with label-free detection systems (e.g., SPR, surface plasmon resonance) (Sassolas et al., 2011). E.g. an RNA aptamer was selected and used as a detector ligand in a sandwich assay to recognize vesicular endothelial growth factor (VEGF) (Drolet et al., 1996). (2) Electrochemical aptasensors depend on the immobilization of the aptamer on an electrode surface. Then the binding conditions with their targets can be monitored by the electrochemical current variations (Willner and Zayats, 2007). E.g. a novel electrochemical sensor system based on two different aptamers recognizing different epitopes of thrombin was developed. The first aptamer was thiol–modified and immobilized on a gold electrode for capturing thrombin while the second indicator aptamer was labelled with a pyrroloquinoline quinone glucose dehydrogenase (Ikebukuro et al., 2005).

Several aptasensors have been developed to detect microorganisms and viral proteins e.g. RNA aptamer chip was successfully manufactured for detecting HCV core antigen (Lee et al., 2007) and also RNA aptamer developed for the detection of the HIV-1 Tat protein (Tombelli et al., 2005).

2.2.4.2 Flow cytometry

The binding of aptamers to their target proteins presented on either cell surfaces or microspheres can be detected by flow cytometry. E.g. a fluorescently labelled DNA aptamer with high affinity to human neutrophil elastase (HNE) was used to stain HNE-coated beads for flow cytometry (Lin et al., 1994; Davis et al., 1997) and also a fluorescently labelled RNA aptamer with a high binding affinity to mouse CD30 proteins had been evaluated for human CD30 protein recognition on intact cells by both, flow cytometry and fluorescence microscopy (Zhang et al., 2009).

2.2.5 Aptamers in therapeutics

Aptamers have been proven to be a promising class of novel drug as they are characterized by small size, low or no immunogenicity, high stability, high specificity and high affinity to their targets. In addition, the synthesis and selection of aptamers is relatively easy and inexpensive. Aptamers have been validated as therapeutics in the areas of anti–infectives, anticoagulation, anti–inflammation, antiangiogenesis, antiproliferation, and immune therapy (Nimjee et al. 2005). The first approval of an aptamer as a therapeutic agent was in 2004; Macugen (pegaptamib, by Pfizer and Eyetech) is the first aptamer drug approved by FDA against the age related macular degeneration, AMD (Ng et al. 2006). An anti–obesity drug was also produced by the NOXXON company, its active principle is spiegelmers which act against a hormone associated with an increase of appetite called ghrelin (Shearman et al. 2006).

2.2.6 Aptamers against hepatitis viruses

Butz and his colleagues selected a peptide aptamer, named C1-1, targeting the core protein of the hepatitis B virus (Butz et al., 2001). This aptamer was delivered *in vitro* and *in vivo* using adenoviral systems where it could inhibit viral DNA replication and consequently the viral infectious cycle (Zhang et al., 2009). An RNA aptamer with high affinity to hepatitis B virus surface antigen (HBsAg) has been successfully selected (Liu et al., 2010). The replication of HBV inside HepG2 cells has been inhibited by using an RNA aptamer which was selected against the ε RNA stem-loop on pgRNA (Feng et al., 2011).

Aptamers have been selected against the NS3 protein of the hepatitis C virus (HCV) and showed *in vitro* inhibition of the viral protease activity by up to 90% (Urvil et al., 1997; Fukuda et al., 2000). The aptamer was then elongated at the 3’ end by a poly-14-U tail which showed binding affinity to the helicase portion of NS3. This longer version inhibited both, the proteinase and the helicase activity of NS3 (Kanai et al., 1995; Fukuda et al., 2004). Aptamers have also been selected against a conserved internal ribosome entry site (IRES) in the 5′ UTR of HCV (Kikuchi et al., 2003). High affinity ssDNA aptamers were successfully selected against the HCV envelope glycoprotein E2 which is proposed to be essential for viral attachment (Chen et al., 2009).

3 Objectives

The goal of this research project was to select and characterize ssDNA aptamers specifically binding to the matrix binding domain (MBD) of HBV capsids, and to evaluate a potential *in vitro* inhibition of the capsid envelopment process by these aptamers which would be a potential therapeutic application. The MBD of the HBV capsid is extremely conserved and single amino acid substitutions usually block virion formation. The MBD mediates a very specific interaction with the matrix domain (MD) of L envelope protein of the virus and this interaction is essential for the envelopment process.

Firstly, it was necessary to overexpress and purify HBV WT capsids carrying the native MBD as well as HBV I126A mutant capsids, a capsid variant with a single mutation in the MBD that blocks the envelopment process of HBV (Pairan and Bruss, 2009). Both capsid variants should be expressed in *E. coli* to be used as a target and counter target, respectively, in SELEX.

Secondly, ssDNA aptamers against the MBD of the HBV capsid should be selected *in vitro* by SELEX with counter selection. After enrichment, the aptamers should be cloned and characterized.

Thirdly, after the isolation and characterization of the selected aptamers, the structure of the aptamers should be characterized and the binding dissociation constants (K_d) should be measured.

Finally, the inhibitory effect of selected aptamers on the HBV infectious cycle in HuH 7 cell culture should be determined.

4 Material and methods

4.1 Material

4.1.1 Antibodies

Antibody	***Application***	***Description***	***Origin***	***Provided by***
H800	Primary antibody Western blot & immunoprecipitation	Polyclonal anti-HBc antibodies	Rabbit	Heinz Schaller, university of Heidelberg
Anti-rabbit	Secondary antibody Western blot	Horseradish peroxidase conjugated anti-rabbit IgG antibody	Goat	Dianova, Hamburg
Anti-HB_S	Immunoprecipitation	Polyclonal serum	Sheep	W.Gerlich, Gießen

4.1.2 Aptamers

Type	ssDNA library
Length	55-mer (with two fixed ends of 15-mer and random sequence of 25-mer)
Complexity	10^{15} molecules
Fixed sequence	5`GCGGGTCGACGTTTG……N (25)……CACATCCATGGGCGG´3
Random sequence	(N25) represents random oligonucleotides based on equal incorporation of A, G, C and T at each position.
Provided by	PURIMEX

Negative aptamer (AO-0N)	5`GCGGGTCGACGTTTGATATGTGGTATACGCTTGGGTGTTAC-ACATCCATGGGCGG´3

4.1.3 Bacterial strains

E.coli BL21 Star (DE3) pRARE2	RNaseE (rne 131) Mutant, with plasmid codons for argU, argW, AegX, GlyT, ileX, leuW, metT, proL, thrT, thrU, TyrU.
ElectroMAX *E.coli* DH10B cells	F^-, mcrA, Δ(mrr-hsdRMS-mcrBC), F80dlacZ ΔM15, ΔlacX74, endA1, recA1, deoR, Δ(ara, leu)7697, araD139, galU, galK, nudG, rps; provided by Life Technologies.

4.1.4 Bacterial media and antibiotics

Product	***Company***	***Concentration***
LB medium	Roth	
TB medium	Roth	
2xYT medium	Roth	
LB Agar	Roth	
Ampicillin	Sigma	100 µg /ml
Canamycin	Sigma	50 mg /ml
Chloramphenicol	Sigma	33 mg /ml

4.1.5 Capsids of HBV

E. coli expressed C terminal deleted HBV capsid (its C protein of 149 aa)	Dr.Tanja Bauer, Institute of Virology, Technical University Munich (TUM).

4.1.6 Cell line

HuH7	Human hepatoma cell line derived from the liver tumor of 57 years old Japanese (Nakabazashi et al., 1982).

4.1.7 Cell culture media

Product	***Company***
DMEM	Lonza
Fetal bovine serum (FBS)	Sigma
MEM Non essential amino acids (NEAA)	PAA
Penicillin-Streptomycin (PS)	PAA
Sodium pyruvate	PAA
Trypsin/EDTA	Biochrom AG

4.1.8 Chemicals and Reagents

Acrylamide mix (30%)	National diagnostics
Acetate	Fluka
Acetic acid	Merck
Ammonium acetate	Sigma
Ammonium persulfate (APS)	Amersham Bioscience
Ammonium sulfate	Roth
Bromophenolblue	Sigma

Butanol	Roth
Canamycin disulfide	Sigma
Chloramphenicol	Fluka
Chloroform	Roth
Coomassie Brilliant Blue (R-250)	Thermo Scientific
Developer A	Agfa Healthcare
Developer B	Agfa Healthcare
Dimethyl Sulfoxide (DMSO)	Sigma
DL-Dithiothreitol (DTT)	Sigma
Ethanol	Merck
Ethidium bromide	Sigma
Ethyelene- Diamine –Tetra- Acetic acid (EDTA)	Sigma
Glucose	Sigma
Glycerin	AppliChem
Glycerol	Fluka
Glycine	Sigma
Glycogen	Fermentas
Hydrochloric acid	Sigma
Isopropanol	Merck
Isopropyl-β-D-thiogalactopyranoside	Roth
KH_2PO_4	Fluka
KOH	Fluka
LE- Agarose	Biozym
LB-broth Base	20 g/l Gibco
Magnesium acetate	Sigma
Magnesium chloride	Fluka
Methanol	Merck
Na_2HPO_4	Fluka
NaCl	Merck
NaOH	Roth
Nonidet P40 (NP-40)	AppliChem
Phenol-Chloroform-Isoamylalcohol	Roth
Polyethylenglycol (PEG 6000)	Merk
Potassium acetate	Fluka
Rapid fixer	Agfa Healthcare
Skim milk powder	Sigma
Sodium acetate	Sigma
Sodium citrate dihydrate	Sigma
Sodium dodecyl sulfate (SDS)	Fluka
Streptavidin	Thermo Scientific
Sucrose	Sigma
Terrific Broth Medium	Roth
Tetramethylethylendiamin (TEMED)	Biorad

Tris(hydroxymethyl) aminomethane	Merck
Tween 20	Sigma
Urea	Sigma
Xylencyanol	Sigma

4.1.9 Enzymes

4.1.9.1 Restriction enzymes

Eco RI-HF	New England Biolabs
Eco RV	New England Biolabs
NcoI	New England Biolabs
Sal I-HF	New England Biolabs

4.1.9.2 Other enzymes

CIP	New England Biolabs
DNaseI	Qiagen
Lysozyme	Serva
Proteinase K	Applichem
RNaseA	Qiagen
T4 DNA Ligase	Fermentas

4.1.10 Devices

Acta Purifier (UV-900, pH/C-900, P-900)	GE Healthcare
Aspiration system Vacusafe and Vacuboy	Integra Biosciences
Balance 2200-2NM	Kern&Sohn
Biofuge fresco (small centrifuge)	Heraeus, Thermo electron Corporation
Biorad Mini Protean Tetra System	Biorad
CO2 incubator HERAcell 150i	Thermo Scientific
Deep freezer - 20 °C	Liebherr
Developer machine Curix 60	Agfa
DISCOVERY Comfort mono-channel pippets	Abimed
Electroblot apparatus	Biometra
Electroporator E.*coli* Pulser Typ I	Biorad
Film cassette 18×24	Agfa
Fine scale CP153	Sartorius
Gel documentation Gel Doc	Biorad
Gene Amp PCR System 2700	Applied Biosystems
Hybridisation glass tubes	Biometra
Hybridisation oven Compact Line OV4	Biometra
Incubator	Memmert

Running chamber SDS	OWI Separation Systems
Labofuge 400	Heraeus
Labogaz 206	Campingaz
LightCycler 480 II	Roche
Magnetic stirrer Variomag	Neolab
Megafuge 1.0 R	Heraeus Instruments
Microplate Reader Mode 550	Biorad
Microscope Primo Vert	Zeiss
Microwave	Siemens
Millipore's Amicon Ultra-2 filters (100K)	Millipore corporation
Millipore 30000	GE Heathcare
Nanodrop ND 2000c	Peqlab
NanoVue 4282 V1.7.1 Spectrophotometer	GE Healthcare
Optima -80K Ultrazentrifuge	Beckman
PD-Desalting Column	GE Healthcare
Pipette	Abimed
Power supply Model200/2.0	Biorad
pH-Meter	inolab WTW
Precision balance AC 100	Mettler
Quartz cuvette Spectrophotometer Cell Micro	Biorad
Refractometer	Krüss Optronic
Refrigerator	Liebherr
Rotor JA 10	Beckman
Rotor SLC 6000	Thermo Scientific
Rotor SW28	Beckman
Rotor SW55 Ti	Beckman
Safety bench Laminar Air HLB 2448	Heraeus
Shaking incubator	Infors AG
Sample rotator	Fröbel
Thermocycler	Eppendorf
Thermomixer comfort	Eppendorf
Thermomixer compact	Eppendorf
Transilluminator	Bachofer
Ultra-centrifuge	Beckman
Ultra-temperature deep freeze (-80^{o}C)	Heraeus
Vertical Polyacrylamide Gel Electrophoresis	Biometra
Vortexer MS3 basic	IKA
Water bath 37^{o}C	Köttermann
Western blot developer machine Curix 60	Agfa

4.1.11 Kit systems

AmpliTaq Gold DNA polymerase	Applied Biosystems
DC Protein Assay	Biorad
Fugene 6/ HD / Extreme	Roche
Lumi-Light Western Blotting Substrate	Roche
PCR Mastermix	Promega
Protein G PLUS Agarose	Santa Cruz Biotechnologies
SYBRGreen I Master	Roche
QIAEX II Gel Extraction Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen

4.1.12 Laboratory consumables

6-well cell culture dish	Nunc
10 cm cell culture dish	Nunc
Cellstar tubes 15 ml and 50 ml	Greiner bio-one
Cryotubes	Nunc
Culture tube polypropylene round-bottom 14 ml	Falcon
Fitler papers	Whatman
Gel Tip 100 ART	Molecular Bio Products
Gene Pulser / *E. coli* Cuvette	Biorad
Gloves Gentle Skin Aloecare	Meditrade
Gloves Purple-Nitrile powder free	Kimtech
LightCycler 480 Multiwell Plate 96	Roche
LightCycler 480 Sealing Foil	Roche
Petri dishes	Greiner bio-one
Pipette tips 10 µl, 200 µl and 1000 µl Tip-One	SteriLab
Plastic pipettes 5 ml, 10 ml, 25 ml and 50 ml	Greiner bio-one
Plastic cuvettes	Braun
Quali-PCR tubes	Kisker
Silanized Glass Wool	PerkinElmer
Sterile filter (0.22 µm)	Millipore
Syringes	Becton-Dickinson
Trans-Blot Pure Nitrocellulose Membrane (0.45 µm)	Biorad
Tubes 1.5 and 2 ml	Eppendorf

4.1.13 DNA and protein markers

λ DNA/Eco911 Marker (*BstEII*) (Fig. 7A)	Fermentas
Gene Ruler 100 bp DNA Ladder (Fig. 7B)	Fermentas
Gene Ruler 10 bp DNA Ladder (Fig. 7C)	Invitrogen
Page Ruler Prestained Protein Ladder (Fig. 8A)	Fermentas
Page Ruler Plus Prestained Protein Ladder (Fig. 8B)	Fermentas

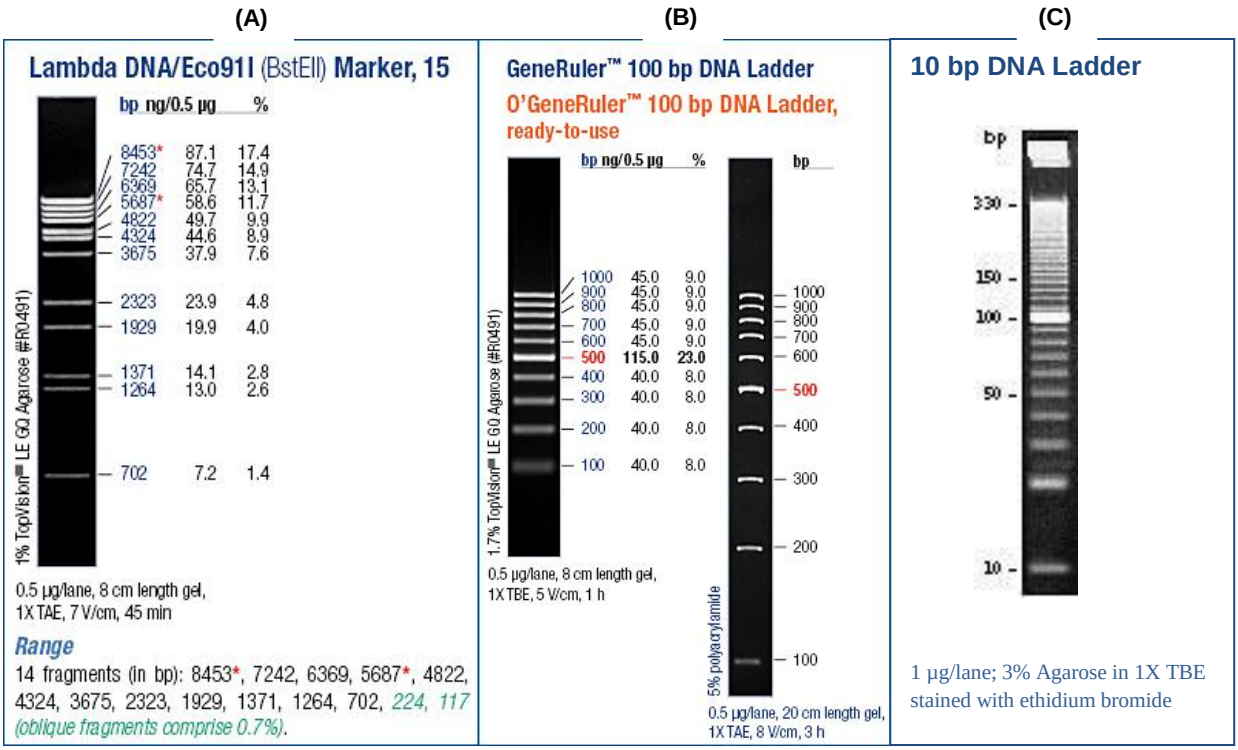


Fig. 7: DNA ladders (A) λ DNA/Eco911 Marker (*BstEII*), Fermentas (B) Gene Ruler 100 bp, Fermentas (C) Gene Ruler 10 bp, Invitrogen

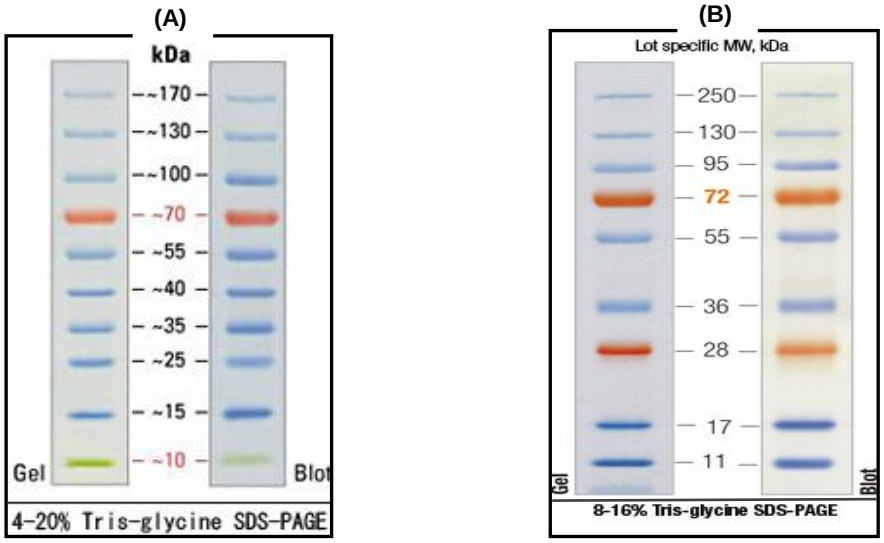


Fig. 8: Protein ladders (A) Page Ruler, Fermentas (B) Page Ruler Plus, Fermentas

4.1.14 Plasmids

Plasmid	***Purpose***	***Figure***
pETM13	Vector for HBV-WT and mutant capsid expression	figure 9
pBluescript II KS (+)	Vector for aptamers' sequencing	figure 10
pRVHBV $^{+}$	HBV-WT genome	figure 11
pSVHBV 1.1LE-	HBV nucleocapsid production in HuH7 cells	figure 12
pSVHBV1.1LE-I126A	HBV- I126A mutant capsid expression	figure 13
pSV45-57	HBV envelope protein production in HuH7 cells	figure 14
pSVBX24H	HBV small envelope protein production in HuH7 cells	figure 15

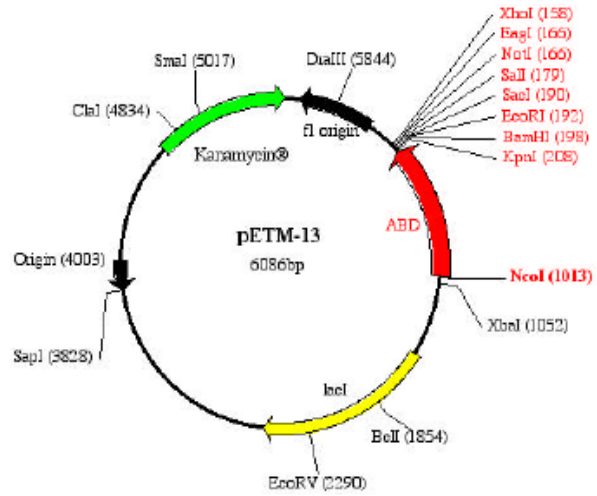


Fig. 9: pETM13 (EMBL)

Expression vector with Lac repressor (lacI), a canamycin resistant gene and the both replication origins (f1 origin, Origin). The actin binding domain (ABD) gene is a stuffer gene. It was replaced in the work by the sequence encoding the C-terminal deleted WT and I126A mutant HBV core protein.

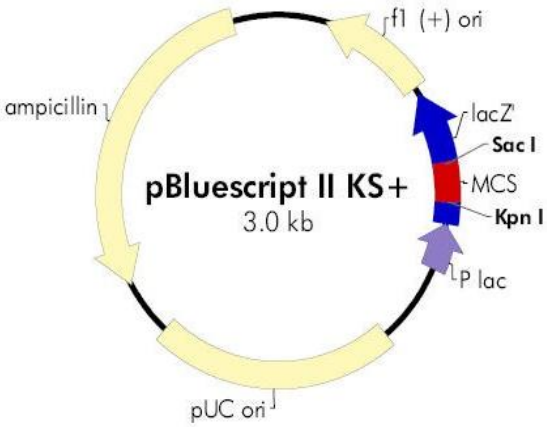


Fig. 10: pBluescript II KS (+)

The plasmid was used for aptamer sequencing and contains the f1 (+) origin of ss-DNA replication, β-galactosidase α-fragment coding sequence (lacZ'), pUC origin of replication and ampicillin resistance (bla) ORF.

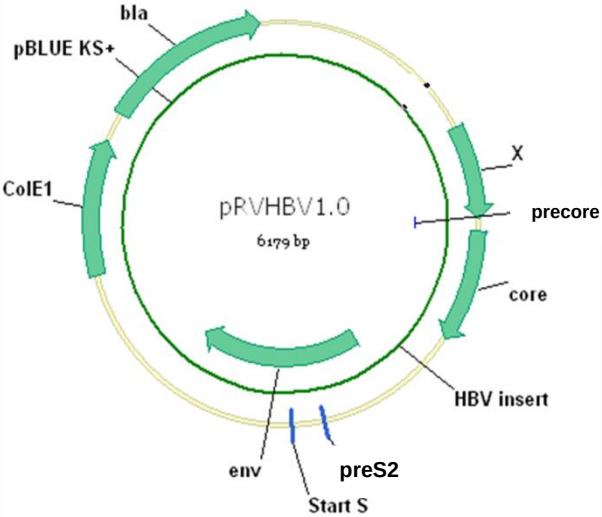


Fig. 11: Plasmid pRVHBV1.0 +

The plasmid contains the whole HBV genome.

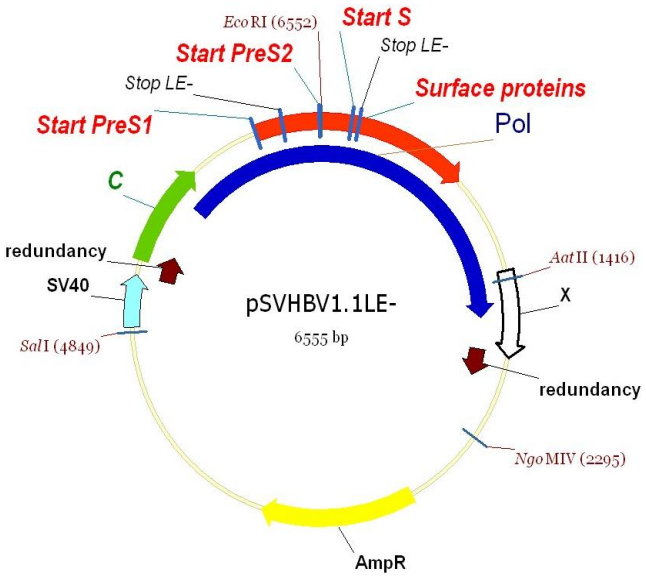


Fig. 12: pSVHBV1.1LE-

The plasmid was derived from plasmid pSVHBV1.5LE- and carries the entire HBV genome with 2 stop codons in the preS2 and S domains, respectively which leads to a non-functional envelope protein ORF (polymerase ORF not affected). After transfection of cells with this plasmid, only capsids and no virions are produced.

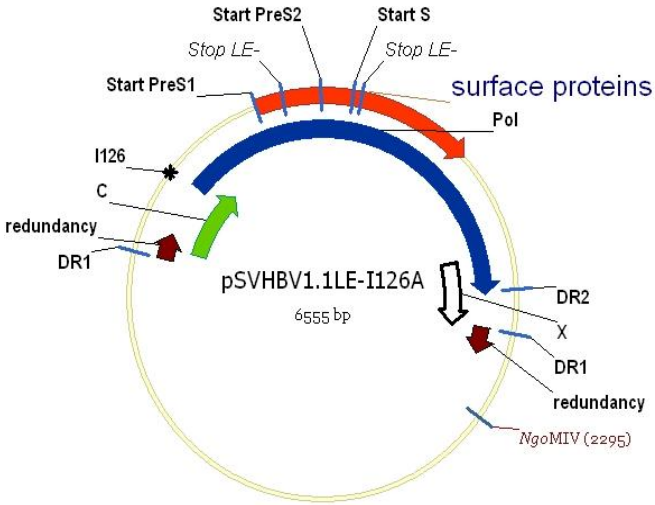


Fig. 13: pSVHBV1.1LE-I126A

The plasmid is the same like the plasmid pSVHBV1.1LE- except for 2 point mutations (A5608G and T5609C) changing codon 126 of the core gene and amino acid 126 of the core protein from isoleucine to alanine. Capsids with this point mutation can not be enveloped.

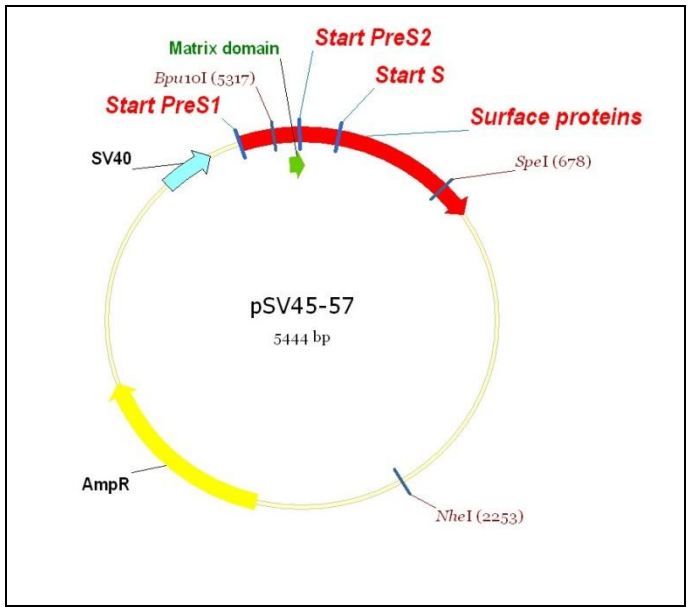


Fig. 14: pSV45-57

The plasmid codes for all three HBV surface proteins under control of a SV40 promoter but it lacks the first 30 codons for the large envelope protein. The shortened L sequence allows more efficient secretion of viral particles. This plasmid carries an ampicillin resistance gene.

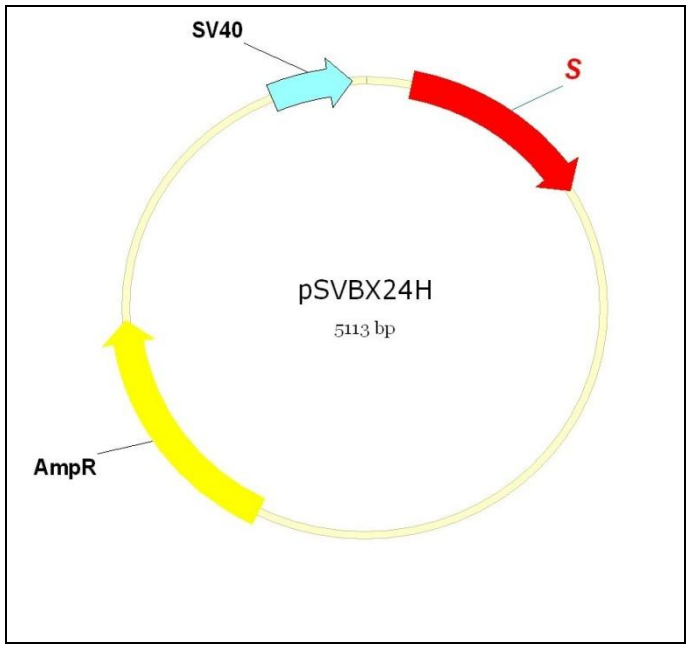


Fig. 15: pSVBX24H

The plasmid contains the ORF for the HBV small surface protein under control of the SV40 promoter. The vector backbone also carries an ampicillin resistance for selection in bacteria. It is used in combination with plasmid pSV45-57 and plasmid pSVHBV1.1LE- for production of virions upon transfection in HUH7 cells.

4.1.15 Solutions and Buffer systems

100x BSA	New England Biolabs
10x DNA-loading buffer	50 % Glycerin, 0.5% Bromphenolblau, 0.5% Xylencyanol
5x Loading buffer for SDS-PAGE	250 mM Tris HCl pH6,8, 10 % SDS, 7.5% Glycerin, 0.5 % Bromphenolblue, 1,6 M DTT
10x NEB-buffer 3	New England Biolabs
10x PBS	180 mM NaCl, 8.5 mM Na_2HPO_4, 2.2 mM KH_2PO_4
2x Proteinase K buffer	2% (w/v) SDS, 20 mM Tris-HCl, 20 mM EDTA pH 7.5
6x Protein loading buffer for	50 % Glycerine (v/v), 0.05 % Bromphenolblue (w/v),

native gels	0.05 % Xylenecyanol FF (w/v), 6x TAE-Puffer
10x SDS-running buffer	0.25M Tris, 1.92 M Glycine, 1% SDS
20x SSC-buffer	3 M NaCl, 0.3 sodium Citrate Dihydrate
50x TAE	2 M Tris HCl pH 6.8, 50 mM EDTA
1000x Trace elements	50 mM FeCl3, 20 mM CaCl2, 10 mM MnCl2, 10 mM ZnSO4, 2 mM CoCl2, 2 mM CuCl2, 2 mM NiCl2, 2 mM Na2MoO4, 2 mM Na2SeO3, 2 mM H3BO3
10 x Transfer buffer salts	28.4 g Tris, 144g Glycine into 1l H_2O
Blocking buffer	10% skim milk powder (w/v), 0.1% Tween 20 in 1x PBS
Cathode transfer buffer	Transfer buffer, 0.5% (w/v) SDS
Coomassie Brilliant Blue staining solution	0.25 g Coomassie Brilliant Blue (R-250), 90 ml methanol : H_2O (1:1); 10 ml glacial acetic acid
Coomassie Brilliant Blue destaining solution	90 ml methanol : H_2O (1:1), 10 ml glacial acetic acid
Diffusion buffer	0.5 M Amm.acetate, 10 mM Mg acetate, 1 mM EDTA, 0.1 % SDS
Gel drying solution	4% glycerol, 20% ethanol in H_2O
Li- Cl	Fluka
Lysis buffer	5 mM EDTA, 50 mM Tris HCl pH 8.0, 2 mg/ml Lysozyme
NaOH/SDS	440 µl 10M NaOH, 1.2 ml 20% SDS, 20.46 ml H_2O
Poncceau S	Sigma
Solution I (Plasmid preparation)	50 mM Glucose, 25 mM Tris HCl pH8, 10 mM EDTA
Solution II (Plasmid preparation)	0.2 NaOH, 1 % (w/v) SDS
Solution III (Plasmid preparation)	3 M Potassium, 5 M acetate pH 5.5
Solution IV (Plasmid preparation)	13 % (w/v) PEG 6000,1.6 M NaCl
Streptavidin buffer	10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA
T4 DNA Ligase buffer	Fermentas
TBS	50 mM Tris HCl pH 7.4, 150 mM NaCl
TE-buffer	10 mM Tris HCl pH 8.0, 1mM EDTA
TNE-buffer	10 mM Tris HCl pH 7.5, 10 mM NaCl, 20 mM EDTA
Trans-Blot Transfer-Medium	Biorad
Transfer buffer	20 % Methanol, 1x Transfer buffer salts
Washing buffer (PBS-T)	0.1% Tween 20 in PBS

4.1.16 Primers

VBAO01	5´ GCGGCCATGGACATTGACC ´3
VBAO02	5´ GCGGGTCGACTTATTAAACAACAGTAGTTTCC ´3
VBAO03	5´ CCGCCCATGGATGTG ´3
VBAO04	5´ GCGGGTCGACGTTTG ´3
VBAO05	5´ GGCGAGAAAGGAAGGGAAGAA ´3
VBBS8	5´ GCTGAGGCGGTGTCTAGGAGA ´3
VBBS18	5´ GGCATAAATTGGTCTGCGCACC ´3

4.1.17 Software

Ammoniumsulfate calculator	www.encorbio.com/protocols/AM-SO4.htm
ClustalW2	http://www.ebi.ac.uk/Tools/clustalw2/index.html
Mfold (version 3.2)	http://mfold.bioinfo.rpi.edu/ cgi-bin/dna-form1.cgi
Microplate Manager 4.0	Biorad
Microsoft Excel 2007	Microsoft
Oligo Calc	http://www.basic.northwestern.edu/biotools/oligocalc.html
Quantity One 4.1.1.	Biorad
Reverse Complement	http://www.bioinformatics.org/sms/rev_comp.html
SigmaPlot12.0	Sigma
Simple Reads Online	GE Healthcare
Unicorn 5.20	GE Healthcare
Vector NTI Advance 10	Invitrogen

4.2 Methods

4.2.1 DNA Technology

4.2.1.1 Conventional polymerase chain reaction (PCR)

The technique was developed in 1983 by Kary Mullis (Bartlett and Stirling, 2003). *Taq* polymerase was used in this work. All PCR reactions were carried out in a volume of 50 µl. Primers were used in a concentration of 1 µM. The annealing temperature was calculated by taking the mean value of the melting temperature of the primers and subtracting 5°C. Extension time was approximately 1 minute per 1000 bp.

4.2.1.1.1 PCR during HBV WT and mutant capsid expression

The sequences in the plasmids pRVHBV+ and pSVHBV 1.1LE- which encode for WT and I126A-mutant HBV capsids, respectively were amplified by PCR using specific primer set VBAO01 and VBAO02. This primer set allows the amplification of the sequences which encode for HBV capsids but with deleted 36 aa at the C-terminal end (Table 1 and 2).

Table 1: PCR reagents used during WT and mutant HBV capsids gene amplification:

Reagents	**Amounts**
pRVHBV^{+}/ pSVHBV 1.1LE-	10 pg
Primer VBAO 01 (50 pmol/µl)	1 µl
Primer VBAO 02 (50 pmol/µl)	1 µl
2x Roche Mastermix	25 µl (0.05 U *Taq*/µl)
H_2O	variable
Total volume	50 µl

Table 2: PCR thermal profile used during WT and mutant HBV capsids expression:

PCR steps	**Test**	**Number of cylcles**
Denaturation	94 °C 5 min	1
Denaturation	94 °C 30 sec	30
Anealing	54 °C 30 sec	
Elongation	72 °C 1 min	
Elongation	72 °C 7 min	1
Cooling	4 °C	

4.2.1.1.2 PCR during aptamer selection and sequencing

For the improvement of the random DNA library amplification and to reduce the formed single strand-double strand hybrids and the PCR by-products, higher concentration of *Taq* polymerase (0.1 U / reaction) was used as well as fewer PCR cycles (15 cycles) were performed (Table 3 and 4).

During the aptamer selection, reverse biotinylated primer (VBAO03) and forward non-biotinylated one (VBAO04) were used while for the molecular cloning of the selected aptamers for sequencing the same both primers were used but in a phosphorylated form.

Table 3: PCR reagents used during aptamer selection and sequencing:

Reagents	**Amounts**
Aptamers	10 ng
Primer VBAO 03 (50 pmol/µl)	1 µl
Primer VBAO 04 (50 pmol/µl)	1 µl
2x Promega Mastermix (0.05 U/µl)	25 µl
AmpliTaq Gold DNA Polymerase	1 µl (5 U)
H_2O	variable
Total volume	50 µl

Table 4: PCR thermal profile used during aptamer selection:

PCR steps	**Test**	**Number of cylcles**
Denaturation	95 °C 5 min	1
Denaturation	95 °C 20 sec	15
Anealing	51 °C 15 sec	
Elongation	72 °C 10 sec	
Elongation	72 °C 2 min	1
Cooling	4 °C	

4.2.1.2 Purification and concentration of DNA

4.2.1.2.1 Phenol-chloroform extraction

DNA can be purified efficiently from proteinaceous and fatty impurities by using phenol/ chloroform/ isoamyl alcohol mixture. The samples were mixed well with equal volumes of phenol/ chloroform/ isoamyl alcohol, then by centrifugation a biphasic mixture was formed where the fats collected in the lower organic phase and the DNA solved in the upper phase while the denatured proteins aggregated in the interphase. The upper phase containing the aimed DNA was transferred to another new tube. Then the same procedure was repeated three times, and finally the DNA was precipitated by ethanol.

4.2.1.2.2 Ethanol precipitation of DNA

The extracted, restricted or ligated DNA molecules in aqueous solutions were purified and/or concentrated by ethanol precipitation. The sample was mixed with 1/10 volume of 3 M sodium acetate pH 5.6 and two volumes of 96% ethanol and be frozen at -80^{o}C for one hour, then centrifuged at 13000 rpm for half an hour. The supernatant was discarded and the pellet washed once with 70% ethanol and air dried and finally resuspended with an adequate volume of DNase free water.

4.2.1.2.3 Purification of DNA solutions and PCR products

The QIAEX II Gel Extraction kit (Qiagen) was used to purify and concentrate DNA solutions and PCR products. According to the manufacturer instructions, three volumes (for DNA from 100 bp – 4 kb) or six volumes (for DNA ≤ 100 bp) of buffer QX1 were added to the sample. 10 µl from the pre-vortexed QIAEX II was added and the mixture incubated at room temperature for 10 min with intermittent vortexing every 2 min to keep QIAEX II in a suspension form. The mixture was centrifuged and then the pellet was washed twice using buffer PE. The pellet was air dried for 10 min and finally, the DNA was eluted by resuspending the pellet in 20 µl Tris.Cl, pH 8.5 and incubating it at room temperature for 5 min. beforecentrifugation. The elution step was repeated to improve the DNA recovery.

4.2.1.3 Gel electrophoresis

4.2.1.3.1 Agarose gel electrophoresis

In this work, 1% agarose gels were prepared for large DNA fragments. The gels were prepared with 1×TAE buffer and 0.5 µg/ml ethidium bromide (EtBr). The gels were run in 1×TAE buffer with a voltage of 8 V/cm. The gels were visualized under ultraviolet (UV) light.

4.2.1.3.2 Denaturing urea–polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) was used to separate and purify ssDNA where the denatured DNA migrates through these gels at a rate that is almost completely independent of its base composition and sequence because these gels polymerized in the presence of urea to suppress base pairing in nucleic acids. In this work, gels containing 6 M urea and 10% acrylamide were prepared (Table 5). The gels were run at 15 V/cm in 1×TBE buffer. The gels were stained by soaking in EtBr solution (1 µg/ml) for 30 min and visualized on an ultraviolet transilluminator.

Table 5: Contents of denaturing urea–polyacrylamide gel:

Acrylamid concentration	**10 %**
30% Acrylamid/bisacrylamide (29:1)	36 ml
10×TBE buffer	10 ml
Urea	36 g
H2O	Up to100 ml
APS (10%)	0.5 ml
TEMED	200 µl

4.2.1.4 Extraction of DNA from gels

4.2.1.4.1 Extraction from agarose gel

The QIAquick Gel Extraction Kit (Qiagen) was used to isolate and extract DNA from gels. Following the manufacturer instructions, three volumes of QG buffer were added to the weighted excised gel piece (300 µl QG buffer /100 mg gel) and incubated at 50^{o}C for 10 minutes with intermittent vortexing every 2-3 minutes till the gel was completely dissolved. Then one gel volume isopropanol was added with proper mixing. The mixture was applied onto a QIAquick column and centrifuged for one minute to allow the DNA binding to the column matrix. This was repeated twice. Then, for removing all gel traces, three steps of washing was done, one with 500 µl of buffer QG followed by two times with 0.75 ml of buffer PE. An additional centrifugation step was done to dry the column. Finally, the DNA was eluted by applying 30 µl water to the column before centrifugation. The elution step was repeated using the flow through of the first one to enhance DNA recovery.

4.2.1.4.2 Extraction from polyacrylamide gel

The QIAEX II Gel Extraction Kit (Qiagen) was used to isolate and purify aptamers from denaturing urea–polyacrylamide gel. Firstly, the excised gel was weighted and crushed into very small pieces using a sterile pestle, then two volumes of the diffusion buffer were added (200 µl diffusion buffer /100 mg gel). The mixture was incubated overnight at 37^{o}C in the thermomixer with shaking at 350 rpm. A centrifugation step at 10,000 rpm for 5 min at 4^{o}C was done and the supernatant was carefully aspirated and passed through packed, silanized glass wool to remove any residual polyacrylamide. The volume of the recovered supernatant was calculated, then the same steps of purification of DNA solutions and PCR products were done (see 4.2.1.2.3).

4.2.1.5 Streptavidin induced electrophoretic mobility shift for ssDNA preparation

Streptavidin induced electrophoretic mobility shift is an efficient and rapid method that allows the purification of ssDNA of uniform size from PCR products. This method exploits the remarkable stability of the biotin–streptavidin interaction under strongly denaturing conditions. The DNA was PCR amplified using a biotinylated primer, complementary to the target single stranded DNA (the forward strand), together with an unmodified primer. The PCR product was purified using the QIAEX II Gel Extraction kit to remove unincorporated primers and also to reduce the volume, and then the recovery was resuspended with an adequate amount of streptavidin buffer to be incubated with streptavidin at room temperature for 30 min (1:4 molar ratio of biotinylated primer to streptavidin). The binding mix is then denatured and electrophoresed on a 10% polyacrylamide, 6 M urea gel (Fig. 16). The free ssDNA (non-biotinylated lighter, lower band) is then purified by passive elution from the crushed acrylamide gel together with the QIAEX II kit as in 4.2.1.4.2.

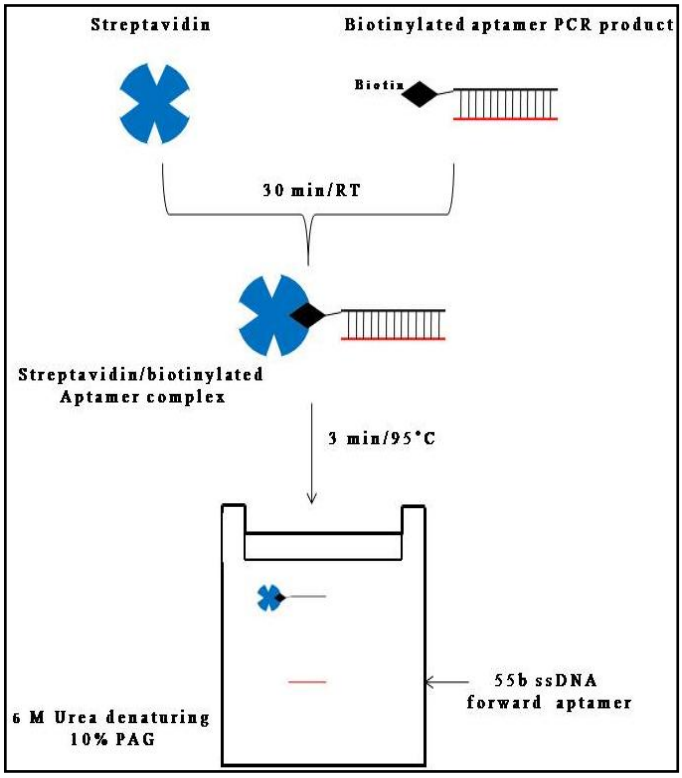


Fig. 16: Streptavidin induced electrophoretic mobility shift for ssDNA preparation

4.2.1.6 Determination of DNA concentrations

The absorbance at wavelength 260 nm (A260) can be used for determination of nucleic acid concentrations in solutions. The concentration to absorbance relation can be defined by the

Lambert–Beer Law, OD = e * c * d. The optical density (OD) is the product of the substance specific extinction coefficient (e), the concentration of the absorbing sample (c), and the optical path length in cm (d). The absorbance to concentration conversion are 1 OD260= 50 µg/ml for dsDNA, 1 OD260= 33 µg/ml for ssDNA and 1 OD260= 40 µg/ml for RNA. The absorbance at wavelength 280 nm (A 280) was used to measure protein concentration. A ratio of A260/A280=2 supposed to be the value for pure DNA.

In this work, a nanodrop machine was used for determining DNA concentrations where 2 µl of the undiluted sample were measured two times. A photometer was also used for determining DNA concentration where three measurements were made for 100-fold diluted sample.

4.2.1.7 DNA Cloning

4.2.1.7.1 DNA restriction

DNA can be cut into desired fragments using specific restriction enzymes. These specific restriction enzymes are chosen to cut the DNA at a specific palindromic site. Depending on the enzyme used, the cut DNA either has blunt or overhanging ends (sticky ends).

4.2.1.7.1.1 Single digestion and dephosphorylation of the plasmid DNA

For molecular cloning of a DNA molecule the vector is cut using restriction enzymes. The vector is after dephospohrylated by using alkaline phosphatase to prevent vector self-ligation. Finally, the target DNA molecule was amplified by PCR using 5′ phosphorylated primers so that the product can be ligated to the dephosphorylated, linearized vector.

During the aptamer cloning, the vector was singly cut by the restriction enzyme *EcoRV* and dephosphorylated simultaneously by using Calf Intestinal Phosphatase (*CIP*). The mixture (Table 6) was incubated at 37^{o}C overnight and finally the restricted dephosphorylated vector was purified using the QIAEX II kit as described in 4.2.1.2.3 to be ready for the ligation step.

Table 6: DNA restriction scheme during aptamer cloning:

Reagents	**Amounts**
Vector	4 µg
EcoRV (10 U/µl)	5 µl
CIP	1 µl
10x NEB-Buffer 3	5 µl
10x BSA	5 µl
H2O	30 µl
Total volume	50 µl

4.2.1.7.1.2 Double digestion of DNA

Another approach for molecular cloning of a DNA molecule is to doubly cut the vector using two restriction enzymes which cut the DNA leaving sticky ends (overhangs) that are complementary to the other sticky ends of the restricted DNA molecule for insertion.

During the construction of expression vector for WT and mutant HBV capsids, the vector and the DNA sequences that encode for the capsids were doubly cut by the restriction enzymes *NcoI* and *SalI*. The mixture (Table 7) was incubated at 37^{o}C for 2 hours and then the restricted DNA was purified by the QIAquick Gel Extraction kit as described in 4.2.1.4.1 to be ready for the ligation step.

Table 7: DNA restriction scheme during WT and mutant HBV capsids preparation:

Reagents	**Vector**	**Insert**
DNA	0.1 µg	3 µg
SalI (20 U/µl)	0.5 µl	1 µl
NcoI (10 U/µl)	1.0 µl	2 µl
10x NEB-Buffer 3	2.0 µl	5 µl
10x BSA	2.0 µl	5 µl
H2O	variable	variable
Total volume	20 µl	50 µl

4.2.1.7.2 DNA Ligation

The *in vitro* ligation between the DNA molecule to be inserted into the vector and the vector was carried out by T4 DNA ligase which builds phosphodiester bonds between 3'and 5′ ends. The mixture (Table 8) was incubated at 16 ^{o}C overnight and then the ligated DNA was purified by ethanol precipitation as described in 4.2.1.2.2 prior to the bacterial transformation.

Table 8: DNA insert/vector ligation scheme during WT and mutant HBV capsids preparation:

Reagents	**Amounts**
Vector	3 µl
Insert	8 µl
T4 DNA Ligase	1 µl
10x T4 DNA Ligase Buffer	2 µl
H2O	7 µl
Total volume	20 µl

4.2.1.7.3 Transformation of bacteria with ligated DNA

The introduction of exogenous DNA molecules into bacterial cells requires an artificial competence of these cells to make them passively permeable to DNA. This can be done by exposing them to conditions that do not normally occur in nature. In this work, two types of competent cells were used, electrocompetent and thermocompetent cells.

4.2.1.7.3.1 Preparation of electrocompetent bacteria

The electrocompetent *E. coli* DH10B bacterial cells were produced by inoculating 1.5 ml of a dense DH10B culture into 250 ml of LB medium free from any antibiotics and by incubation at 37^{o}C overnight. Once the OD of the culture reached 0.6-1 at 578 nm, the cells were harvested as this means that the bacterial growth reached the logarithmic phase. The harvested bacteria were then kept on ice for 20 min and subsequently centrifuged at 5000 rpm for 15 min at 4^{o}C to be washed twice with 250 ml of ice-cold water and finally once with 10 ml of ice-cold, sterile 10% glycerol. The centrifugation during the washing steps was done at 5000 rpm for 12 min at 4^{o}C. Finally the cell pellets were resuspended in 1.5 ml of ice-cold and sterile 10% glycerol and divided into aliquots of 55 μl and then stored at -80^{o}C.

4.2.1.7.3.2 Transformation of electrocompetent cells

The frozen electrocompetent *E. coli* DH10B cells were thawed on ice and only 25 μl of thawed cells were mixed with 1 μl prediluted DNA (1/10000 – 1/100000 dilution of large scale plasmid preparation or 1/5 dilution of DNA samples from ligation). The mixture was loaded into a ice precooled 0.1 cm pulser cuvette (Biorad) and electroporated at 1.8 kV then immediately 1 ml of 37^{o}C prewarmed LB was added on them. The mixture was transferred into a 1 ml Eppendorf tube and shaken in a thermomixer at 450 rpm and 37^{o}C for 30 min. The cells were collected by centrifugation at 3000 rpm for 5 min, the supernatant was removed and then the cells were resupended into 50 μl LB to be streaked out on agar plates containing the proper antibiotic (Canamycin 30 mg/ml or Ampicillin 100 μg/ml). Finally, the plates were incubated overnight at 37^{o}C.

4.2.1.7.3.3 Transformation of thermocompetent cells

The frozen thermocompetent *E. coli* BL21 Star (DE3) pRARE2 cells were thawed on ice and only 100 μl of thawed cells were mixed with 1 μl prediluted DNA. Then the mixture was incubated on ice for 30 min. The cells were heat shocked by heating them at 42^{o}C for 45 seconds. The heat shocked cells were kept on ice for 2 minutes to allow the closure of the

cellular pores. Finally, 4-volumes of prewarmed LB were added and the mixture incubated at 37^{o}C for 1 hour. The cells streaked out on agar plates containing Canamycin 30 mg /ml and then the plates were incubated overnight at 37^{o}C.

4.2.1.7.4 Plasmid preparation

4.2.1.7.4.1 Low scale preparation (Miniprep)

A single colony containing the desired plasmid from transformed *E. coli* bacteria was subcultured in 4 ml of LB containing the appropriate antibiotic (Canamycin 30 mg/ml or Ampicillin 100 µg/ml) and incubated overnight at 37^{o}C with shaking at 260 rpm. Then 3 ml were pelleted by centrifugation at 9000 rpm for 3 min. The cells were taken up in 100 µl of solution I, lysed by adding 200 µl of freshly prepared solution II with gentle shaking and incubation at room temperature for 5 min. After that 150 µl of solution III were added to the mixture and cooled on ice for 5 min to allow the neutralization of the pH. The mixture was centrifuged at 13000 rpm for 10 min at 4^{o}C and then the supernatant was transferred to new eppendorf tube to be centrifuged once more to assure the removal of all cellular debris and proteinaceous wastes. Finally, the plasmid DNA was concentrated by ethanol precipitation and the pellet was resuspended into 30 µl of water containing 0.5 µl of RNase A. The prepared plasmid was stored at -20^{o}C.

4.2.1.7.4.2 Medium scale preparation (Midiprep)

This was done by using the Qiagen Plasmid Midi kit. A single colony containing the desired plasmid was inoculated into 100 ml of LB medium with 100 µg/ml ampicillin and incubated overnight at 37^{o}C with shaking at 260 rpm. The DNA was isolated and purified according to the manufacturer's protocol. At the end the DNA pellet was dissolved in 100 µl of water.

4.2.1.7.4.3 Large scale preparation (Maxiprep)

A single colony containing the desired plasmid was subcultured in 30 ml of TB medium containing the appropriate antibiotic (canamycin 30 mg/ml or ampicillin 100 µg/ml) and incubated overnight at 37 ^{o}C with shaking at 260 rpm. The cells were collected by centrifugation at 4000 rpm for 15 min at 4^{o}C. The sedimented cells were resuspended in 5 ml of cold solution I. Then 10 ml of freshly prepared solution II was added with gentle mixing and the mixture left at room temperature for a period not exceeding 5 min to allow cell lysis without DNA damage by the high pH of solution II. The high pH was neutralized by adding 7.5 ml of solution III with cooling the mixture on ice for 5 min. Then 2 centrifugation steps

were performed at 4000 rpm for 10 min at 4°C to remove all the cellular debris from the supernatant which contains the plasmid DNA. The DNA was precipitated by adding 0.6 volumes (13.5 ml) of isopropanol on the supernatant, mixing and incubation for 3 minutes at room temperature. After that the solution was centrifuged at 4000 rpm for 10 min at 4°C. The pellet was dried on air and resuspended in 3 ml of TE buffer. 3 ml of -20°C precooled 5 M LiCl were mixed with the resuspended pellet and centrifuged at 4000 rpm for 15 min to precipitate the RNA. The DNA in the supernatant was precipitated with an equal volume of isopropanol at 4000 rpm for 15 min. After air drying of the pellet, it was dissolved in 500 µl of TE buffer containing 1 µl of RNase and left at room temperature for half an hour to assure the complete digestion of RNA in the sample. Then 500 µl of solution IV was mixed with the mixture and a centrifugation step at 13000 rpm for 15 min was done. The pellet was air dried, then dissolved in 400 µl of TE buffer. The solution was extracted by phenol choloroform and ethanol precipitated (see above). Finally, the desired plasmid DNA pellet was dissolved in 100-300 µl of water and preserved at -20°C.

4.2.1.8 DNA sequencing

DNA sequencing was performed by the company GATC Biotech. The results were analyzed by the help of computer programs, Vector NTI (Invitrogen) for identifying the plasmids' genetic maps, ClustalW2 of the European Molecular Biology laboratory – European Bioinformatics Institute (EMBL-EBI; http://www.ebi.ac.uk/Tools/clustalw2/index.html) for aligning the sequenced DNA and Reverse Complement (http://www.bioinformatics.org/sms/rev_comp.html) for converting the sequences to the complementary sequence.

4.2.1.9 DNA quantification by specific Real-Time PCR

HBV viral genomes isolated from virions or the selected aptamer were quantified by quantitative PCR (qPCR). The dye used was SYBR Green which binds unspecifically to double stranded but not the single stranded DNA.

4.2.1.9.1 qPCR standards

For the quantification of HBV viral genomes isolated from virions, five 10-fold serial dilutions (10^2-10^6) of plasmid pSVHBV1.5LE- were used as standard because the qPCR primer set (VBBS8 and VBBS18) produces the same 222 bp DNA fragment as the HBV genome. These plasmid dilutions were made after adjusting the plasmid concentration using spectrophotometer and according to the plasmid molecular weight which was calculated using

a oligonucleotide properties calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html). During the aptamer selection process, six 10-fold serial dilutions (10^2-10^7) of the ordered random DNA aptamer library (PURIMEX) was used as a standard while during the evaluation of the selected aptamers, six 10-fold serial dilution (10^2-10^7) of one ordered selected aptamer (AO-01) was used.

4.2.1.9.2 qPCR setup and program

All qPCR reactions were carried out in a volume of 20 µl. Primers were used in a concentration of 1 µM. The reactions were carried out in 96-well plates which were sealed with adhesive foil (Roche). The qPCR reagents were used in concentrations according to the manufacturer instructions (Table 9). To run and analyze the qPCR, the Roche LightCycler 480 II and its software release 1.5.0 SP4 (version 1.5.0.39) were used. In this work, the "SYBR Green I 96-II" program was used with minor modifications (Table 10).

Table 9: qPCR reagents used during the quantification of HBV genomes or selected aptamer:

Reagents	Amounts
DNA diluted in Roche PCR grade water	9.6 µl
Primer VBBS8 (100 pmol/µl) or Primer VBAO 03 (100 pmol/µl)	0.2 µl
Primer VBBS18 (100 pmol/µl) or Primer VBAO 04 (100 pmol/µl)	0.2 µl
Roche SYBR Green I Master	10 µl
Total volume	20 µl

Table 10: qPCR thermal profile used during the quantification of HBV genomes or selected aptamer:

PCR steps	HBV genome	Aptamers	Cylcles
Denaturation	95 °C 5 min	95 °C 5 min	1
Denaturation	95 °C 20 sec	95 °C 20 sec	30
Anealing	60 °C 10 sec	51 °C 15 sec	
Elongation	72 °C 10 sec	72 °C 10 sec	
Melting curve of PCR products			

4.2.2 Protein Technology

4.2.2.1 Protein expression

The HBV wild type and mutant (I126A) capsids were over-expressed into *E. coli* BL21 Star (DE3) pRARE2 cells. Firstly, a single colony from the transformed cells was subcultured into 5 ml LB-Medium containing antibiotics (Canamycin 30 mg/ml, Chloramphenicol 33 mg/ml) and incubated overnight at 37^{o}C with shaking at 220 rpm. In the next day, the cultured cells were transferred to 500 ml 2XYT medium with antibiotics (Canamycin 30 mg/ml, Chloramphenicol 33 mg/ml) and incubated at 37 °C with shaking at 220 rpm with intermittent checking (every 1-2 hours) of the OD using a spectrophotometer. When the bacterial culture OD reached 0.7-1 it was cooled down to 20 °C, then 200 µM isopropyl-β-D-thiogalactopyranoside (IPTG) were added to induce the protein expression and finally the cell culture was incubated overnight at 20 °C with shaking at 220 rpm.

Finally, the cultured cells were centrifuged at 6000 rpm for 20 minutes at 4°C (Thermo Scientific Rotor SCL 6000). The sedimented cells were dissolved in an adequete amount of medium and centrifuged again but at 14000 rpm for 15 min at 4°C. Then the precipitated cells were preserved at -80°C.

4.2.2.2 Protein Purification

4.2.2.2.1 Cell lysis

The bacterial pellet was dissolved in 20 ml of lysis buffer then it was incubated on ice for 30 min and subjected to 4 steps of repeated freezing (at -80°C) and thawing (at 37°C in a water bath). The lysed cells were left on ice and 1 ml of DNase/$MgCl_2$ solution (200 µg DNase in 0.1 M $MgCl_2$) was added and the mixture was incubated at room temperature for 15 min to allow the destruction of cellular DNA. Finally, the cell lysate was centrifuged at 15000 rpm for 10 min at 4 ^{o}C (Thermo Scientific Rotor SS34) and the supernatant which contained the capsids was preserved at -20^{o}C.

4.2.2.2.2 Protein precipitation

Proteins can be extracted from a solution by ammonium sulphate as ammonium sulphate can change the interaction between the ionizing groups of amino acids and the H_2O molecules leading to aggregation of proteins which then can be separated easily by centrifugation.

In this work, 50% ammonium sulphate was used for precipitating the HBV capsids. The amount of the ammonium sulphate was determined by using a software, Ammonium Sulphate Calculator, (www.encorbio.com/protocols/AM-SO4.htm). The calculated amount of ammonium sulphate was added gradually to the protein solution with gentle mixing using a magnetic stirrer in a cold room at 4^{o}C and the mixture left for additional 30 min at 4^{o}C with continuous shaking. Finally, the protein was precipitated by centrifugation at 19000 rpm for 30 min at 4^{o}C (Rotor SS34) and then the pellet was resuspended in 10 ml of TBS/0.1 %NP-40.

4.2.2.2.3 Protein concentration

The protein was concentrated by using a concentrator (Millipore 30000) with a cut off of 30 KDa. The protein solution was loaded into the concentrator and then centrifuged at 4000 rpm for 20 min at 4 °C. Finally, the concentrated protein was preserved at -80^{o}C.

4.2.2.2.4 Chromatography

4.2.2.2.4.1 Sephacryl S-500 HR column

For the purification of large molecules, Hiprep 26/60 Sephacryl S-500 HR (GE Healthcare) columns can be used (size exclusion chromatography). The used column was connected to the Akta Purifier apparatus (GE Healthcare), and the purification conditions were adjusted and controlled using the software Unicorn 5.20 (GE Healthcare).

The sample was loaded into the TBS-preequilibrated column and then it was pumped automatically by the pump (P-900) in a rate of 2.6 ml/min. Throughout the sample partitioning, the absorption of the elutes were measured at wavelengths 254 and 280 nm (UV-900). Furthermore, the conductivity of the sample was measured (C-900). Finally, the elutes were collected in fractions, each of 5 ml. The elutes showing high absorbance at wavelength 280 nm were pooled. The final volume was 100 ml. The sample was preserved at 4^{o}C.

4.2.2.2.4.2 PD-10 desalting column

The purification, desalting or even changing the buffer of a sample can be done by using PD-10 columns (GE Healthcare). The cut off of this column is 5 KDa. The procedure was done according to the manufacturer protocol (Gravity protocol).

4.2.2.2.5 Sucrose gradient ultracentrifugation

One method for virus purification is sucrose gradient ultracentrifugation. In this work, six different sucrose fractions were prepared (10 %, 20 %, 30 %, 40 %, 50 %, 60 % (w/v)) with TBS buffer pH 7.4 and then 6 ml from each fraction were loaded slowly into ultraclean centrifugation tube (Beckman) starting with the highest concentration. The sample (2.5 ml) was loaded on the top of the gradient. The centrifugation was done in a SW28 rotor at 10 °C and 25000 rpm for 24 h. Finally, the fractions were collected carefully from the top; each fraction was 1 ml in volume. They were preserved at 4^{o}C.

4.2.2.3 Protein detection

4.2.2.3.1 SDS-PAGE

Sodium Dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) is a technique to separate proteins according to their electrophoretic mobility. SDS, an ionic detergent, which denatures secondary and non–disulfide–linked tertiary structures, applies a negative charge to each protein in proportion to its mass. So SDS–protein complexes can migrate through the gel in accordance to the size of the protein.

In this work, a gel composed of 15% separating gel and 5% stacking gel was used (Table 11). The samples were mixed with 5x SDS-loading buffer with DTT (0.125 g DTT/500 µl) and heated at 95 °C for 5 min before being loaded into the gel to allow the protein disintegration into its monomer structure. Furthermore, the SDS ensures that the proteins is uniformly charged negatively and thus can be separated by means of gel electrophoresis. The predenatured protein samples and 10 µl of the protein marker „Page Ruler or Page Ruler Plus prestained Protein Ladder“ (Fermentas) were loaded into the prepared gel and then run in 1x SDS running buffer at 200 V for 2 h. Then it was stained by coomassie brilliant blue staining solution or run at 30 V overnight when it was electro-blotted to be detected by the HBV anti-core antibody.

Table 11: Reagents for15 % separating and 5 % stacking SDS gels

Reagents	**Separating 15 %**	**Stacking 5 %**
1.5 M Tris HCl buffer pH 8.8	2.5 ml	-
1.0 M Tris HCl buffer pH 6.8	-	0.63 ml
10 % SDS buffer	0.1 ml	0.05 ml
30 % Acrylamide Mix	5 ml	0.83 ml
10 % Ammonium persulphate	0.1 ml	0.05 ml
TEMED	0.004 ml	0.005 ml
H_2O	2.3 ml	3.4 ml
Total volume	10 ml	5 ml

4.2.2.3.2 Agarose gel electrophoresis

The separation of the native HBV capsids was done by electrophoresis in 1 % agarose gels. The samples were diluted in TNE-buffer pH 7.5 and mixed with 6x native protein loading buffer without any reducing agent to keep the capsids intact without any disintegration. The samples, the positive control and the marker were loaded into the gel and run at 65 V for 2 h. Finally, the loaded samples were capillary blotted (see 4.2.2.3.3) and detected using an HBV anti-core antibody (see 4.2.2.3.5).

4.2.2.3.3 Capillary blotting

The protein samples on agarose gels were transferred to nitrocellulose membranes by capillary blotting which depends on the diffusion of the protein particles within the buffer flow from the gel to the nitrocellulose membranes which have high protein-binding affinity.
In this work, a long 3 mm Whatman blotting paper (Schleicher und Schüller) was cut to the gel width and immersed in 10X SSC buffer and then left on a glass plate while its both ends still immersed in the buffer to allow the continuous buffer flow through the paper. In the middle of this paper, 5 small 10X SSC prewetted Whatman blotting papers were located and over them the gel was laid but in an inverted position. A nitrocellulose membrane (0.45 µm) was wetted with water, laid over the gel and on it the lanes were marked using a pen. Over the membrane additional 5 small 10X SSC prewetted Whatman blotting papers were placed and the air bubbles among the different layers were removed by rolling them out with a pipette. To avoid the loss of the buffer on the sides, long pieces of parafilm were placed on the glass plate and around the layers. Finally, a stack of dry papers was put on the top. To facilitate the buffer flow through the layers a 1 kg weight was put on the top and then the whole layers were left overnight at room temperature. In the next day, the blotted membrane was carefully removed and incubated in the blocking buffer overnight at 4^{o}C.

4.2.2.3.4 Electro blotting

The electroblotting is a simple and fast method used to transfer proteins from polyacrylamide gel to a nitrocellulose membrane depending on the electrophoretic properties of the protein.
In this work, the semi dry electroblotting was done using the Fastblot Semi-Dry Electrophoretic Transfer Apparatus from Biometra. Firstly, the SDS-gel containing samples and the nitrocellulose membrane (0.45 µm) were immersed in an adequate amount of transfer buffer and left at room temperature for 10 min. 5 transfer buffer prewetted Whatman blotting papers were put onto the anode of the electroblot machine and over them the nitrocellulose

membrane then the gel and finally additional 5 cathode buffer prewetted Whatman blotting papers were put on the top. The air bubbles between the different layers were removed by rolling them out with a pipette. The lid was closed and a 1 kg weight was put on the top of the lid. Then an electric current of 350 mA was applied for 1 h. Finally, the blotted membrane was carefully removed and incubated in the blocking buffer overnight at 4^{o}C.

4.2.2.3.5 Immunostaining

The blotted nitrocellulose membrane was transferred to a hybridization tube. Its upper surface holding the proteins was exposed to the inside of the tube to make the proteins accessible for the antibodies. The primary H800 rabbit anti-HBc-antibody (1:10000 in blocking buffer) was added to the tube containing the membrane and an incubation step was done in the hybridization oven at 25^{o}C for 2 h. The membrane was washed then 3 times by incubation with washing buffer (PBS-T) for 15 min at RT and with shaking to remove the non-bounded antibodies. The washed membrane was reintroduced again into the hybridization tube and incubated with the secondary antibody (goat anti-rabbit-horseradish-peroxidase coupled antibody) in the hybridization oven at 25^{o}C for 1.5 h. Three washing steps were done by incubating the membrane twice with washing buffer and once with PBS for 15 min at RT and with shaking to remove the free non bounded antibodies which may give false positive results. The detection of the blotted proteins was done by placing the membrane on a transparent foil. Then an adequate amount of freshly prepared Luminol Enhancer/Substrate Mixture (1:1 ratio, Roche) was added. Then the membrane was covered by another transparent foil and incubated in a dark place for 2 min to allow the action of the peroxidase enzyme on the substrate. Finally, the excess of the substrate and the air bubbles were squeezed out and the membrane was exposed to a film in a film cassette. The film was developed in the Curix 60 developer machine (Agfa) after different exposure time.

4.2.2.3.6 Coomassie staining

Proteins in SDS-polyacrylamide gels can be stained non-specifically using Coomassie Brilliant Blue stain.

In this work, Coomassie Brilliant Blue (R-250) was used. The gel containing the denatured samples was immersed in 5 volumes of Coomassie Brilliant Blue staining solution and left at RT for 4 h with gentle shaking. The stained gel was then removed and incubated into an adequate volume of destaining solution at RT for 4-8 h with gentle shaking and with intermittent changing of the destaining solution with a new one. When the gel background

appeared clear a photo was taken. The gel was dried by immersing it in an adequate amount of gel drying solution for a few minutes. Then it was placed between 2 warm water prewetted cellophane papers. Finally, the air bubbles were squeezed out to avoid gel crashing and then the cellophane papers containing the gel were fixed into the gel drying frame (24×24 cm) which left in a vertical position for a few days till the gel was completely dry.

4.2.2.4 Determination of protein concentration

The DC-protein assay (Biorad) was used for the determination of the amount of expressed HBV capsids. The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. As with the Lowry assay, there are two steps which lead to colour development: The reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein. Colour development is primarily due to the amino acids tyrosine and tryptophan, and to a lesser extent, cystine, cysteine, and histidine. Proteins effect a reduction of the Folin reagent by loss of 1, 2, or 3 oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue colour with maximum absorbance at 750 nm and minimum absorbance at 405 nm.

In this work, the standard to which the protein samples were calibrated was BSA. The procedure of the test was done according to the manufacturer Microplate Assay protocol. The OD of the samples was measured in a photometer at wavelength 655 nm and by using „Microplate Manager 4.0“(Biorad) software.

4.2.3 In vitro Selection of aptamers

4.2.3.1 Filteration partition method

Amicon Ultra-2 mL Centrifugal Filters (100 K) were used to separate the aptamer–HBV capsid complexes from the unbound ssDNAs. The basis of the methodology is the fact that HBV capsids (WT and mutant) have a much high molecular weight (~4 MDa) than aptamer (~17 KDa). So, the filtration using these filters with a cut off of 100 KDa will lead to the separation between the aptamer-capsids complex and free non bounded aptamers. To reduce the non–specific adsorption of nucleic acids to the filters, the filters were pre–treated with alkali as described (McENtee et al. 1980). The filters were filled with and soaked in 0.5 M KOH at RT for 20 min, then washed extensively with distilled H_2O. The filters were filled

with and immersed in binding buffer (PBS) and left at RT for 45 min with gentle shaking and finally the old buffer was replaced by a fresh one. The filters were stored at 4°C.

4.2.3.2 Selection procedure

The selection method used in this work was SELEX with counter selection. The target molecule of *in vitro* selection was HBV WT capsids while the counter target was HBV- I126A mutant capsids. The binding buffer was phosphate buffered saline (PBS), pH 7.0. A ssDNA library (10 nmol, about 6.0×10^{15} molecules) was used. Thirteen rounds of consecutive positive and negative selections were made (Fig. 3). To induce a sort of selective pressure, different concentrations of aptamers, WT capsids and mutant capsids were used throughout the different rounds of selection as well as different incubation times and different volumes of PBS during washing steps were used (Table 12).

4.2.3.2.1 Snap cooling and pre-selection of aptamers

To allow the aptamers to be folded into their 3 dimensional structures, the DNA library was dissolved in 100 µl binding buffer (PBS, pH 7), heated to 85°C for 15 min and immediately placed on ice for 15 min and then finally equilibrated at RT for 15 min. The renatured DNA was filtrated through the alkali pre-treated filter in the absence of target or counter target molecules to remove the matrix binders.

4.2.3.2.2 Positive selection of aptamers

The pre-selected aptamers were incubated with the target molecule (HBV-WT capsids) at RT. PBS were added to the aptamers/WT capsids mixture. After incubation the mixture was loaded onto an alkali pre-treated filter and centrifuged in a swinging bucket rotor at 4000 xg for 30 min at 25^{o}C. The retained material (aptamers/WT capsids complex) was collected by inverting the filter and centrifugation at 1000 xg for 5 min at 25^{o}C. The bounded aptamers were extracted by phenol/chloroform extraction (see 4.2.1.2.1) and then precipitated by using the QIAEX II Kit (see 4.2.1.2.3). The precipitated aptamers were resuspended in water and amplified by standard PCR using a forward non-biotinylated primer and a reverse biotinylated one (see 4.2.1.1.2). The PCR product was purified by the QIAEX II kit. Then it was mixed with streptavidin inducing an electrophoretic mobility shift for the preparation of ssDNA molecules (see 4.2.1.5). Three consecutive positive selection steps were done in the beginning of the selection process to reveal higher concentrations of the capsid best binders for the negative selection.

4.2.3.2.3 Negative selection of aptamers

The positively selected aptamers were incubated with the counter target molecule (HBV-I126A mutant capsids) at RT. PBS were added to the aptamers/mutant capsids mixture. After incubation, the mixture was loaded into an alkali pre-treated filter and centrifuged in swinging bucket rotor at 4000 xg for 30 min at 25^{o}C. The non bounded aptamers which came into the flow through were directly precipitated using a QIAEX II kit after that amplified by PCR with reverse biotinylated primer. The PCR product was purified by the QIAEX II kit. Then it was subjected to streptavidin induced electrophoretic mobility shift to prepare ssDNA molecules to be used for the next round of selection.

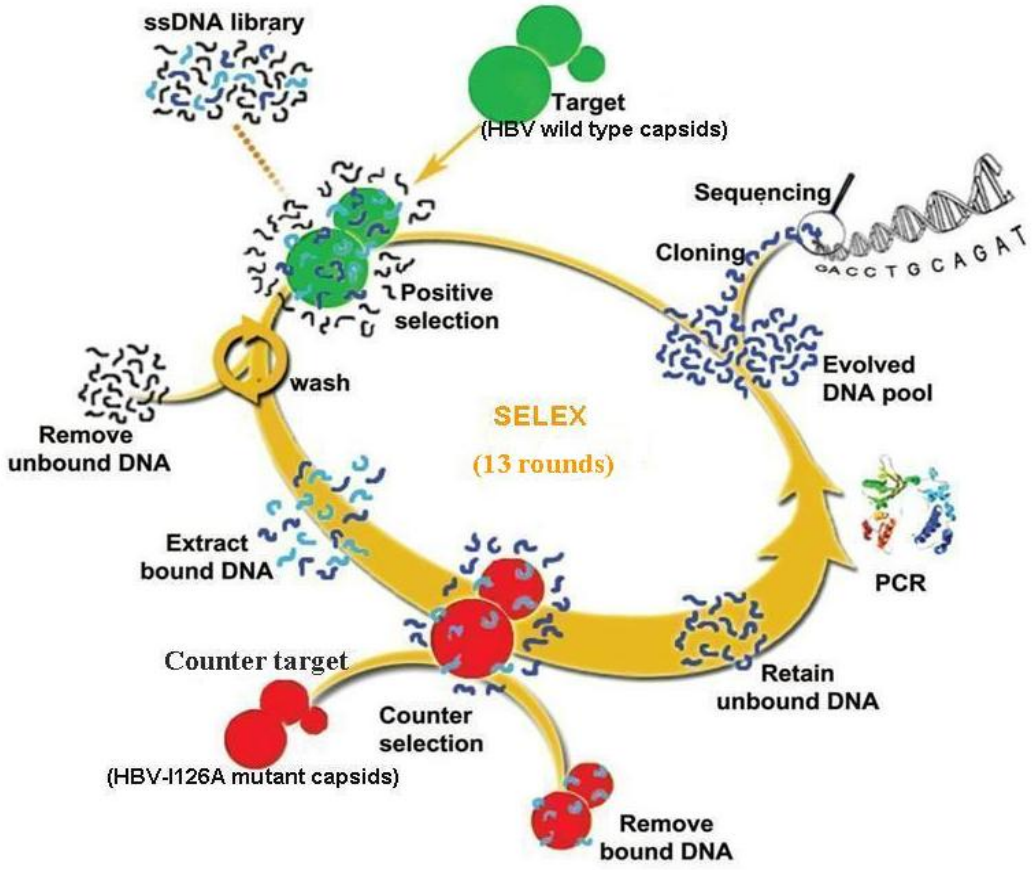


Fig. 17. General scheme of the SELEX with counter selection. 13 rounds of consecutive positive selection (against WT-HBV capsids) and negative selection (against I126A-HBV mutant capsids) were done**.** At the end aptamers having an affinity to the MBD of HBV were selected. *Modified from http://www.springerimages.com/Images/LifeSciences/1-10.1007_978-1-61779-188-8_9-0.*

Tabel 12: Conditions during 13 selection rounds.

Round	1st, 2nd& 3rd	4th, 5th&6th	7th, 8th&9th	Last 4
Aptamer amount	400 ng	400 ng	200 ng	100 ng
WT - capsids amount	7 µg	3 µg	3 µg	1 µg
Mutant capsids amount	3 µg	5 µg	5 µg	7 µg
IT[1] /WT-capsids	1 hour	1 hour	30 min	15 min
IT / mutant capsids	30 min	30 min	1 hour	1 hour
WR[2] / + selection	1/10	1/10	1/20	1/20
WR /- selection	1/10	1/10	1/4	1/4

1 IT is the incubation time

2 WR is the washing ratio

4.2.3.2.4 Aptamer sequencing

After 13 rounds of selection, the selected aptamers were cloned into the *EcoRV* site of pBluescript II KS (+). Purified plasmids were sequenced using primer VBAO05 (see 4.2.1.8).

4.2.3.3 Aptamer secondary structure prediction

Secondary structures of the selected aptamers were predicted by the Zuker algorithm (Zuker, 2003), using Mfold (version 3.2, http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi) with conditions set up at 0.15 M NaCl and 25°C.

4.2.4 Immunological & Biophysical Assays

4.2.4.1 Immunoprecipitation assay

The immunoprecipitation method can be used to qualitatively and quantitatively determine protein–nucleic acid affinities. The principle of this assay depends on the separation of the aptamer-protein complexes from the free aptamers by precipitating it using agarose beads on which polyclonal antibodies against the protein were fixed.

4.2.4.1.1 Preincubation of agarose beads and antibody

The protein G coupled agarose beads were undergoing coating by rabbit polycolonal antibodies against the HBV core protein. 25 µl of beads (Santa Cruz Biotechnologies) were washed 3 times with 1 ml PBS by centrifugation at 2500 rpm for 5 min at 4 ^{o}C. Then 1 µl of

undiluted rabbit anti-HBV core were added. The volume in the tube was filled up to ~800 ul with PBS to ensure proper mixing. The mixture was incubated on the sample rotator at 4 ^{o}C overnight to allow binding of the antibodies to the beads. In the morning, the coated beads were washed 3 times with PBS and resuspended into an adequate volume of PBS and left at 4 ^{o}C until usage.

4.2.4.1.2 Incubation of aptamers and HBV capsids

10 pmol of the pre-snap cooled selected aptamer (AO-01) were incubated with 0.1 pmol HBV-WT capsids in the binding buffer (100 µl) at RT for 1 h. 2 negative controls were made with the same conditions, the first one by incubating the pre-snap cooled selected aptamer (AO-01) with HBV-I126A mutant capsids, and the second one by incubating the pre-snap cooled randomly constructed aptamer (AO-0N) with the HBV-WT as well as with mutant capsids. To calculate the background of the assay, the same concentration of the pre-snap cooled aptamer (AO-01 and AO-0N) was incubated alone with the antibody coated beads.

4.2.4.1.3 Immunoprecipitation

The antibody coated beads were mixed with the aptamer/capsid mixture and the volume in the tube was filled up to ~700 µl with PBS. The mixture was incubated on the sample rotator at RT for 2 hours to allow binding of the antibodies to HBV capsids. To remove non bounded aptamers, the mixture was washed 3 times with 1 ml PBS by centrifugation at 2500 rpm for 5 min at 4 ^{o}C. Finally, the pellet was resuspended into adequate volume of water.

4.2.4.1.4 Extraction of bounded aptamers

The bounded aptamers were extracted from the capsids by two steps of phenol/chloroform extraction (see 4.2.1.2.1) and one chloroform extraction to remove any residues of phenol from the sample. Then the upper aqueous phase containing the aptamers was transferred to an eppendorf tube. The sample was then heated in a thermomixer to 85 ^{o}C for 15 min at 350 rpm while the lid was open to evaporate any residues of chloroform that may affect the PCR reaction. To determine the percent of aptamer loss during this step, the same extraction steps were done for the same concentration of the pre-snap cooled aptamer alone.

4.2.4.1.5 Aptamers quantification

The extracted aptamers were firstly diluted by PCR grade water and then quantified by real time PCR (see 4.2.1.9.2) using VBAO03 and VBAO04 as a primer set.

4.2.4.2 HBV virion immunoprecipitation

The cell culture supernatant of transfected HuH 7 cells was harvested and centrifuged for 5 min at 4000rpm to pellet the cells. The supernatant (750 µl) was transferred into a new tube. Then anti-HBs antibody coated beads (see above) were added and the mixture was incubated overnight with rotation at 4°C. On the next day, the immunoprecipitate was washed 3 times with 1 ml of PBS. The supernatant was carefully removed and the pellet was resuspended in PBS. To degrade any free DNA molecule in the solution, DNAseI (Qiagen) was added with its specific RDD buffer and then the mixture was incubated at RT for 25 min. After that a washing step was performed using 1 ml of PBS. The virion DNA was liberated by incubating the mixture with proteinase K (17 nM) in the presence of its specific buffer at 56°C for 3h to allow destruction of the capsids. Finally, a phenol/chloroform treatment was done followed by ethanol precipitation to get DNA molecules which could be quantified by an HBV genome specific qPCR.

4.2.4.3 Filteration assay

The binding affinity of the aptamer–target complex can be determined by a filtration method. In this work, the pre-snap cooled selected aptamer (AO-01) and the negative control aptamer (AO-0N) were incubated with HBV WT and mutant capsids in the same concentrations as in immunoprecipitation assay (see 4.2.4.1.). The bounded aptamers were separated from the free ones as in the positive selection of the SELEX-last 4 rounds (see 4.2.3.2.2) and then recovered and quantified as in the immunoprecipitation assay.

4.2.4.4 Determination of dissociation constant for the binding of aptamers to capsids

The constant of dissociations (K_d) of the selected aptamers capsid complex were calculated by the immunoprecipitation method (see above). Different concentrations of the aptamers (from 5 pM to 1 µM) and a fixed concentration of the HBV WT capsids (1 nM) were used. The obtained data were analyzed and then the K_d values of the different selected aptamers were estimated by the Sigma Blot 12.0 software program.

4.2.5 Cell Culture Techniques

4.2.5.1 Cultivation of HuH7

The cells which had been allowed to grow to a confluent monolayer were divided into several plates by splitting in appropriate ratios. Firstly, the cells were washed once with an adequate amount of 37°C warm PBS. After that the cells were trypsinized by covering the cells with trypsin/EDTA solution at RT for a few seconds. Then the trypsin/EDTA solution was removed and the cells were subsequently incubated at 37°C and 5 % CO2 with intermittent observation until the cells appeared to be detached from the plate. The detached cells were taken up in 6 ml 37°C warm growth medium (DMEM with 10 % FBS, 1x NEAA, 1x sodium pyruvate, 1 % Penicillin/Streptomycin), diluted in the desired split ratio in the same media and transferred to new cell culture dishes. To maintain the cells in culture, they were split every four days with a ratio of 1:6. For transfection, the cells were seeded in 10 cm dishes or 6 well plates. They were either split 1:2 (10 cm dishes) or 1:15 (6 well dishes) the day before transfection. The total volume of growth medium in 10 cm dishes was 10 ml, in 6 well plates 2 ml per well.

4.2.5.2 Freezing of cells

The plates with densely grown cells were trypsinized (see above). Then the cells were taken up in 900 µl warm FCS and transferred to a cryotube. Then, they were supplemented with 10 % DMSO (100 µl) which is a cryoprotectant by adding it slowly and dropwise while swirling the tube to avoid an osmotic shock. The cells were firstly frozen in an ultra temperature deep freezer at -80°C. After a few days, they were transferred to liquid nitrogen where they were stored for unlimited time.

4.2.5.3 Thawing of cells

The frozen cells were thawed by immersing the cryotube into a water bath at 37°C until only a small frozen remainder was left. Then the cells were transferred to a 15 ml Falcon tube and 10 ml of warm growth medium was added in a dropwise manner. Then the cells were settled by centrifugation at 1000 rpm for 5′ at 4°C. The supernatant was replaced by fresh growth medium and the cells were resuspended and distributed to two or three cell culture dishes. They were incubated at 37°C and 5 % CO2 until the cell layer was dense. Then they were split for at least two times and finally used for experiments.

4.2.5.4 Transfection of HuH7

In this work, the transfection was done by using the Roche Fugene system (Fugene 6, Fugene HD or X-tremeGene HP) which forms complexes with DNA allowing the entrance of the vectors into the cells. 1 µg of DNA plasmid used for transfection of cells grown in 6 well plates. When cotransfecting several DNA plasmids, they were used in the same molecular ratio making up 1 µg in total. The DNA was diluted in medium w/o any additives to reach 47 µl volume in total, and then 3 µl of Fugene reagent were added directly into the liquid without prior touching the tube wall with the pipette tip. The batch was mixed gently and incubated at RT for at least 40 min to allow the transfection complex to be formed. For transfection, preconfluent cells (~80 % density) split the day before were used and this was done by splitting the cells in a ratio 1:20. Before transfection the cells had been washed once with PBS then the growth medium was replaced by 0.7 ml medium w/o any additives. The transfection complex was transferred dropwise and evenly onto the cells and the plates were swirled afterwards to ensure a proper distribution. The cells were then incubated for 6 h at 37°C and 5 % CO2. Thereupon, the transfection medium was removed and the cell layer was washed once with PBS to remove remaining transfection complex. Two ml fresh growth medium was dispensed and the transfected cells were incubated for 3 days at 37°C and 5 % CO2.

4.2.5.5 Harvest of supernatant

The supernatant of transfected cells was harvested 3 days after transfection. It was transferred to 2 ml tubes and dead cells were pelleted by centrifugation at 13000 rpm for 10 min. 750 µl aliquots of the supernatant were preserved at -80°C until usage for immunoprecipitations.

5 Results

5.1 *Production and purification of recombinant HBV capsids*

The expression and purification of HBV WT capsids was done by Maira Bieringer during her master thesis (Bieringer, 2010). Her results are shown here again just for comparison with expression and purification of HBV I126A mutant capsids which was part of this work.

5.1.1 Construction of expression vectors

The DNA sequences for the C-terminally of codon 150 truncated core protein (delta-C) and its point mutant I126A (each of 455 bp nucleotide length) were present in the plasmids pRVHBV+ and pSVHBV1.1LE-I126A, respectively. They were amplified by conventional PCR using VBAO01 as a forward primer harbouring a start codon and a *NCOI* restriction site, and VBAO02 as a reverse primer containing a stop codon (TAA) and *SALI* restriction site (see 4.2.1.1.1). Both amplified sequences were doubly digested by the enzymes *NCOI* and *SALI* (see 4.2.1.7.1.2), gel extracted (see 4.2.1.4) and finally ligated with the doubly *NCOI* / *SALI* digested, gel extracted and purified T7 polymerase dependant expression vector pETM 13 (see 4.2.1.7.2).

The resulting plasmids were tested by double restriction with *NCOI* and *SALI* of 10 minipreparations. In addition, two samples of the expression vector exhibiting the stuffer gene (ABD, 834 bp), one without restriction and the other doubly restricted by *NCOI* and *SALI* were used as controls. All samples were electrophoresed in 1% native agarose gels containing EtBr to be visualized in the gel documentation system (see 4.2.1.3.1). In addition, to check the correct sequence of the PCR products, two samples of the miniprepared plasmids, one containing the gene for delta-C HBV WT core protein and the other containing the gene for delta-C HBV I126A mutant core protein, were sequenced using two primers: (1) The reverse primer VBAO02 allows the sequencing of the target sequence (455 bp) from bp 225 until bp 900. (2) The forward primer VBAO01 allows the sequencing of the target sequence from its beginning until bp 595 (see 4.2.1.8).

The WT and I126A mutant constructs showed two bands in all 10 minipreparations (Fig. 18; lanes: 1-10), the band of higher molecular weight refered to the backbone of the double digested vector (5252 bp) while the one of lower molecular weight had the same length as the target sequences that encoded for HBV WT and I126A mutant delta-C core

proteins (455 bp). The doubly restricted expression vector exhibiting the ABD gene showed also two bands, the first was the same backbone of the double restricted vector (5252 bp) while the second band refered to the ABD sequence (834 bp) that encoded for a different protein, Actin Binding Domain (Fig. 18; lane: P1). The non-restricted expression vector showed a strong band of 6086 bp refered to the open circular form of the plasmid and another lighter band refered to the supercoiled form (Fig. 18; lane: P2). The sequencing revealed that the both target sequences were exactly as expected and contained no unintentional mutations.

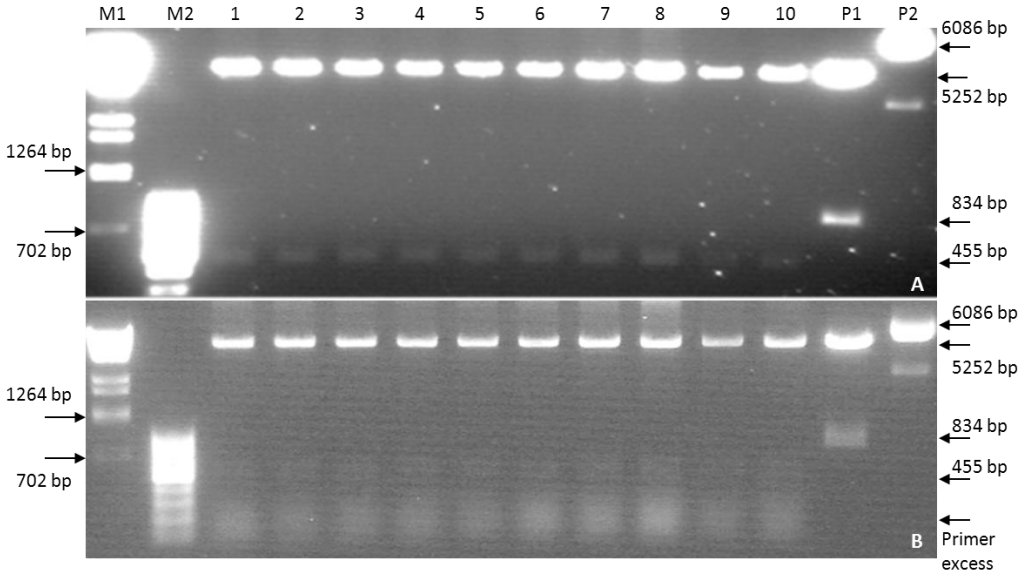


Fig. 18. Double, *NCOI* and *SalI*, restriction of DNA constructs encoding for HBV WT and its I126A mutant delta-C core protein. Double restriction of 10 HBV WT ligated constructs **(A)** and 10 HBV I126A- mutant ligated constructs **(B)** by *NCOI* and *SalI* enzymes showed a 5252 bp fragment (double restricted vector, pETM 13) and a 455 bp fragment (target gene sequence encoding for both HBV capsids). **P1:** *NCOI* and *SalI* enzymes double restricted pETM 13 exhibiting a stuffer gene (ABD). **P2:** The non restricted pETM 13 vector. **M1:** Lambda marker and **M2:** 100 bp marker.

5.1.2 Capsid overexpression

HBV WT and I126A mutant delta-C core proteins were successfully overexpressed in *E.coli* BL21 Star (DE3) pRARE2 cells. The cells were transformed with the expression vectors containing the gene sequences encoded for both, HBV WT and I126A mutant delta-C core proteins (see 4.2.1.7.3.3). In addition, a negative control was carried out by transforming the cells with the same expression vector (pETM 13) exhibiting the ABD gene that encoded for a different protein, Actin Binding Domain. The transformed bacterial cells were highly enriched in 500 ml culture media and simultaneously forced to express the proteins by using the

inducer (IPTG) as described in 4.2.2.1. The over-expressed proteins were then liberated by lysing the cells using repeated freezing and thawing with 1 ml of lysis buffer (see 4.2.2.2.1).

A preliminary test for the expression of HBV WT and I126A mutant delta-C core proteins, their molecular weight is nearly 16.8 KDa, was done and in comparison with the negative control protein (ABD, its molecular weight is 30.9 KDa) by electrophoresing a small volume of the supernatants of the lysed cells (1/100) in a 15% SDS gel to be stained by Coomassie Brillian Blue stain (see 4.2.2.3.6).

The results (Fig. 19) revealed signals in the expected positions (16.8 KDa) for C-terminally deleted HBV WT core protein, the I126A mutant and the positive control indicating successful expressions of both HBV WT and its I126A mutant delta-C core proteins. The negative control showed a signal in the expected position for a different protein, actin binding domain (30.9 KDa).

The capsids assembled from both expressed HBV core proteins, WT and I126A mutant, (240 core proteins, its molecular weight is nearly 4 MDa) were also immunodetected by western blotting.

The supernatants of the lysed cells that expressed HBV WT delta-C core proteins, HBV I126A mutant delta-C core proteins and actin binding domain (as a negative control) were run in a 1% native agarose gel to be capillary blotted against a nitrocellulose membrane. Then the capsids were immunodetected by the anti-HBc antibody H800 (see 4.2.2.3.3/5).

The results (Fig. 20) showed signals for both expressed HBV capsids, WT and I126A mutant capsids, parallel with the positive control while the negative control showed no signal indicating the successful assembly of the expressed HBV delta-C core proteins into capsids.

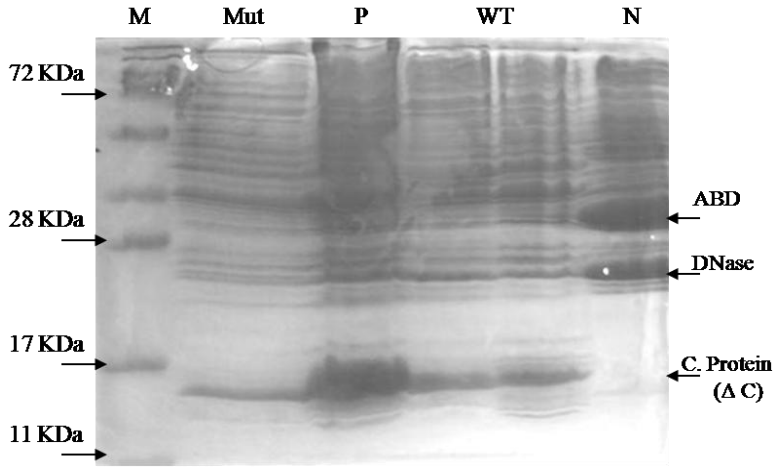


Fig. 19. Coomassie Brilliant Blue stained *E.coli* expressed HBV WT and I126A mutant delta-C core proteins in 15% SDS gel. M: Page Ruler Plus Prestained Protein Ladder, **WT:** HBV wild type delta C, **P:** cell lysate harbouring HBV WT delta-C core proteins as a positive control, **Mut.:** HBV I126A mutant core proteins and **N:** negative control, Actin Binding Domain (ABD) protein.

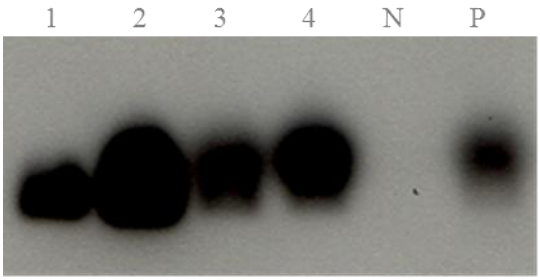


Fig. 20. Immunoblotting detection of *E.coli* expressed HBV WT and I126A mutant delta-C capsids in a 1% native agarose gel. 1&2: 0.01 and 0.1% diluted cellular supernatant containing HBV I126A mutant capsids, **3&4:** 0.01 and 0.1% diluted cellular supernatant containing HBV WT capsids, **N:** negative control (ABD) and **P:** Positive control (WT HBV delta-C capsids). *Exposure time was 5 seconds.*

5.1.3 Capsid purification

5.1.3.1 Precipitation of proteins

The total protein including the expressed capsids in the cellular lysates were precipitated by ammonium sulphate (see 4.2.2.2.2) to remove non protein like impurities and to concentrate the sample for the next step of the purification procedure, size exclusion chromatography. The delta-C core proteins of both, HBV WT and I126A mutant, were detected in the supernatant and the resuspended pellets after ammonium sulphate precipitation and also in the whole cell lysates before ammonium sulphate precipitation using Coomassie Brillian Blue staining in 15% SDS gel.

The results showed signals for delta-C core proteins (16.8 KDa) in the precipitate and also in the supernatant but its concentration appeared to be higher in the precipitate than in the supernatant (Fig. 21).

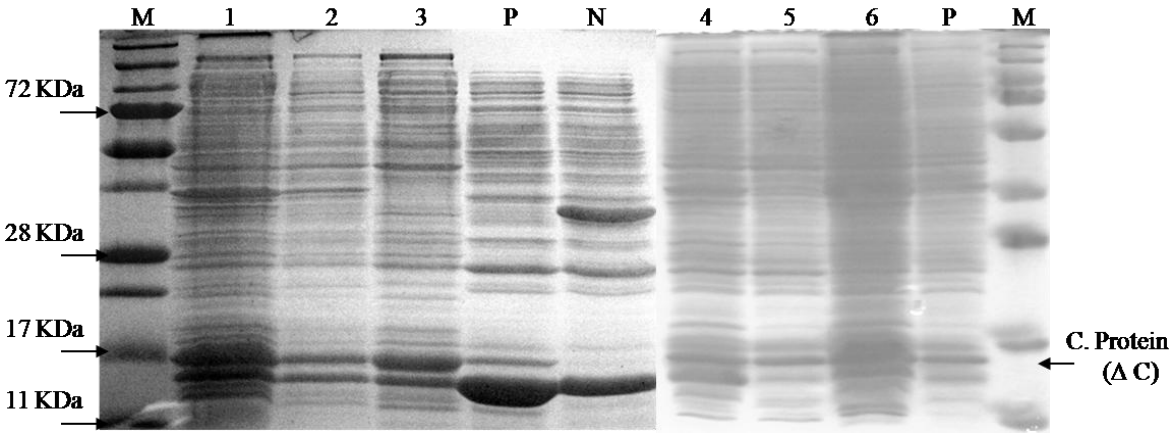


Fig. 21. Coomassie Brilliant Blue stained HBV WT (lanes 1 to 3) and HBV I126A mutant (lanes 4 to 6) delta-C core proteins after ammonium sulfate precipitation in 15% SDS gel. M: Page Ruler Plus Prestained Protein Ladder, **1&6:** whole cell lysate before precipitation, **2&5:** cell lysate supernatant, **3&4:** resuspended precipitated proteins, **P:** cell lysate harbouring HBV WT core proteins & **N:** negative control (ABD).

5.1.3.2 Size exclusion chromatography

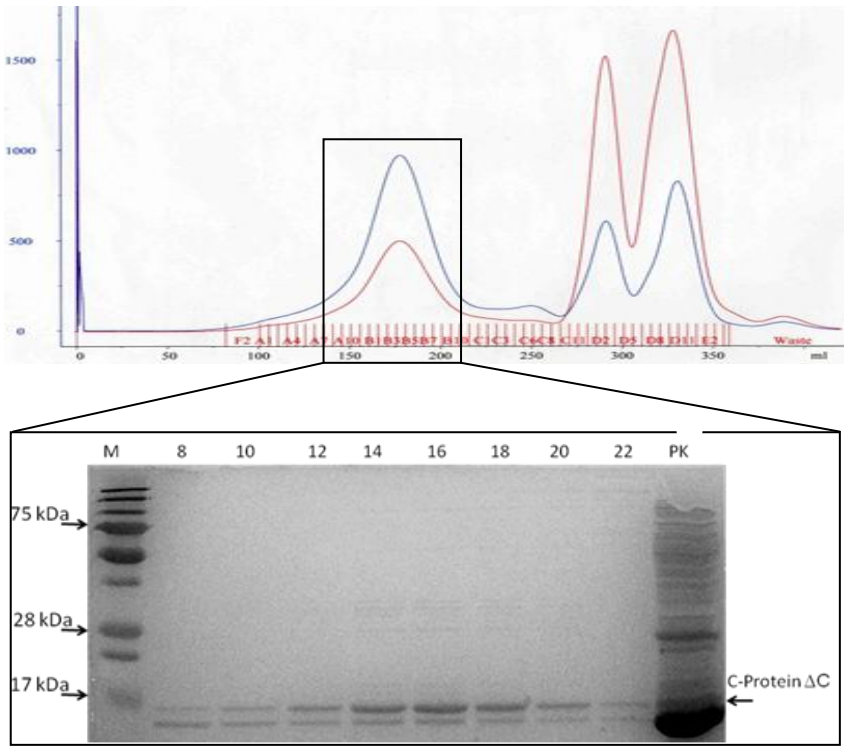
The HBV delta-C capsid has a large molecular weight (~ 4 MDa). This fact was used to purify the capsids by size exclusion chromatography using a column filled with Sephacryl S-500 HR (GE Healthcare). The capsids should pass faster through the column matrix and should be collected earlier in the first fractions than the impurities with smaller molecular weight that were lodged in the matrix pores and were collected later (see 4.2.2.2.4.1). The sample fractions (each of 5 ml) that showed high absorbance at wave length 280 nm (fractions of higher protein content) were tested by Coomassie Brilliant Blue staining in 15% SDS gel (see 4.2.2.3.1/6) to define the fractions which harbour the highest concentration of the expressed capsids and with less impurities to be used afterwards.

The results showed bands of both, HBV WT and its I126A mutant delta-C core proteins (~ 16.8 KDa), with much less impurities. On the other hand, the whole fractions showed a similar non specific band of lower molecular weight (~ 14 KDa) which appeared to be correlated with the delta-C core proteins (Fig. 22).

To check if the capsids were still intact without deassembly or degradation after the size exclusion chromatography, small amounts (1 µl of 1/100 dilution) from fractions containing the delta-C core proteins were electrophoresed in 1% native agarose gel and also into 15% SDS gel to be immunodetected by western blotting using anti-HBc (H800).

All fractions in the native gel showed distinct signals in the same line as the positive controls indicating that there is no extensive capsid degradation. In the denaturing gel, the delta-C core proteins of both, HBV WT and its I126A mutant, showed the expected bands (~ 16.8 KDa) and the non specific bands which appeared after Coomassie Brilliant Blue staining (see above) disappeared completely indicating that these bands are non specific and not related to the expressed core proteins (Fig. 23).

(A)



(B)

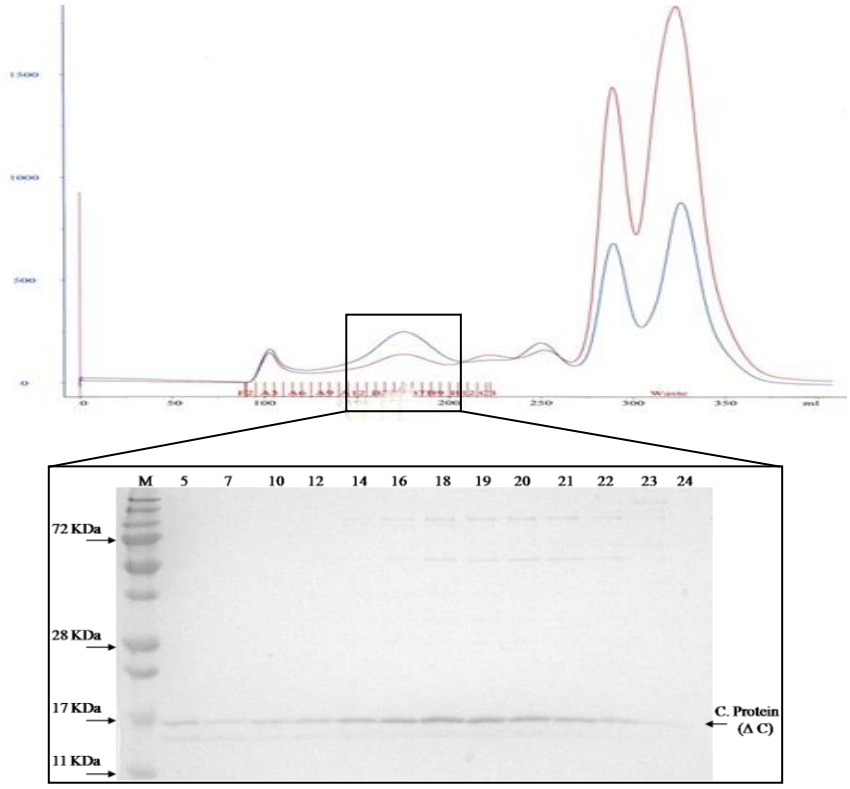


Fig. 22. Chromatogram and Coomassie Brillian Blue staining of fractions with high core protein content after size exclusion chromatography during HBV capsid purification. After the size exclusion chromatography, both HBV WT capsids containing fractions (**8-22**) (**A)** and HBV I126A mutant capsids containing fractions (**5-22**) **(B)** showed high absorbance (blue lines) in the chromatogram, ~ 1000 and 300 mAU, respectively, and bands in the same position as the positive control (PK, HBV delta-C WT capsid) in 15% SDS polyacrylamide gel after the Coomassie blue staining. *0.1% of each fraction was used in PAGE.*

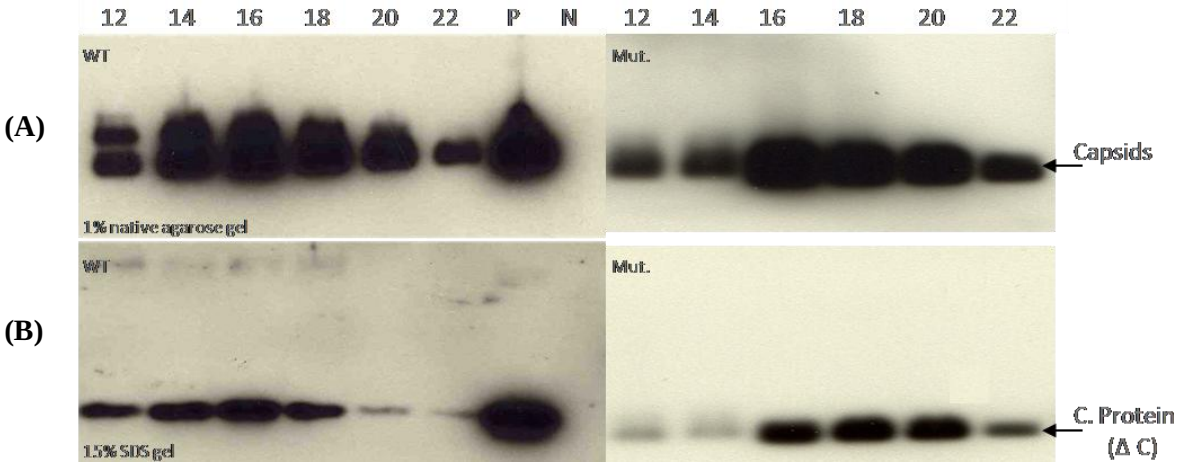


Fig. 23. Western blot validation of HBV WT and I126A mutant delta-C capsids and their core proteins after size exclusion chromatography. Some fractions harbouring high concentrations of HBV WT and I126A mutant delta-C core proteins **(fractions no. 12, 14, 16, 18, 20 &22 in Fig. 22 A and B)** showed signals in the expected positions as the positive control in both 1% native agarose gel **(A)** and 15% SDS gel **(B)**. **P:** positive control (WT delta-C capsids) and **N:** negative control (ABD).

5.1.3.3 Sucrose gradient ultracentrifugation

After the size exclusion chromatography, the fractions 8 to 22 showed high concentrations of HBV WT core proteins (8:22) and the similar fractions showed high concentrations of the I126A mutant core proteins (5 to 22). They were pooled and then concentrated by using PD-desalting columns (see 4.2.2.2.4.2) to be further purified by sucrose gradient ultracentrifugation (see 4.2.2.2.5) for further removing non specific proteins and other impurities which were still accompanied the capsids. Small amounts (1 µl of 1/100 dilution) of the collected fractions were immunodetected by western blotting in a 1% native agarose gel (see 4.2.2.3.2/3/5) to determine the fractions which contain the expressed capsids.

The results showed positive signals for HBV WT and I126A mutant delta-C capsids in fractions 25 to 39 and fractions 24 to 39, respectively (Fig. 24).

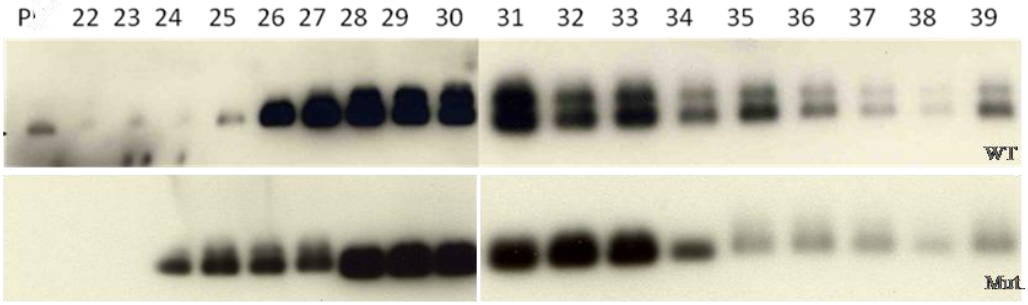


Fig. 24. Western blot detection of HBV WT and its I126A mutant delta-C capsids in different fractions after sucrose gradient ultracentrifugation. Fractions harbouring delta-C capsids of HBV WT **(25 to 39)** and its I126A mutant **(24 to 39)** showed signals in the expected positions as the positive control in a 1% native agarose gel. **P:** positive control (HBV WT delta-C capsids).

The fractions containing WT capsids (25 to 39) were pooled and the same also was done with the fractions containing I126A mutant capsids (24 to 39). Then the pooled fractions were filtered through a Millipore concentrator for removing the sucrose and also to concentrate the samples (see 4.2.2.2.3). To check the purity of the overexpressed capsids, small amounts from the solutions containing the purified capsids of both, WT and its I126A mutant, were electrophoresed in 15% SDS gel to be stained by Coomassie Brillian Blue. The results revealed that the impurities were greatly reduced. The signal of the purified WT capsids appeared higher in intensity than that of the I126A mutant capsids (Fig. 25).

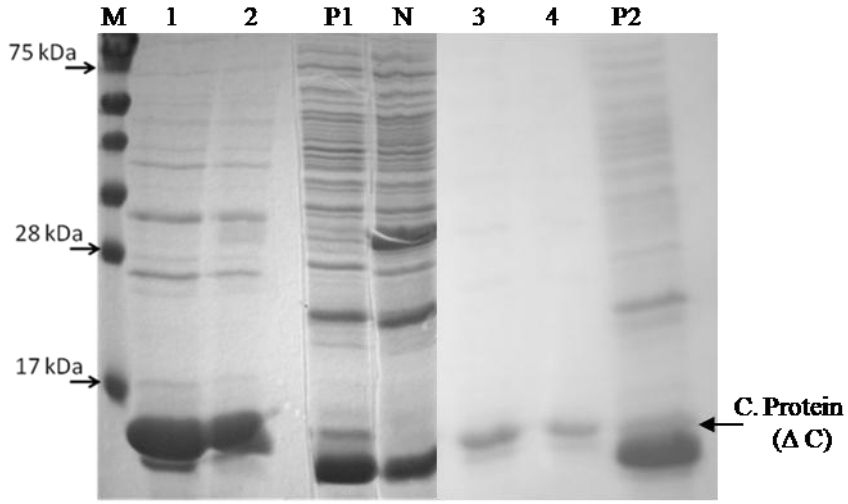


Fig. 25. Comparative Coomassie Brillian Blue detection of delta-C core proteins of both HBV WT and I126A mutant before and after purification in 15% SDS gel. M: Page Ruler Plus Prestained Protein Ladder, **1:** 20 µl purified WT capsids, **2:** 10 µl purified WT capsids, **P1:** whole cell lysate containing HBV WT delta-C capsids (positive control) and **N:** whole cell lysate containing ABD protein (negative control), **3:** 20 µl purified I126A mutant capsids, **4:** 10 µl purified I126A mutant capsids and **P2:** whole cell lysate containing HBV I126A mutant delta-C capsids (positive control).

5.1.4 Determination of the concentration of purified capsids

The concentrations of both purified capsids, HBV WT and I126A mutant, were measured by DC-protein assay (see 4.2.2.4). BSA was used as a standard. The final concentration of HBV WT capsids was 1.4 mg/ml. The total amount was 4.2 mg. The final concentration of HBV I126A capsids was 0.12 mg/ml. The total amount was 0.36 mg.

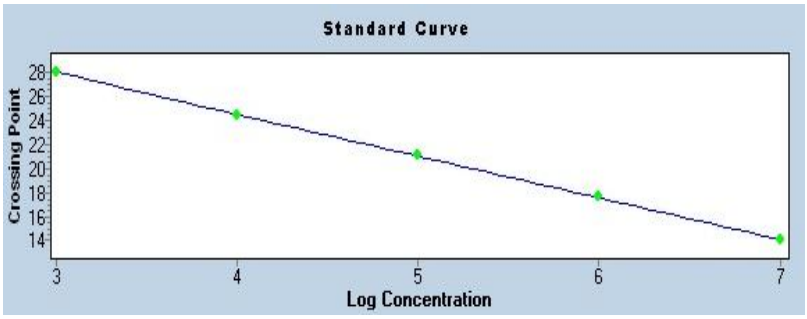
5.2 *In vitro selection of aptamers*

5.2.1 Aptamers quantification by real-time PCR

The ability of quantifying aptamers from heterogenous ssDNA library of 55 nt length using real-time PCR was tested. Five samples of the aptamers' stock solution containing 10^3, 10^4, 10^5, 10^6 and 10^7 aptamers were amplified by real-time PCR using the primer set VBAO03 and VBAO04 and with conditions described in paragraph 4.2.1.9.2. CT values/crossing points measured for the samples were plotted against the logarithm of the input amount of aptamers (Fig. 26A). To analyze the real time reactions, small volume (1/10) of the PCR product from each sample was electrophoresed through a 10% non-denaturing polyacrylamide gel, then the gel was immersed in a EtBr bath (100 µg/500 ml H_2O) to visualize the DNA in the gel documentation system.

The results revealed a linear fitting curve indicating that the PCR reaction was suitable for measuring the amount of the aptamers (Fig. 26A). In the gel, the PCR products showed signals with strong intensity in the expected position (55 bp). However, PCR by-products were detected especially in the samples containing 10^6 and 10^7 aptamers but with very low intensity (Fig. 26B).

(A)



(B)

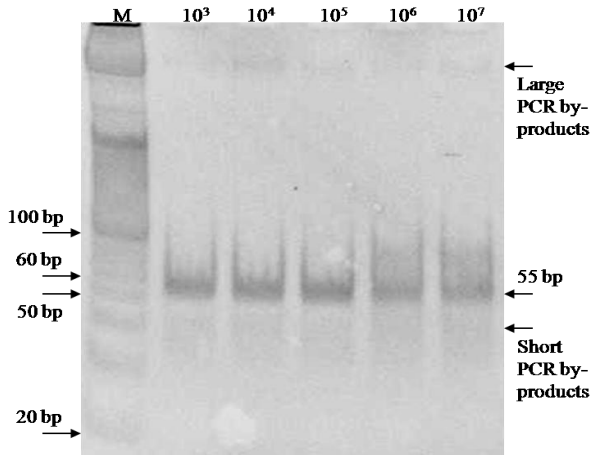


Fig. 26. Aptamers quantification by real-time PCR. (A) CT values/crossing points of five different aptamers amounts (**10^3-10^7**) show a linear curve against the logarithm of input amount of aptamers. **(B)** The real-time reactions of the aptamers' five amounts after 10% PAGE. **M:** Marker, Gene Ruler 10 bp.

5.2.2 Efficacy of alkaline pretreated filters

Amicon Ultra-2 mL Centrifugal Filters (100 K) that were used during SELEX with counter selection were firstly alkaline treated using 0.5 M KOH (see 4.2.3.1). To check the effect of the alkaline treatment on the filteration efficiency against aptamers, 100 pmol (6.0×10^{13} molecules) of the presnap cooled ssDNA library (see 4.2.3.2.1) were filtered through the alkaline pretreated filters and the same amount also filtered through non treated filters. Then the aptamer concentrations in both, filtrated and recovered solutions, were calculated by real-time qPCR (see 4.2.1.9). The results revealed that the alkaline treatment of the filters has a significant effect on the filteration efficacy against ssDNA aptamers. Less than 5% of the aptamers that could pass through the alkaline non-treated filters and this percentage rose to be more than 97% after alkaline treatment (Fig. 27).

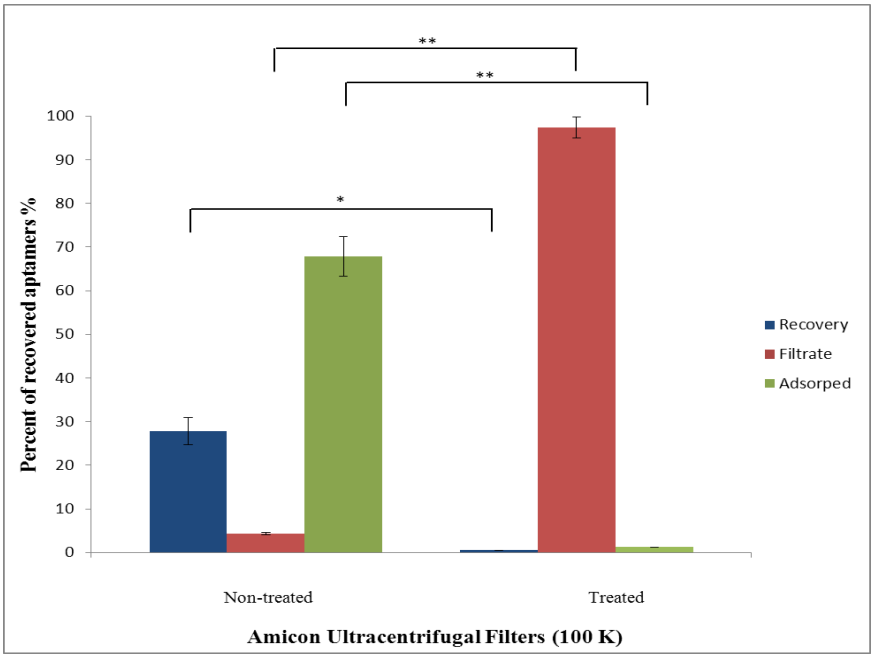


Fig. 27. Evaluation of filter alkaline treatment on the filteration efficiency. The efficiency of Amicon Ultra-2 mL Centrifugal Filters (100 K) against 55 nt length heterogenous ssDNA aptamers was greatly enhanced (~25 fold more) after the alkaline treatment using 0.5 M KOH. Mean values and standard deviations were calculated from 3 independent experiments. **: $P \leq 0.05$; **: $P \leq 0.01$.*

The alkaline pretreated filters were also checked against the expressed HBV WT capsids to be sure that the filter matrix was still intact without any damage and also without any destructive effects on the capsids. A large amount of capsids (20 µg) were filtered through the alkaline pretreated filter, then the capsids were checked in the filterate by western blotting using anti-HBc antibody (H800) and also 0.1% of the recovery (the expected

amount, 20 ng) was double fold serially diluted. Then the first four dilutions (the expected amounts 20, 10, 5 and 2.5 ng) were electrophoresed in 1% native agarose gel to be immunodetected by western blot. The results showed no capsids at all in the filterate while in the recovery the concentration of the capsids is nearly the same as the intial capsid concentration used in the experiment (Fig. 28). These results indicated that no damage or holes were performed in the filter matrix and also no harmful effects on the capsids were induced by the alkaline treatment of the filters.

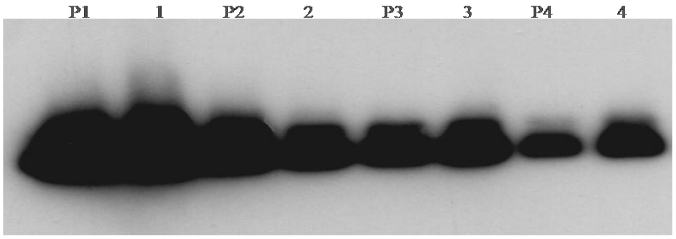


Fig. 28. Western blot evaluation of the effect of filter alkaline treatment on HBV WT capsids. The signals of the different dilutions of the recovered capsids after filteration **(1, 2, 3 & 4)** are of nearly the same intensity as the signals of the positive controls (**P1:** 20, **P2:** 10, **P3:** 5 & **P4:** 2.5 ng).

5.2.3 Optimization of aptamers PCR amplification

The efficiency of PCR amplification of aptamers (library of heterogenous DNA sequences) was checked to find out if there are any differences from PCR amplification of the usual homogenous DNA templates.

Six samples of two different concentrations of the ssDNA library (10 and 1000 pM) were amplified by conventional PCR using the primer set VBAO03 and VBAO04 (see 4.2.1.1.2) but with 3 different numbers of amplification cycles (35, 25 and 15 cycles). The six PCR products of each concentration were pooled together (300 µl), then concentrated and purified by a QIAEX II Kit (see 4.2.1.2.3). A small volume of each purified PCR product (1/10) was electrophoresed in 10% non-dentauring polyacrylamide gel then the gel was immersed in a EtBr bath (100 µg/500 ml H_2O) to be visualized in the gel documentation system.

The results revealed that with rising numbers of PCR cycles, the more and more PCR by-products were produced, the higher was the possibility for losing the aptamer library. This was very obvious in the PCR product after 35 cylces as the aptamer PCR product (55 bp) was nearly lost (Fig. 29). Concerning the difference in the initial concentrations of the aptamers used in PCR, non significant effects on the produced PCR products and by-products were observed.

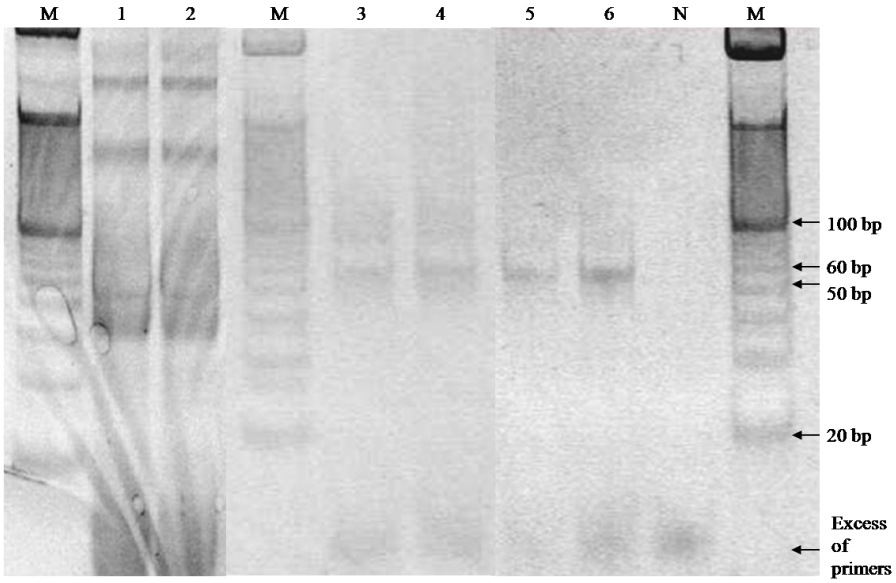


Fig. 29. Dependance of molecular weight fragment length of PCR products on the number of PCR cycles. After 35 PCR cycles, the PCR product appeared heterogenous with almost no product of 55 nt length using initial aptamer concentrations of 10 and 1000 pM, **1** & **2**, respectively. By decreasing the number of PCR cycles to 25 (**3** & **4**) and 15 (**5** & **6**) cycles using the same initial concentrations, 10 and 1000 pM, respectively, the PCR by-products were reduced and nearly disappeared leaving more PCR products with the length of the original apamers. **M:** Marker, Gene Ruler 10 bp. **7:** Negative control.

5.2.4 SELEX with counter selection

The snap cooled ssDNA library (10 nmol, ~ 6.0×10^{15} molecules) was initially preselected by filteration alone through the alkaline pretreated filter to remove all binders of the filter matrix. Then the concentration of aptamers which could pass through the filter was measured by a nanodrop machine and qPCR. Less than 2% of the aptamers was bound to the filter matrix and the ones which could pass (more than 98%) were recovered. To remove the huge amount of capsid non-binders, the preselected aptamers were used in three consecutive positive selections (see 4.2.3.2.2) against a constant amount of HBV WT capsids (7 µg, ~ 10^{12} capsids) without any PCR amplification in between.

After the third positive selection, the recovered aptamers (~1 pmol, 6.0×10^{11} molecules) were divided into six aliquots to be amplified by conventional PCR using a non-biotinylated forward primer (VBAO03) and a biotinylated reverse primer (VBAO04) (see 4.2.1.1.2) followed by the purification of the pooled PCR products using a QIAEX II Kit (see 4.2.1.2.3). Then the purified dsDNA molecules were exposed to streptavidin, denatured by heat and separated by denaturing PAGE in the presence of 6 M urea to prepare ssDNA molecules (Fig. 30A). To check the successful preparation of ssDNA molecules, small volumes (1/20) from

the gel purified ssDNA molecules were electrophoresed in 10% non-denaturing polyacrylamide gel. The gel was immersed in a EtBr bath for 30 min to visualize the DNA in the gel documentation system (Fig. 30B).

The figure shows the streptavidin induced an electrophoretic mobility shift after electrophoresis of the streptavidin/aptamer complex in 10% denaturing polyacrylamide gel containing 6 M urea. The ssDNA molecules formed a sharp distinct band (Fig. 30A) while in the non-denaturing 10% polyacrylamide gel they formed to some extent a smear like band (Fig. 30B).

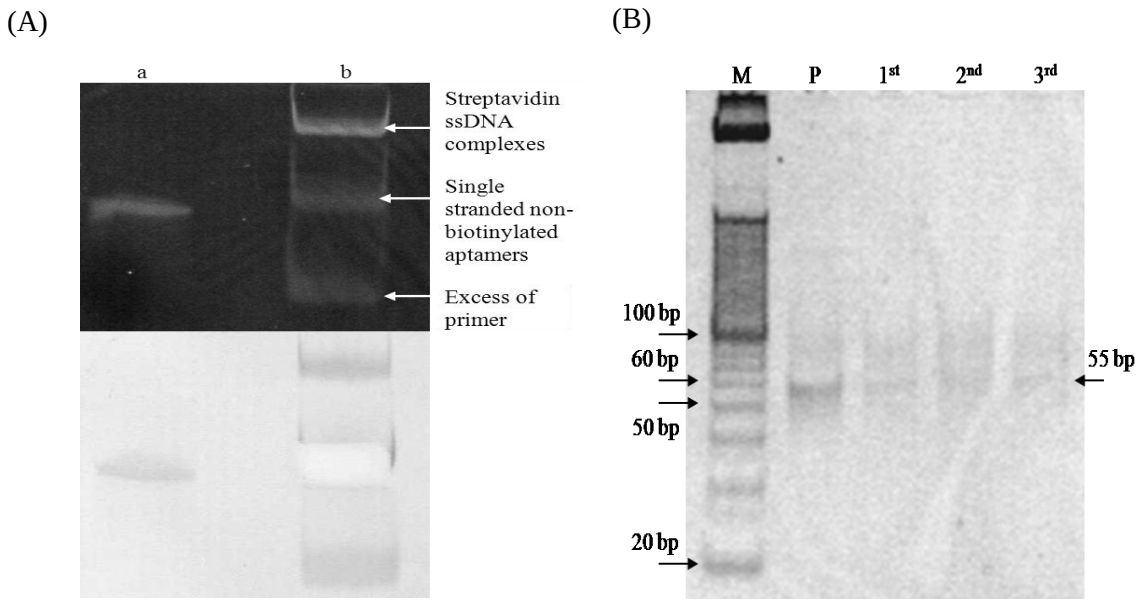


Fig. 30. Positively selected aptamers in 10% non-denaturing and denaturing (6 M urea) polyacrylamide gels. (A) The upper photo shows ssDNA molecules of both, aptamer stock solution **a** and the positively selected aptamer PCR product-streptavidin complex **b,** that formed sharp distinct parallel bands in the denaturing gel. The lower photo shows the excised gel piece containing the non biotinylated ssDNA aptamers to be recovered and used in the first SELEX round. **(B)** ssDNA aptamers from the aptamers'stock solution (**P**) and the recovered aptamers after 3 consequtive positive selections (**1st, 2nd & 3rd**) showed smear like bands in the non-denaturing gel indicating the high heterogenicity and multiple conformations of aptamers. **M:** marker, Gene Ruler 10 bp.

The ssDNA aptamers in the excised gel piece were recovered by the crush and soak method and by a QIAEX II Kit (see 4.2.1.4.2). Their amount was calculated by a nanodrop machine and qPCR, the results revealed 1.4 µg and 4.2×10^{13} molecules, respectively. Theoretically and according to the molecular weight of the aptamer library (17 KDa), 1.4 µg should contain 5×10^{13} aptamers. This small variance (16%) between the nanodrop and qPCR readings was owed to the efficiency of PCR amplification of heterogenous DNA sequences which seems to be lower than that of the homogenous templates. 400 ng (~ 1.5×10^{13} molecules) were used in the positive selection step (see 4.2.3.2.2) of the first SELEX round against HBV WT capsids (7 µg, ~ 10^{12} capsids). The amount of the resulting ssDNA aptamers was also calculated by a

nanodrop machine and qPCR, the results revealed 1.1 µg and 3.2 × 10^{13} molecules, respectively. Then the same amount of aptamers used in the positive selection of the first SELEX round (400 ng) was used in the first negative selection (see 4.2.3.2.3) against HBV I126A mutant capsids (3 µg, ~ 4.5×10^{11} capsids). In the next rounds of selection the same procedure was followed but different concentrations of aptamers, WT capsids and mutant capsids were used as well as different incubation times and different volumes of PBS during washing steps were used (Table 12) to induce a selective pressure which could improve the selection of aptamers with high affinities.

After each complete round of *in vitro* selection with counter selection and before the production of ssDNA molecules using the streptavidin induced electrophoretic mobility shift, a small volume of each PCR product (1/50) was electrophoresed in 10% non-denaturing polyacrylamide gel to be visualized in the gel documentation system (Fig. 31).

The results revealed bands in the expected position (55 bp) indicating a successful aptamer selection. Because of the high heterogenicity of the aptamer library in the first rounds of selection (1-4), PCR by-products were more prominent than in the subsequent rounds. Throughout the subsequenct rounds of selection, the amount of PCR by-products declined gradually until they nearly disappeared at the last round. In addition, the aptamer band (55 bp) became sharper indicating the gradual reduction of library heterogenicity.

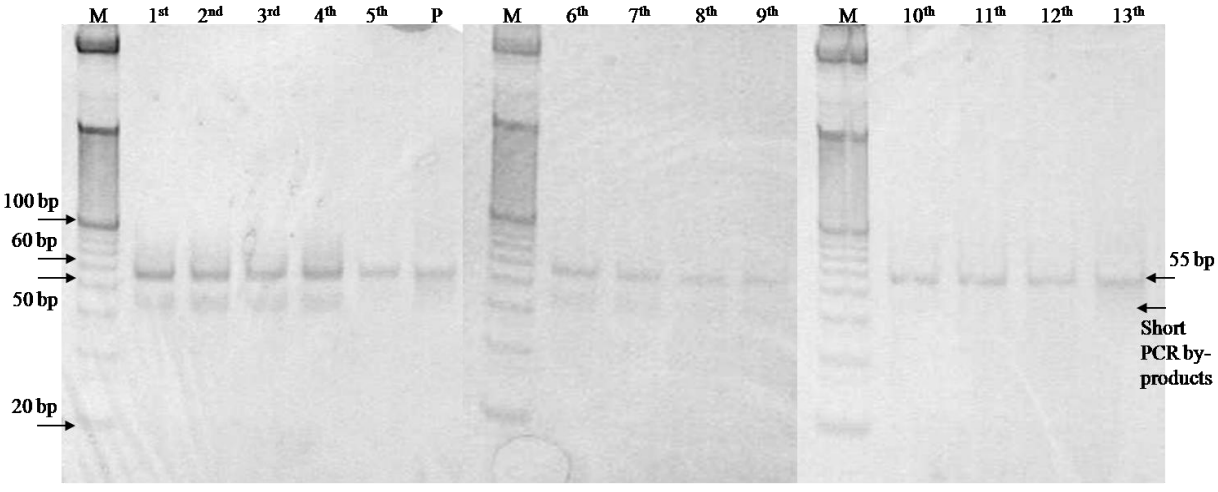


Fig. 31. PCR products of thirteen rounds of 55 nt length aptamer selection against the MBD of HBV WT capsids. All rounds of aptamer selection **(1^{st} – 13^{th})** showed 55 bp bands at the same position as the PCR product of the aptamer stock solution (positive control, **P**) in 10% non-dentaturing PAGE. The first rounds of selection (1^{st} – 4^{th}) showed large (> 55 bp) and short (~ 45 bp) PCR by-products which gradually decreased and nearly disappeared in the last rounds of selection (10^{th} – 13^{th}). **M:** marker, Gene Ruler 10 bp.

5.3 *Characterization of selected aptamers*

5.3.1 Aptamer sequencing

The ssDNA aptamers recovered from the final round (13th) of SELEX with counter selection were PCR amplified using a phosphorylated primer set (VBAO03 and VBAO04). The phosphorylated PCR product was purified by a QIAEX II Kit and an aliquot was ligated with *EcoRV* restricted, dephosphorylated and gel purified pBluescript II KS + (see 4.2.1.7.1.1, 4.2.1.4.1 and 4.2.1.7.2). The ligated product was ethanol precipitated and introduced into *E.coli* DH10B cells (see 4.2.1.7.3.2). Fifty clones were taken, enriched and subjected to the low scale plasmid preparation (see 4.2.1.7.4.1). Finally, the obtained plasmids were visualized in a 1% native agarose gel and sequenced (see 4.2.1.8).

Out of 50 clones, 12 clones (24%) showed concatemers of aptamers which increased the number of sequenced aptamers to 73. Only 16 different variants were obtained. The lengths of the sequences ranged from 52 to 56 nucleotides. One selected aptamer (AO-08, 56 nt) was longer and 4 selected aptamers (AO-09, 11, 15 & 16) were shorter than the original length (55 nt). Based on the sequence analysis, the first four aptamers represented more than 50% of all clones. The aptamer AO–01 was represented most frequently among the pool (~ 18%).

Alignment of the selected aptamers revealed four distinct regions of conservation (…CG…N0,1,2,3,5,7...**CCA**…N0,2,5…TG…N0,2,3,5…TG…). These four conserved sequences were found in all aptamers except both shorter aptamers, AO-11 and AO-16, which missed only one TG of the conserved sequences. Eleven different aptamers (69%) of all sequences including the most frequent one (AO-01) contained no nucleotide between the first two conserved sequences. So they contained a pentameric consensus motif (CG**CCA**). Out of these eleven aptamers, six aptamers (38%) showed a GC nucleotide sequence between the last two conserved sequences generating a hexamer consensus motif (TGGCTG). This hexamer motif followed directly to the former pentamer consensus motif (CG**CCA**) forming an undecamer conserved motif (CG**CCA**TGGCTG). Surprisingly, the best binder had also the same undecamer conserved motif but only one nucleotide (G) of the GC nucleotide sequence between the last two conserved sequences was replaced by a T nucleotide. The sequences of the aptamers AO-03 and AO-04 that have nearly the same frequency, showed great similarities as 10 consequent nucleotides (**CCA**TTCCGTG) are present in both of them. Finally, it was obvious that the consensus motifs were located mostly at the same positions (closer to the 3' end) in the original random region of selected aptamers (Table 13).

Tabel 13: Frequency and consensus sequences of selected aptamers.

Aptamer	Random sequence	No.	Frequency (%)	L (nt)
AO-01	CACACGCGAGCCG**CCA**TGTCTGGGC	**13**	**17.8**	**25**
AO-02	GGGACCGCAGAAGA**CCA**CATGTGCC	**11**	**15.1**	**25**
AO-03	GGGACGGCCCG**CCA**TTCCGTGTGGC	**7**	**9.6**	**25**
AO-04	GTCGACGCGC**CCA**TTCCGTGGGGTG	**6**	**8.2**	**25**
AO-05	GGCACACAACGTCG**CCA**TGGCTGTG	4	5.5	25
AO-06	CCCACGCAACGGCG**CCA**TGGCTGTG	4	5.5	25
AO-07	GCGTCGGCGCGCG**CCA**TTGTGGTGC	4	5.5	25
AO-08	GGGCAGGGTCGACCG**CCA**TGGCTGTG	4	5.5	26
AO-09	GGCACAAACGCG**CCA**TGGCTGC	4	5.5	22
AO-10	GCCAACGACGGGCCG**CCA**TGGTCTG	3	4.1	25
AO-11	GGCACAAACGCGGG**CCA**TCCATGC	3	4.1	24
AO-12	GGCACCCAACGCCC**CCA**TGGGTGTG	2	2.7	25
AO-13	GGGCAGGGTCGACCG**CCA**TGGCTGG	2	2.7	25
AO-14	CCGAGGGGCAACGGCG**CCA**TGGCTG	2	2.7	25
AO-15	CATAACGTTGCCCC**CCA**TGTGTTG	2	2.7	24
AO-16	GGCAGCCTCGACCCC**CCA**TGGC	2	2.7	22
Total		73	100	

5.3.2 Secondary structure prediction

Secondary structures of the selected aptamers were predicted by the Zuker algorithm (Zuker 2003), using Mfold (version 3.2) with conditions set up at 0.15 M NaCl and 25°C.

The potential secondary structures revealed that five variants (AO–01, AO–02, AO–03, AO–04 & AO–10) could be folded into very similar helix like structures with a large bulge close to the 3' fixed sequence. For the other aptamers different folds were predicted. However by forcing the conserved sequences within these variants to be single stranded using corresponding constraint in the software program, they could be folded into structures very similar to the proposed structure of AO–01 (Fig. 32). In all variants, the second consensus sequence (**CCA**) appeared mostly closer to the 5′ end of the bulge and the first consensus sequence (CG) formed the stem 5′ of the bulge while the two last consensus sequences (TG) are mostly positioned at end of the large bulge (Fig. 32).

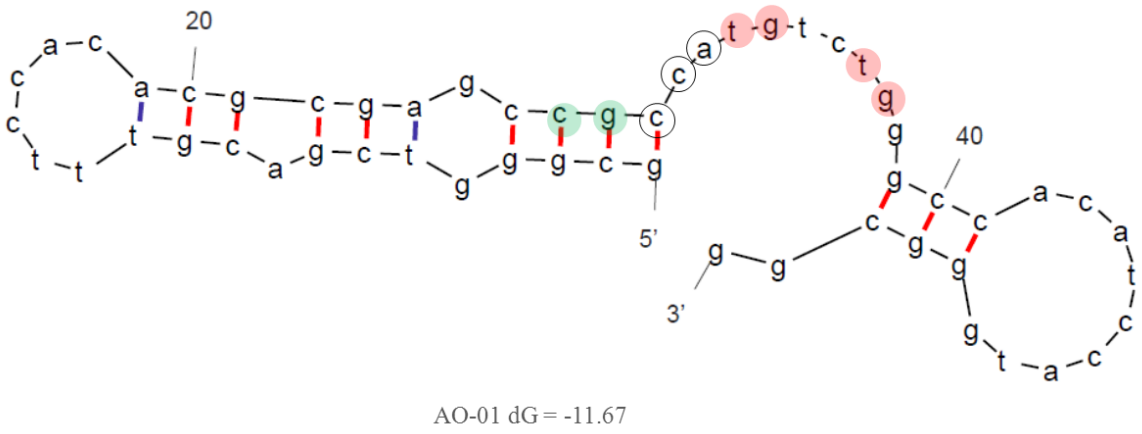


Fig. 32. Potential secondary structure of aptamer AO–01. Positions 1–15 and 41–55 are the primers sequences. The conserved nucleotides are green and red colored and serrounded by circles. All aptamers containing the conserved motif could be folded into similar secondary structures (after removing the possible base pairing of their conserved sequences) based on the folding algorithm of Zuker.

5.3.3 Binding characteristics

5.3.3.1 Binding affinity and specificity

The binding affinity of aptamer AO-01 (showed the highest frequency) to the HBV WT capsid was evaluated in comparison to its binding affinity to the HBV I126A mutant capsids. The presnap cooled aptamers (10 pmol) were incubated with the capsids (0.1 pmol) in binding buffer (PBS, 100 µl) at room temperature for one hour. For separating the free aptamers from the bounded ones, two different techniques, immunoprecipitation and filteration, were used. In the immunoprecipitation technique, the volume of the aptamer/capsid mixture (100 µl) was rised by binding buffer to 700 µl. The mixture was incubated with anti-HBc antibody coated agarose beads for the immunoprecipitation (see 4.2.4.1). In filteration technique, the volume of mixture was raised by binding buffer upto 2 ml and filtered through the alkaline pretreated filter (see 4.2.4.3). Finally, the bound aptamers were recovered by phenol / chloroform extraction, precipitated using a QIAEX II Kit (see 4.2.1.2.1/3) and their concentration were defined using a nanodrop machine and qPCR. As a negative control, the binding affinity of an aptamer with random region (AO-0N) against both capsids was evaluated using the same concentrations and conditions as with aptamer AO-01. The background was calculated in both immunoprecipitation and filteration assays by either incubating the same amount (10 pmol) of the presnap cooled

aptamers (AO-01 and AO-0N) with the anti-HBc antibody coated beads directly for one hour or by filtering them directly through the alkaline pretreated filters, respectively. Then they were recovered by the same procedure and their amount was calculated by a nanodrop machine and qPCR.

The background of both immunoprecipitation and filteration assays showed relatively low binding of the presnap cooled aptamers AO-01 and AO-N with the anti-HBc antibody coated beads (appr. 1%) and also with the filter matrix (appr. 2%), respectively. The results revealed that the number of AO-01 aptamers that bounded to WT capsids was 69.4 and 41.8 aptamers per capsid while the number bound to I126A mutant capsids was 12.8 and 12.5 aptamers per capsid were measured by filtration and immunoprecipitation assays, respectively. On the other hand, the number of AO-N aptamers that bound to WT capsids was 6.5 and 2.9 aptamers per capsid while the number of bound AO-N aptamers to I126A mutant capsid was 4 and 3.6 aptamers per capsid were calculated from filtration and immunoprecipitation assays, respectively (Table 14). The ratio of WT capsid bound negative aptamers (AO-N) to I126A mutant capsid bound aptamers was 0.8 and 1.6 fold (immunoprecipitation and filteration assays, respectively) indicating that there was only a low degree of unspecific binding of the negative aptamer to both capsids. The ratio of aptamer AO-01 bound to WT and I126A mutant capsids was 3.3 and 5.4 (immunoprecipitation and filteration assays, respectively) indicating that there was a specific binding of aptamer AO-01 to WT capsids (Fig. 33).

Tabel 14: Calculation of the number of bound aptamers per WT and I126A mutant capsid by filtration and immunoprecipitation (IP) partitioning techniques.

Aptamer / **HBV capsid**	AO-01		AO-N	
	filtration	IP	filtration	IP
WT	69.4 ± 11.2	41.8 ± 8.2	6.5 ± 2.2	2.9 ± 0.8
mutant	12.8 ± 2.30	12.5 ± 1.2	4.0 ± 1.7	3.6 ± 2.1

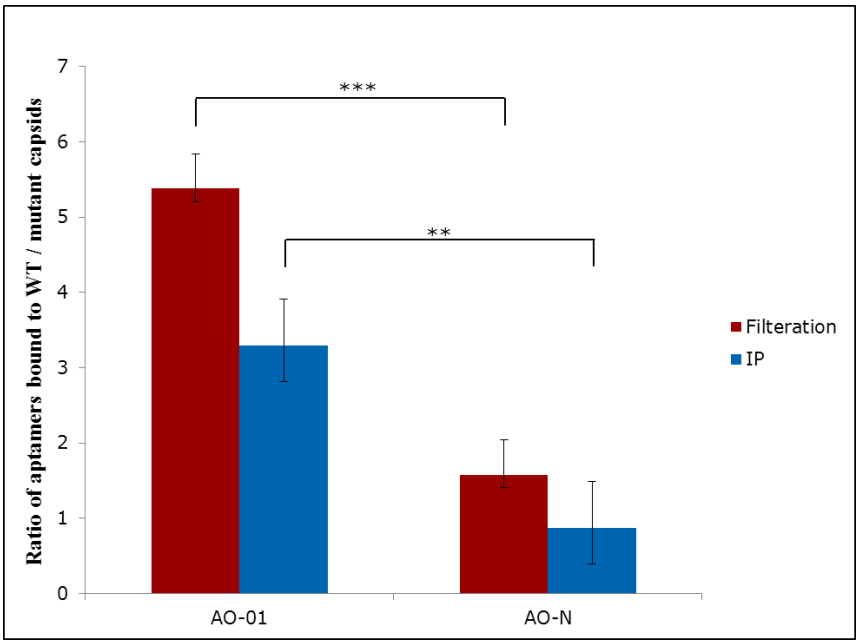


Fig. 33. Binding affinity and specificity of aptamer AO-01 to HBV WT delta-C capsids. The binding ratio of aptamer AO-01 to HBV WT and I126A mutant capsids was 3-4 fold more than that of the negative aptamer (AO-0N) using two different partitioning techniques, the filteration and immunoprecipitation. The binding affinity of the aptamer AO-01 to WT capsids was 3.3-5.4 fold more than its binding affinity to I126A mutant capsids. Mean values and standard deviations were calculated from 3 independent experiments. ***: $P \leq 0.01$; ***: $P \leq 0.001$.*

5.3.3.2 K_d determination

The K_d values of the four aptamers that had showed the highest frequencies (> 50%) after SELEX (AO-01, 2, 3 & 4) were determined against HBV WT and I126A mutant capsids, using the immunoprecipitation assay and the results were determined and analyzed by the SigmaPlot 12 software program (see 4.2.4.4, Fig. 34 and Fig. 35).

The results revealed that the K_d value of the aptamer AO-01 against WT capsids (180 ± 82 nM) was at least 7 fold less than its K_d value against I126A mutant capsids (1306 ± 503 nM) and also it was the lowest among the K_d values of the four aptamers (Table 14).

Tabel 15: Dissociation constants (K_d) of four selected aptamers against HBV WT and I126A capsids.

Aptamer	Frequency (%)	K_d / WT (nM)	K_d / Mut (nM)
AO-01	17.8	180 ± 82	1306 ± 503
AO-02	15.1	335 ± 96	1041 ± 449
AO-03	9.6	284 ± 169	925 ± 426
AO-04	8.2	369 ± 285	780 ± 452

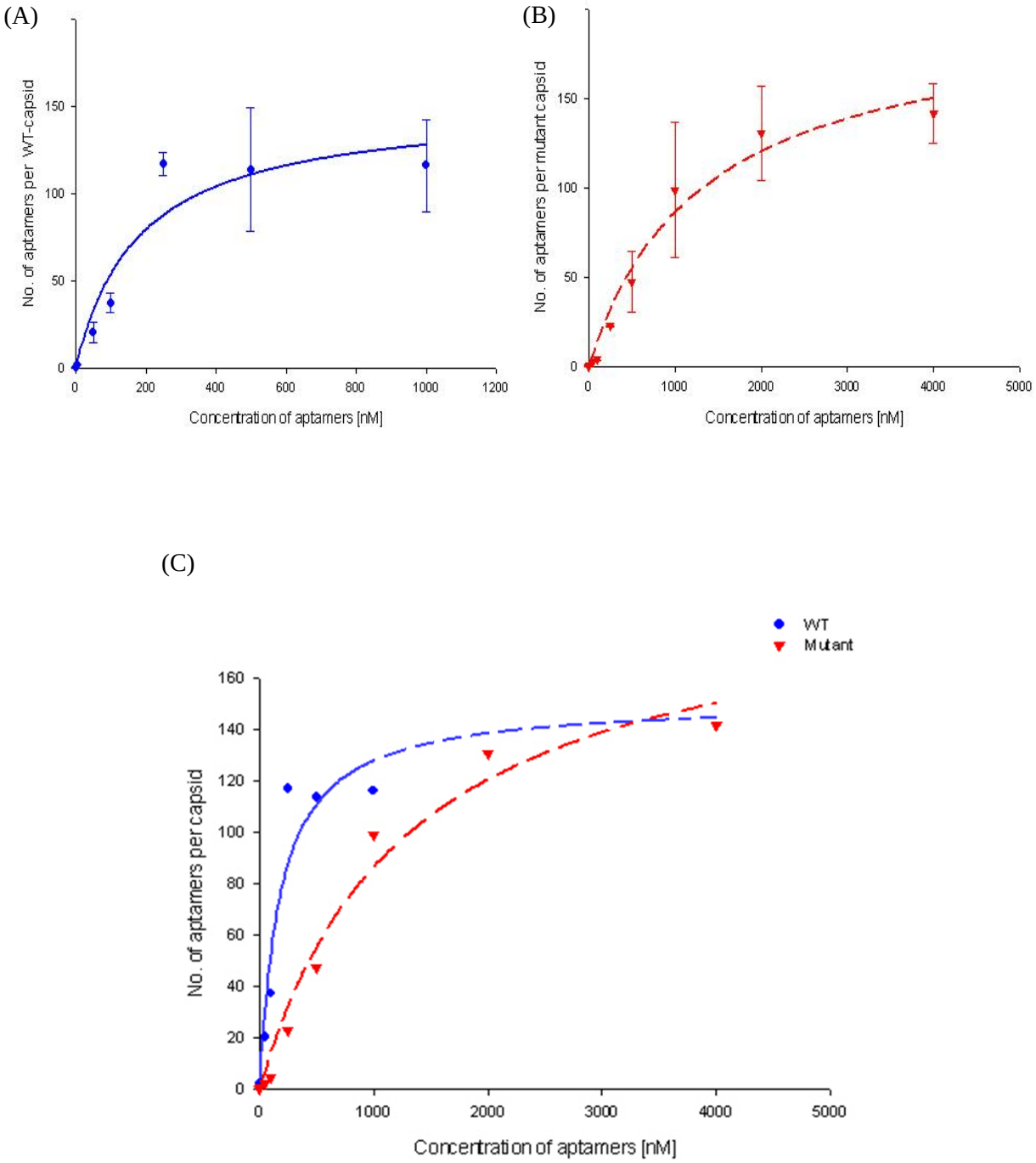


Fig. 34. SigmaPlot global curve fitting to measurements for determining K_d of best binder AO-01 against HBV WT and I126A mutant capsid. Increasing the concentration of the aptamer showed reproducible binding to a fixed concentrations of HBV WT and mutant capsids but the binding ratio to WT capsids **(A)** was much higher than that to the mutant one **(B)** and this could be easily observed in the merge of the figures **(C).** Mean values and standard deviations were calculated from 3 independent experiments.

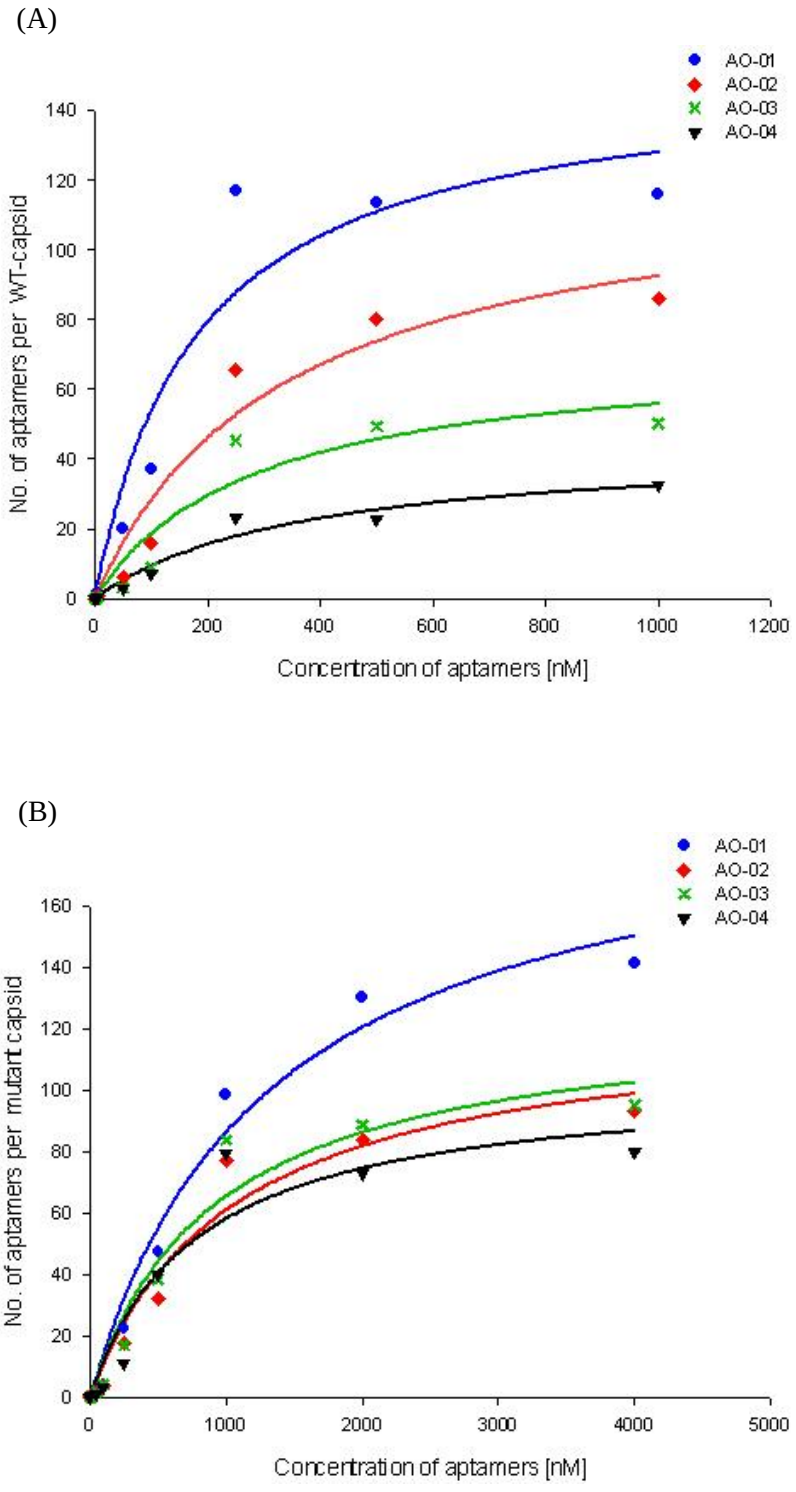


Fig. 35. Comparative SigmaPlot global curve fitting to measurements for determining K_d values of four selected aptamers against HBV WT and I126A mutant capsids. The binding ratios of the four selected aptamers to WT capsids was correlated with the aptamer frequency and this could be seen in the best binder AO-01 (of the highest frequency) which showed the highest binding ratio to WT capsids **(A).** On the other hand, the binding ratios of the four selected aptamers to the mutant capsids showed no marked correlation with aptamer frequency and also no great variance **(B)**. Mean values and standard deviations were calculated from 3 independent experiments and they are not shown here for better visualization.

5.4 *In vitro inhibition of nucleocapsid envelopment*

The inhibitory effect of the selected best binder (AO-01) on HBV capsid envelopment was preliminary tested in HuH-7 cell culture. In order to produce HBV virions in HuH-7 cells, the cells were transiently transfected with three trans-complementary plasmids: (1) pSVHBV1.1LE- contains the HBV genome carrying two stop codons in the surface protein ORF. Therefore it can not express the envelope proteins and consequently can not induce production of virions, (2) pSVBX24H carrying the gene for SHBs and (3) pSV45-57 harbouring genes encode for LHBs besides MHBs and SHBs.

The HuH-7 cells were transfected (see 4.2.5.4) by five different mixes. Three of them (P, S & N1) contained the same amount (1 µg, 1:1:1 molar ratio) of the three plasmids (pSVHBV1.1LE-, pSVBX24H and pSV45-570). The first sample (P) had only these 3 plasmids as a positive control, the second one (S) had the pre-snap cooled best binder AO-01 (1 µg) beside the three plasmids, the third one (N1) had the pre-snap cooled negative aptamer AO-0N (1 µg) together with the three plasmids as a first negative control, the fourth one (B) had pSVHBV1.1LE- only which could not produce virions alone as a background and the fifth one (N2) had no constructs at all as a second negative control. The cell supernatants were collected 3 days post-transfection. The secreted virions were immunoprecipitated with antibodies against SHBs and their genomes were recovered to calculate their concentrations using an HBV genome specific qPCR (see 4.2.4.2 & 4.2.1.9). The inhibition of HBV replication was calculated by the following equation:

$$\text{Inhibition \%} = 100 - \left[\frac{\text{No. of recovered genomes from the sample}}{\text{No. of recovered genomes from the positive control (P)}} \text{ X } 100 \right]$$

Nearly no signal was detected in the negative control (N2). The background (B) showed relatively high readings (nearly the half of the positive control). The results revealed that the best binder AO-01 (S) could inhibit virion secreation by 47.1 – 61.9 % while the negative aptamer AO-N (N1) show non significant degree of inhibition (< 5.5 %) (Fig. 36).

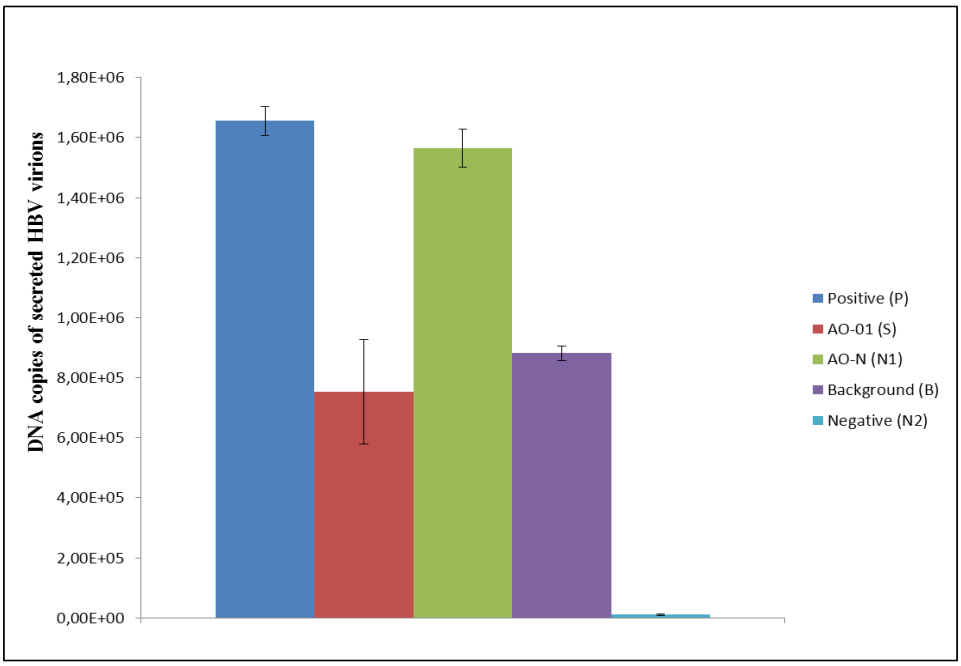


Fig. 36. The inhibitory effect of the best binder AO-01 on HBV virion production in HuH-7 cells. (S) The number of HBV virions secreted from cells cotransfected with the best binder besider AO-01 and the three HBV trans-complementary plasmids pSVHBV1.1LE-, pSVBX24H and pSV45-57. **(P)** The number of HBV virions secreted after transfection by the three HBV trans-complementary plasmids. **(N1)** The number of HBV virions secreted after cotransfection with the negative aptamer AO-0N and the three HBV trans-complementary plasmids. **(B)** The number of HBV virions secreted after transfection by the plasmid pSVHBV1.1LE- **(N2)** The number of HBV virions secreted after adding the transfection reagent on cells without any DNA molecules. The number of the secreted virions were measured 3 days post-transfection. Mean values and standard deviations were calculated from 4 independent experiments.

6 Discussion

In this project *in vitro* selection with counter selection was used to identify ssDNA aptamers that could bind to the MBD of HBV capsid to inhibit its interaction with the MD of the L envelope protein and consequently inhibits the HBV capsid envelopment and virion release. For *in vitro* selection with counter selection, both WT and I126A mutant HBV capsids were successfully expressed and purified to be used as a target and a counter target, respectively. The selected aptamer of the highest frequency (AO-01) bound to the target with a K_d of 180 nM in a very specific manner. Sequence alignment of selected aptamers revealed four conserved sequences (CG, CCA, TG & TG) among all high–affinity aptamers. These consensus sequences could be folded into a large bulge with a stem like structure by secondary structure prediction. In cell culture, the selected best binder (AO-01) could inhibit HBV virion production by at least 47 %.

6.1 *E.coli HBV expressed capsids*

To select ssDNA aptamers having high binding affinities to the MBD of HBV capsids, it was essential to over-express two HBV capsids, the WT and an envelopment-deficient mutant. As the capsid mutant the version I126A carrying a point mutation in the MBD residues. This mutant forms comparable amounts of capsids relative to the WT but can not be enveloped (Ponsel and Bruss, 2003). For assembly of the HBV capsid, the N-terminal 140 amino acids of the core protein are sufficient (Birnbaum and Nassal, 1990). The arginine-rich C terminus of the HBV core protein is not believed to be directly involved in capsid formation because core subunits without the basic C terminal region still assemble into shells. Furthermore, it was proposed that the C terminal region is important mainly for pregenome encapsidation (Gallina et al., 1989; Nassal, 1992; Zlotnick et al., 1997). No morphological differences can be observed between HBV capsids that were assembled in *E. coli* and the authentic capsids from virions of infected liver (Kenney et al., 1995).

The C terminal region has binding capability to nucleic acids. Therefore, to reduce the possibility of non-specific binding of aptamers, C terminal deleted HBV core proteins of both WT and mutant I126A were decided to be expressed in *E. coli*. The used expression plasmid was controlled by the *lac* operon which suppresses the expression of the desired protein (HBV core protein) until IPTG is added. After adding IPTG during the mid-log

phase of growth, HBV core protein could be successfully expressed in large amounts (see 5.1.2) (Wynne et al., 1998).

The Coomassie blue staining of both WT and I126A mutant core proteins in 15% SDS-denaturing gels after treatment with a reducing agent (DTT) could not distinguish between them. This is owed to the molecular weights of their delta-C core derivatives, 16847 and 16805 Daltons, respectively, which showed a narrow difference (only 42 Daltons). Moreover, the isoelectric point of both core proteins became nearly similar by the action of SDS that is present in both, denaturing gel and running buffer. Therefore, they produced nearly parallel bands in the gel (see Fig. 19). Under non-denaturing conditions in native agarose gels, however, a difference in the position of the band after electrophoresis was observable (see Fig. 20). This difference is propably not due to the difference in the molecular weight which is still relatively small (7560 Da for a 4 MDa particle), but the difference might be due to changes in the isoelectric point.

6.2 *Purification of the expressed capsids*

Throughout the purification steps, the immunodetection of both purified WT and mutant I126A capsids in 1% native agraose gels showed two bands (see Fig. 23/24). This is probably owed to different conformations of HBV capsids, T=3 and T=4. Two conformations of HBV capsids, T=3 and T=4, composed of 180 and 240 core proteins, respectively, can be found not only in the natural liver infections but also in the laboratory setting (Cohen and Richmond, 1982; Kenny et al., 1995). In this project, both WT and I126A mutant capsids consist of truncated core protein (149 aa in length). However, they could produce both conformations, T=3 and T=4, which is the same as the results described in the literature (Newman et al., 2003). Newmann and his colleagues showed that HBV capsids constructed from either full-length (183 aa) or truncated (149 aa) core proteins can produce the T=3 and T=4 conformations with a difference in their molecular weighs by at least 1 MDa which could be easily detected in the native agarose gel.

After purification of the both capsids by chromatography, the preparations containend high concentrations of the capsids with low amounts (nearly disappeared) of impurities except for a protein of approximaterly 14 KDa molecular weight (see Fig. 22). The Coomassie blue staining of all purified fractions in 15% SDS polyacrylamide gels revealed this protein. This protein was not detected by the immunodetection of the purified fractions in the denaturing gel using anti-HBc (see Fig. 23) indicating that it is a non specific protein and is not

correlated to the HBV core protein. Another possibility is that this 14 KDa protein is a fragment of the core protein loosing the epitope. Therefore, additional purification step, sucrose gradient ultracentrifugation, was used to remove this protein.

6.3 *Optimization of the in vitro selection protocol*

There are many different types of *in vitro* selection of aptamers. The choice of a suitable *in vitro* protocol for selecting aptamers against a target molecule depends largely on the target properties and the requirements of selected aptamers. In this project, the target is a small domain (MBD, 2.3 KDa) present numerously on the surface of a large particle (HBV capsid, 4 MDa). Moreover, there is a mutated form of the HBV capsid carrying only a point mutation (I126A) in the target domain inhibiting its binding to another protein (MD of L protein) which is an essential step in HBV capsid envelopment (Bruss, 2007). *In vitro* selection with counter selection is a specific type of SELEX depending on sequential steps of a positive selection against a protein containing the target epitope and a negative selection against a mutant protein devoid of this epitope or contains a mutated functionless one (Andreola et al., 2001). Thus, in this project, the method of counter selection was used for selection of aptamers against the MBD of HBV capsid (see Fig. 17).

The type of oligonucleotide library is an important aspect for the selection process. There is no difference between DNA and RNA aptamers in terms of affinity and specificity. However, DNA aptamers have certain advantages over RNA aptamers: (1) DNA aptamers are more stable than RNA aptamers in a broad range of conditions including biological fluids, which improve their effects in different clinical applications. (2) The *in vitro* selection procedure for DNA aptamers is much faster and easier than that for RNA aptamers (Gold et al., 1995a; Hermann and Patel, 2000). Therefore, a ssDNA library was chosen instead of a RNA library in this work.

In general, the selected specific binders in each round of the *in vitro* selection are accompanied with a number of non-specific binders. These non-specific binders are selected either by their non specific binding to the target by electrostatic interactions alone or due to the effect of incomplete separation between bounded and non-bounded sequences which is called in another term as background partitioning (Vant-Hull et al., 1998; Gerland et al., 2002). Therefore, multiple rounds of selection are generally needed in order to eliminate non-specific binders. The number of selection rounds depends largerly on the method of partitioning used in the experiment. For example, to complete the entire selection process in

which nitrocellulose membranes are used, 12-15 cycles are usually required. After that the selected molecules can be cloned into an appropriate vector and sequenced (Gopinath, 2007). In this project, the partitioning step was carried out by filteration using nitrocellulose membranes. Thus, 13 rounds of *in vitro* selection with counter selection were performed.

One problem facing the usage of nitrocellulose membranes during the partitioning step is that these membranes can adsorb nucleic acids to a large extent. The alkaline treatment of these membranes could greatly reduce the non–specific adsorption of ssDNA aptamers improving the filtration efficiency (see Fig. 27) (McENtee et al. 1980). Moreover, the pre-selection step against the membrane matrix removed the filter matrix binders from the ssDNA library. Therefore, almost 98% of the sequenced variants obtained after thirteen cycles of *in vitro* selection with counter selection usually turned out to be target binders (Gold et al., 1995).

For the in vitro selection, a chemically synthesized ssDNA library was used directly without PCR amplification. A different protocol was applied by some researchers on the aptamer library before starting the selection process. This protocol depends on PCR amplification of the random library in a large scale before initiating the selection process in order to eliminate damaged DNA synthesis products, which can not be amplified by PCR. However, the main drawback of this protocol is the possibility for losing some of the target–binding sequences in the original library due to the variance of PCR efficiency (Marshall & Ellington 2000; Dubertret et al., 2001). In the denaturing gel, the oligonucleotides in original ssDNA library showed the expected length (see Fig. 30A). Thus, a library with about 6×10^{15} sequences was used directly in the first round of selection.

For the overall *in vitro* selection procedure to be efficient, PCR amplification of the selected aptamers within the rounds of selection must be highly efficient. Fundamental differences could be detected between PCR amplification of homogeneous DNA templates and PCR amplification of random DNA libraries. In the PCR reaction, the homogenous DNA template is producing PCR products until primers are exhausted (plateau phase) while for the random DNA library the production of PCR products stops when PCR primers are still in excess. At this point PCR products convert rapidly to by-products and virtually disappear after a few additional PCR cycles. The conversion of PCR product to by-product is mostly induced by product-product hybridization within the heterogenous DNA libraries (Musheev and Krylov, 2006). It was confirmed that, by increasing the number of PCR cycles, the by-products were accumulated and their amounts increased gradually untill they dominated the

PCR products and the 55 bp long molecules nearly disappeared (see Fig. 29). Therefore, 15 PCR cycles were used. This showed the lowest concentration of PCR by-products in this project.

Streptavidin induced electrophoretic mobility shift offered an efficient and fast method for ssDNA preparation. The partitioning of biotinylated-DNA/streptavidin complex and the band purification of non-biotinylated ssDNA can be combined in a single step resulting in higher yield with less effort (see Fig. 30A). This technique was also suggested in the literature (Pagratis, 1996).

A risk during the *in vitro* selection process is the elimination of the strongest binders from the initial random pool in the first selection steps because the concentration of the strongest binders is extremely low at this time. Since the fraction of strong binders increases with the number of rounds, the risk of eliminating them is getting lower during the later rounds of the procedure. So, a higher protein to aptamers ratio should be used in the first rounds of selection, but then in the subsequent rounds this ratio should be reduced gradually (He et al., 1996). Therefore, in the positive selection, a higher WT capsid/aptamer ratio was used in the first 3 selection rounds and this ratio was reduced gradually untill it reached the lowest level in the last 4 selection rounds. On the contrary, in the negative selection the ratio of I126A mutant capsid/aptamer was increased gradually untill it reached the highest level in the last 4 selection rounds. Moreover, in the positive selection and contrary to the negative selection, the incubation time for the aptamer capsid binding was reduced gradually throughout the subsequent selection rounds until it reached the lowest level in the last 4 rounds. In addition, in the positive selection and contrary to the negative selection, the volume of the washing buffer was increased gradually throughout the subsequent selection rounds untill it reached the highest level in the last 4 steps (see Table 12). The aim of this procedure was to decrease the stringency of selection in the early rounds of the experiment, but to increase it later in the experiment, when the strongest binders are present in larger quantity. Such a procedure could induce a selective pressure and reduces the risk of losing the best binders during the *in vitro* selection (see Fig. 31). This procedure was also proposed by numerical simulations (Irvine et al., 1991).

6.4 *Structural features of the aptamers*

The aim of the *in vitro* selection is to identify the strongest binders for a given target molecule. Usually, the selection process reveals more than one selected aptamer. By comparing their sequence it is often possible to define a consensus sequence present in all or a major fraction. The existence of consensus sequence/s among the selected oligonucleotides is an indication for the successful selection. Usually, an oligonucleotide binder that is far from the consensus shows sequence-independent interaction with the target protein (Winter et al., 1981). In this work, sixteen variants were selected, and all showed four conserved sequences (CG, CCA, TG & TG) which indicated a successful selection. Four out of the sixteen selected variants showed nucleotide lengths different from the initial library (55 nt length) (see Table 13). This owed to insertions and deletions within the randomized region that presumably arose during PCR amplification or all of these variants were present already in the original library.

In principle, the interactions of proteins with single-stranded oligonucleotides are more complex than protein-dsDNA interactions. This is due to the fact that interactions of proteins with single-stranded oligonucleotides depend largely on nucleic acid secondary and tertiary structures (Jones et al., 2001). This was also supported by Nagai who showed that the proteins generally tend to interact with RNA in locations where the RNA forms secondary structure elements such as stem-loops and bulges (Nagai, 1996). In this project, the conserved sequences among all selected variants could be folded into a common motif of a bulge like structure by secondary structure prediction.

6.5 *Binding affinity and specificity of the aptamers*

After a certain number of *in vitro* selection rounds are performed, the affinity of the selected binders reaches an upper limit. The upper limit is determined by the affinity of the strongest binder in the initial random library. At the upper limit, most of the sequences in the selected pool will consist of the strongest binders (Vant-Hull et al., 1998; Djordjevic and Sengupta, 2006). In this project, four selected aptamers (AO-01, AO-02, AO-03 and AO-04) represented more than 50% of all sequenced clones. The aptamer AO–01 was represented most frequently among all selected sequences (~ 18%) (see Table 13). Therefore, the constant of dissociation (K_d) of these four selected aptamers to WT capsids was measured. Furthermore, to evaluate the specific binding of the selected aptamers to WT capsids, the K_d values of the four selected aptamers to I126A mutant capsids were also tested. The K_d

values of the four selected aptamers to WT capsids were correlated to a large extent with the aptamer frequency. E.g. the aptamer AO-01 had the highest frequency (~ 18%) and showed the lowest K_d to WT capsids (180 nM). On the contrary, the K_d values of the four selected aptamers to the mutant capsids showed no marked correlation with aptamer frequency and also no great variance (see Fig. 35 and Table 13). This indicates that the selected aptamers have specific binding affinity to WT capsids rather than to the mutant capsids. The I126A mutant capsid is the same as the WT capsid except only for its MBD. So, the selected aptamers have specific binding affinities to the MBD of HBV WT capsids.

In addition, the binding affinity of aptamer AO-01 to both WT and I126A capsids was tested in comparison with a negative aptamer (AO-N) which has not any of the four conserved sequences. The binding ratio of aptamer AO-01 to HBV WT versus I126A mutant capsids was around 4 whereas the ratio of the negative aptamer (AO-0N) was around 1 measured by two different partitioning techniques, immunoprecipitation and filteration. So, the random oligonucleotide did not distinguish between WT and mutant capsids while aptamer AO-01 preferentially bound to the WT capsid. In general, during the evaluation of aptamer AO-01 binding affinity to WT capsids, the filteration partitioning technique showed higher readings than the immunoprecipitation technique (see Table 14). This is owed to the background of the partitioning which seems to be higher for the filteration than for the immunoprecipitation method. Therefore, the immunoprecipitation technique was used during the K_d determination of selected aptamers.

6.6 *Inhibition of HBV nucleocapsid envelopment in HuH 7 cells*

To test the inhibitory potential of the best binder AO-01 on HBV release in HuH 7 cells, it was essential to monitor virions in the supernatant of transfected cells. One method with relatively strict constraints and drawbacks to achieve this purpose. This method was created by Landers and his colleauges and called endogenous polymerase reaction (Landers et al., 1977). It depends on the *in vitro* completion of the gap in the incomplete double stranded viral DNA genome which is contained in mature capsids by the viral polymerase which is packed within the same capsid using radioactive labelled nucleotides. These radioactively labelled genomes purified from the capsids of secreted virions can be easily visualized after gel electrophoresis by autoradiography. The usage of radioactive substances was considered as the major disadvantage of this method. Moreover, the measurement of HBV concentrations by e.g. southern blotting of the viral genome is relatively unsensitive and

hampered by the large amount of plasmids carrying HBV sequences in the culture supernatant resulting from the transfection.

A recent method for measuring HBV virions directly in the supernatant of transfected cell culture without using radioactive elements has been established (Schittl, 2012). This method is characterized by its high sensitivity as it relies on the quantification of the purified genomes from immunoprecipitated mature HBV virions using a specific real time qPCR discriminating between genomic and plasmid DNA. Small amounts of virions can be detected by this technique as only 5% of normal virion production was sufficient to get a clear positive signal. Therefore, this assay seemed to be fitted for estimating the potential inhibition of aptamer AO-01 on HBV virion release.

The results revealed a promising inhibition by the best binder AO-01 (at least 47%) in comparison with that of the negative aptamer AO-N (appr. 5.5%) (see Fig. 36). The number of MBD on the surface of one HBV capsid is probably between 90-120 MBDs. However, it is open whether almost all of the MBDs must be occupied by aptamers to block the capsid envelopment process or whether the binding of aptamers to some of these domains is sufficient for complete blocking of the envelopment process.

In conclusion, this study marks the first one showing the successful selection of an aptamer (AO-01) having a high binding affinity (180 ± 82 nM) to the MBD of HBV capsid. Moreover, this aptamer (AO-01) is also considered to be the first one that could preliminarly inhibit the envelopment process of a virus (HBV) in cell culture.

7 Summary

The hepatitis B virus (HBV) is a member of the family *Hepadnaviridae.* It causes human B-type hepatitis. Its high prevelence and the large incidence for severe liver diseases in HBV infected individuals make HBV infection a major global health problem. HBV is a hepatotropic enveloped DNA virus consisting of a nucleocapsid and a surrounding envelope containing three surface proteins refered as small (S), middle (M) and large (L) protein. During the formation of hepatitis B virions a small domain of a viral L envelope protein (matrix domain, MD) interacts specifically with a binding domain (matrix binding domain, MBD) on the capsid surface. This interaction can be blocked by introducing point mutations in any of the both domains resulting in the blocking of capsid envelopment and consequently the abortion of virion release. The binding of an aptamer to the MBD with high affinity and specificity could be an approach to inhibit this interaction. This would open new possibilities in producing antiviral substances against HBV infection.

In this project, specific aptamers for MBD on the HBV capsid surface were selected using *in vitro* selection with counter selection from an initial pool of 6×10^{15} different ssDNAs with a 25–nt central block of randomized sequence. A wild type version of a C-terminally deleted HBV capsid protein C and an envelopment deficient point mutant C-I126A were overexpressed in *E.coli*. They assembled into capsids which were purified by size exclusion chromatography and sucrose gradient ultracentrifugation. Thirteen rounds of SELEX using the wild type capsids for positive selection and the I126A mutant capsids for counter selection were performed. Twelve aptamers (AO-01 to AO-12) were enriched within 73 sequenced clones. All twelve aptamers showed four conserved sequences (CG, CCA, TG & TG) which form a loop like structure in models for the fold of most of the oligonucleotides. Four out of twelve selected aptamers (AO-01, AO-02, AO-03 and AO-04) represented more than 50% of all sequenced clones. Contrary to their dissociation constants (K_d) against mutant capsid, K_d values of these four aptamers against WT capsid showed direct correlation with their frequency.

The aptamer AO-01 with the highest frequency (18 %) showed the lowest K_d against WT capsids (180 ± 82 nM). Its K_d value against the I126A mutant capsids was at least 7 fold higher (1306 ± 503 nM). The best binder AO-01 was proven to be specific for the MBD. Its binding affinity to WT capsids was 3.3-5.4 fold more than that to the I126A MBD point mutant capsids. Testing of the inhibitory potential of the best binder AO-01 on HBV release in transiently cotransfected HuH-7 cell revealed 47 % inhibition by AO-01 and no inhibition by an aptamer with random sequence AO-N.

8 Zusammenfassung

Das Hepatitis B Virus (HBV) gehört zur Familie Hepadnaviridae. Es verursacht die Type B Hepatitis beim Menschen. Wegen seiner hohen Prävalenz und der hohen Inzidenz von schweren Lebererkrankungen in HBV-infizierten Personen stellt die HBV-Infektion ein bedeutendes globales Gesundheitsproblem dar. Das HBV ist ein hepatotrophes, umhülltes DNA-Virus, das aus einem Nukleokapsid und der umgebenden Hülle besteht, die drei Oberflächenproteine enthält, die als kleines (S), mittleres (M) und großes (L) Protein bezeichnet werden. Während der Bildung des Hepatitis B Virions interagiert eine kleine Domäne des viralen L Proteins (Matrix-Domäne, MD) spezifisch mit einer bindenden Domäne (Matrix-bindende Domäne, MBD) auf der Kapsidoberfläche. Diese Interaktion kann durch die Einführung von Punktmutationen in einer der beiden Domänen blockiert werden, was in einer Blockade der Kapsidumhüllung und daraufhin einer Inhibition der Virusfreisetzung resultiert. Die Bindung eines Aptamers an die MBD mit hoher Affinität und Spezifität könnte ein Ansatz sein, diese Interaktion zu inhibieren. Dies würde neue Möglichkeiten eröffnen, antivirale Substanzen gegen die HBV-Infektion zu entwickeln.

In diesem Projekt wurden spezifische Aptamere gegen die MBD auf der HBV-Kapsidoberfläche durch in vitro Selektion mit Gegenselektion von einem initialen Pool von 6 x 10^{15} verschiedenen ssDNAs mit einem zentralen Block von 25 nt zufälliger Sequenz selektiert. Eine Wildtyp-Version eines C-terminal deletierten HBV-Kapsidproteins C und eine umhüllungsdefiziente Punktmutante C-I126A wurden in E. coli überexprimiert. Sie assemblierten zu Kapsiden und wurden durch Ausschlusschromatographie und Sucrosegradientenzentrifugaiton gereinigt. Dreizehn SELEX-Runden mit dem Wildtyp-Kapsid für eine positive Selektion und der I126A-Mutante für die Gegenselektion wurden durchgeführt. Zwölf Aptamere (AO-01 bis AO-12) waren angereichert unter 73 sequenzierten Klonen. Alle zwölf Aptamere enthielten vier konservierte Sequenzen (CG, CCA, TG und TG), die eine schleifenartige Struktur in den Faltungsmodellen für die meisten Oligonukleotide bildeten. Vier der zwölf selektionierten Aptamere (AO-01, AO-02, AO-03 und AO-04) repräsentierten mehr als 50 % aller sequenzierten Klone. Im Gegensatz zu den Dissoziationskonstanten (K_d) gegen das mutierte Kapsid, zeigten die K_d—Werte gegen das Wildtyp-Kapsid von diesen vier Aptameren eine Korrelation mit der Häufigkeit ihres Auftretens innerhalb der 73 sequenzierten Klone.

Das Aptamer AO-01 mit der höchsten Frequenz (18 %) zeigte den geringsten K_d—Werte gegen das Wildtyp-Kapsid (180 +/- 82 nM). Sein K_d—Wert gegen das I126A-mutierte Kapsid war mindestens 7-fach höher (1306 +/- 503 nM). Es wurde gezeigt, dass das Aptamer AO-01 spezifisch an die MBD bindet. Seine Bindungsaffinität zu Wildtyp-Kapsiden war 3,3 – 5,4-fach höher als zu der I126A MBD-Punktmutante des Kapsids. Die Bestimmung des inhibitorischen Potentials von AO-01 auf die Freisetzung des HBV in transient kotransfizierten HuH7-Zellen zeigte eine 47% Inhibition, aber keine Hemmung durch das Aptamer AO-N mit zufälliger Sequenz.

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10 List of Figures

Figure 1: Morphology and structure of HB associated particles. 6

Figure 2: Transmembrane topology of the HBV surface proteins (Schittl, 2012) 7

Figure 3: Morphology of HBV capsid and the core homodimer (Pairan and Bruss, 2009; Conway et al., 1997) 8

Figure 4: HBV DNA genome showing the circular arrangement of the four overlapping but frame-shifted open reading frames (Schittl, 2012) 10

Figure 5: Life cycle and envelopment process of HBV 13

Figure 6: General scheme of the standard SELEX procedure. 17

Figure 7: DNA ladders 29

Figure 8: Protein ladders 29

Figure 9: pETM13. 30

Figure 10: pBluescript II KS (+) 30

Figure 11: pRVHBV + ... 30

Figure 12: pSVHBV 1.1LE- 31

Figure 13: pSVHBV1.1LE-I126A 31

Figure 14: pSV45-57 32

Figure 15: pSVBX24H. 32

Figure 16: Streptavidin induced electrophoretic mobility shift for ssDNA preparation 39

Figure 17: General scheme of the SELEX with counter selection. 53

Figure 18: Double, NCOI and SalI, restriction of DNA constructs encoding for HBV WT and its I126A mutant delta-C core protein. 60

Figure 19: Coomassie Brilliant Blue stained E.coli expressed HBV WT and I126A mutant delta-C core proteins in 15% SDS gel. 61

Figure 20: Immunoblotting detection of E.coli expressed HBV WT and I126A mutant delta-C capsids in a 1% native agarose gel. 62

Figure 21: Coomassie Brilliant Blue stained HBV WT (lanes 1 to 3) and HBV I126A mutant (lanes 4 to 6) delta-C core proteins after ammonium sulfate precipitation in 15% SDS gel. 62

Figure 22: Chromatogram and Coomassie Brillian Blue staining of fractions with high core protein content after size exclusion chromatography during HBV capsid purification. 64

Figure 23: Western blot validation of HBV WT and I126A mutant delta-C capsids and their core proteins after size exclusion chromatography 65

Figure 24: Western blot detection of HBV WT and its I126A mutant delta-C capsids in different fractions after sucrose gradient ultracentrifugation. 65

Figure 25: Comparative Coomassie Brillian Blue detection of delta-C core proteins of both HBV WT and I126A mutant before and after purification in 15% SDS gel. 66

Figure 26: Aptamers quantification by real-time PCR 67

Figure 27: Evaluation of filter alkaline treatment on the filteration efficiency 68

Figure 28: Western blot evaluation of the effect of filter alkaline treatment on HBV WT capsids 69

Figure 29: Dependance of molecular weight fragment length of PCR products on the number of PCR cycles 70

Figure 30: Positively selected aptamers in 10% non-denaturing and denaturing (6 M urea) polyacrylamide gels. 71

Figure 31: PCR products of thirteen rounds of 55 nt length aptamer selection against the MBD of HBV WT capsids 72

Figure 32: Potential secondary structure of aptamer AO–01 75

Figure 33: Binding affinity and specificity of aptamer AO-01 to HBV WT delta-C capsids 77

Figure 34: SigmaPlot global curve fitting to measurements for determining Kd of best binder AO-01 against HBV WT and I126A mutant capsid 78

Figure 35: Comparative SigmaPlot global curve fitting to measurements for determining Kd values of four selected aptamers against HBV WT and I126A mutant capsids 79

Figure 36: The inhibitory effect of the best binder AO-01 on HBV virion production in HuH-7 cells 81

11 List of Tables

Table 1: PCR reagents used during WT and mutant HBV capsids gene amplification..... 35

Table 2: PCR thermal profile used during WT and mutant HBV capsids expression..... 35

Table 3: PCR reagents used during aptamer selection and sequencing..... 36

Table 4: PCR thermal profile used during aptamer selection..... 36

Table 5: Contents of denaturing urea–polyacrylamide gel..... 38

Table 6: DNA restriction scheme during aptamer cloning..... 40

Table 7: DNA restriction scheme during WT and mutant HBV capsids preparation 41

Table 8: DNA insert/vector ligation scheme during WT and mutant HBV capsids preparation..... 41

Table 9: qPCR reagents used during the quantification of HBV genomes or selected aptamer..... 45

Table 10: qPCR thermal profile used during the quantification of HBV genomes or selected aptamer 45

Table 11: Reagents for15 % separating and 5 % stacking SDS gels..... 48

Table 12: Conditions during 13 selection rounds..... 54

Table 13: Frequency and consensus sequences of selected aptamers 74

Table 14: Calculation of the number of bound aptamers per WT and I126A mutant capsid by filtration and immunoprecipitation (IP) partitioning techniques..... 76

Table 15: Dissociation constants (K_d) of four selected aptamers against HBV WT and I126A capsids..... 77

12 Appendix

12.1 *Nucleotide sequence encodes for delta-C WT core proteins*

Atggacattgacccttataaagaatttggagctactgtggagttactctcgtttttgccttc
tgacttctttccttccgtcagagatctcctagacaccgcctcagctctgtatcgagaagcct
tagagtctcctgagcattgctcacctcaccatactgcactcaggcaagccattctctgctgg
ggggaattgatgactctagctacctgggtgggtaataatttggaagatccagcatccaggga
tctagtagtcaattatgttaatactaacatgggtttaaagatcaggcaactattgtggtttc
atatatcttgccttacttttggaagagagactgtacttgaatatttggtctctttcggagtg
tgg**att**cgcactcctccagcctatagaccaccaaatgcccctatcttatcaacacttccgga
aactactgttgtt (447 bp)

12.2 *Amino acid sequence of delta-C WT core proteins*

MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCW
GELMTLATWVGNNLEDPASRDLVVNYVNTNMGLKIRQLLWFHISCLTFGRETVLEYLVSFGV
W**I**RTPPAYRPPNAPILSTLPETTVV (149 aa)

12.3 *Nucleotide sequence encodes for delta-C I126A mutant core proteins*

Atggacattgacccttataaagaatttggagctactgtggagttactctcgtttttgccttc
tgacttctttccttccgtcagagatctcctagacaccgcctcagctctgtatcgagaagcct
tagagtctcctgagcattgctcacctcaccatactgcactcaggcaagccattctctgctgg
ggggaattgatgactctagctacctgggtgggtaataatttggaagatccagcatccaggga
tctagtagtcaattatgttaatactaacatgggtttaaagatcaggcaactattgtggtttc
atatatcttgccttacttttggaagagagactgtacttgaatatttggtctctttcggagtg
tgg**gct**cgcactcctccagcctatagaccaccaaatgcccctatcttatcaacacttccgga
aactactgttgtt (447 bp)

12.4 *Amino acid sequence of delta-C I126A mutant core proteins*

MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCW
GELMTLATWVGNNLEDPASRDLVVNYVNTNMGLKIRQLLWFHISCLTFGRETVLEYLVSFGV
W**A**RTPPAYRPPNAPILSTLPETTVV (149 aa)

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