MASTER OF SCIENCE THESIS 2014

Development of a Hepatitis B virus capsid as a nanocontainer and a carrier for protein delivery into cells

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1. Abstract

Encapsulation systems have long been studied, and the designs have varied in both capsule material as well as the type of cargo. The intended use have ranged from nanoreactors to delivery systems.

This work presents an encapsulation system consisting of protein capsule and a protein cargo. The capsule is a redesigned hepatitis B virus capsid protein, in which the luminal extension has been altered to bind the protein calmodulin instead of the viral genome. The cargo is consequently a calmodulin fused protein.

It is shown that co-expression of the redesigned capsid protein together with a capsid protein lacking a luminal extension greatly aids the solubility and assembly of redesigned capsids. Furthermore it is shown that the approximate 1:1 ratio resulting from the co-expression is maintained throughout disassembly and reassembly of the capsids.

2. Introduction

2.1 Purpose of the Project

2.1.1 General Goal

The purpose of the project is to create a capsid protein that can selectively encapsulate a specified protein. The idea is to accomplish this by redesigning the Hepatitis B virus capsid protein to selectively bind to Calmodulin.

The ability to encapsulate protein can be useful for several different applications. One such application could be protein delivery, where the virus' infectious properties could be utilized to release the encapsulated protein inside living cells. Another application could be nanoreactors, where enzyme studies could be carried out by encapsulating one or more enzymes within the confined space of the capsid.

The concept builds on 5 key components (Fig. 1):

1, an assembly competent capsid protein
2, a protein binding peptide
3, a linker sequence
4, a peptide binding protein
5, a protein of interest, fused to 4

These 5 components come together to encapsulate a protein of interest. Component 1-4 will be explained in further detail during the introduction.

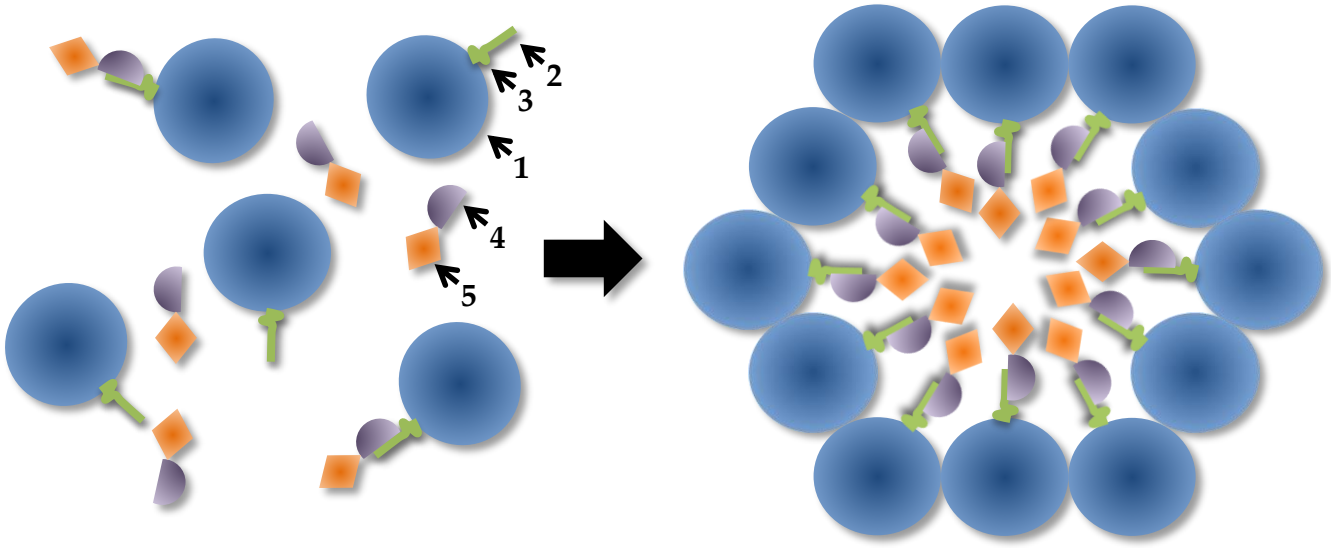

Figure 2.1. Concept Overview

Overview showing the 5 key components and how they come together to encapsulate the protein of interest.
1, capsid protein; 2, protein binding peptide; 3, linker sequence; 4, peptide binding protein; 5, protein of interest.

2.1.2 Specific Goal

This master's thesis continues the work from two previous master's projects carried out in the André Lab. The specific goal of this part of the project is twofold. The first part is to evaluate capsid forming potential of a capsid protein developed in a previous part of the project. The second part is to set up and evaluate a co-expression strategy in response to the results of the first part.

The co-expression strategy was developed as an attempt to increase the solubility of the capsids, after concluding that neither of the two previously developed constructs were soluble. We speculated that perhaps the c-terminal extensions were clashing as the capsids assemble, preventing the capsid formation and resulting in insoluble aggregates. By expressing the modified and truncated constructs simultaneously in the same cell, it is possible that this potential clashing issue could be circumvented.

2.2 Encapsulation

Systems for encapsulations have been developed in many different forms, the main application usually being either a nanoreactor system or a protection/delivery system. A nanoreactor could for example be used for various enzymatic studies and a delivery system could be used for drug delivery or gene therapy. The cargo molecules in these delivery systems can range from small molecules to proteins and nucleic acids, the encapsulating molecules have also shown a great variety from DNA[1, 2] to proteins[3] to various organic molecules[4, 5].

Work has also already been done on the Hepatitis B Virus, encapsulating the cargo either through C-terminal fusion[6] or with C-terminally truncated capsid proteins and random encapsulation through proximity inclusion[7].

Our contribution to the field of encapsulation will be the ability to target and encapsulate a specified cargo molecule, and allowing the encapsulation system to target various cargo molecules by modifying the cargo (through a fusion partner) rather than the capsid protein itself.

2.3 Hepatitis B Virus

2.3.1 Overview

The hepatitis B virus (HBV) is a human enveloped icosahedral RT-dsDNA virus, primarily infecting the human liver. HBV causes the disease known as hepatitis B and 240 million people are chronically infected resulting in more than 780 000 yearly deaths[8].

The viral particles used in this project lack envelope, viral genome and RNA binding capabilities and are hence completely harmless.

2.3.2 Structure

The HBV capsid protein is 183 AA long, weighs 21 kDa and consist of two main parts; a core domain (1-149) responsible for capsid assembly, and a protamine domain (150-183) responsible for nucleic acid binding[9]. It has been shown that a truncated capsid protein, consisting of the core domain alone, is still capable of capsid assembly[10]. The HBV capsids are approximately 30 nm in diameter, constructed from a collection of capsid protein dimers, and appear in two different sizes know as T=3 and T=4, containing 90 and 120 dimers respectively[9]. (See Fig 2 for a schematic overview of the structure)

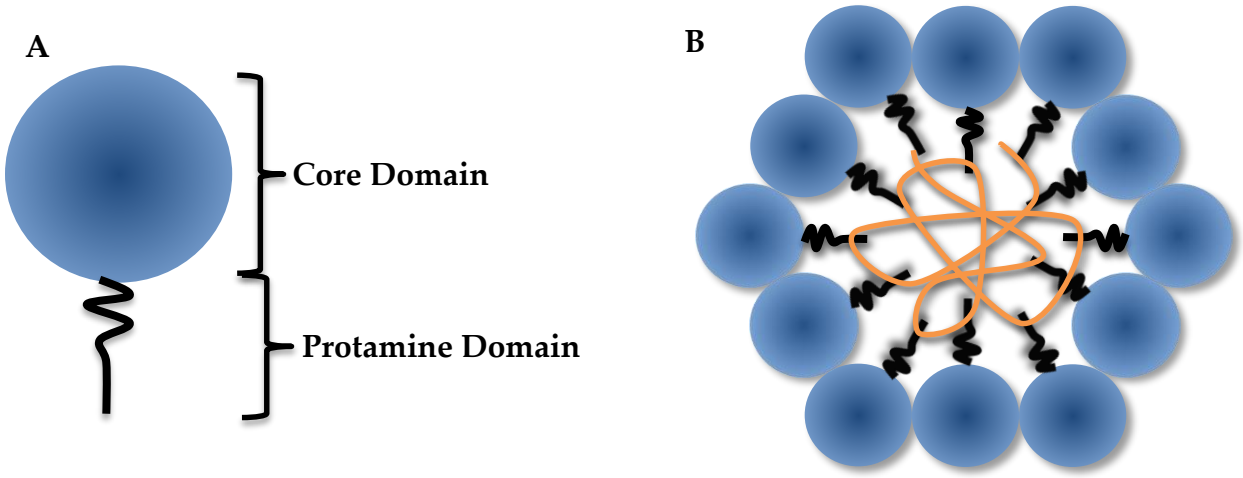

Figure 2.2. Schematic Overview of the Structure

A. Capsid protein monomer, and its two main parts; core domain and protamine domain.

B. Capsid proteins assembled into a capsid, surrounding the viral genome (orange).

2.3.3 Assembly and Disassembly

During HBV infection capsid assembly occurs in the human cytoplasm and is tightly coupled with the packing of the viral genome[11]. It has also been shown that the HBV core antigen, here referred to as HBVcore, spontaneously assemble into capsids when expressed in the *E. coli* cytoplasm in the absence of the viral genome[12].

These spontaneously assembled capsids can in turn be disassembled through treatment with urea. By adjusting the time and concentration it is possible to control whether the capsids disassemble into dimeric or monomeric form[7].
Disassembled HBVcore dimers can then be reassembled into functional capsids again, by the addition of NaCl followed by incubation on ice[13].

2.4 Calmodulin

2.4.1 Overview

Calmodulin is a calcium sensing signal protein expressed in eukaryotic cells, and consists of two globular domain, referred to as the C- and N-terminal domain. In the presence of sufficient levels of calcium ions calmodulin undergoes a conformational change exposing a hydrophobic patches and wrapping calmodulin around a target peptide (Fig. 3A), signaling to the bound protein elevated levels of Ca^{2+}. In the eukaryotic cell calmodulin interacts with a range of various intracellular proteins, such as kinases and Ca^{2+} pumps[14].

2.4.2 C20W

Upon studying a Ca2+ pump, a 20 AA long calmodulin binding peptide (C20W) was discovered. C20W was shown to interact only with the C-terminal domain of calmodulin (Fig. 3B). In solution the C20W peptide maintains an unstructured conformation, but upon binding to calmodulin it adopts a helical structure[14].

The combination of strong interaction and small size makes the ‘C20W-peptide’-‘C-terminal calmodulin domain’ pair an excellent choice for mediating selective protein encapsulation.

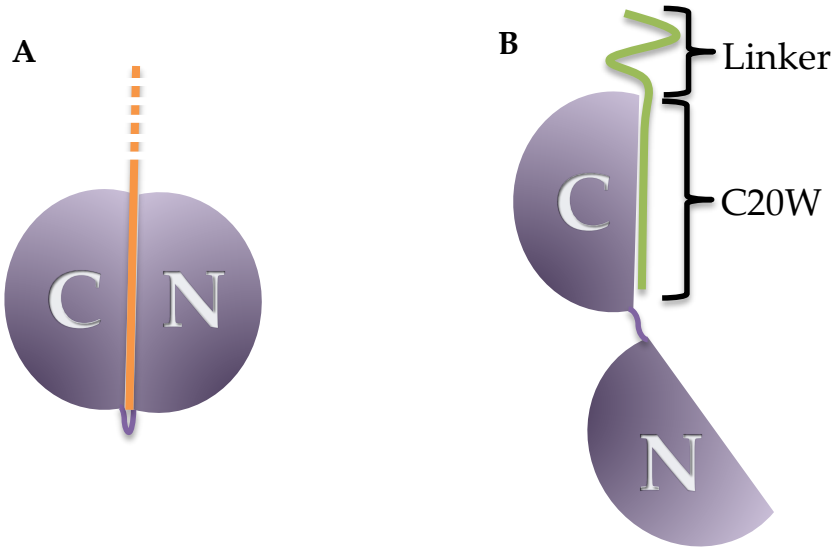

Figure 2.3. Schematic Overview of Calmodulin-Peptide Interactions
A. Ordinary calmodulin-peptide binding. B. Calmodulin binding to the C20W peptide.

2.5 Linker

A linker sequence is needed in order to distance the protein binding peptide from the capsid proteins core domain. During the linker design three different strategies were considered. The first was to model different linkers using computer simulation and choosing a linker that was predicted to present the protein binding peptide in a desired way. The second was to make the linker as flexible as possible, allowing it to adopt a large number of different conformations. The third was to mimic the C-terminal residues of the native protamine domain, possibly minimizing the risk of incompatibility with the capsid protein.
In this project we are evaluating a designed, computer simulated, linker and a native-like linker.

2.6 Specialized Techniques

2.6.1 Rosetta

Rosetta is a software for macromolecular structure prediction and design. It builds on the observation that most macromolecules adopt the conformation with the lowest free energy. Two of the keystones in the Rosetta software is the energy function, which simulates the physical interaction between atoms and conformational sampling, which allows it to detect local minima[15].

Rosetta was used for the prediction and identification of the simulated linker being evaluated in this project.

2.6.2 Ligase Independent Cloning (LIC)

Standard restriction enzyme based cloning can sometimes be a laborious process. An alternative method is that of ligase independent cloning (LIC), which builds on the fact that the base pairing between long single stranded overhangs is strong enough to survive transformation without the need for ligation.

LIC utilizes the dual nature of the T4 DNA polymerase's exonuclease/polymerase activity to create long single stranded overhangs (≥12 bp). In the absence of dNTPs the 3'-5' exonuclease activity would degrade any DNA it comes across. But if only a single type of dNTP (eg dGTP) is added the T4 DNA polymerase starts at the DNA's 3'-end and degrades the strand until it comes across the added nucleotide at which point the polymerase activity replaces the removed nucleotide faster than the exonuclease activity can remove the next nucleotide, effectively stalls the nuclease propagation. Resulting in a single stranded overhang, which's length is determined by the position of the 'stop nucleotide'[16] (See fig. 2.4)

Figure 2.4. Schematic Overview of LIC Overhang Generation

Arrow indicating the position of the 'stop nucleotide'. LIC site in orange.

2.6.3 Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) is a technique used to determine the size of particles (e.g. virus capsids). This is achieved by shining a laser into a solution with the particles suspended within it, and measuring the scattered light bouncing of the particles. DLS utilizes Brownian motion by correlating the effect that the moving particles have on the scattered light to the size of the particles.

2.6.4 Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy (TEM) is an imaging technique that irradiates a thin sample with an electron beam. The electron beam is recorded after passing through the sample, and the changes in the beam intensity is used to generate an image. Due to the small wavelength of electrons, electron microscopy can achieve a far higher resolution than standard light based microscopy.

Negative staining is a technique that stains the background rather than the sample itself. This is done by adding a solution containing a heavy metal (eg vanadium) which spreads out uniformly, but is excluded from the space occupied by the sample. This results in a dark background with light areas outlining the sample.

2.7 Previous Work

The work in this project builds upon two previous master projects carried out by Anna Bille and Robert Lizatović.

2.7.1 Anna Bille

During the master project of Anna Bille capsid formation of the truncated HBV capsid protein (Cp149/ HBVcore) was verified. A set of different designs of C-terminal extensions containing a linker sequence and the calmodulin binding peptide C20W were modeled in Rosetta, the most promising structure (HBVmod-A) was chosen. HBVmod-A was cloned, expressed and shown to aggregate, initial refolding attempts were made[17].

2.7.2 Robert Lizatović

During the master project of Robert Lizatović extensive refolding attempts of HBVmod-A were made without any success, refolding was concluded to be an ineffective approach. A new design was made (HBVmod -R), where the linker sequence was redesigned to mimic the corresponding part in the native HBV C-terminal extension[18].

2.7.3 Summary

HBVcore: Truncated version of the native HBV capsid protein that has been shown to independently form capsids. The removed residues correspond to an unstructured 'tail' extruding from the main domain (residues 1-149) and is responsible for the binding of viral RNA.

HBVmod-A: Modified HBVcore protein with a C-terminal extension designed to present a calmodulin binding peptide (C20W) to the capsid lumen. The protein is prone to aggregation upon overexpression.

HBVmod-R: Modified HBVmod-A protein where the linker sequence of the extension has been redesigned to mimic the native HBV structure. The protein has not yet been tested.

Amino acid sequence for all protein can be found in Appendix A and vector maps for all plasmids used in the project can be found in Appendix C.

3. Results

3.1 Cloning

Three plasmids were generated during this project. pET11a_HBVmod-R encoding the protein HBVmod-R. pDual-A/R encoding HBVcore as well as HBVmod-A or HBVmod-R. The reasoning for the pDual plasmids are elaborated under the expression results section.

3.1.1 Construction of pET11a_HBVmod-R

The construction of pET11a_HBVmod-R was successful and resulted in a 6160 bp large plasmid carrying the gene for HBVmod-R under a LacI repressed T7 promoter. The plasmid retained the normal features of pET11a, such as the genes for LacI and β-lactamase (Ampicillin resistance) and is kept at low copy number (Fig. 3.1). Colony PCR and sequencing results came back positive.

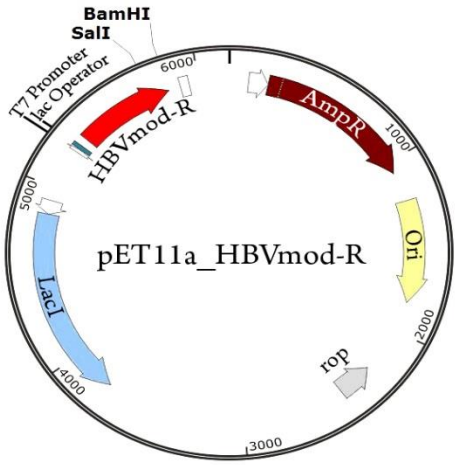

Fig. 3.1, Vector pET11a_HBVmod-R
Containing the gene for HBVmod-R under a LacI repressed T7 promoter. Plasmid contains genes for Ampicillin resistance and the LacI repressor. Plasmid is kept at low copy number. Restriction sites used in the cloning are included for reference.

3.1.2 Construction of pDual-A and pDual-R

The construction of pDual-A and pDual-R was successful. The pDual plasmids encode genes for both HBVcore and HBVmod-A/HBVmod-R for Dual-A/Dual-R respectively (Fig. 3.2). The gene for HBVcore is separated from the gene for HBVmod by 106 bp counting from the end of the T7 terminator to the beginning T7 promoter. Both genes are under the control of identical LacI repressed T7 promoters as well as identical ribosome binding sites (RBS).

Colony PCR, digestion verification and sequencing results came back positive.

Heating the mixture and allowing it to slowly cool during the annealing step didn't seem to have any significant impact of transformation efficiency.

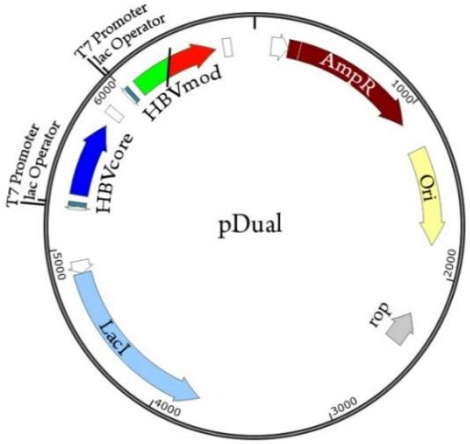

Fig. 3.2, pDual Vectors
Containing genes for both HBVcore and HBVmod. Where HBVmod corresponds to HBVmod-A/-R for pDual-A/-R respectively.
Features from pET11a are preserved.

See Appendix C for vector maps of all vectors used in the project.

3.2 Expression

3.2.1 HBVmod-R

In order to evaluate the modified capsid protein (HBVmod-R) from Robert Lizatović's master project the pET11a_HBVmod-R was expressed.

Analysis by SDS-PAGE (Fig. 3.3) of the expression revealed that HBVmod-R mainly ends up in the insoluble fraction, likely due to aggregate formation. The capsid proteins migrate faster across the gel than what normally would be expected from their size. The band of the HBVcore (16.8 kDa) lies slightly below the 15 kDa band of the ladder, and the band of the HBVmod-R (19.6 kDa) lies just above the ladder's 15 kDa band.

Furthermore cells carrying the pET11a_HBVmod-R vector showed a slower growth rate after induction compared to cells carrying the pET11a_HBVcore vector.

Lysis using the Bugbuster® detergent cocktail resulted in all capsid proteins becoming insoluble, both for HBVmod-R and HBVcore.

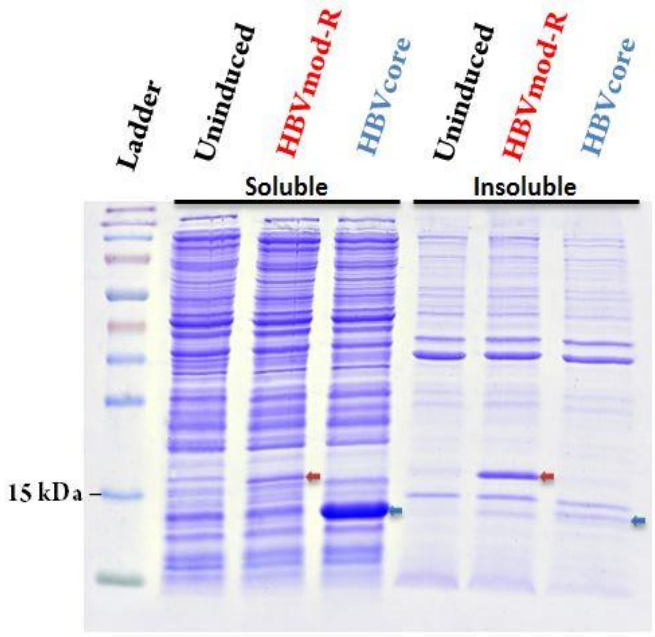

Fig. 3.3, SDS-PAGE - Expression of HBVmod-R
Expression of HBVmod-R. Uninduced culture and cells expressing HBVcore included for reference. Divided into soluble and insoluble fractions.
Red arrows indicate the position of the HBVmod-R and blue arrows indicate the position of HBVcore.

3.2.2 Dual Expression Strategy

Since HBVmod-R was shown to aggregate a new strategy had to be constructed. Previous refold attempts of HBVmod-A had proven unsuccessful and refolding was deemed an unsuitable approach. Instead a dual expression strategy was formulated, in which the modified capsid proteins were co-expressed from the same plasmid as the HBVcore protein. The construction of these plasmids is described in the cloning results section. Dual-A/Dual-R is used to signify the co-expression of HBVcore and HBVmod-A/HBVmod-R respectively.

The main reasoning behind the dual expression was our hypothesis that perhaps the C-terminal extensions (Linker and Calmodulin binding peptide) were clashing during capsid assembly. Some support to this hypothesis is given by capsid 3D structures showing the C-terminals of HBVcore proteins being in close proximity (See Fig 3.4)[19]. In the fully assembled capsids 5 dimers C-terminals interact at the same point, rather than only the two shown in the figure. Mixing the modified capsid proteins with HBVcore proteins would potentially give the C-terminal extensions some additional space during capsid assembly.

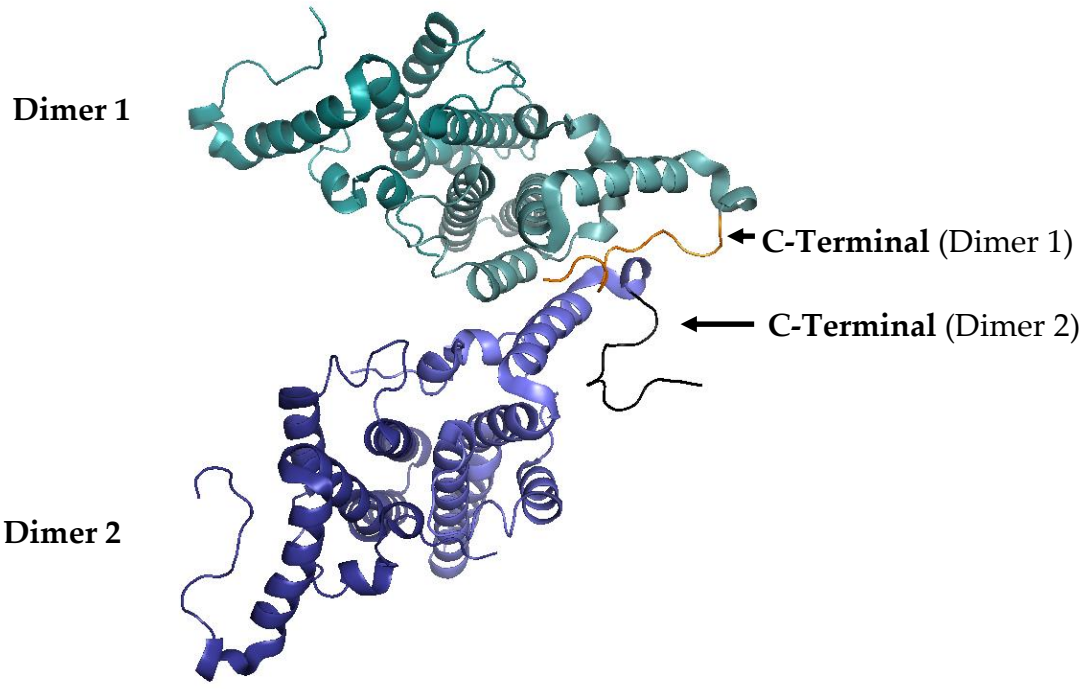

Fig. 3.4, CryoEM 3D structure of HBVcore Dimers
3D structure of two interacting HBVcore dimers from a CryoEM structure of an assembled HBVcore capsid. Two of the C-terminals are shown in orange and black respectively. The structure has a resolution of 3.50 Å (PDB: 3J2V).

3.2.3 Dual Expression Results

Analysis by SDS-PAGE (Fig. 3.5) of the dual expression revealed that the co-expression of the protein pairs had a solubilizing effect on the modified capsid proteins. The effect was greatest on the Dual-A co-expression, where nearly all capsid proteins seemed to remain in the soluble fraction. The co-expression of Dual-R did increase the percentage of HBVmod-R proteins remaining in the soluble fraction. However, it also increased the percentage of HBVcore proteins ending up in the insoluble fraction. It is worth noting that the resolubilization technique used is imperfect, and the percentage of insoluble capsids may be greater than what the gel shows.

The growth rate of cells carrying pDual-R was slower than that of cells with pET11a_HBVcore, but not as slow as cells with pET11a_HBVmod-R. Carrying pDual-A also seemed to slow the growth rate when compared to pET11a_HBVcore, but to a far lesser degree than pDual-R. The growth rate data are only derived from preliminary results and further testing should be carried out in order to draw any reliable conclusions.

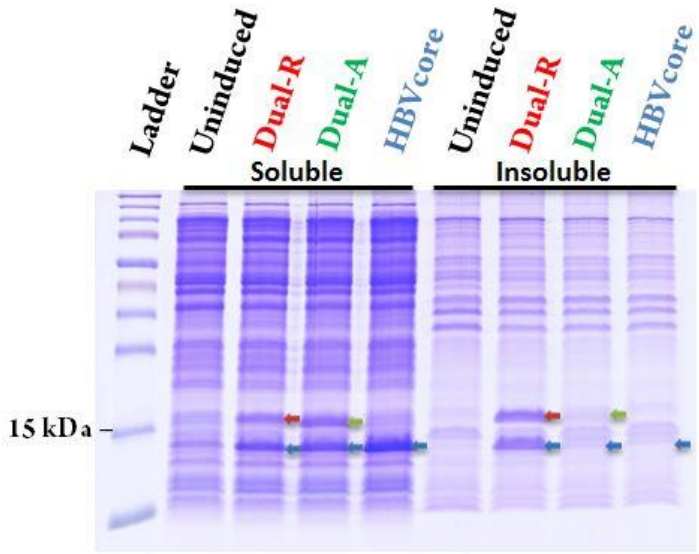

Fig. 3.5, SDS-PAGE – Dual Expression

Expression of Dual-R and Dual-A. Uninduced culture and cells expressing HBVcore included for reference. Divided into soluble and insoluble fractions. Arrows indicate the positions of the proteins: Blue = HBVcore, Red = HBVmod-R, Green = HBVmod-A

3.3 Purification

3.3.1 Purification strategy

In order to obtain pure dimers a two step size exclusion purification strategy was implemented, utilizing the unique property of capsid protein complexes to drastically change their molecular weight between the assembled/disassembled states.
The purification strategy can be sumerized in the following way:

0. Crude Lysate

- Content: In vivo assembled capsids + soluble cytoplasmic proteins

1. High MW size exclusion chromatography (Sepharose CL-4B)

- Content: Assembled capsids + High MW proteins/complexes

2. Urea disassembly

- Content: Disassembled capsid dimers + High MW proteins/complexes + disassembled complexes

3. Low MW size exclusion chromatography (Supradex 75)

- Content: Pure disassembled capsid dimers + minor contaminants

The purified dimers were subjected to concentration and buffer exchange by centrifugal filtration and either used for reassembly directly or stored at -80 °C until needed.

3.3.2 Purification results

The cell lysates from the various expression cultures were purified by high MW size exclusion chromatography and portioned into fractions, which in turn were analyzed by UV spectroscopy (Fig. 3.6) and SDS-PAGE (Fig. 3.7). All the different constructs showed similar analysis results (figures showing HBVcore capsid purification).

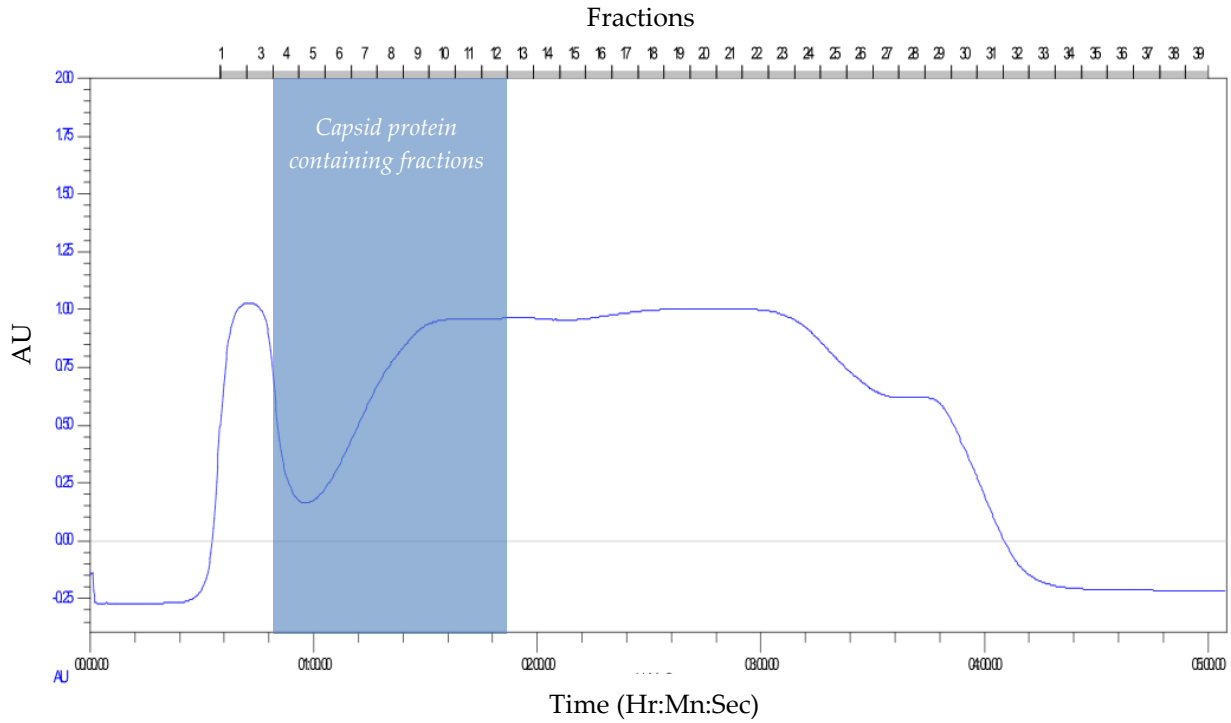

Fig. 3.6, FPLC Chromatogram – Capsid Purification
Chromatogram from UV spectroscopy analysis of HBVcore capsid purification.
Blue overlay showing position of the capsid protein.

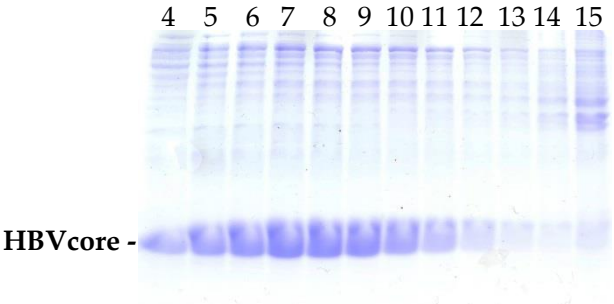

Fig. 3.7, SDS-PAGE – Capsid purification
Purification of HBVcore capsids.
Numbers corresponding to fractions in Fig. 3.5.

Following the capsid purifications, the constructs were disassembled and purified again by low MW size exclusion chromatography, separating on smaller sizes as to purify the dimers resulting from the disassembly. The elute was fractionated and analyzed by UV spectroscopy (Fig. 3.8) and SDS-PAGE (Fig. 3.9). All the constructs showed similar analysis results, (figures showing Dual-A dimer purification).

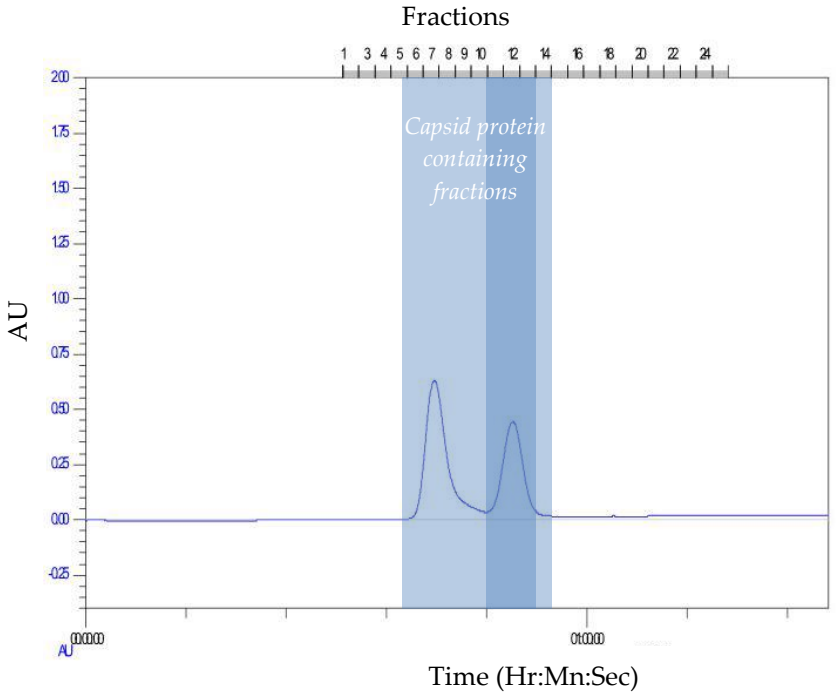

Fig. 3.8, FPLC Chromatogram – Dimer Purification
Chromatogram from UV spectroscopy analysis Dual-A dimer purification. Blue overlay showing position of the capsid protein.

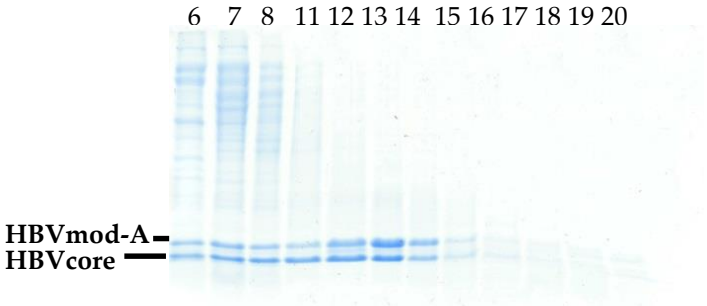

Fig. 3.9, SDS-PAGE – Dimer Purification
Purification of Dual-A dimers. Numbers corresponding to the fractions on Fig. 3.7.

3.4 Reassembly

Reassembly Overview

Reassembly was carried out on concentrated dimers from the purification step thawed from storage at -80°C. Dimers were mixed with NaCl and incubated on ice, in order to trigger the capsid reassembly.

Three different reassemblies were performed. The first one on HBVcore and Dual-A. The second one on a new batch of proteins, testing Dual-A at different protein ratios as well as Dual-R and HBVcore at standard protein ratios. The third one on stored dimers measured without any treatment.

Reassembly of HBVcore and Dual-A

Following the dimer purification HBVcore and Dual-A were to be reassembled. HBVcore reassembled just as expected and was confirmed by size exclusion chromatography (Fig. 3.10). The purity was assessed by SDS-PAGE (Fig. 3.11). No contaminants could be detected.

Dual-A did not reassemble and aggregates were confirmed by visual inspection.

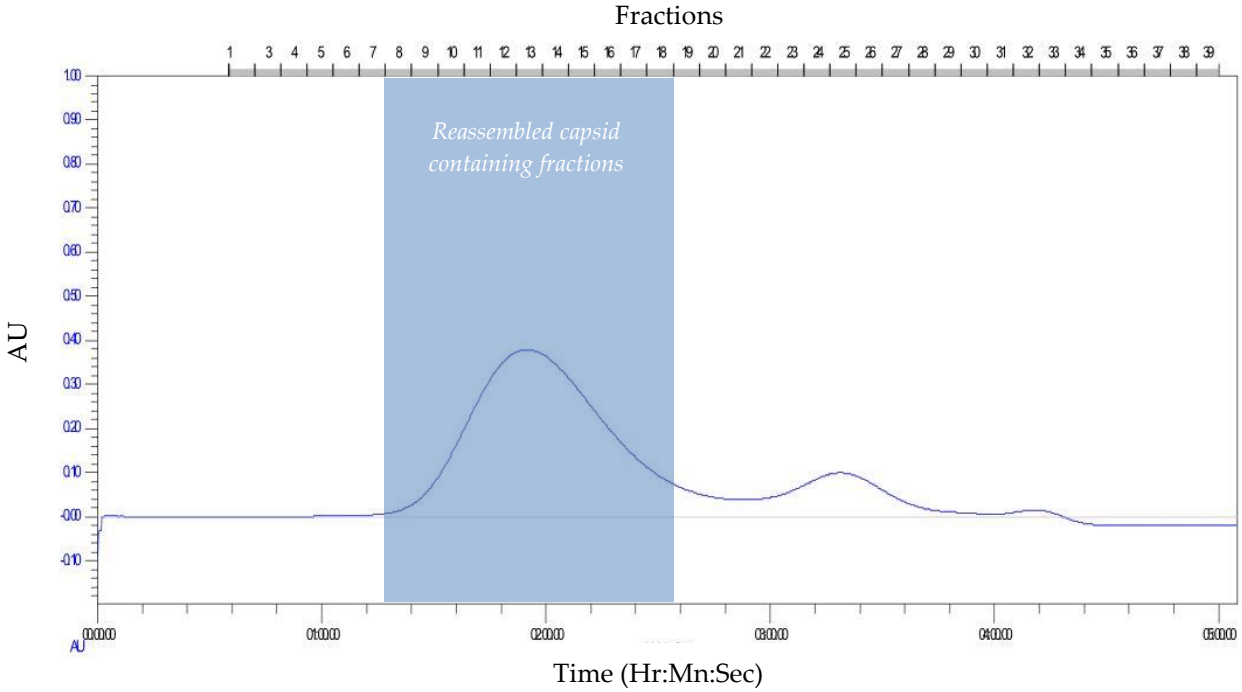

Fig. 3.10, FPLC Chromatogram of HBVcore Reassembly

Chromatogram from UV spectroscopy analysis of reassembled HBVcore capsid purification. Blue overlay showing position of the reassembled capsids.

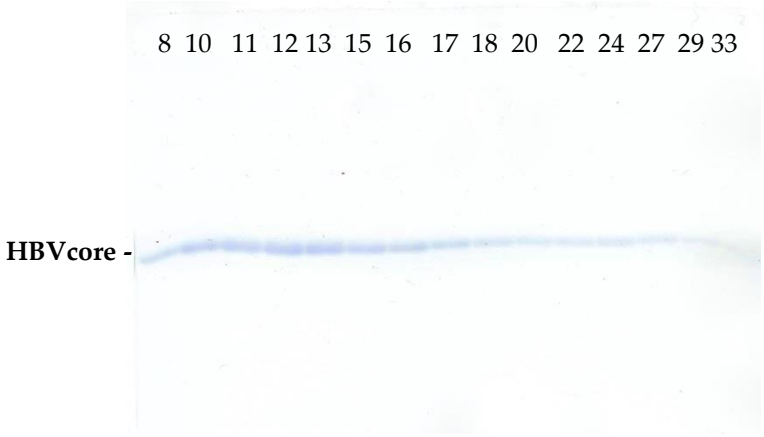

Fig. 3.11, SDS-PAGE of FPLC Fractions
Numbers 8-28 corresponds to the fractions seen in Fig. 3.9. Samples appear to be completely free of contaminants.

Ratio Dependent Reassembly

In order to work around the aggregation of Dual-A, a ratio dependent reassembly was performed. Dual-A was mixed with HBVcore to create protein mixtures containing HBVmod-A at different concentrations (25% / 5%). Dual-R and HBVcore was reassembled for comparison.

DLS was performed on the samples and it was confirmed that all samples were in the capsid state, the reassembly induced as well as the negative controls that were expected to be in a dimeric state (See fig 3.12 for a representative example). The size of the reassembled capsid corresponded to the expected size, and measured approximately 17 nm in average radius.

Sample supernatants were loaded onto SDS-PAGE (Fig 3.13) and it was confirmed that the Dual-A capsids did indeed consist of both HBVcore and HBVmod-A. However, the Dual-R capsids only contained HBVcore, while HBVmod-R aggregated.

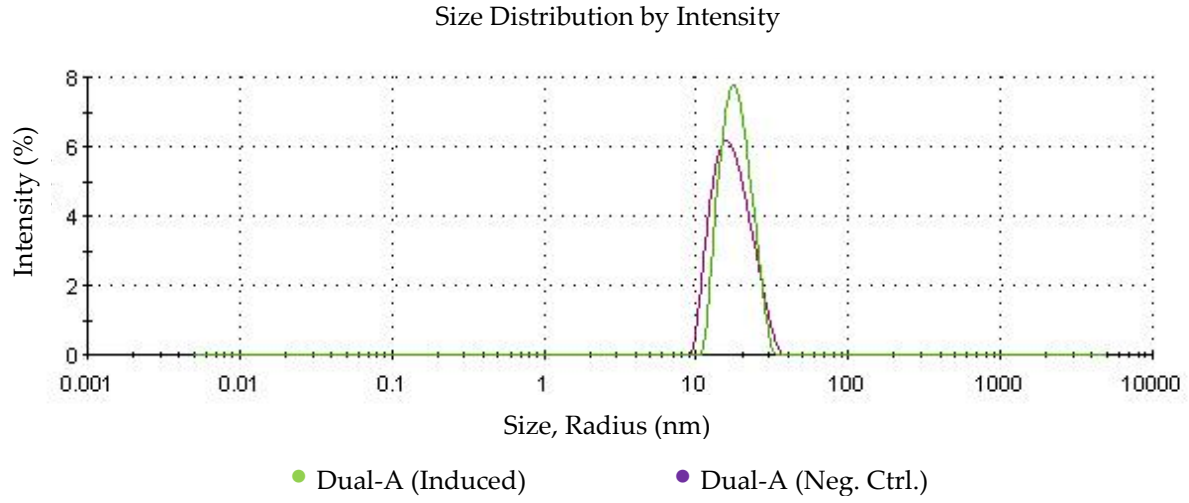

Fig. 3.12, DLS of Reassembled Capsids
Representative result from DLS measurement.
Purple; Dual-A (Neg. Control), Green; Dual-A (Induced)

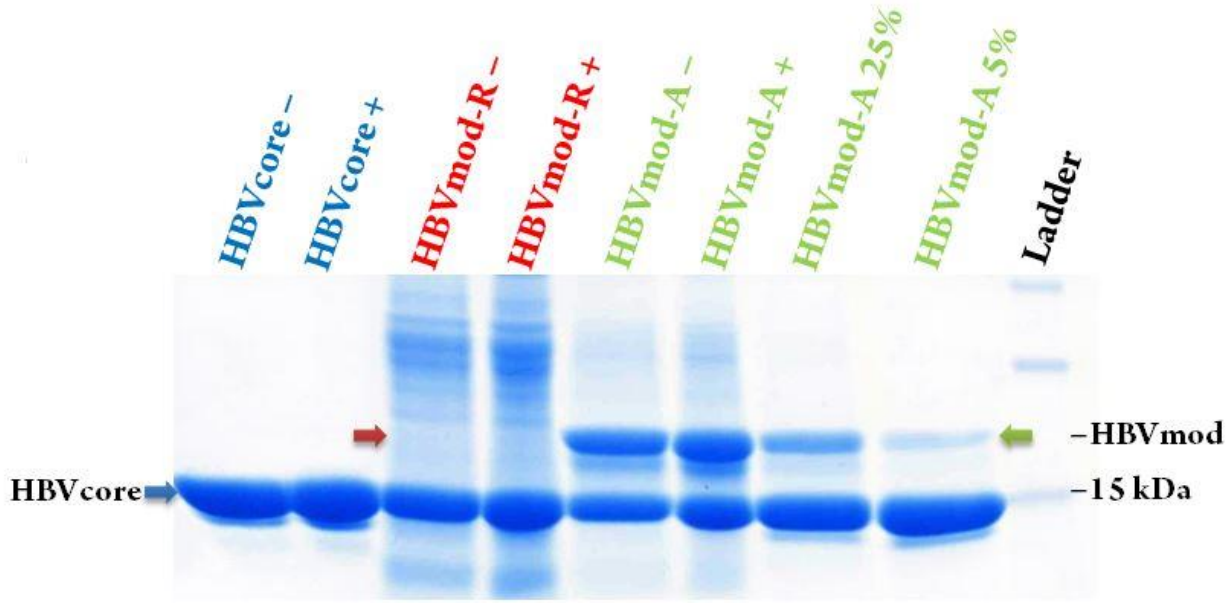

Fig. 3.13, SDS-PAGE - Supernatant of Reassembly mixtures
"+" indicates that NaCl was added to induce reassembly and "-" indicates a negative control, in which only H_2O was added. 25% and 5% indicates the fraction of the capsid proteins that where HBVmod-A. Blue arrow shows the position of HBVcore. Green arrow shows the position of HBVmod-A. Red arrow indicates the expected position of HBVmod-R.

DLS Measurement of Stored Dimer Samples

Since the negative controls in the previous experiment all showed unwanted capsid formation, the stored dimer samples were tested in order to investigate whether the dimers had reassembled before being stored at -80 °C. DLS measurements were made directly on freshly thawed samples (Fig. 3.14), without any incubation or further treatment.

The DLS reported a clear peak with a maximum around 18 nm, consistent with previous measurements. Hence, indicating that the dimers most likely had reassembled before being frozen at -80 °C.

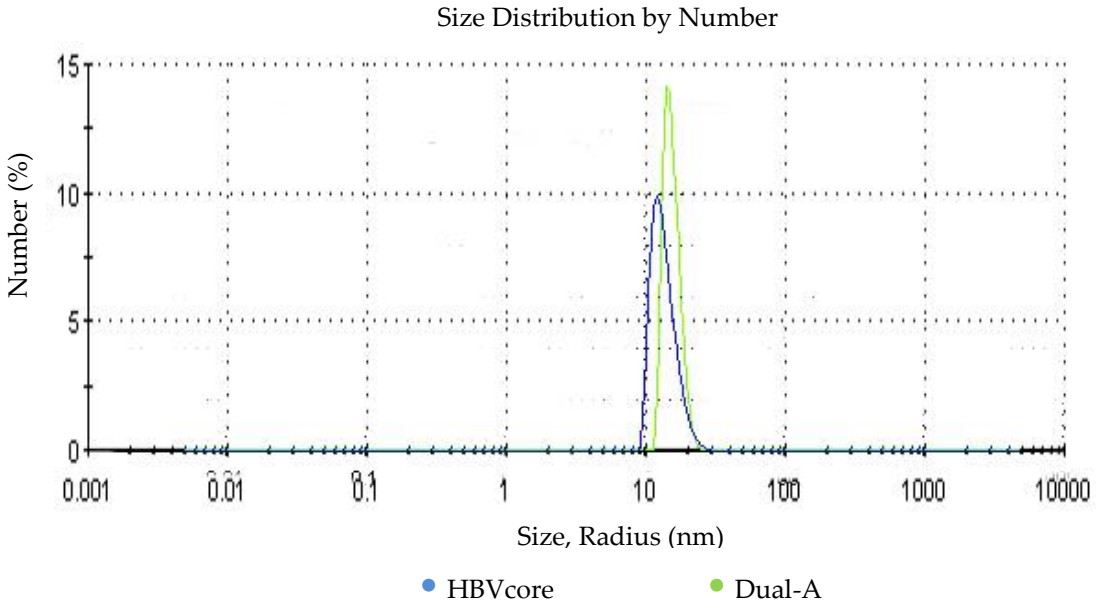

Fig 3.14, DLS - Freshly thawed samples
Sample measured directly after being thawed from -80 °C storage.
Blue; HBVcore, Green; Dual-A

3.5 Electron Microscopy Inspection

Negative stain TEM inspection of the reassembled Dual-A sample confirmed that the sample does indeed assemble into spherical capsids (Fig. 3.15). The reassembled HBVcore sample was used as a positive control.

The sizes of the capsids were measured to approximately 30 nm, which correlates well with previously reported data of HBVcore capsids[9]. The capsids appear in two distinct sizes, corresponding to the T=3 and T=4 morphologies, with the majority assembling into the slightly larger capsid (T=4).

Furthermore many capsids seemed to remain intact despite the harsh conditions of negative stain TEM, such as drying and vacuum exposure.

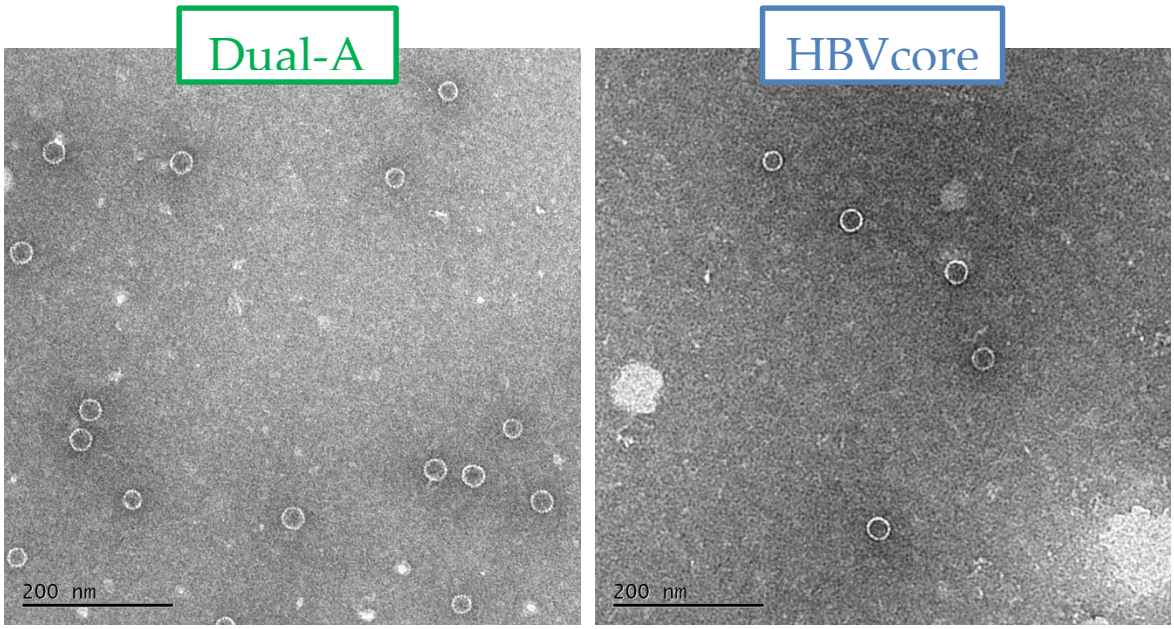

Figure 3.15, TEM picture of Assembled Capsids
Left: Dual-A Capsids, Right: HBVcore capsids (Positive Control).
Capsid diameters: ~30 nm. Scale bars included for reference.

4. Discussion

4.1 Expression

Dual Expression

The analysis of the dual expression (Fig. 3.4) revealed that Dual-R is prone to aggregation. Furthermore the aggregating HBVmod-R seem to bring HBVcore proteins down with them, while still maintaining what appears to be a 1:1 ratio. More thorough studies of the insoluble fractions would need to be performed before any real conclusions can be drawn. But if the ratio truly is maintained at 1:1 it could potentially indicate that the cause of aggregation is not luminal crowding or altered capsid structure, but rather an effect of the C-terminal extension occasionally blocking the capsid protein's interaction sites. There is also a few other explanations that potentially could result in a maintained 1:1 ratio.

4.2 Purification

Calmodulin Affinity Chromatography

The purification procedure is rather time consuming and wasteful. One way to simplify the process could be to utilize the calmodulin binding peptide of the HBVmod-A/-R to perform a calmodulin affinity chromatography. By adding urea to the cell lysate it might be possible to shorten the purification protocol to a one step procedure. This could potentially reduce the time invested and increase the number of capsid proteins retained. However, HBVcore cannot be purified using this method and if applied to Dual-A/-R it would reduce the HBVcore : HBVmod ratio from 1:1 to 1:2 if purified as dimers, and to 0:1 if purified as monomers. A 1:2 ratio might be enough to form stable capsids, or separately purified HBVcore could be added to create any desired ratio. Either way, calmodulin affinity chromatography could potentially help decrease purification time as well as increase purity and purification yield.

C-terminal Cysteine

Regardless of the purification method used it might be a good idea to do a point mutation in the HBVcore gene. Originally the genes had been modified to replace all cysteines with alanines. However, a cysteine was left at the very end of the HBVcore C-terminal. Replacing this cysteine would simplify the purification procedure by eliminating the need to use a reducing agent, such as DTT. The cysteine has been

replaced by the extensions in the HBVmod constructs, but it still remains a problem while expressing the pDual vectors due to its presence in the HBVcore gene.

4.3 Reassembly

Premature Reassembly

During the capsid reassembly studies it was reviled that the dimers most likely had reassembled before being frozen and stored at -80 °C. The most likely cause is the preceding buffer exchange step, in which the sample buffer is changed from buffer N (pH 9.6) to buffer H (pH 7.5) using centrifugal spin filters. It is possible that during this buffer exchange the concentration increased enough for the dimers to spontaneously reassemble in the absence of NaCl. This could be avoided in two different ways, either by using regular dialysis filters or by ensuring that the concentration never exceeds 2 mg/ml during the spin filtration.

Reassembly by concentration might be a useful method. It would be fairly straight forward to mix capsid dimers with the cargo or protein of interest and then concentrating the mixture by spin filtration, ideally resulting in the cargo being encapsulated by the capsid protein. This method also have the benefit of being proven to work as where the NaCl based reassembly seemed to cause Dual-A to aggregate.

Final Verification

Even though all evidence (DLS, SDS-PAGE, EM) appear to indicate that the reassembly has been successful, a final verification must be made to eliminate all alternative explanations. Dual-A must be reassembled at larger scale, and purified by size exclusion chromatography followed by SDS-PAGE analysis. Only when this has been done can it be concluded that HBVmod-A is indeed a part of the assembled capsid.

4.4 Electron Microscopy

Choice of Protocol

The staining protocol used for the EM gave a clear image of the reassembled capsids. However, it did result in a slightly high background staining. If EM is to be used in the future, it might be worth to revise the staining protocol in order to obtain even better images.

4.5 Future work

Final Verification

As mentioned previously, one final verification step remains in order to eliminate any doubt about the capsid reassembly.

Calmodulin binding

Once the capsid has been confirmed the next step would be to confirm that the capsid can bind and encapsulate calmodulin. This would be achieved by mixing Dual-A dimers with calmodulin, reassembling the mixture and verify encapsulation. HBVcore should be used as a negative control to ensure that encapsulation is specific and caused by the extension.

Calmodulin fusion proteins

As a final verification of the system calmodulin would be fused to a protein of interest followed by a protein specific encapsulation. Once this has been confirmed the primary goal of the project has been achieved. A good starting point for proteins of interest would be enzymes or fluorescent proteins. For an enzyme the reassembled capsids could be purified and enzyme activity measured. For a fluorescent protein (e.g. GFP) the reassembled capsids could be loaded onto a native PAGE. Potentially the encapsulation could even be confirmed by EM.

Alternative Extension Design

Even though the Dual-A construct seem to work, it might still be interesting to have a system consisting only of a modified capsid protein. If the aggregation is due to binding site blockage, a redesign of the calmodulin binding peptide might be of interest. Upon binding to calmodulin the peptide forms an alpha helix, with one face interacting with calmodulin while the rest is solvent exposed. It is likely possible to redesign the binding peptide to be more hydrophilic while still preserving the key residues for the binding interaction.

5. Material and Methods

5.1 General Methods

Agarose Gel Electrophoresis

Gels were cast with 0.5x TBE buffer containing GelStar™ (Lonza) stain and agarose concentrations varying from 0.8% to 3% depending on desired separation. Samples were loaded using a 10x sample buffer and run under constant voltage of 130 V, in 0.5x TBE buffer.

DNA was extracted from the agarose gels using GeneJet gel extraction kit (Thermo Scientific).

Calculations

Ammonium Sulfate Saturation

Ammonium Sulfate Saturation ($(NH_4)_2SO_3$ calculations were performed using EnCor Biotechnologies' 'Ammonium Sulfate Calculator'[20].

Primer T_m

Primer melting temperatures (T_m) calculations were performed using Thermo Scientific's 'Tm Calculator'[21].

Concentration Determination

Protein concentration was determined by measuring the absorbance at 280 nm using a NanoDrop 2000 Spectrophotometer.

Extinction coefficients (ε) and molecular weights (MW) used:

HBVcore: $\varepsilon = 29\,400\ cm^{-1}\ M^{-1}$
MW= 16.81 kDa

HBVmod-A: $\varepsilon = 35\,090\ cm^{-1}\ M^{-1}$
MW= 19.74 kDa

HBVmod-R: $\varepsilon = 34\,970\ cm^{-1}\ M^{-1}$
MW= 19.63 kDa

Dual-A: $\varepsilon = 32\,245\ cm^{-1}\ M^{-1}$
MW= 18.28 kDa

Dual-R: $\varepsilon = 32\,185\ cm^{-1}\ M^{-1}$
MW= 18.22 kDa

DNA sequencing
DNA sequences were obtained by Sanger sequencing carried out by LGC Genomics ltd.

Growing Liquid *E. coli* Cultures
Cells were grown in complex media at 37 °C at 220 rpm using baffled flasks and a liquid volume no more than 20% of the flask volume.

PCR
PCR were carried out using Phusion® HF polymerase (Thermo Scientific) in 1x Phusion HF buffer with 0.2 mM dNTPs and 0.5 μM of each primer. The thermal cycling was performed by a PTC-200 Peltier Thermal Cycler. For specifications on each program see Appendix B.
Amplified DNA was purified using GeneJet PCR purification kit (Thermo Scientific)
Colony PCR was carried out using DreamTaq polymerase (Thermo Scientific) in 1x DreamTaq buffer with 0.2 mM dNTPs and 0.5 μM of each primer, using a colony suspension as template.

Plasmid Purification
Plasmids were purified using GeneJet Plasmid Miniprep Kit (Thermo Scientific).

Protein Concentration and Buffer Exchange
Protein Concentration and Buffer Exchange were carried out at 4 °C using Vivaspin® 20 centrifugal concentrators with filter sizes of either 10 or 100 kDa.

SDS-PAGE
Gels used were either cast in-house with 15% acrylamide in a tris-glycine buffer or supplied as tris-glycine precast gels (BioRad) containing 10-20% acrylamide. Gels were run for approximately 55 min at 180 V in a Tris-glycine running buffer.
Samples were loaded using a 4x sample buffer.
The ladder used was Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific)

Transformation

Chemical transformation was performed using calcium competent *E. coli* cells.
50 µl competent cells were thawed on ice for 30 min. 1-5 µl DNA was added to the cells and incubated on ice for 1 hour. The cell-DNA mixture was heat shocked for 45 s at 42 °C and then incubated on ice for 5 min before being transferred to 1 ml SOC media and incubated for 1 hour at 37 °C. Cells were plated on LA plates containing appropriate antibiotic and incubated at 37 °C overnight.
Cloning constructs were cloned into ER2566 cells.
Purified plasmids were then transformed into BL21*(DE3)pLysS and BL21 Tuner™.

5.2 Materials

5.2.1 Enzymes

PCR

Phusion® HF (Thermo Scientific)

Colony PCR

DreamTaq (Thermo Scientific)

Standard Cloning

BamHI and SalI(Fast digest) (Fermentas)

T4 DNA Ligase (Fermentas)

FastAP - Alkaline Phosphatase (Fermentas)

Lysis

Benzonase® Nuclease (Novagen)

LIC

T4 DNA Polymerase (NEB)

DpnI (Fermentas)

BamHI – Construct verification

5.2.2 Primers

All primers were supplied by Life Technologies.

5.2.3 Other

Bugbuster® (Novagen)

Protease Inhibitor Cocktail (Roche)

Ladders

Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific), 10 – 260 kDa

GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific), 75 – 20 000 bp

5.3 Cloning

5.3.1 Construction of pET11a_HBVmod-R

Construction of Insert

The insert was constructed by running a PCR using a self-priming primer pair (See Appendix B, Reaction 1), with each primer being a template for the other (Fig. 5.1) yielding a DNA fragment containing the HBVmod-R C-terminal extension as well as an overlap with a portion of HBVcore's C-terminal end. The resulting fragment was purified.

Fig. 5.1, Primer Pairing
Primers annealing to eachothers 3′ end, resulting in the full insert after amplification.

Preparation of Vector and Insert

Both the pET11a_HBVcore vector (for vector map, see Appendix C; v.1) and the purified insert were digested by BamHI for 4 hours at 37 °C. The digested DNA was purified again, followed by a second digestion by SalI (FastDigest) where the vector and insert was treated for 60 min at 37 °C, an alkaline phosphatase (FastAP) was included in the vector digestion.

Ligation of Vector and Insert

The digested vector and insert were then ligated by T4 DNA ligase during incubation at 22 °C for 14 hours. The ligation mixture was used to transform chemically competent *E. coli* cells, and the plasmid uptake was confirmed by colony PCR. Sequence confirmation was done by DNA sequencing of the CDS.

5.3.2 Construction of pDual-A and pDual-R

The construction of the dual expression plasmids (pDual-A/pDual-R) was carried out through ligation independent cloning (LIC). See appendix C for vector maps.

Preparation of Vector and Inserts

A LIC enabled vector was created by PCR amplification of the whole pET11a_HBVcore vector, using a pair of primers each carrying an extension containing a unique LIC site. (For specifications, see Appendix B, reaction 3).

The inserts were amplified by PCR using a pair of primers targeting the T7 promoter and the T7 terminator. Each primer also had an extension containing appropriate LIC sites complementary to that of the LIC enabled vector. pET11a_HBVmod-A/pET11a_HBVmod-R were used as templates to generate inserts carrying the genes for HBVmod-A/HBVmod-R. (For specifications, see Appendix B, reaction 2).

The PCR products were treated with DpnI for 16 hours at 37 °C to ensure that none of the templates remained in the mixtures. The mixtures were purified to remove any residual dNTPs from the PCR reaction.

T4 DNA Polymerase Treatment

The LIC enabled vector was incubated for 30 min at room temperature in a reaction mixture containing 3 U/µl T4 DNA polymerase, 1x NEBuffer 2, 2.5 mM dCTP, 5 mM DTT. The T4 DNA polymerase was inactivated by heating to 75 °C for 20 min.

The inserts were subjected to the same treatment, with the exception that the reaction mixture contained dGTP instead of dCTP.

Construct Annealing

The vector mixture was mixed with each of the insert mixtures at a 1:2 ratio and incubated for 5 min at room temperature. 10 mM EDTA was added and the mixtures were heated to 80 °C for 5 min and were left to slowly cool to room temperature. Samples without heating during the annealing step were added for comparison.

Annealed mixtures were used for transformation. Transformants were verified by colony PCR and purified plasmids were verified by BamHI digestion and sequencing. The resulting plasmids are here on referred to as Dual-A and Dual-R.

5.4 Expression

5.4.1 Transformation into Expression Strains

BL21* (DE3)pLysS was used as host strain for the expression test of HBVmod-R.

For the dual expression pDual-A, pDual-R and pET11a_HBVcore were transformed into both BL21* (DE3)pLysS and BL21 Tuner. The expression was compared and BL21 Tuner was used for the remainder of the experiments.

5.4.2 Expression

Single colonies were used to inoculate flasks containing LB with 100 µg/ml ampicillin, 30 µg/ml chloramphenicol. The flasks were incubated overnight. The overnight cultures were used to inoculate flasks of 2x LB with 2% glucose, trace metals, 100 µg/ml ampicillin, 30 µg/ml chloramphenicol. The cultures were induced with 2 mM IPTG at OD ~0.6 and incubated for three additional hours. The cultures were harvested at 6000 g for 30 min at 4 °C. Cell pellets were weighed and either sonicated or stored at -20 °C for later use.

An uninduced culture was used as a negative control.

5.5 Purification

The purification protocol used is a slightly modified version of the protocol developed by Zlotnick et al[13]. Samples were kept on ice or at 4 °C to the extent this was possible.

Lysis

E. coli cell pellets were resuspended in 4 ml Lysis buffer[A] (pH 7.5) per 1 g of pellet. The cell suspensions were sonicated in batches of 10 ml using a pulsing sonication mode at 80% output with 1 s on 1 s off for 15 s followed by 15 s of rest for a total of 10 min. An ice water bath was used for cooling during the sonication.

To clarify the lysate the sonicated suspensions were centrifuged at 27 000 g for 30 min at 4 °C.

Samples from the supernatant and the resuspended pellet (lysis buffer + 1% SDS) were analyzed by SDS-PAGE.

A, Lysis buffer: 50 mM Tris (pH 7.5), 5 mM DTT, 1x Protease Inhibitor, 1 U/ml Benzonase®

Precipitation

The proteins were precipitated from the supernatant by slow addition of ammonium sulfate to 40% saturation (0.233 g/ml) while slowly stirring at 8 °C for 1 hour. The precipitated protein suspensions were centrifuged at 25 000 g for 1 hour at 4 °C. The resulting supernatants were discarded and the precipitate pellets were resuspended in Buffer A[B] and centrifuged again at 25 000 g for 15 min at 4 °C. The supernatants were concentrated to a volume of 6 ml each using centrifugal concentrators.

Capsid Purification

6 ml of the concentrated supernatant were loaded onto a Buffer A-equilibrated size exclusion column (Sepharose CL-4B, ~300 ml bed volume, 2.6 cm diameter). The column was run at a flow rate of 1 ml/min at room temperature. Several 7 ml fractions were collected and pooled based on analysis by the chromatographic profile, A_{280} and SDS-PAGE. The pooled fractions were subjected to centrifugal buffer exchange and concentration. The buffer was exchanged for Buffer N[C], resulting in a residual Buffer A concentration of less than 1% and the sample was concentrated to a final volume of ~5.5 ml.

Dimer Purification

The capsids in the sample were disassembled by the addition of Urea to a final concentration of 3 M (0.209 g/ml), and incubated on ice for 2 hours, intending to get the majority of the capsid proteins into a dimeric state.

6 ml of the dimeric sample were loaded onto a Buffer N-equilibrated size exclusion column (Supradex 75, ~300 ml bed volume, 2.6 cm diameter). The column was run at a flow rate of 2.6 ml/min at 22 °C. Several 5 ml fractions were collected and pooled based on analysis by the chromatographic profile, A_{280} and SDS-PAGE. The pooled fractions were subjected to centrifugal buffer exchange and concentration. The buffer was exchanged for Buffer H[D], resulting in a residual Buffer N concentration of less than 1% and the sample was concentrated to a final concentration of 2.0 mg/ml.

The purified dimers were stored at -80 °C until needed.

B, Buffer A: 100 mM Tris (pH 7.5), 100 mM NaCl, 2 mM DTT
C, Buffer N: 50 mM $NaCO_3$ (pH 9.6), 2 mM DTT
D, Buffer H: 50 mM HEPES (pH 7.5), 2 mM DTT

5.6 Reassembly

Reassembly of HBVcore and HBVmod-A

Dimers were thawed on ice. Reassembly was carried out by adding cold NaCl (5 M) to HBVcore dimers (2.0 mg/ml) to a final NaCl concentration of 0.5 M. The mixture was incubated at +8 °C overnight.

The same procedure was performed on Dual-A dimers (2.0 mg/ml).

The reassembled HBVcore was loaded onto a Buffer N-equilibrated size exclusion column (Sepharose CL-4B, ~300 ml bed volume, 2.6 cm diameter). The column was run at a flow rate of 1 ml/min at room temperature. Several 7 ml fractions were collected and pooled based on analysis by the chromatographic profile. A SDS-PAGE was performed on the fractions to assess purity. The pooled fractions were concentrated and stored at -80 °C.

Ratio Dependent Reassembly

Dimers for the reassembly of HBVcore were acquired by disassembling the capsids from the previous reassembly. Dimers for the reassembly of Dual-A/-R were acquired through the purification and disassembly of capsids from frozen cell pellets.

HBVcore, Dual-A and Dual-R dimers were thawed on ice. Reassembly was carried out by adding cold NaCl (5 M) to dimers (2.0 mg/ml) to a final NaCl concentration of 0.5 M. Negative controls were made by adding dH_2O instead of NaCl. Furthermore HBVcore and Dual-A dimers were mixed to investigate the HBVmod-A : HBVcore ratio's influence on reassembly. Dual-A was mixed with HBVcore at ratios of 1:1, 1:4 and 1:9 yielding HBVmod-A concentrations of approximately 25%, 10% and 5% respectively.

All samples were centrifuged at 12 000 g for 30 min at room temperature. The supernatants were loaded into a 12 µl quartz cuvette and measured by DLS. To ensure that both HBVcore and the HBVmod were present in the capsids the supernatants were loaded onto a gel for SDS-PAGE analysis.

DLS Measurement of Stored Dimer Samples

Dimers of HBVcore and HBVmod-A were thawed on ice. Samples were loaded into a 12 µl quartz cuvette and measured by DLS.

5.7 Electron Microscopy Inspection

Reassembled HBVcore and Dual-A samples were inspected by negative stain TEM.

The samples in fig 3.x was prepared with the following protocol:

Lay grid on Sample drop for 30 s.
Dry using filter paper.
Lay grid on Stain drop for 30 s.
Dry using filter paper.
Ready for EM.

Stain: NanoVan® (Vanadium based).
Grid: Carbon on 300 mesh (Cu).

A more rigorous protocol was used for preparation of additional samples:

Lay grid on Buffer drop for 30 s.
Dry using filter paper.
Lay grid on Sample drop for 60 s.
Dry using filter paper.
Lay grid on H_2O drop for 30 s.
Dry using filter paper.
Lay grid on Stain drop for 3 s.
Dry using filter paper.
Lay grid on Stain drop for 30 s.
Dry using filter paper.
Ready for EM.

Stain: 1% Uranyl acetate.
Grid: Carbon on 300 mesh (Cu).
This did however yield a more noisy background and was not included in the results.

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Appendix A – Amino Acid sequences

HBVcore:

Theoretical pI: 4.89

Mw: 16 777 Da

Length: 150 AA

MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTAAALYRDALESPEHASPHHTALRQAILAWGD
LMTLATWVGTNLEDPASRDLVVSYVNTNVGLKFRQLLWFHISALTFGRETVLEYLVSFGVWIRT
PPAYRPPNAPILSTLPETTVVC

HBVmod-A:

Theoretical pI: 5.32

Mw: 19 399 Da

Length: 171 AA

MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTAAALYRDALESPEHASPHHTALRQAILAWGD
LMTLATWVGTNLEDPASRDLVVSYVNTNVGLKFRQLLWFHISALTFGRETVLEYLVSFGVWIRT
PPAYRPPNAPILSTLPEKLTDEALTERRGQILWFRGLNRIQTQ

HBVmod-R:

Theoretical pI: 7.95

Mw: 19 626 Da

Length: 172 AA

MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTAAALYRDALESPEHASPHHTALRQAILCWGD
LMTLATWVGTNLEDPASRDLVVSYVNTNVGLKFRQLLWFHISALTFGRETVLEYLVSFGVWIRT
PPAYRPPNAPILSTLPETTVVRRRGRLRRGQILWFRGLNRIQTQ

Native HBV:

Theoretical pI: 9.93

Mw: 21 116 Da

Length: 183 AA

MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCWGE
LMTLATWVGVNLEDPASRDLVVSYVNTNMGLKFRQLLWFHISCLTFGRETVIEYLVSFGVWIRT
PPAYRPPNAPILSTLPETTVVRRRGRSPRRRTPSPRRRRSQSPRRRRSQSRESQC

Appendix B – PCR Programs

Reaction 1:

Construction of HBVmod-R Insert

The following reaction creates a DNA fragment encoding the C-terminal extension of HBVmod-R.

Forward Primer (5′-3′):

TCCTGTCGACACTTCCGGAGACCACCGTTGTTCGTCGTCGTGGTCGTCTGCGTCGTGGTCAGATCC

Reverse Primer (5′-3′):

GCGGGATCCCTACTGGGTCTGGATACGGTTCAGACCACGGAACCACAGGATCTGACCACGACGCAGACGA

Template:

Primers templating each other, creating the following annealing structure:

```
TCCTGTCGACACTTCCGGAGACCACCGTTGTTCGTCGTCGTGGTCGTCTGCGTCGTGGTCAGATCC
                                  ||||||||||||||||||||||||
                                  AGCAGACGCAGCACCAGTCTAGGACACCAAGGCACCAGACTTGGCATAGGTCTGGGTCATCCCTAGGGCG
```

PCR Program

Step #:	Cycle Step:	Temp (°C):	Time (s):	
1	Denaturation	98	30	
2	Denaturation	98	10	← Return to #2
3	Annealing	63	20	29 times
4	Elongation	72	10	
5	Elongation	72	300	
6	Hold	4	∞	

[Appendix continues on the next page]

Appendix B – PCR Programs (Continued)

Reaction 2:

LIC Insert Preparation

The following reaction extracts the HBVmod-A/R genes from the T7 promoter to the T7 terminator, and adds LIC extensions at the ends compatible with the LIC vector constructed below.

Preparation of LIC Enabled HBVmod-A Gene:

T7-Promoter-LIC (5′-3′):

GGTTAATTGATGAGGCTAATACGACTCACTATAGGGGAATTGTGAGCGG

T7-Terminator-LIC (5′-3′):

GGATGATTTGAATGGCCAAAAAACCCCTCAAGACCCGTTTAGAGG

Template:

pET11a_HBVmod-A

Preparation of LIC Enabled HBVmod-R Gene:

T7-Promoter-LIC (5′-3′):

GGTTAATTGATGAGGCTAATACGACTCACTATAGGGGAATTGTGAGCGG

T7-Terminator-LIC (5′-3′):

GGATGATTTGAATGGCCAAAAAACCCCTCAAGACCCGTTTAGAGG

Template:

pET11a_HBVmod-R

PCR Program

Step #:	Cycle Step:	Temp (°C):	Time (s):	
1	Denaturation	98	30	
2	Denaturation	98	10	
3	Annealing	60	20	Return to #2
4	Elongation	72	15	2 times
5	Denaturation	98	10	Return to #5
7	Elongation	72	15	26 times
8	Elongation	72	600	
9	Hold	4	∞	

[Appendix continues on the next page]

Appendix B – PCR Programs (Continued)

Reaction 3:

LIC Vector Preparation

The following reaction amplifies the whole HBVcore plasmid, and adds LIC extensions at the ends compatible with the LIC inserts constructed above.

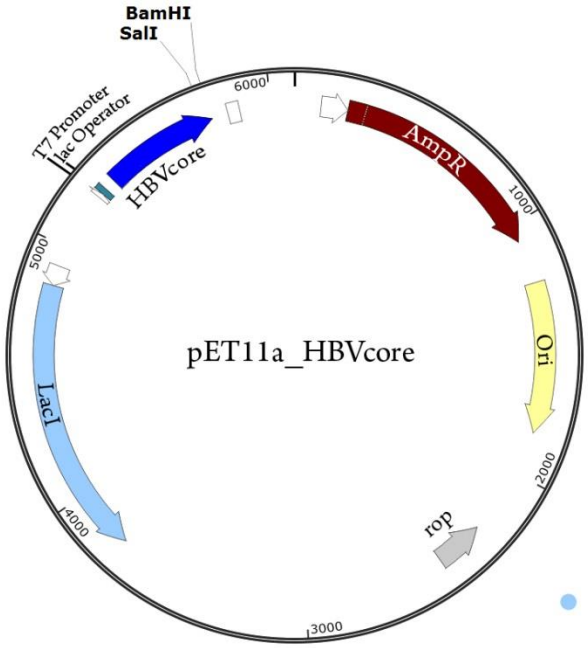
Plasmid-LIC_Forward (5′-3′):
CCATTCAAATCATCCGGTGCCGAGGATGACGATGAG
Plasmid-LIC_Reverse (5′-3′):
CCTCATCAATTAACCGCGTCACCCTGGATGCTGTAGGC
Template:
pET11a_HBVcore

PCR Program

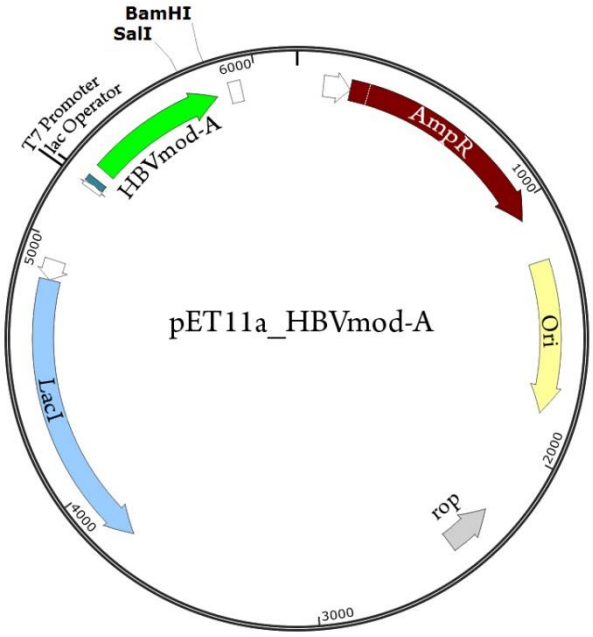
Step #:	Cycle Step:	Temp (°C):	Time (s):	
1	Denaturation	98	30	
2	Denaturation	98	10	← Return to #2
3	Annealing	60	20	
4	Elongation	72	120	2 times
5	Denaturation	98	10	← Return to #5
6	Elongation	72	120	26 times
7	Elongation	72	600	
8	Hold	4	∞	

Appendix C – Vector Maps

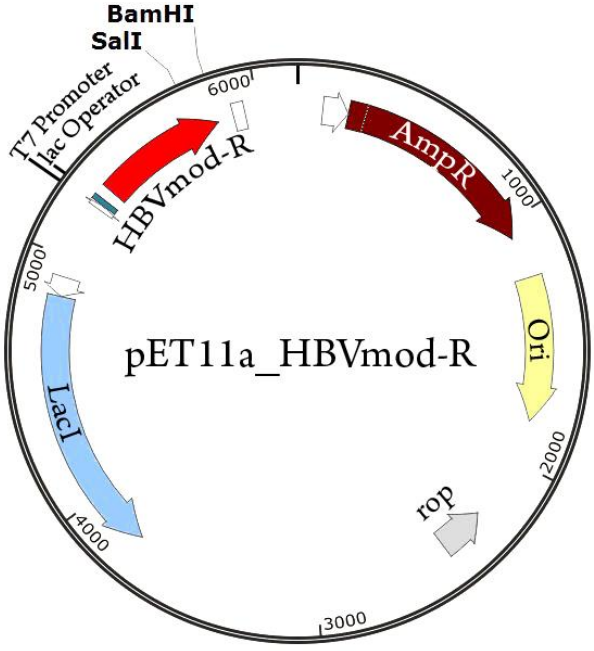
v.1 - pET11a_HBVcore (6093 bp):



v.2 - pET11a_HBVmod-A (6154 bp):



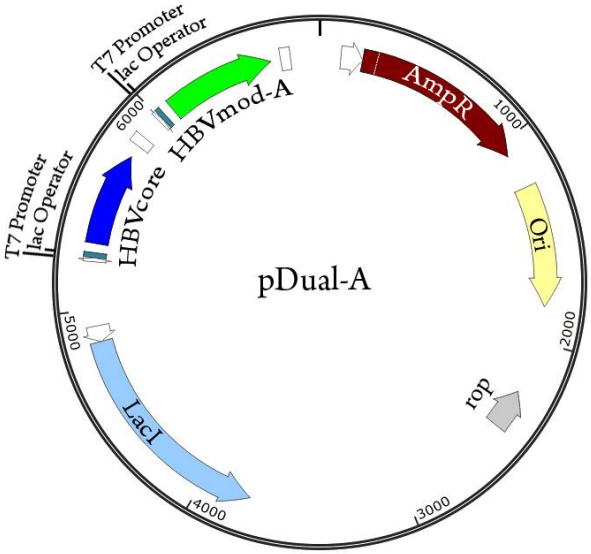
v.3 - pET11a_HBVmod-R (6160 bp):



[Appendix continues on the next page]

Appendix C – Vector Maps (Continued)

v.4 – pDual-A (6840 bp):



v.5 – pDual-R (6846 bp):