Hepatitis C virus RNA-dependent RNA-polymerase NS5B

Overexpression, purification and characterization

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Summary

Hepatitis C virus is a pathogen that causes chronic hepatitis, cirrhosis and liver cancer worldwide. The virus is transmitted by blood-to-blood, for example, by intravenous drug use, unsafe medical intervention, blood transmission, etc. The infection is usually asymptomatic, which makes it difficult to diagnose at an early stage. Nowadays, high prevalence of the virus, the absence of a vaccine and the inefficient therapy are currently big medical concerns.

Hepatitis C virus is a positive-stranded RNA virus, which belongs to the Flaviridae family. It was discovered in 1989 and was previously known as “non-A, non-B hepatitis”. Hepatitis C virus infects humans and chimpanzees. Hepatocytes are the main target, however other cell types can also be infected, for example B cells.

RNA-dependent RNA-polymerase NS5B plays a very important role in hepatitis C virus replication and is thus one of the main drug targets and thereby of big interest for drug discovery. This project aimed to overexpress, purify and characterize the viral polymerase for further studies and drug discovery campaigns by using surface plasmon resonance technology.

Truncated forms of NS5B, with the C-terminal membrane spanning region excised, were overexpressed in E.coli cells and purified with various chromatographic techniques, according to the fused affinity tags. The most efficient protein capture was achieved with His6Cys and Lys10 affinity tags and required only one step purifications. Overexpression and purification procedures of truncated NS5B were optimized to achieve reasonable protein yields and purities. Various affinity tags served not only to facilitate the purification of the enzyme but can also be utilized for immobilization mechanisms to the biosensor chip surface. The production of full-length NS5B by using a cell-free based expression system did not reach desirable results. But some optimization strategies might be applied to solve the problems with the overexpression. To overexpress the soluble full-length variant of NS5B in insect cells, a recombinant bacmid was generated. The bacmid contains the gene encoding the full-length NS5B under control of a strong polyhedrin promoter and all necessary viral elements to generate recombinant baculovirus.

A non-radioactive, homopolymer end-point enzyme assay was established to check the functionality of the polymerase. The activity of truncated NS5B was tested with a coupled enzymatic reaction using a pyrophosphate detection kit, and RNA content measurement. The assay proved that the purified NS5B was active.

To further characterize NS5B polymerase, the protein has been immobilized on a sensor chip surface and interaction experiments with a known inhibitor and RNA have been performed using a Biacore surface plasmon resonance system. Preliminary interaction kinetic parameters for NS5B and an inhibitor were determined that can serve as a starting point for further characterization of NS5B and drug discovery.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Bis-Tris</td>
<td>Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E1 and E2</td>
<td>Envelope protein 1 and Envelope protein 2</td>
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<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride</td>
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<tr>
<td>GTP</td>
<td>Guanosine tri-phosphate</td>
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<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>IMAC</td>
<td>Immobilized metal ion affinity chromatography</td>
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<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
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<tr>
<td>MES</td>
<td>4-Morpholineethanesulfonic acid</td>
</tr>
<tr>
<td>MOPS</td>
<td>4-Morpholinepropanesulfonic acid</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NS</td>
<td>Non-structural protein</td>
</tr>
<tr>
<td>NTA</td>
<td>Nitrilo-triacetic acid</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PolyC</td>
<td>Polycytidylic acid</td>
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<tr>
<td>RU</td>
<td>Response unit</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
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<tr>
<td>Tris</td>
<td>2-Amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride</td>
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Introduction

Hepatitis C

According to the World Health Organization (WHO), about 3% of the human population worldwide is infected with the hepatitis C virus (HCV). Around 70% of these individuals can develop a chronic HCV infection, one of the main causes of liver cirrhosis and hepatic cancers. The virus is transmitted by blood-to-blood route, for example, by intravenous drug use, an unsafe medical intervention, blood transmission, etc. The infection is usually asymptomatic, which makes it difficult to diagnose at an early stage. Today no vaccines are available against HCV and existing therapy with pegylated interferon-α and ribavirin shows only 50% efficiency. It is expensive and poorly tolerated. Development of new drugs for treatment of HCV is therefore highly desirable (WHO homepage, 2010).

Hepatitis C virus

Hepatitis C is a positive-stranded RNA virus, which belongs to the Flaviridae family. It was discovered in 1989 and was previously known as “non-A, non-B hepatitis”. Using electron microscopy it was confirmed that the virus is an enveloped spherical particle, around 40-70 nm in diameter. HCV infects humans and chimpanzees. Hepatocytes are the main target, but it can infect other cell types, for example B cells (Maradpour et al, 2007). The HCV genome is about 9.6 kb long and contains a single large open reading frame encoding a polyprotein, flanked by 5’ and 3’ non-coding regions (NCR). The viral RNA at the 5’NCR contains an internal ribosomal entry site (IRES). After the virus has entered into the host cells, its RNA is released and translated by cellular ribosomes into the polyprotein (Figure 1.).

Figure 1. HCV life-cycle. The virus attaches to the cell surface and enters into the host cell by endocytosis (a); the virion is unpacked and the RNA is released into the cytoplasm (b); RNA is translated into the polyprotein. The polyprotein is processed and associated with the endoplasmatic reticulum (c); initiation of RNA replication in the membraneous web (d); the virus particles are assembled (e); and the mature viruses are realized from the cell (f). (Maradpour et al, 2007) (Reprinted with permission from Nature Publishing Group)
The polyprotein precursor is cleaved by host and viral proteases into 10 structural and non-structural proteins. These proteins are associated with the endoplasmatic reticulum (ER), forming a replication machinery (Figure 2.). The structural proteins, i.e. the core protein (C), envelope proteins 1 (E1) and 2 (E2), and p7, are used for assembly of new virus particles, whereas most of the non-structural (NS) proteins, i.e. NS2, NS3, NS4A, NS4B, NS5A and NS5B participate in viral genome replication. After formation of the replication complex, the viral RNA synthesis is initiated. The packaging and assembly of new viruses occur in a so called membranous web. The mature viruses are then released from the host cell by exocytosis (Figure 1.).

Figure 2. Organization of HCV genome, polyprotein processing and ER membrane association scheme. The viral RNA, illustrated at the top, is translated into the polyprotein. The structural proteins and p7 polypeptide are subsequently cleaved by endoplasmatic reticulum signal peptidases (diamonds and scissors), whereas non-structural proteins are processed by NS2-3 and NS3-4A viral proteases (arrows). The proteins with known structures are depicted in ribbon, while the proteins with unknown structures are shown in spheres and cylinders. The core protein in red is forming a viral nucleocapsid; E1 and E2 protein are glycosylated (green dots) envelope proteins; p7 possesses an ion channel activity; NS2-3 is autoprotease; NS3 together with NS4A form a complex. NS3 is a multifunctional protein. It has a serine protease and a helicase domain. NS4A functions as a cofactor; NS4B participates in membranous web formation; NS5A is highly phosphorylated protein and it is believed that it modulates RNA replication, but exact function is still unclear; NS5B is RNA-dependent RNA-polymerase.

(Maradpour et al, 2007) (Reprinted with permission from Nature Publishing Group)
**HCV genotypes**

There are six major genotypes and several subtypes of HCV. The genotypes are differing according to geographic regions. The genotype 1 is predominant in North America and genotypes 1, 2 and 3 in Europe. In Scandinavia, for example, 50% of HCV infected individuals have genotype 3, which is also prevalent in Asia along with genotype 6. Genotype 4 is found in Middle East, genotype 5 in South Africa, and genotype 6 mainly in Hong Kong. Up to 30% of sequence difference is observed among the genotypes (Davis, 1999). Probably, the detailed study of HCV genotypes in a particular geographic area would be beneficial in order to propose more successful and effective treatment regime and avoid resistance issues in the future.

**RNA-dependent RNA-polymerase NS5B**

The RNA-dependent RNA-polymerase NS5B (RdRp) is an endoplasmatic reticulum membrane associated, 66 kDa big protein. The membrane spanning helix consists of 21 C-terminal amino acid residues. NS5B shares a common right hand structure with other RdRps and have three main domains: fingers, palm and thumb. However, it has a distinct feature, that is, close interaction between fingers and thumb domains, resulting in closed active site structure (Maradpour et al, 2007). NS5B catalyses the synthesis of both positive and negative RNAs during the virus replication, and functions in oligomeric form (Wang et al, 2002). NS5B is able to initiate the RNA synthesis by two different mechanisms: 1) primer dependent mechanism, exogenous or self priming (Behrens et al, 1996) and 2) de novo synthesis on the template by joining free NTPs and extend them to long RNA chain (Kao et al, 2000). Moreover, the NS5B is an error-prone RdRp, lacking proof reading activity, which explains the great variation in terms of number of virus genomic subtypes (Le Pogam et al, 2008). Thus, NS5B plays a fundamental role in HCV replication and is therefore an important drug target.

**Surface Plasmon Resonance (SPR) technology**

The characterization of NS5B, in terms of immobilization capability on sensor chip surface and interaction with RNA and inhibitor, was investigated by using a Biacore surface plasmon resonance system. The Biacore system is a label-free technique, based on the surface plasmon resonance (SPR) phenomenon, which enables to monitor the interactions of molecules in real time and determine necessary interaction characteristics, such as specificity, kinetics, affinity, and concentration analysis, multiplicity of interaction, thermodynamics and stoichiometry. SPR technology is applicable in various areas, for example, food analysis, immunogenicity, when proteins as drugs are used, in proteomics, in drug discovery to identify the target to optimize and screen the lead candidate, etc (Karlsson, 2004).

The principle is based on attachment of one interaction partner to the chip surface, for example, a protein, whereas another interaction partner, for example, inhibitor is delivered into the flow cell with the solution. The binding of molecules generates the response, which is detected and recorded as a sensorgram by the Biacore instrument (Figure 3).

The typical sensorgram is illustrated in Figure 3 (right). From the association and dissociation phases the kinetic rate constants $k_{on}$ and $k_{off}$ can be derived and the equilibrium dissociation constant $K_D$ can be determined.
Figure 3. **Left:** Illustration of the principle of Biacore SPR technology. The interaction occurs in the flow channel of the sensor chip, where the ligand is attached on the surface and the analyte is passed in the solution over the ligand. If an interaction takes place it changes the refractive index of the solution on the interface of gold film and medium resulting in alteration of resonance angle. This causes the change of an angle of the reflected polarized light, which is subsequently detected by optical detection unit. **Right:** When the injection starts the binding is detected as an increase in the number of response units. The kinetic association rate constant $k_{on}$ can be extracted from association phase of the sensorgram. When the injection stops and the complex is washed by buffer, an exponential decay of the signal is observed, which corresponds to the dissociation phase of the analyte. From dissociation phase a dissociation rate constant $k_{off}$ can be determined. The affinity parameter $K_D$ can be obtained from the ratio of kinetic association and dissociation rate constants.

The key component of SPR technology is the sensor chip – a glass surface covered with a thin layer of gold. It provides necessary physical conditions to generate a SPR signal. Three different approaches to attach the molecules on the chip surface such as covalent attachment, affinity capture and hydrophobic absorption can be used. In my experiments two of three approaches were utilized: for polylysine-tagged NS5B immobilization a CM5 sensor chip has been used for covalent attachment by amine coupling and His$_6$Cys tagged NS5B was immobilized by affinity capture on nitrilotriacetic acid (NTA) sensor chip.

**Aims**

The first aim was to obtain NS5B in sufficient amounts for enzymatic studies. Second, the overexpressed protein should be obtained in an active form. Therefore, an assay needed to be developed in order to assess functionality of the NS5B. And last but not least, the protein should be characterized to understand its properties and thus create a basis for further studies and drug discovery.
Results

Plasmids containing truncated and full-length forms of NS5B with various fusion tags were obtained from Johan Winquist in the laboratory of prof. Helena Danielson. The experiments were aimed to overexpress protein with different tags and to verify which of the chromatographic techniques that would result in pure sample preparation after a one step purification procedure. Moreover, the NS5B with different fusion tags was to be tested to verify which of these tags would provide better immobilization on the biosensor chip surface.

Since the full length form of NS5B with its transmembrane region tends to aggregate, leading to protein insolubility when using bacterial cells as a host for overexpression, the *E.coli* expression system was chosen to overexpress truncated forms of NS5B with the 21 amino acids C-terminal membrane spanning region excised (NS5BΔ21). The genes were from the BK or Con1 strains of HCV.

Overexpression and Purification NS5BΔ21-His6Cys (Hepatitis C, Con1 strain)

After several trials, NS5BΔ21-His6Cys was finally expressed and purified using an optimized buffer composition. The protein was purified by a one step purification procedure using immobilized metal affinity chromatography (IMAC). Two purification procedures were investigated: First, purification by batch centrifugation: raw lysate was mixed with Ni-NTA agarose slurry and the protein was eluted with lysis buffer containing 250 mM imidazole, lane 6 (Figure 4). As can be seen from the SDS-PAGE picture (Figure 5, lane 6), the protein sample still contains some contaminations. The concentration was not measured for this sample. The second approach was purification using an Äkta Explorer system with gradient elution (Figures 4 and 5). Purified NS5B from both purification procedures was confirmed by western blot (WB) (Figure 5B) and by mass spectroscopy (MS) analysis (not shown). The measured concentration of the protein in pooled fractions 17/18 was 130 µg/ml in 3 ml fraction volume. All in all, the protein after IMAC purification is considered as pure and resulted in very efficient protein capture as a single step purification.

Figure 4. NS5BΔ21His6Cys purification chromatogram. The peak on the chromatogram represents NS5BΔ21-His6Cys elution (black arrow).
Overexpression and purification of NS5AΔ21-Lys10 (Hepatitis C, Con1 strain)

Truncated NS5B with a polylysine tag was expressed as described below. The protein was purified by cation-exchange chromatography using a negatively charged SP-Sepharose matrix as well as by affinity chromatography using Heparin Fast Flow resin packed into a “home made” columns. After purification, samples were analyzed by SDS-PAGE and WB (Figure 6). As shown in Figure 6, the ion-exchange chromatography technique allows performing one-step purification and results in pure protein sample. It should be noticed that the protein was eluted at rather high salt concentration, suggesting strong binding (lanes 5-6, Figure 6), however, a subsequent sample desalting step is necessary. The purification by using the Heparin column did not result in pure protein. (Figure 6, lanes 8-15). The NS5BΔ21-Lys10 measured concentration of 800 mM NaCl eluted sample from SP-Sepharose column was 490 µg/ml in 3 ml elution volume.
It can be concluded that use of heparin column purification as a first or single step purification procedure is not optimal. However it is very suitable for polishing the purity of the NS5B protein after IMAC or ion-exchange chromatography.

*Overexpression and purification of NS5BA21-Strep II (Hepatitis C, Con1 strain)*

The Strep II tag is a short sequence that consists of 8 amino acids (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys). It is considered as a very specific affinity tag, which can lead to very pure protein sample after purification. In Figure 7 the last attempt of purification of Strep II tagged NS5BA21 is shown. Initial trials did not result in any protein capture and purification. In this attempt, a short linker (Serine and Alanine) between protein and Strep II tag has been introduced. As shown in figure, it has improved the purification of the protein a bit, however, a lot of protein is still lost without attaching to the matrix (Figure 7). A longer linker between NS5B and the Strep II tag will probably improve the accessibility of the tag and result in more efficient purification of the protein.

![Figure 7](image)

*Figure 7. Overexpression and purification profile of NS5BA21-StrepII. Lanes: 1. Size marker, 2. E.coli raw lysate, 3. column flow through, 4 and 5 lysis buffer wash, 6 and 7 2,5 mM d-Desthiobiotin elution. A: SDS-PAGE stained with Coomassie blue. Arrow indicates the band that corresponds to NS5BA21-StrepII. B: Detection of NS5B on the WB.*

*Cell free expression and purification of full-length NS5B (Hepatitis C BK strain)*

The choice of a cell-free expression system to produce full-length NS5B relied on the idea of providing conditions more similar to the natural environment of NS5B – that is the membrane anchorage. The MembraneMax cell-free expression kit is designed especially to produce soluble membrane proteins. The key component of this expression kit is the MembraneMax Reagent – a lipid bilayer surrounded by scaffold protein. The synthesized membrane protein can be directly inserted into such particles.

The cell free expression and purification profile of full-length NS5B is illustrated in Figure 8. The protein was expressed, as detected by western blot analysis (Figure 8, B), however in low amount (not detected by Coomassie staining) and probably incomplete. The IMAC procedure did not result in any purification.
Recombinant bacmids for full-length NS5B (Hepatitis C, BK strain) overexpression in insect cells

The baculovirus expression system can be used for the production of recombinant proteins in cultured insect cells or insect larvae. To generate the recombinant baculovirus a recombinant bacmid should first be obtained. This recombinant bacmid contains all necessary elements to generate a virus and is propagated in DH 10 E.coli cells. The bacmid is chemically delivered into the insect cells where the recombinant baculovirus is generated.

In contrast to bacterial expression systems, baculovirus expression system provides better conditions for correct folding, solubility and glycosylation of heterologous proteins. Therefore, this system was selected to obtain soluble and active full-length form of NS5B in high yield for further studies.

The recombinant bacmids containing a gene, encoding full-length NS5B with His₆Cys and StrepII affinity tags under strong polyhedrin promoter for overexpression in Sf9 insect cells were generated. The insert was verified by PCR using pUC/M13 amplification primers. The PCR product produced by M13 primers is about 2300 bp plus 1700 bp of the full-length NS5B protein results in the band on the agarose gel of about 4000 bp (Figure 9). This confirms the presence of the insert in the bacmid. The bacmid from the first sample (lane 2, Figure 9) was discarded due to presence of an unspecific PCR product.

Thus, the generated bacmids containing full-length form of NS5B are ready to produce recombinant baculovirus stock and overexpress NS5B in insect cell culture.
Figure 9. Agarose gel of PCR analysis to verify the presence of full-length NS5B encoding gene in the bacmid. Lane 1 DNA molecular weight marker X. Lanes 2-7 samples from PCR.

Activity measurement (homopolymer assay)

To check if the overexpressed NS5B was active, an activity assay needed to be established. The activity of truncated NS5B was examined by using a pyrophosphate assay kit. During RNA polymerization, the pyrophosphate (PPi) is released upon addition of a nucleotide into the RNA chain. Schematically, the reaction can be described as follows:

$$(RNA)_n + NTP \rightarrow (RNA)_{n+1} + PPi$$

The pyrophosphate kit contains the enzyme inorganic pyrophosphatase that catalyses the conversion of PPi into inorganic phosphate (Pi). The released Pi is then involved in conversion of the substrate 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) by purine nucleoside phosphorylase to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine. This enzymatic conversion of the MESG substrate into the product leads to a shift in absorbance from 330 to 360 nm. (Upson et al, 1996)

As shown in Figure 10, the NS5B is active and is very stable. The polymerization reaction was still ongoing prior the next measurement after 20 hours incubation with the pyrophosphate kit components at room temperature. Non desalted protein sample showed less activity or the activity was retarded. This can be explained as the final volume together with the kit components reached 1ml and thus the salt concentration is reduced 10 times. BSA served as control reaction and no free PPi was detected.
Figure 10. Activity measurement of NS5BΔ21-His6Cys by pyrophosphate assay kit after 5 minutes and 20 hours incubation at room temperature with kit components.

In order to validate that the detected pyrophosphate was a product of polymerase reaction and not from another source, the RNA content was also measured. After 20 hours of incubation the RNA content was measured at the wavelength of 260 nm (Figure 11) and the absorbance is clearly higher in samples that contain NS5B. The background signal here should be taken into account since some proteins can also absorb at this wavelength. The experiment showed that there is a correlation between detected free pyrophosphate and amount of synthesized RNA.

Figure 11. Measurement of the RNA content of polymerase reactions with pyrophosphate assay kit components mixture after 20 hours of incubation.

Interaction analysis

SPR biosensor based interaction analysis was performed to characterize the properties of NS5B in terms of immobilization efficiency and its interactions with RNA and inhibitor.
Characterization of NS5BΔ21-Lys10

In order to immobilize the protein by amine coupling, the enzyme is first pre-concentrated by injecting it at a pH below its pI value, allowing electrostatic attraction to the negatively charged dextran surface on the sensor chip. The pH scouting procedure showed that pre-concentration of NS5BΔ21-Lys10 on the biosensor chip surface was most efficient at the pH 6.9 and 7.4 (Figure 12, red and blue lines). pH 7.4 was chosen for further immobilization and interaction experiments.

![Figure 12](image)

**Figure 12.** Overlay plot of pH scouting of NS5BΔ21-Lys10 showing that best pre-concentration of the protein achieved at the pH 6.9 and 7.4

NS5BΔ21-Lys10 was immobilized on the CM5 chip surface by an amine coupling procedure, with 1 minute contact time and the flow rate 5 µl/min. The immobilization of NS5BΔ21-Lys10 gave a final response after surface deactivation of 6016 RU (Figure 13).

![Figure 13](image)

**Figure 13.** Immobilization of NS5BΔ21-Lys10 on CM5 biosensor chip surface. 1. Surface activation with EDC/NHS; 2. Immobilization of the protein; 3. Deactivation with ethanolamine. Immobilization resulted in 6016 RU. Dashed line indicates the baseline level prior protein immobilization.
After immobilization of NS5BΔ21-Lys₁₀, two-fold dilution series of increased concentrations (from 0.078 µM to 2.5 µM) of inhibitor were injected over the enzyme surface. The inhibitor used for the interactions studies is a benzimidazole derivate. It is a non-nucleoside inhibitor, which binds to an allosteric site of the enzyme, resulting in an inactive conformation.

Blank sample corrected sensorgrams are represented in Figure 14. Curves were fitted to a 1:1 binding model (P+L↔PL). Obtained kinetic parameters are shown in Table 1. Notably, a baseline drop was observed prior inhibitor injection.

**Figure 14.** Sensorgram representing interaction between NS5BΔ21-Lys₁₀ and concentration series of inhibitor. The curves fitted to a 1:1 binding model.

The immobilization of NS5B by amine coupling in a single injection step, resulted in sufficient amount of NS5B on the chip surface to perform the interaction experiment with an inhibitor. The inhibitor showed a strong interaction with NS5B, but none of the curves were perfectly described by a 1:1 interaction model.

**Characterization of NS5BΔ21-His₆Cys**

The immobilization of NS5BΔ21-His₆Cys on NTA sensor chip was performed at the flow rate of 5 µl/min, with contact time of 7 minutes and required several steps of protein injection, probably due to low concentration of the protein. The immobilization resulted in 2700 RU (Figure 15). However, a continuous baseline decline (baseline drift) was observed which became more or less stable after 6 hours.

**Figure 15.** Immobilization of NS5B NS5BΔ21-His₆Cys on NTA chip. The protein was injected 3 times and reached an immobilization level of 2700 RU. Dashed line indicates the baseline level prior protein immobilization.
An inhibitor was injected in different concentration series as described previously. The curves were fitted to 1:1 binding model (Figure 16) and again a non-perfect curve fitting was obtained with this interaction model.

**Figure 16.** Sensorgram representing interaction between NS5BΔ21-His$_6$Cys and an inhibitor concentration series. The curves fitted to a 1:1 binding model.

*Interaction between NS5BΔ21-His$_6$Cys with bound RNA and benzimidazole*

The protein was immobilized with 4 steps and resulted in 3838 RU of final response. A drifting baseline was observed in this experiment as well (Figure 17). After protein immobilization, 1 mg/ml of poly C was injected 2 times over the immobilized enzyme surface for 3 minutes at 5 µl/min flow.

**Figure 17.** Immobilization of NS5BΔ21-His$_6$Cys with 4 times of protein injection. Obtained final response was 3838 RU. Dashed line indicates the baseline level prior protein immobilization.

The concentration series of the inhibitor were injected as described above. The curves were fitted to a 1:1 drifting baseline interaction model. Obtained kinetic parameters of protein/inhibitor interaction are summarized in table 1.
Figure 18. Sensorgram of the interaction between NS5BΔ21-His6Cys with Poly C and an inhibitor concentration series (0.078-2.5 µM). Curves fitted to a 1:1 drifting baseline interaction model.

The kinetic parameters of protein / inhibitor interactions differ between two NS5B variants. However, the dissociation constant $k_{off}$ was almost similar. Such differences could be explained that the baseline after immobilization of the protein on NTA chip surface was constantly declining and/or different immobilization strategy probably may have an impact on the enzyme causing small conformational changes. Generally, equilibrium dissociation constants $K_D$ are low, suggesting strong binding of the inhibitor to NS5B. But NS5B in complex with RNA significantly decreased the affinity of the inhibitor (Table 1).

Table 1. Interaction kinetic constants of the inhibitor with NS5B

<table>
<thead>
<tr>
<th>Protein</th>
<th>Immobilized</th>
<th>$k_{on}$ (s$^{-1}$ M$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$K_D$ (µM)</th>
</tr>
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<tr>
<td>NS5BΔ21-Lys10 (CM 5 chip)</td>
<td>6016 RU</td>
<td>56000</td>
<td>0.0029</td>
<td>0.053</td>
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<tr>
<td>NS5BΔ21-His6Cys (NTA chip)</td>
<td>2700 RU</td>
<td>28600</td>
<td>0.0032</td>
<td>0.112</td>
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<tr>
<td>NS5BΔ21-His6Cys + PolyC</td>
<td>3838 RU</td>
<td>9590</td>
<td>0.009</td>
<td>0.942</td>
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</table>

*Immobilization of full-length NS5B-His6 (Hepatitis C, BK strain)*

The immobilization of full-length NS5B-His$_6$ from cell free expression system was not successful (Figure 19). The protein was injected over the NTA chip surface 2 times with the contact time 10 minutes and flow rate 5µl/min. The final response after immobilization trial reached only 112 RU.
Figure 19. Immobilization sensorgram of full-length NS5B-His6 (BK) on NTA chip surface with final response 112 RU. Dashed line indicates the baseline level prior protein immobilization.
Discussion

Purification of NS5B

The purification by centrifugation is a fast and easy method to obtain pure protein sample in one step. The contamination observed after this purification procedure (Figure 5) can be easily removed by another purification step, such as, for example, heparin purification or gel filtration. However, in other batches of NS5B-His6Cys purification, this contamination was not observed or appeared in low amount. Purification by using heparin columns (Heparin allows to capture DNA binding proteins) as first and single purification step is not optimal; the sample is not pure after heparin purification and lots of overexpressed protein can be lost. In general, one step-purification is preferable, since the NS5B is still expressed in small amount and more steps of purification will lead to the loss of expressed protein. Similarly, when performing protein desalting or buffer exchange, it is better to use spin columns with molecular weight cut-off filters rather than spin columns with resins. However, the last is less time consuming.

Purification of NS5B with Strep II tag did not succeed as it was expected, considered that StrepII tag provides high specificity and affinity in protein purification (Schmidt et al, 2007). This might be due to sheltering of the affinity tag by the NS5B protein and therefore making it inaccessible to the ligand on the matrix. Longer linker between protein and the Strep II tag could probably solve the problem.

Cell-free expression

The idea to use the in vitro expression system was to increase the full-length NS5B protein yield and provide a close physiological environment for the protein since the MembraneMax protein expression kit contains nanolipoprotein discoidal particles. These particles represent scaffold protein rings that encloses lipid bilayer, so that expressed NS5B with its C-terminal membrane spanning region can be inserted into such disc. The NS5B protein synthesis was detected by western blot analysis. However, I was not able to purify and quantify the expression of the NS5B. There might be several reasons for that: 1) Not optimal expression vector, 2) Possibility of the presence of internal ATG codon in the context of ribosomal binding site sequence. 3) The protein is too large, thus the reaction conditions should be optimized, for example, by increase feeding buffer volume. 4) Contamination with RNAses.

Full-length NS5B overexpression

Unfortunately, time did not allow me to overexpress full-length NS5B in insect cells. The work was stopped on the stage of generated bacmids, which are ready for recombinant baculovirus stock generation and overexpression in Sf9 insect cells.

NS5B activity measurement

EnzCheck pyrophosphate assay kit provides easy and convenient way of checking the activity of RNA polymerase by end point type method. This kit can also be used for continues determination of pyrophosphate released in reaction, but special conditions should be applied. (Lloyd et al, 1995). The disadvantage of using this kit is low sensitivity, since the assay can detect as little as 1 µM of pyrophosphate (Upson et al, 1996). Therefore, a high amount of RNA polymerase is required in the reaction as well as high concentrations of template, primer and substrate needed to stimulate the enzymatic activity of NS5B (Lahser et al, 2004; Simister
et al, 2009). However, a high GTP concentration can inhibit the enzyme activity significantly when using heteropolymer assay for truncated NS5B (Vo et al, 2004). The high salt concentration in the reaction inhibits the binding of the template to the polymerase by elevated ionic environment. Also, Vo et al reported that truncated NS5B possesses remarkable stability (Vo et al, 2004). These observations were seen also in my experiments (Figures 10 and 11).

**Biacore SPR analysis**

Since the protein was not immobilized by covalent coupling on the NTA chip, it results in baseline drifting, which is a disadvantage of using this type of sensor chip. The advantage of using the NTA sensor chip is that it can be regenerated and reused several times (Willard et al, 2006).

The obtained kinetic protein/inhibitor interactions data are not in accordance with those obtained by others using Biacore technology (Geitmann et al, unpublished; Hang et al, 2009). However, the authors investigated NS5B from another HCV genotype and NS5B with resistant mutations as well as different buffers were used for immobilization and interaction studies. Notably, the decrease of inhibitors affinity after NS5B-RNA complex formation was also observed by the authors in their experiments (Geitmann et al, unpublished). This probably suggests that RNA binding stabilizes NS5B and the protein with bound RNA locks allosteric binding pocket that prevent binding of allosteric inhibitors. It is known that benzimidazole inhibitor binds to the allosteric region resulting in inactive conformation state of NS5B. But in complex with RNA the polymerase is still active even with high inhibitor concentration (Tomei et al, 2003)
Materials and Methods

Expression of truncated form of NS5B in E.coli cells

Plasmids and E.coli strain

The plasmid pET11a containing a gene segment coding for NS5B protein lacking 21 C-terminal amino acids (membrane spanning region) with several C-terminally fused tags (His6Cys, Lys10, Strep II) as well as pT7-7 vector containing full-length NS5B with hexahistidine tag are used for overexpression in E.coli BL21(DE3) strain and in vitro cell free expression system.

Overexpression and purification

Bacterial cells were grown to OD600=0.7-0.9 in LB medium (10 g/l trypton, 5 g/l yeast extract, 10 g/l NaCl, pH 7.5) and overexpression was induced by addition of 1mM isopropyl-β-thiogalactopyranoside (IPTG) followed by incubation at room temperature for 4 h and/or continued at 4°C over night. Cells were harvested by centrifugation at 6000 x g for 15 min at 4°C and resuspended in 120 ml of lysis buffer 1 (LB1: 100 mM Tris HCl pH 8, 100 mM NaCl, 1 mM MgCl2, 2% Triton X-100, 2 mg/ml Lysozyme, 1 U/ml Benzonase). Incubated for 30 min on ice and centrifuged for 15 min, 20 000 x g at 4°C. Supernatant was discarded and the pellet resuspended in 40 ml of lysis buffer 2 (LB2: 20 mM Tris HCl pH 7.5, 500 mM NaCl (IMAC) or 200 mM (ion-exchange), 0.1 % β-octylglucopyranoside, 10 mM Imidazole (IMAC), 50% v/v Glycerol, 10 mM 2-mercaptoethanol. The cells were lysed by cell disruptor (Constant Cell Disruption Systems) and subsequently centrifuged for 45 min, 20 000 x g at 4°C. According to introduced tags at C-termini, NS5BΔ21 and flNS5B were purified by IMAC, cation-exchange, and affinity chromatography techniques. Purified NS5BΔ21 was analysed by SDS-PAGE, western blot (WB) and mass spectroscopy (MS) as well as quantified by Bradford protein assay and stored at -20°C.

Cell free expression and purification

Full-length hexahistidine tagged NS5B (genotype 1b, strain BK) was expressed by using MembraneMax expression kit (Invitrogen) as described in manufacturer instruction. 1 µg of pT7-7 plasmid containing flNS5B-His6 used as DNA template for in vitro overexpression in 100 µl reaction volume. IMAC was performed to purify full-length NS5B.

Chromatography

Immobilized metal affinity chromatography (IMAC)

Batch centrifugation

20 ml of obtained raw E.coli cell lysate was mixed with 5 ml Ni-NTA agarose slurry (Qiagen), incubated for 30 minutes at 4°C with mild shaking, centrifuged for 3 min at 1000 x g. The supernatant was discarded and the Ni-NTA agarose slurry washed two times with 30 ml LB 2 containing 50 mM Imidazole for 3 min at 1000 x g. Bound NS5BΔ21-His6Cys was eluted with 3 ml LB 2 containing 250 mM Imidazole for 3 min at 1000 x g.
Purification with automated purification system

NS5BΔ21-His6Cys was purified by using the ÄktaExplorer (GE Healthcare) system. 20 ml of E.coli cell lysate was applied onto a pre-packed and pre-equilibrated Ni-NTA agarose XK16 column (GE Healthcare) with the flow rate 2 ml/min. NS5BΔ21-His6Cys was eluted with an imidazole gradient.

Cation-exchange chromatography

Cation exchange chromatography technique was used to purify poly-lysine tagged NS5BΔ21. 5 ml of SP-sepharose Fast Flow slurry (GE Healthcare) was used to pack “home made” column. The resin was equilibrated with the LB2 buffer containing 200 mM NaCl. 20 ml of E.coli lysate applied into the column and NS5BΔ21-Lys10 was eluted with step-wise increasing concentration of NaCl.

Affinity chromatography

NS5BΔ21-Lys10 was also subjected to purification with affinity chromatography by using pre-packed HiTrap Heparin HP column (GE Healthcare). Column equilibrated with 10 column volumes of lysis buffer LB2 with 200 mM NaCl. 10 ml of E.coli cell lysate injected into the column at a flow rate of around 5 ml/min. The protein was eluted with step-wise increase of NaCl concentration.

To purify NS5BΔ21 with Strep II fusion tag StrepTactin Sepharose High Performance (GE Healthcare) chromatography medium was used. 5 ml of the medium was used to pack “home made” column. Prior application of E.coli cell lysate, the column was equilibrated with lysis buffer (LB2), containing 25% v/v Glycerol and 500 mM NaCl. Bound NS5BΔ21-Strep II was eluted with 2,5 mM d-desthiobiotin (Sigma) in LB2.

Expression of full-length form of NS5B in insect cells

Recombinant bacmid generation

The primers were designed to introduce the fusion tags His6Cys and Strep II to the full-length NS5B by PCR. Full-length NS5B-His6Cys and Strep II PCR products were purified and cloned into the pFastBac1™ (Invitrogen) shuttle vector. Obtained pFastBac1/flNS5B-His6Cys and pFastBac1/flNS5B-StrepII constructs were transformed into DH10 E.coli cells to generate a recombinant bacmid. After blue/white colony selection the bacmid was isolated and analysed by PCR.

SDS-PAGE and Western-blot

To verify the overexpression and purification of NS5B protein, pre-cast NuPAGE 4-12% Bis-Tris gels and Novex Gel system (Invitrogen) were used. Prior to loading, the samples were mixed with a loading buffer, and heated to 100 °C for 10 minutes. The gels were run in MOPS buffer (Invitrogen) at 200V constant voltage for 1 hour and stained with Coomassie blue dye.

For western blot detection the proteins were transferred from the gel onto nitrocellulose membrane in NuPAGE transfer buffer (Invitrogen) for 1 hour at 30V constant voltage using Xcel II Blot Module (Invitrogen). After transfer, the membrane washed 3 times with TBST
buffer (pH 7.5), blocked with TBST+3% BSA for 1 hour at 4 °C and incubated with 1:12000 diluted primary polyclonal rabbit antibodies to NS5B (AbCam) overnight at 4 °C. Afterwards, the membrane washed 3 times with TBST followed by one hour incubation with 1:12000 diluted secondary donkey anti-rabbit HRP-conjugated antibodies (AbCam) at room temperature. To detect HRP-conjugated antibodies a color development substrate (Promega) was added onto the membrane until protein bands became visible. The reaction was stopped with tap water.

**Protein yield quantification (Bradford protein assay)**

The overexpressed and purified NS5B protein was quantified by Bradford protein assay (Bio-Rad). Seven dilutions of BSA concentrations were prepared in the range from 0.125 to 2 mg/ml and the standard curve was obtained using Microsoft Excel. 10 µl of standard and sample pipetted into each microtiter plate well followed by addition of 200 µl of diluted reagent and incubation for 5 min at room temperature. The absorbance measured at 595 nm using SpectraMax plus 384 spectrophotometer (Molecular Devices).

**Activity measurement**

**Polymerase assay**

The polymerase reaction was assembled as described by Lahser et al, with some modifications (Lahser et al, 2004). The polymerase reaction components such as polycytidylic acid (Poly C), guanosine 5′-triphosphate (GTP) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, USA). RNAsin was from Promega (Madison, USA) and Oligo G₁₂ from Eurogentec (Liège, Belgium). The assay was performed in 100 µl reaction containing 20mM Hepes, pH 7.1, 7.5 mM DTT, 10 mM MgCl₂, 60 mM NaCl, 100 µg/ml BSA, 20 U/ml RNAsin, 50 µg/ml Poly C, 5 µg/ml Oligo G₁₂, 10 µM GTP, and 500 µg/ml NS5B. NS5B sample was desalted by using Microcon YM-30 filter unit (Millipore) with molecular weight cut-off 30 kDa. The homopolymer components Poly C and Oligo G₁₂ were preincubated with NS5B for 24 h prior to the addition of GTP to initiate the reaction. Then, the reaction mixture was incubated for 1 h at 30 °C in a heating block.

**Pyrophosphate content and RNA measurement**

The pyrophosphate content was measured using the EnzCheck pyrophosphate assay kit (Molecular Probes/Invitrogen) with SpectraMAX Plus 384 spectrophotometer (Molecular Devices). The 100 µl polymerase assay reaction was mixed with kit components, incubated for 5 min and 20 h at room temperature followed by absorbance measurement at 360 and RNA at 260 nm, respectively.

**Surface Plasmon Resonance (SPR) analysis**

The immobilization of NS5B and NS5B/RNA/inhibitor interactions experiments were performed with a BIAcore 2000 surface plasmon resonance biosensor instrument (GE Healthcare).
To investigate the optimal pH for NS5BΔ21-Lys10 immobilization a pH scouting procedure has been performed. The protein was diluted in Hepes buffers (10 mM Hepes, pH 7.4 and pH 6.9), in MES buffers (10 mM MES, pH 6.5 and pH 6.0) and in acetate buffers (10 mM Acetate, pH 5.5, 5.0, 4.5 and 4.0) and injected over the CM5 chip (GE Healthcare) surface. Prior protein immobilization the surface of CM5 chip was activated by injecting EDC and NHS in 1:1 ratio. After NS5BΔ21-Lys10 immobilization the chip surface was deactivated by injecting 10% ethanolamine solution for 7 minutes at 5 µl/min flow. NS5BΔ21-Lys10 was immobilized by amine coupling on the chip surface in modified LB2 buffer (10 mM Hepes, pH 7.4, 50 mM NaCl, 0.1% n-octylglycopyranoside, 50% v/v glycerol, 10 mM 2-mercaptoethanol) with contact time 1 minute and the flow rate 5 µl/min. Protein/inhibitor interactions experiments were performed in Tris buffer (20 mM Tris HCl, pH 7.4, 130 mM NaCl, 0.005% P-20 and 3% v/v DMSO). Six concentrations of benzimidazole inhibitor in the range from 0.078 µM to 2.5 µM were used and injected for 60 s over the immobilized NS5B.

NS5BΔ21-His6Cys

The NTA chip surface was prepared by injecting regeneration buffer (10 mM Hepes, 150 mM NaCl, 350 mM EDTA, 0.005% P-20), followed by charging with 500 µM NiSO₄ solution for 10 minutes at the flow rate 5 µl/min each. NS5BΔ21-His6Cys in modified LB2 buffer was immobilized into the flow channel of the NTA-chip (GE Healthcare). The interaction experiments were performed in Hepes running buffer (10 mM Hepes, pH 7.4, 50 mM NaCl, 50 µM EDTA, 0.005% P-20 (GE Healthcare), 3% v/v DMSO). Concentration series of benzimidazole inhibitor in Hepes running buffer were injected for 60 s over the immobilized NS5B. Poly C in 1 mg/ml concentration was injected over the immobilized enzyme surface for 5 minutes at the flow rate 5 µl/min.

Full-length NS5B-His6

Full-length NS5B-His6 from cell free expression was immobilized in running buffer (10 mM Hepes, pH 7.4). The NTA chip surface was prepared as described above.

Data analysis

The interaction data were analyzed with BIAevaluation software (GE Healthcare/Biacore). Sensorgrams from blank surface were subtracted from experimental data and the curves were fitted to a 1:1 binding model.
Conclusion

I was able to overexpress and purify truncated HCV RNA-dependent RNA-polymerase with different affinity tags in reasonable amounts. The various affinity tags served not only to facilitate the purification of the protein but also to use and evaluate different immobilization strategies on Biacore sensor chips for further studies. The cell-free expression system is promising tool to obtain the NS5B polymerase in its natural environment. But the optimization of the system is required. A bacmid encoding full-length NS5B was generated and ready to produce a recombinant baculovirus to overexpress the protein in insect cells. With developed non-radioactive, end-point polymerase activity measurement assay it is possible to check the functionality of overexpressed NS5B as well as effect of potential inhibitors. The preliminary NS5B immobilization and interaction results obtained by using biosensor technology can be used as a starting point for further characterization of NS5B and drug discovery.
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