universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Lectin-based virus-detecting assays“

verfasst von / submitted by

Cornelia Trinker, BSc.

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2017 / Vienna 2017

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066838
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Ernährungswissenschaften
Betreut von / Supervisor:	Prof. Dr. Karl-Heinz Wagner

Acknowledgements

First of all, many thanks to all those who motivated and supported me in writing this master thesis.

I would particularly like to thank Univ.-Prof. Mag. Dr. Karl-Heinz Wagner, Dr. Jakub Dostalek and Dr. Irene Maier for creating a pleasant working atmosphere at the Department for Nutrition as well as at the Austrian Institute of Technology.

I would like to express my gratitude to Dr. Irene Maier for her kind offer to supervise my thesis and answering all my questions with patience.

Furthermore, I thank the whole Biosensor Technologies Group at the AIT for taking me into their group with open arms and all their help on the way.

I am very thankful to my loved ones, especially my parents, who have supported me throughout my life. My family was supportive and proud of my role, both behind the scenes and under the spotlight, whether in heaven or on earth. Thank you!

Finally, I would like to thank Stefan C. for all the moral support, encouragement and patience. It was me like “a small boat on the ocean, sending big waves into motion” but you always stand by me. I thank you for the moments of your life and never giving up on me. I thank you from the bottom of my heart ❤.

“If you want to grant your own wish, then you should clear your own path to it.”

- Okabe Rintarou, Steins;Gate

Statutory Declaration

Hereby I declare that this master thesis submitted was in all parts exclusively written on my own. All passages quoted from publications or paraphrased from these sources are properly cited and marked as such. The thesis was not submitted in the same or in a substantially similar version, not even partially, to another examination board and was not published elsewhere.

_____ _____

Date Signature

Table of content

1. Introduction 14

2. Objectives 16

3. Background 17

3.1. Cyanovirin-N 17

3.2. Experimental design 18

3.2.1. Recombinant protein expression - Cell culture 18

3.2.2. Purification of recombinantly expressed protein 19

3.2.3. Quantitative protein analysis - Bradford assay 21

3.2.4. Gel electrophoresis - SDS-Page 21

3.2.5. Quick test – "ELISA" 22

3.2.6. Surface Plasmon Resonance 23

4. Methods 28

4.1. Growing and harvesting a bacterial culture 28

4.2. Recombinant protein purification 29

4.2.1. Preparation of cleared E. coli lysates 29

4.2.2. Protein purification by affinity chromatography 30

4.3. Quantitative protein analysis 31

4.4. Gel electrophoresis 32

4.5. Quick "ELISA" 34

4.6. SPR 36

4.6.1 Optical Setup and Sensor Chips 36

4.6.2. Immobilisation of protein 37

4.6.2.1. Immobilisation of his-tagged ligands 37

4.6.2.2. Immobilisation via amine coupling 38

5. Results 40

5.1. Quantitative protein analysis 40

5.1.1. Standard curve 40

5.1.2. Protein concentrations of samples 40

5.2. SDS-Polyacrylamid-Gelelektrophoresis 41

5.3. Quick Test 43

5.4. SPR Surface Plasmon Resonance 44
6. Discussion 47
7. Conclusion 48
8. Summary 49
9. Kurzzusammenfassung 50
References 51
Appendix 55

List of abbreviations

AIT	Austrian Institute of Technology
CV	Column Volumes
DF	Dilution factor
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
ELISA	Enzyme-Linked Immunosorbent Assay
FT	Flow through
HRP	Horseradish peroxidase
kDa	Kilodalton
LB	Luria-Bertani medium
IPTG	Isopropyl β-D-thiogalactoside
OD	Optical Density
SDS	Sodium dodecyl sulfate
SOC	Super optimal broth with catabolite repression
SDS-Page	Sodium dodecyl sulfate polyacrylamid gel electrophoresis
MW	Molecular Weight
Mv	Mean value
NHS	N-hydroxysuccinimide
PEG	Polyethylene glycol
IgY	Immunoglobulin Y
Ab	Antibody
Ag	Antigen
SPR	Surface Plasmon Resonance
SPs	Surface Plasmons
SAM	Self-assembled monolayer
SPFS	Surface plasmon-enhanced fluorescence spectroscopy
SPS	Surface Plasmon Spectroscopy
SUMO	Small ubiquitin-related modifier

List of tables

Table 1: Pipetting scheme of BSA solutions 32
Table 2: Recipe for a 10% mini gel for SDS-PAGE..... 33
Table 3: Final concentration of the samples V 41
Table 4: Materials 56
Table 5: Equipment..... 57
Table 6: Buffers for purification 58
Table 7: Bacterial growth I 60
Table 8: Bacterial growth II 60
Table 9: Bacterial growth III..... 60
Table 10: Absorbance of BSA at 595 nm 61
Table 11: Absorbance values I..... 62
Table 12: Absorbance values II..... 62
Table 13: Absorbance values III 62
Table 14: Final concentration of the samples II..... 62
Table 15: Absorbance values IV 63
Table 16: Final concentration of the samples IV 63
Table 17: Absorbance values V 63
Table 18: Material for preparing protein standards..... 64
Table 19: Equipment for purification by batch method..... 65
Table 20: Pipetting scheme of direct ELISA 66
Table 21: Absorption data of Quick test 66

List of figures

Figure 1: Structure of influenza viron 15
Figure 2: Flow chart of the experiment 16
Figure 3: The domain-swapped dimer of CVN 17
Figure 4: Binding of CVN-SUMO to varying concentrations of HA H3 18
Figure 5: Cell lysis (Brown, 2010) 20
Figure 6: Interaction between Ni-NTA matrix and neighbouring 20
Figure 7: Scheme of SDS-Page (Bio Rad, 2016) 22
Figure 8: Diagram of common ELISA formats (Abnova, 2016) 23
Figure 9: Principle of SPR (Biosensing Instruments, n.d.) 24
Figure 10: SPR Scanning Angle Response 24
Figure 11: Schematic of a sensogram 25
Figure 12: Scheme of Ni^{2+}/Cu^{2+}-mediated immobilisation of His-tagged ligands 26
Figure 13: Amine reaction (Johnsson et al., 1991) 27
Figure 14: Centrifugation to remove cell debris (Brown, 2010) 30
Figure 15: Direct ELISA (modified from Abnova, 2016) 35
Figure 16: BSA standard curve 40
Figure 17: SDS-Page analysis of rCVN (3.11.2015) 41
Figure 18: SDS-Page analysis of rCVN (13.4.2016, 2^{nd} culture batch) 42
Figure 19: Quick Test I 43
Figure 20: Binding of CVN-SUMO to HA H3 full length protein 44
Figure 21: BSA dilutions standards. 64
Figure 22: Ni2+ mediated immobilisation of his-tagged betagalactosidase 67

1. Introduction

In 1918, at the end of the First World War a global killer emerged causing almost 50 million deaths. This was the global influenza pandemic called Spanish flu, spreading around rapidly from person to person around the world. There were other influenza pandemics throughout the 20th and early 21st centuries. In April 2009, a new influenza virus emerged in Mexico. This virus was identified as a strain of Type A Influenza, subtype H1N1, it was the same subtype as the Spanish flu virus, emerged 90 years earlier (WHO & Global Influenza Programme, 2005).
Besides, the risk of a pandemic, seasonal influenza epidemics represent a year round disease burden, especially during cold seasons in the northern and southern hemispheres and all year round in tropical areas. It is estimated that seasonal epidemics cause 3 to 5 million cases of severe illness and about 25.0000 to 50.0000 deaths a year particularly in people at high risk: the elderly, pregnant women, infants under 5 years and individuals with specific chronic medical conditions (WHO, 2016).
There are three types of influenza viruses: A, B and C. First, type A influenza viruses are classified into the following subtypes: H1N1, H3N2, H5N1, H7N9. The letters and numbers are related to Hemagglutinin (H) and the Neuraminidase (N) proteins, which look like spikes on the surface of the virus (Figure 1). The numbers added after the H and N indicate in which order these proteins were discovered (WHO, 2014). These glycosylated enveloped surface glycoproteins, neuraminidase and hemagglutinin, certainly represent the main focus for vaccine development (Guo & Boons, 2009).
The following figure illustrates the basic structure of the influenza viron with its enveloped glycoproteins.

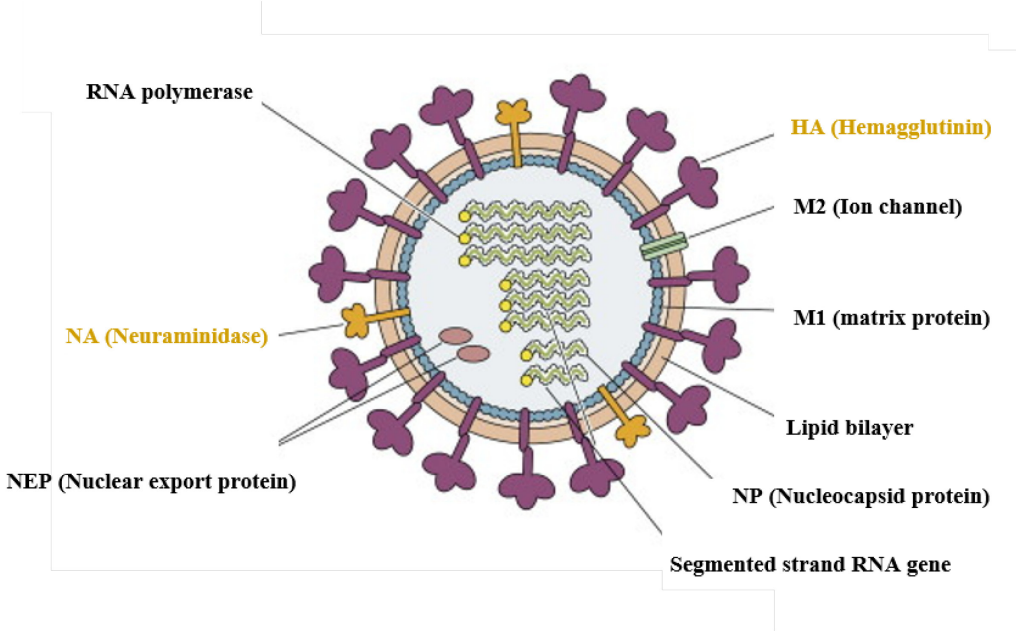


Figure 1: Structure of influenza viron
The hemagglutinin and neuraminidase proteins (coloured in yellow) are shown on the surface of the particle (Virology, 2009)

The glycosylation of hemagglutinin can modulate the immune response to infection. Seasonal influenza virus changes in antigenic structure by altering the number of glycosylation sites on HA. Evolutionary influenza studies (H1N1 and H3N2) show that the number of these glycosylation sites, on the head of hemagglutinin, increased after their emergence in the human population. Therefore, the addition of glycosylation sites might confer a selective advantage for the virus, likely by preventing the binding of antibodies (Tate et. al, 2014).

What is the connection between Influenza and Cyanovirin-N?

Cyanovirin-N is known for its broad neutralization ability against the human immunodeficiency virus (HIV). This mechanism is based on the binding of specific high-mannose oligosaccharides on HIV enveloped glycoproteins (Keeffe et. al, 2011). Structural analysis show similar oligosaccharide structures enveloped on Influenza viruses (Neu et. al, 2016).

Further research shows a potent anti-influenza activity of CVN against strains of Influenza A and B virus targeting the hemagglutinin on its surface (O'Keefe et. al, 2003).

2. Objectives

Neutralization of enveloped viruses and subtypes of viruses by targeting glycans exposed at the surface of viral antigens has become an important “tool” for the design of therapeutic agents to cure viral infections.

Another approach is based on the investigation of carbohydrate-binding proteins (e.g. lectins) that have high and specific affinity for the monosaccharide or oligo-saccharide structures present on viral enveloped glycoproteins.

Therefore, this thesis **aims** to establish a biointerface for SPR analysis to study the interactions between lectins and the viral glycoprotein hemagglutinin.

The thesis is divided into two experimental parts which represent two working phases. The first one mainly deals with the expression, biochemical characterization and quantification of the protein of interest **(1)**. Moreover, it copes with the development of a quick test based on an Enzyme Linked Immunosorbent Assay (ELISA) design **(2)** to confirm the presence of rCVN in solution via the detection of its SUMO-tag.

The second part of this thesis is focused on the instrumentation, assay design and surface modification of a biosensor chip for SPR Analysis. The main objective is to develop a suitable surface **(3)** as a basis for bimolecular interaction analyses between lectins and viral glycoproteins as a next step (see flow chart).

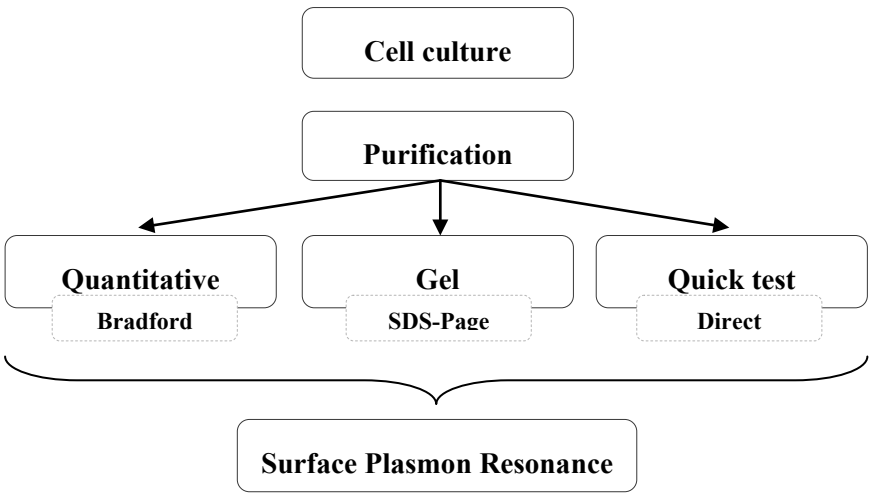


Figure 2: Flow chart of the experiment

3. Background

3.1. Cyanovirin-N

Cyanovirin-N (CVN) is an 11-kDa protein from the cyanobacterium Nostoc ellipsosporum. It has a unique sequence of 101 amino acid residues (European Protein Databank, 2002). Initially, the protein was isolated from the cyanobacterium Nostoc ellipsosporum within the framework of a screening program searching for novel anti-HIV agents (Boyd et. al, 1992).

Furthermore, some other promising lectins show antiviral activity against HIV and other enveloped viruses. The best options seem to be Griffithsin (GRFT), Microcystis virdis lectin (MVL) and scytovirin (SVN), but their physical and chemical stability does not appear to be as good as that of CVN (Xiong et al., 2010).

Structure

CVN has an extremely low sequence homology in comparison with any other known protein. It primarily consists of beta-sheats with an oblate shape and has a pseudosymmetric axis of rotation that relates the two subunits. Each subunit (CVN monomer) contains two binding sites. Additionally, CVN is insensitive to denaturants, detergents, organic solvents, and extreme temperatures (Barrientos, 2002). The following figure shows the domain-swapped dimer of CVN.

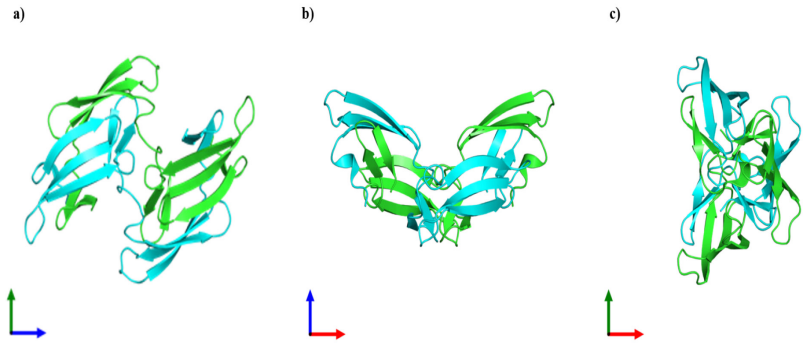


Figure 3: The domain-swapped dimer of CVN
CVN is coloured by chain and viewed from

a) the front b) the side c) the top
(Protein database in Europe, 2002)

Each monomer contains two symmetrical domains. The two domains A and B show a homologous amino acid sequence as well as a remarkable structural semblance. The monomeric CVN displays two binding sites whereas the domain swapped dimeric form shows four binding sites (Protein Databank in Europe, 2002). In solution the protein exists primarily as a monomer; with a domain-swapped dimeric form. The ability of dimerization increases the binding affinity by doubling the number of the binding sites, which are potentially augmenting its antiviral activity (Keeffe et. al, 2011).

The antiviral activity of CVN is mediated through specific, high-affinity interactions with the viral surface envelope glycoproteins, including HA. Gao and co-workers also showed that an attached SUMO-tag can stabilise and facilitate cytoplasmic expression in E. coli, and the corresponding fusion protein has been shown to bind HA H3 in this project (Figure 4) (Gao et al., 2009).

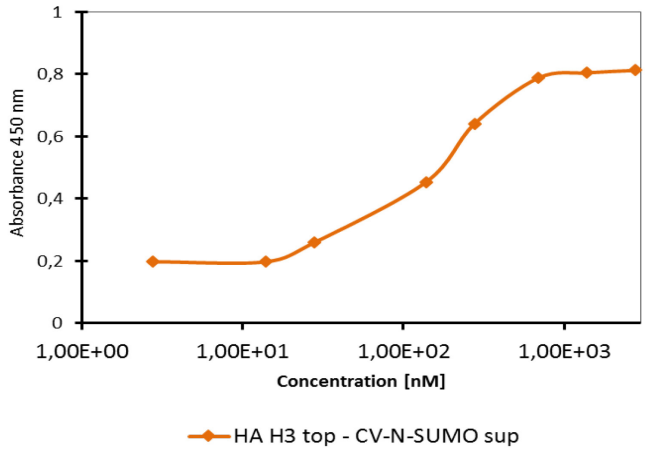


Figure 4: Binding of CVN-SUMO to varying concentrations of HA H3 studied by ELISA; Figure is credited to Irene Maier, *unpublished*

3.2. Experimental design

3.2.1. Recombinant protein expression - Cell culture

Escherichia coli (E.coli) is one of the organisms of choice for the production of recombinant proteins. The advantages of using E. coli as the host organism are

well known. The organism brings along fast growth kinetics and its doubling time is about 20 min. Furthermore, the method is cost efficient (Sezonov & Joseleau-Petit, 2007).

The expression of highly-purified rCVN in the cytoplasma of E. coli yields highest protein production rates. A careful selection of host strains, vectors and growth conditions results in a recombinant protein expression at high level. Naturally, SUMO-fused CVN comprises more than 30 percent of the total soluble protein in the cytoplasm. Along with this, Luria-Bertani medium suggests high growth yields and is optimal for growth at an early log phase (Rosano & Ceccarelli, 2014). In order to determine the growth of bacterial cultures, measuring the optical density at 600 nm (OD_{600}) is a common technique in microbiology. The growth of the cell culture should be monitored periodically until the culture reaches the OD of about 1.8 – 2.0. The cell pellets are harvested by centrifugation (Brown, 2010).

3.2.2. Purification of recombinantly expressed protein

Cell lysis

First, cell lysis results in extracting the protein of interest (Figure 5). Non-mechanical methods can be used in combination with mechanical force to ensure an optimal disruption of a cell.

Examples of physical disruption methods are liquid homogenization, sonication, freeze-thawing method, manual grinding and mechanical disruption with a mortal or a pestle. Alternatively, the addition of Lysozyme causes digestion of the polysaccharide components of yeast and bacterial cell walls. Protease inhibitors should be added to protect the proteins from degradation. The viscosity can be reduced by adding RNase or DNase to the samples (Labome, 2015 & Thermo Fisher Scientific, 2015).

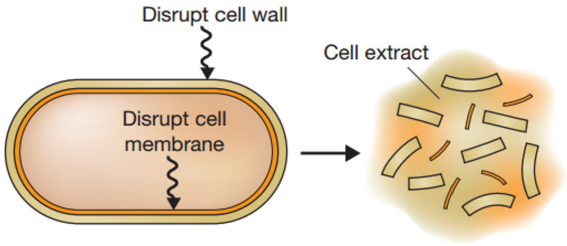


Figure 5: Cell lysis (Brown, 2010)

The addition of small amounts of imidazole to the lysis buffer minimizes nonspecific binding and reduces the amount of contaminating proteins. To prevent protein degradation, the cell pellet as well as the protein solutions should always be kept from 0 to 4 degrees Celsius.

Purification by affinity chromatography

The Co/Ni-NTA Agarose Purification system is an affinity chromatography matrix for purifying recombinant his-tagged proteins. It promotes an efficient binding of the his-tagged protein even if it is not fully accessible or the proteins concentration is low (QIAGEN, 2003). In general, cell lysates are loaded onto the packed column and the histidine residues of the proteins his-tag bind to the Co/Ni-nitrilotriacetic acid (NTA). Ni-NTA binding reaction shown in Figure 6.

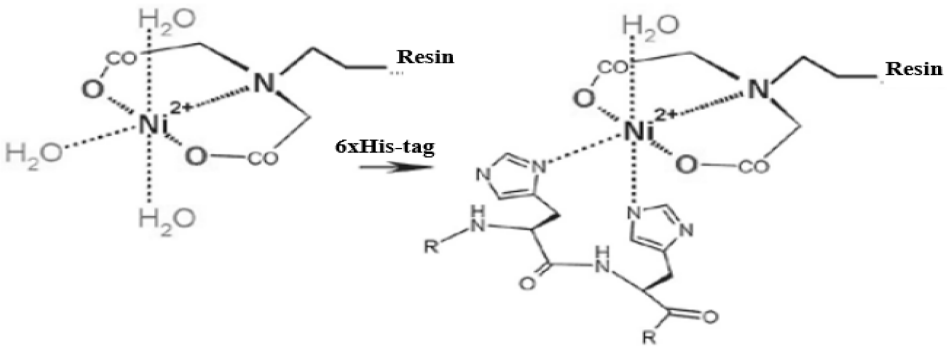


Figure 6: Interaction between Ni-NTA matrix and neighbouring pseudo electrophile residues in the his-tag (Kinesis, 2016)

After a washing step, the his-tagged proteins are eluted in buffer under native conditions. The addition of low concentration of imidazole to the lysis and wash buffer minimizes nonspecific bindings. Finally, an elution buffer is added to disrupt the binding interaction by a high concentration of imidazole. This leads to the release of the target molecule so that the 6 x his-tagged proteins can be collected in its purified form. This is followed by the removal of imidazole by a sephadex column and sequentially, the collected fractions can be directly used for further biochemical applications (Thermo Fisher Scientific, 2010 & Gao et. al., 2009).

3.2.3. Quantitative protein analysis - Bradford assay

Generally, the concentration of proteins is measured by using a UV-Vis spectrophotometer. The Bradford assay is a colorimetric protein assay used to obtain the concentration of proteins in solution. The principle of the Bradford method is simple. The Coomassie brilliant blue dye (which is negatively-charged) binds to the positively-charged proteins (primarily arginine, lysine and histidine), turns blue and absorbs at a wavelength of 595 nm. As a next step, the absorption per sample can then be compared to a standard curve. In most cases, bovine serum albumin serves as reference protein in order to construct a protein standard curve (Bradford, 1976).

3.2.4. Gel electrophoresis - SDS-Page

The sodium-dodecyl-sulfate polyacrylamide-agarose-gel-electrophoresis (SDS-Page) is a laboratory technique used to separate proteins according to their size. On the one hand, protein separation by SDS-Page allows to determine the relative molecular mass of a protein, and on the other hand to monitor the purity of the protein sample. To ensure a successful separation of proteins in an electrical field due to their molecular weight, the addition of SDS as an anionic detergent is required (SIGMA-ALDRICH, 2016).

The following figure shows the principle of a SDS-Page gel electrophoresis.

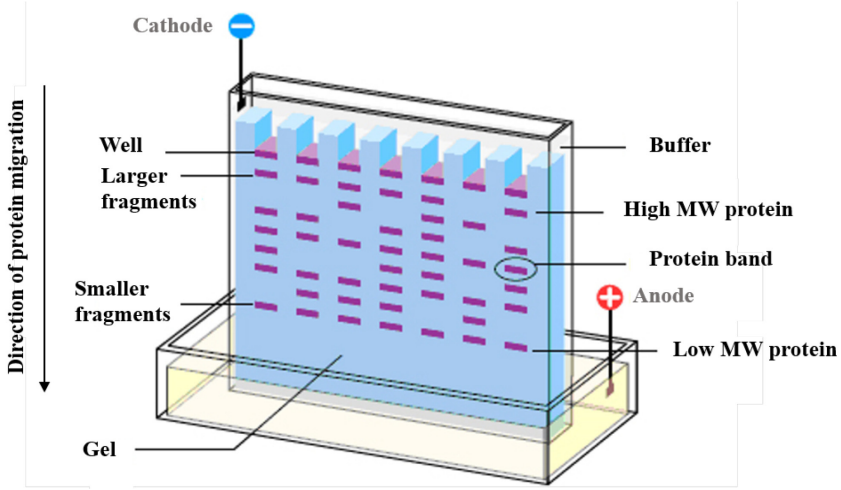


Figure 7: Scheme of SDS-Page (Bio Rad, 2016)

By adding SDS to the sample buffer, along with a boiling step and the addition of 2-Mercaptoethanol as reducing agent, the protein denaturises and SDS imparts an overall negative charge on the proteins according to the size of each protein - the run can start (Bio Rad, n.d.).

Finally, the visualization of the separated proteins can be achieved by Coomassie staining. This is a well-established staining technique, which is highly sensitive and suitable for long-term storage of the polyacrylamide gels. (SIGMA-ALDRICH, 2016).

3.2.5. Quick test – "ELISA"

The abbreviation "ELISA" stands for Enzyme-linked immunosorbent assay. It is a commonly used immunochemical method (golden standard) to detect the presence and to measure the quantity of an analyte (e.g. the biomarkers, peptides, proteins, hormones, bacterial antigens or antibodies) in solution. Nevertheless, this method remains time consuming and labour-intensive.

The different forms of ELISA formats are shown in Figure 8 (Pezzuto et. al., 2007).

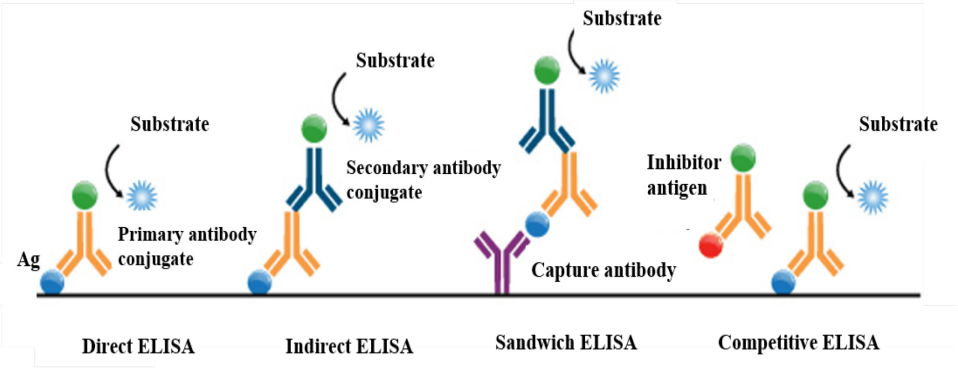


Figure 8: Diagram of common ELISA formats (Abnova, 2016)

In general, a typical assay can be carried out with several modifications to the basic procedure. Briefly, an ELISA consists of four steps: the coating of the antigen, the blocking, the reaction of antigen and antibody and the detection through a colorimetric substrate. The first step can be achieved through a direct adsorption of the antigen to the plate or indirectly through a capture antibody that has been coated to the assay plate. The antigen can be detected either directly through a labelled primary antibody or indirect via a labelled secondary antibody. Finally, the addition of a substrate (usually TMB) triggers an enzymatic colour reaction which has to be stopped with a stop solution changing the colour from blue to yellow (Thermo Fisher Scientific, 2015 & Rehm, 2006).

3.2.6. Surface Plasmon Resonance

Surface plasmon resonance or SPR has emerged as a powerful optical detection technique.

Surface Plasmon Resonance is an optical technique to study and detect bimolecular interactions within a variety of applications such as life sciences, electrobiochemistry, food and environmental safety.

Liedberg et al. first demonstrated a monitoring of biomolecular interactions vie SPR-based sensors in 1983 (Liedberg et al., 1983). In general surface plasmon resonance-based biosensors provide a great potential for real-time detection of biomolecular interactions. Further advantages of this method are the possibility of

a label-free detection, a high sensitivity and the ability to detect analytes at very low concentrations (Wolfbeis & Homola, 2006).

Basically, the method relies on the principle that the binding of biomolecules to biosensors' surface causes a change in the refractive index of the metal film resulting in an angular shift (Figure 9).

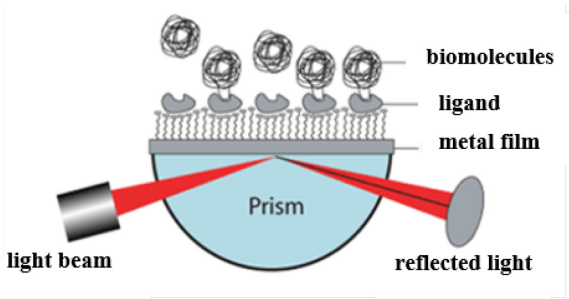


Figure 9: Principle of SPR (Biosensing Instruments, n.d.)

As a basic principle, the sensor chip forms a barrier between the optical system (dry system) and the flow cell (liquid system). The surface, a glass slide coated with a thin metal film, is covered with one more layer to ensure the immobilisation of the analyte which is injected through a flow cell. Polarised light from a laser source is directed through a prism to the under surface of the metal film where surface plasmons occur at a certain angle of incidence. The light is reflected from the surface, acting as a mirror and projected onto a detector (photodiode). As a result of a biomolecular binding event, a shift in the reflectivity curve can be observed (Richard et al., 2008). A typical response is illustrated in Figure 10.

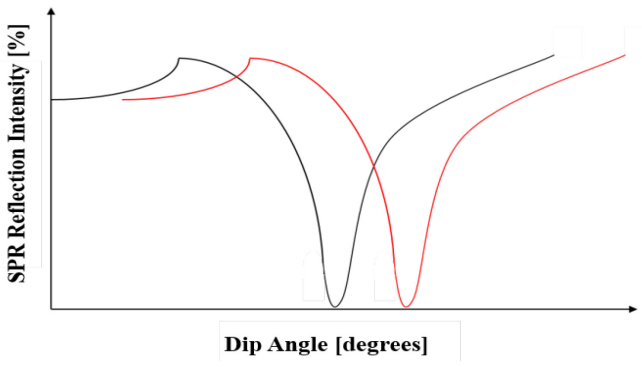


Figure 10: SPR Scanning Angle Response

Another powerful aspect of SPR technology is its ability to observe the time-depending interaction between molecules. Throughout monitoring this change in SPR response over time the kinetics of molecular binding events can be studied. The capture of the target molecules can be estimated from the refractive index change (RIU). First, a buffer baseline of the response is generated, which is followed by the injection of the analyte. The association phase is indicated by an increase in the signal. Once an equilibrium has been reached, the dissociation phase starts by rinsing with buffer again.
A typical response of an SPR real time measurement is shown in the following sensorgram (Figure 11) (Richard et al., 2008).

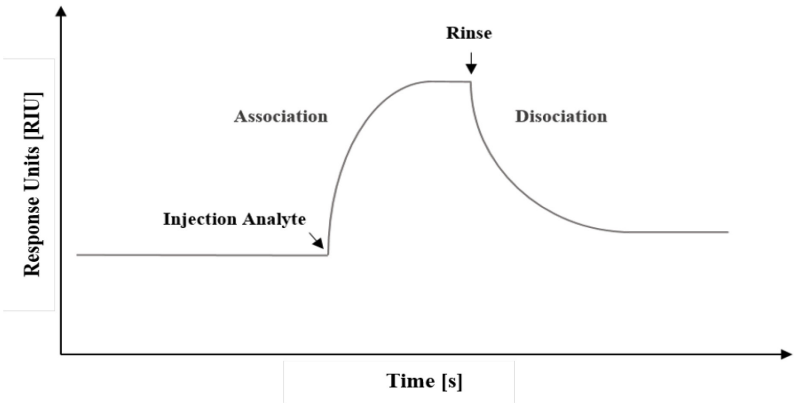


Figure 11: Schematic of a sensogram

The binding constant (KD) can be calculated from the association rate ('on rate', ka) and the dissociation rate ('off rate', kd).

Immobilisation

First and foremost, a glass slide coated with a thin gold film creates the sensors surface. This gold interface is needed as molecules can interact with the surface and cause a fluctuation in the refractive index. Moreover, gold surfaces are commonly used for the excitation of SPs because of their advantageous stability. Considering that the binding strength is relatively weak and that the orientation of the immobilized molecules cannot be controlled that well, this method is not recommendable for complex coupling analysis (De Mol & Fischer, 2010).

The choice of immobilisation methods plays a major role in the establishment of a biointerface suitable for SPR experiments. In the simpliest case, an analyte of interest is captured by a ligand which is immobilised on the sensor chip. The specificity of the surface is determined by the nature of the molecule attached to it. One binding partner is attached to the surface sensor chip while the other is injected in a continuous flow of solution. The attached interacting partner is called "ligand" while the partner in solution is called "analyte" (Richard et al., 2008).
In the particular case of binding rCVN, two different immobilisation methods appear most suitable:

- Protein binding via his-tag
- Amine coupling via reactive esters

Protein binding via his-tag

A Ni^{2+}/Cu^{2+}-mediated immobilisation of his-tagged ligands is one of the common techniques to immobilize proteins (Figure 12).

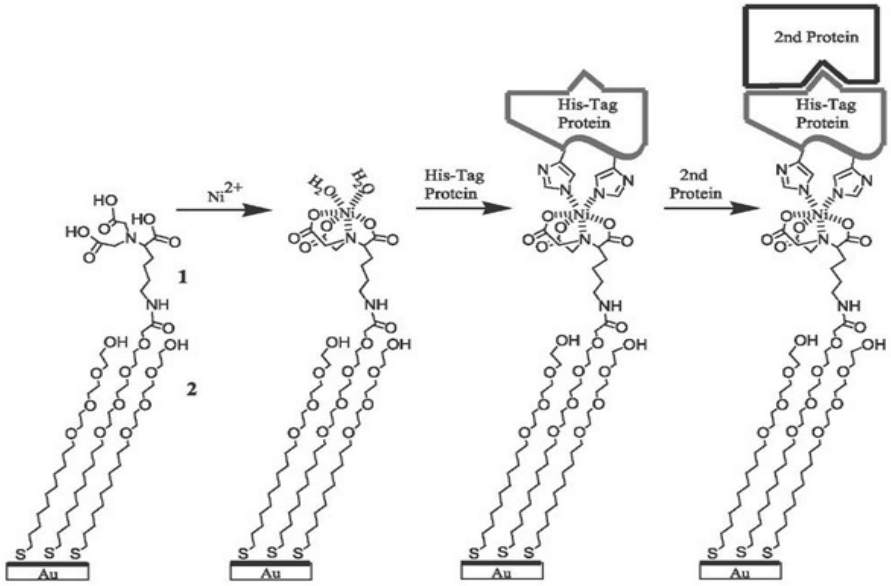


Figure 12: Scheme of Ni^{2+}/Cu^{2+}-mediated immobilisation of His-tagged ligands (The Crankshaft Publishing, n.d.)

At first, the surface is treated with nickel chloride (or optional with copper sulfate), which enables the Ni^{2+} ions to bind to the NTA which is subsequently available for chelating with the his-tag. As a last step, the histidine-tagged protein is able to bind a second protein (Richard et al., 2008).

Amine coupling via reactive esters

A stable linking to a metallic surface can be reached by a covalent coupling. In this case, a self-assembled monolayer of alkane thiol-derivates is immobilized to the gold surface which provide the functional groups needed.
Initially, the surface-bound carboxyl groups (-COOH) need to be activated by EDC/NHS resulting in reactive ester intermediates, which are then aminolyzed by NH_2-groups of the ligand as shown in Figure 13 (De Mol & Fischer, 2010).

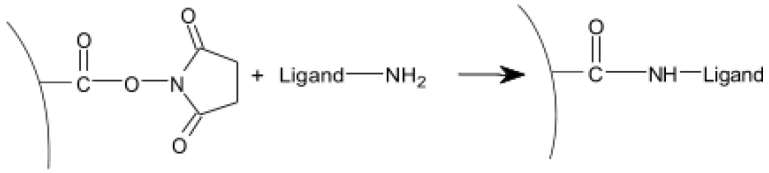


Figure 13: Amine reaction (Johnsson et al., 1991)

4. Methods

All the materials and equipment that were used in the experiments are listed in the appendix (Table 4 and 5).

4.1. Growing and harvesting a bacterial culture

Procedure

The preparation step of chemically competent Escherichia coli cells was carried out in cooperation with the University of Natural Resources and Life Sciences of Vienna (Division of Biochemistry).

For preparing competent E. coli cells, the Inoue method based on the Heat Shock Competent Cells Production Protocol was carried out (Inoue et. al., 1990).

All experiments on day one and two were carried out under sterile working conditions.

Day 1:

A single E. coli colony was picked from an agar plate of the expression host containing the recombinant plasmid (IMC 72), that had been incubated overnight. Afterwards 3 x 50 ml LB was inoculated with picked single colonies. 50 µl of the appropriate antibiotic Ampicillin was added in each flask. For growing a starter culture flasks were incubated overnight at 37° C.

The additional use of 600 µl SOC outgrowth medium maximizes the transformation efficiency of competent cells.

The original method, developed in 1990, suggests growing the E. coli cultures overnight at 18° C. Nonetheless, it works very well if they are grown overnight at 37°C in a shaking incubator. A flask containing 50 ml LB was used as control.

Day 2:

The growth of a preculture (3 flasks) could be observed with the naked eye. 3 x 2 L flasks containing 500 ml LB were prepared. 50ml of the preculture as well as

500 µl Ampicillin were added to each flask. Flasks were placed in the laboratory shaking incubator at 37° C for 1 h.

At this stage the estimated OD_{600} is about 4. However, before starting the induction process an OD_{600} of approximately 0,1 is needed. Therefore, 10 ml of the preculture were added to 500 ml LB again in each case. The optical density was measured with a wavelength of 600 nm, using the Infinite 200 PRO Multimode reader. For the measurement, 1 ml of the sample was pipetted in a glass cuvette and placed in the spectrophotometer at a wavelength of 600 nm.

Due to the fact that the procedure was performed two times, both results are listed in the appendix (Table 7 & 8).

Flasks were placed in the shaking incubator at 37°C to reach an OD_{600} of 0,55 – 0,60 and measured again.

As the next step, the protein expression was induced by the addition of 500 µl IPTG (1:1000 dilution). From this point on the cells didn't grow anymore and use their resources for the production of the target protein. After the induction process, the samples were incubated overnight at 16°C in the lab shaker.

Day 3:

A 1:10 dilution was prepared by the addition of 100 µl of the culture into 900µl of LB to measure the final optical density (Table 9 in the appendix) again.

Finally, the cell pellets were harvested by centrifugation (20min, 4000g, 4°C) and stored in the freezer at -20°C.

4.2. Recombinant protein purification

4.2.1. Preparation of cleared E. coli lysates

Procedure

The cell pellet was thawed on ice for 15-20 min. Cells (~3 g) were resuspended in lysis buffer by mechanical grinding at a ratio of 1g of cells per 5 ml of buffer,

followed by the addition of 1 mg/ml lysozyme. 150 µl of Protease Inhibitor Cocktail was added to the lysis buffer, too (1:100 dilution recommended).
The suspension was incubated for 30 min on ice. If it was observed that the cell lysate was very viscous, 6 bursts, each having the duration of 8 seconds at 110 W with a cooling period of 10 s between each burst using a sonicator was carried out.

Repeated cycles of freezing and thawing were applied to disrupt the cells through ice crystal formation, but this method did not further improve the purification results.

Cell lysate was centrifuged at 10.000 × g for 25 min at 4° C to pellet the cellular debris as shown in Figure 14 (Brown, 2010).

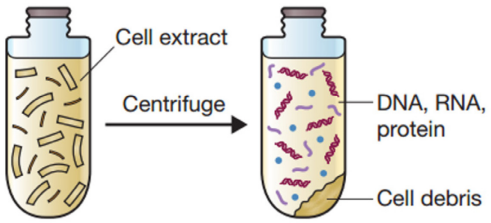


Figure 14: Centrifugation to remove cell debris (Brown, 2010)

Finally, the supernatant was saved and temporarily stored on ice. 1ml of the supernatant was saved for SDS-Page analysis (QIAGEN, 2003 & Gao et al., 2009).

4.2.2. Protein purification by affinity chromatography

Procedure

Incubation step: At first 5 ml (=1 CV) of the Ni-NTA slurry were added to the supernatant and mixed gently on ice on a rotary shaker lasting for 60 min.
Afterwards the lysate-Ni-NTA mixture was loaded into the column.
The stock was opened and the column flow-through (FT) was collected.

Then the resin was washed with wash buffer (2 × 2 CV). These wash fractions (W1, W2) were collected for SDS-analysis again. The target protein was eluted with elution buffer (E1-E4) and collected again (4 × ½ CV) (QIAGEN, 2003). The collected samples from each fraction (CL, FT, W1, W2, E1-E4) were subjected to SDS-Page analysis. Furthermore, the protein concentration was determined by a spectrophometric assay.

Procedure for Ni-NTA resin resignation:
Firstly, Ni-NTA Resin was washed with 10 CV of Lysis Buffer. Secondly, the Ni-NTA Resin was washed with 10 CV of ultrapure water. After that the Ni-NTA Resin was stored in 20% ethanol (Thermo Scientific, n.d.).

Note: As an alternative way to purify the target protein from the cleared lysate more effectively, 1-step batch Midi Plus columns were used. The protocol as well as some pertinent notes can be found in the appendix.

4.3. Quantitative protein analysis

Procedure

Standard curve

First of all, a standard curve based on dilution series of BSA was generated.
A series of protein standard solutions of BSA with concentrations ranging from 0,125 mg/mL to 1,5 mg/ml were prepared (Table 1).

Used formula: $C_1 \times V_1 = C_2 \times V_2$

1 ml of the protein sample and 3 ml of the Bradford reagent were mixed and gently vortexed. After an incubation time of about 5 minutes the absorbance of the solutions was measured in duplicate at the optimum measurement wavelength of 595 nm in the spectrophotometer (SIGMA-ALDRICH, 2000 & Universität Wien, n.d.).

Standard	BSA (µl)	Buffer (µl)	Concentration (mg/ml)
1	750	250	1,5
2	500	500	1,0
3	250 of S1	250	0,75
4	500 of S2	500	0,5
5	500 of S4	500	0,25
6	500 of S5	500	0,125

Table 1: Pipetting scheme of BSA solutions
*Blank already subtracted

The glass cuvettes needed to be rinsed with ethanol. Finally, a standard curve was drawn by plotting the concentration of BSA against absorbance at the given wavelength.

Bradford Assay

For a quantitative analysis of the concentration the protein samples 0.1 ml of the protein sample and 3 ml of the Bradford reagent were pipetted into a tube, vortexed gently and the absorbance was measured again at 595 nm. The approximate protein concentration of the samples was estimated using the plotted equation of the standard curve of BSA.

4.4. Gel electrophoresis

Procedure

Preparation of handcasting gels:
First, the casting frames (two glass plates were clamped in the frames) were set on the casting stands. The separating gels were prepared as described in Table 2. All solutions which were used in this experiment are listed in the appendix.

Note: Solution E and TEMED must be added right before each use.

The separating gel solution was pipetted into the gap between the glass plates (remaining ~ 1.5 cm for the stacking gel). The gel was overplayed with ~2 mm isopropanol to exclude oxygen and ensure a flat interface between the two gels. It takes about 45 min until the polyacrylamide is formed by the reaction of acrylamide and bis-acrylamide.

The preparation of the stacking gel follows the same steps as the separating gel ones. The stacking gel was prepared, poured on top of the gel cassette and the comb was slowly inserted into the stacking gel. It took again about 45 min until the separating gel polymerized.

Solution	Seperating gel	Stacking gel
	10%	4%
A	6.7 mL	1.3 mL
H_2O dest.	8.0 mL	6.1 mL
B	5.0 mL	-
C	-	2.5 mL
D	200 µl	100 µl
E	100 µl	50 µl
TEMED	10 µl	10 µl
∑	20 mL	10 mL

Table 2: Recipe for a 10% mini gel for SDS-PAGE

Sample preparation:

10 µL of each sample were mixed with 10 µL of the loading dye and heated for 5 min at 95° C in a water bath (BIO-RAD, n.d.). The chemical compound 2-Mercaptoethanol in the loading dye is added as reducing agent in order to break all disulphide bonds.

In most cases, the proteins BSA and Taka-Diastase were used as standard proteins (see testing in the appendix). If a commercial protein marker was used, only 5 µL was loaded in the wells directly.

Electrophoresis:

First, the comp was removed and the two gel cassettes were set up in the tank. Then the tank was filled with 500 mL running buffer and the prepared samples were loaded in the wells.

Electrophoresis was started at a constant voltage of 200 V.

Before the dye front reached the bottom of the gel the run was stopped (~ 45 min).

Staining:

The gel was removed from the cassette and dropped into glass bowl with staining solution. Staining was usually done overnight or at least 2 - 4 hours with agitation. The glass bowl was covered during the staining process to avoid contamination and to prevent the evaporation of the solution.

The stain solution was removed and replaced by the destain solution for at least 2 hours. The destain solution was changed several times. The destaining step was continued until the protein bands were seen without any blue background staining of the gel. The gels were stored in fresh, ultrapure water.

4.5. Quick "ELISA"

Procedure

To confirm the presence of the antigen (rCVN) in the sample a type of direct ELISA was carried out (Figure 15).

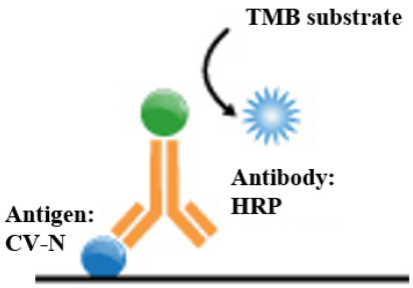


Figure 15: Direct ELISA (modified from Abnova, 2016)

Firstly, the purified protein samples (rCVN) were bound directly to the well through adsorption. Secondly, an antibody (HRP) was used to confirm the presence of the recombinant CVN used to bind the proteins SUMO-tag.

Coating:

The microtiter plate was coated with different dilutions of the purified rCVN using the coating buffer as the diluent. 100 µl of the diluted antigen were added per well. All samples were added in triplicate to ensure the accuracy of the results. Furthermore, the protein samples were tested in three different dilutions (1:1, 1:10, 1:100). Additionally, 100 µl of 1% BSA in PBS were coated as negative control. Afterwards the plate was covered with an adhesive plastic film and incubated overnight at 4° C.

The next day, the coating solution was removed followed by five washing steps (to thoroughly remove unbound material) with washing buffer using a multipipette. After every washing step, the plate was patted dry on a paper towel.

Blocking:

120 µl of blocking buffer were added to each well and incubated for 2 hours at room temperature. The blocking buffer was flicked out and patted dry again followed by five washing steps.

Incubation:

First, an antibody dilution of 1:10.000, according to the manufacturer's instructions, was prepared. Then 100 µl of the diluted antibody were added to

each well or without any antibody as negative control. The plate was covered with an adhesive plastic film again and incubated overnight at 4° C.

Detection and Plate reading:

A further series of five washing steps was performed. Finally, 100 µl of TMB substrate were added to each well inducing peroxidase reaction. The plate was incubated for about 15 minutes, until the wells turned blue, at room temperature. The reaction was stopped by adding 100 µl of stop solution or 0.5 M Sulforic Acid on the other half of the plate by way of comparison. The optical density of each well was determined within 20 minutes. The absorbance was read at $\lambda = 450$ nm using the plate reader.

4.6. SPR

All experiments were performed with a self-assembled SPR-Setup by group members of the Plasmonic Sensor Group at the AIT.

4.6.1 Optical Setup and Sensor Chips

For the excitation of surface plasmons (SPs) a helium-neon laser ($\lambda ex= 632.8$ nm) was used. The prism, consisted of LASFN9 glass, was matched to a sensor chip using immersion oil. The sensor chip was clamped onto a self-assembled flow cell made from fused silica. The flow cell contains a simple inlet and outlet which are connected to tubings. A peristaltic pump is used to pump the liquid through the tubings to the sensor chip surface.

Preparation of Sensor Chips

In general, the coating on a biosensor chip usually consists of the following elements: a mechanic carrier (glass), a metal layer (chrome, gold) and a linking layer.

Procedure

Glass slides were cut to a size of 2.0 x 2.5 mm from standard microscope slides. Then they were washed with Isopropanol, dried and put into a tank. All glass slides were sonicated for 15 min in a soapy solution (50 µl Hellmanex, H_20 dest.), washed with H_20 dest., ethanol and dryed again. The gold layer was evaporated using thermal vacuum evaporation (Auto 306, HHV). The glass slides were coated with 2 nm of chrome and 50 nm of gold.
In order to control the layer thickness of chromium and gold an angular scan (20°-30°) was carried out. The real thickness of the metal layer was calculated using the programm "Winspall".
As a next step, the coated sensor chips were immersed either in a 1 mM solution of COOH-dithiols and OH-dithiols or in a 1mM solution of the NTA-terminated PEG and OH-dithiols, which were dissolved in ethanol at a 1:9 ratio.
The incubation of the sensor chip was carried out overnight in order to form a self assembled monolayer (SAM). On the next day, the sensor chip was washed with ethanol, dried under a stream of nitrogen and stored into a tank under argon atmosphere until use for the analysis (Raether, 1989 & Richard et al., 2008).

4.6.2. Immobilisation of protein

4.6.2.1. Immobilisation of his-tagged ligands

Procedure

Going for a Ni^{2+}/Cu^{2+}-mediated immobilisation of the his-tagged ligand the sensor chips derivatised with NTA was used. Before starting the time measurement, the specific angle was measured. As the measurement of the SPR angle is the most representative parameter of a biomolecular interaction, an angular scan (45°-60°) was measured before and after every injection. All reagents and buffers were used at a flow rate of 10 µl/min. In addition, before starting the

immobilisation phase a calibration step measuring different concentrations of Ethylenglycol (1 %, 2 %, 4 %, 2 %, 1 %) was carried out.
First of all, the surface was washed with PBS for about 20 min. The sensor chip was loaded with 0.3 M $NiCl_2$ or $CuSO_4$ (~20 min) followed by another injection of PBS (~ 2 min). Subsequently the injection of ligand (Beta-galactosidase or rCVN) in immobilisation buffer followed. The dissociation phase was induced by a further injection of PBS after a stable baseline was reached.

An immobilisation of the his-tagged Cyanovirin-N was also carried out in petri dish, but did not result in a better response.

Due to a low response (RU) at this stage the binding experiment of the his-tagged ligand via Ni^{2+}/Cu^{2+}-mediated immobilisation with the sensor chip was stopped.

4.6.2.2. Immobilisation via amine coupling

Procedure

All reagents and buffers were used at a flow rate of 10 µl/min. Before starting the time measurement, the specific angle was measured since the SPR angle is the most representative parameter of biomolecular interactions. An angular scan (45°-60°) was measured before and after every injection, except after the activation step. In addition, a calibration step measuring different concentrations of Ethylenglycol (1%, 2%, 4%, 2%, 1%) was carried out before starting the immobilisation phase.
The surface was washed with acetate buffer for about 20 min. The EDC-containing activation buffer was prepared in the meantime: 21.0 mg NHS in 1 ml H_2O dest. + 75.0 mg EDC in 1 ml H_2O dest. were dissolved, mixed together (1:1) and vortexed. The freshly prepared activation mix was injected for about 15 minutes followed by a brief rinse with acidic coupling buffer.

After the activation step, the experiment continued with the injection of the ligand (beta-galactosidase, rCVN or HA). The equilibrium signal level was checked. If the binding capacity was too low, the coupling procedure was continued with a higher concentration of the ligand. After a stable baseline was reached the dissociation phase was induced once more by the injection of acetate buffer again. When a complete coupling reaction was observed, the system was switched to sample buffer (PBS or HEPES containing 0.01% Tween 20) and the ligand was injected. The equilibrium signal level was checked again. If the binding capacity was too low, the coupling procedure was continued with a higher concentration of the analyte. If an equilibrium was reached, the dissociation phase was induced by a further the injection of sample buffer.

5. Results

5.1. Quantitative protein analysis

5.1.1. Standard curve

A standard curve of bovine serum albumin was generated (Figure 16). The equation of the following standard curve served as a base for the determination of the protein concentration in the samples. The appertaining absorbance rates of BSA at a wavelength of 595 nm are attached in the appendix.

Formula: y = 0.7254 x – 0.0334

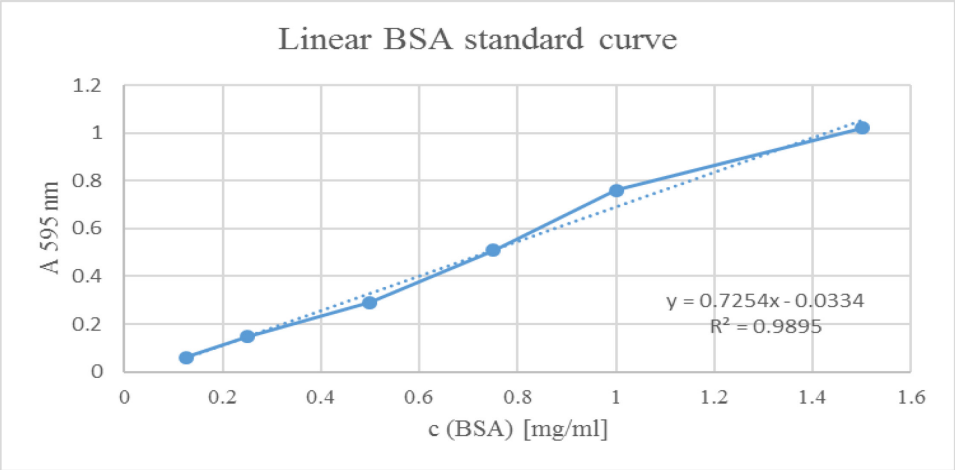


Figure 16: BSA standard curve

5.1.2. Protein concentrations of samples

In all cases the protein concentration was determined with the following equation:

y = 0.7254 x – 0.0334

The following table shows the calculated concentrations for the purification process carried out on April 13th, 2016. The first elution fraction (marked in green) was chosen for some SPR experiments. All absorbance values of the samples can be found in the appendix.

SAMPLES, DATE: 13.04.2016

	LZ	FT	W1	W2	E1	E2	E3	E4
c [mg/ml]	1.910	1.307	1.176	0.164	0.402	0.120	0.111	0.022

Table 3: Final concentration of the samples V
LZ (lysate after centrifugation), FT (flow-through), W (washing), E (elution)

5.2. SDS-Polyacrylamid-Gelelektrophoresis

The following SDS-Page gel shows all samples taken at all stages of the purification.

The predicted molecular mass of CVN as a his-SUMO fusion protein is ~ 43 kDa. The arrow indicates the purified his-tagged SUMO-CVN.

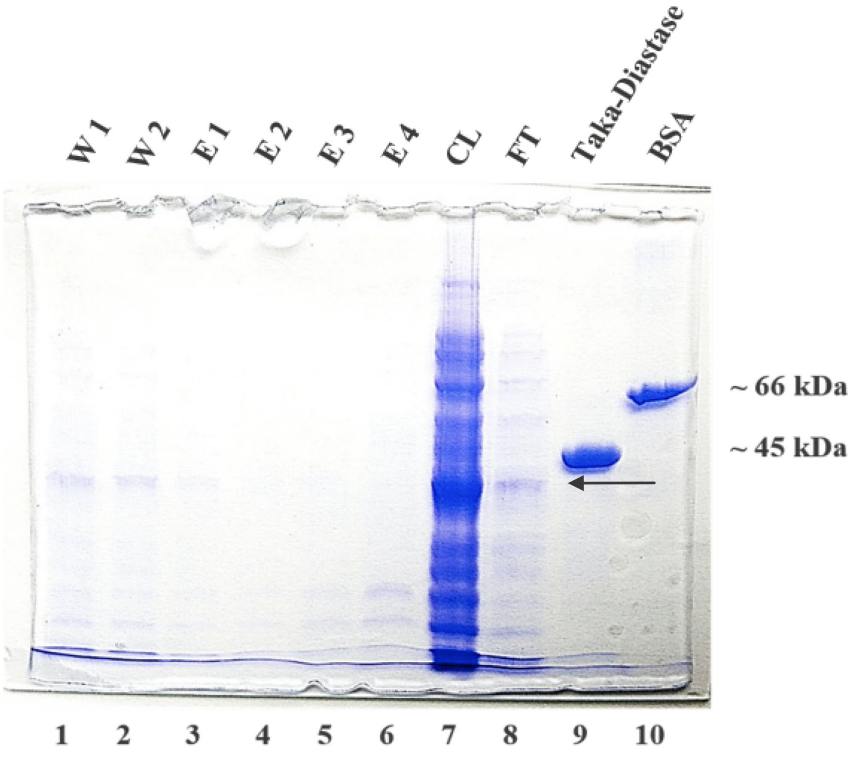


Figure 17: SDS-Page analysis of rCVN (3.11.2015)
All fractions were collected from the purification and resolved using SDS-Page. Lane 1: First wash fraction Lane 2: Second wash fraction Lane 3: First elution fraction Lane 4: Second elution fraction Lane 5: Third elution fraction Lane 6: Forth elution fraction Lane 7: Cleared lysate Lane 8: Flow through Lane 9: Taka-Diastase c = 0,3 mg/ml Lane 10: Bovine serum albumin c = 0,5 mg/ml

How is the molecular weight calculated?

CVN as a dimer has a molecular mass of ~ 22 kDa (monomer ~ 11 kDa). The addition of a his-tag has little effect on the molecular mass by adding ~ 1 kDa to the weight of the protein. The SUMO tag has an expected molecular weight of ~ 20 kDa. Therefore, the calculated molecular mass of CVN as a his-SUMO fusion protein is ~ 43 kDa.

Another representative gel is shown in Figure 18.

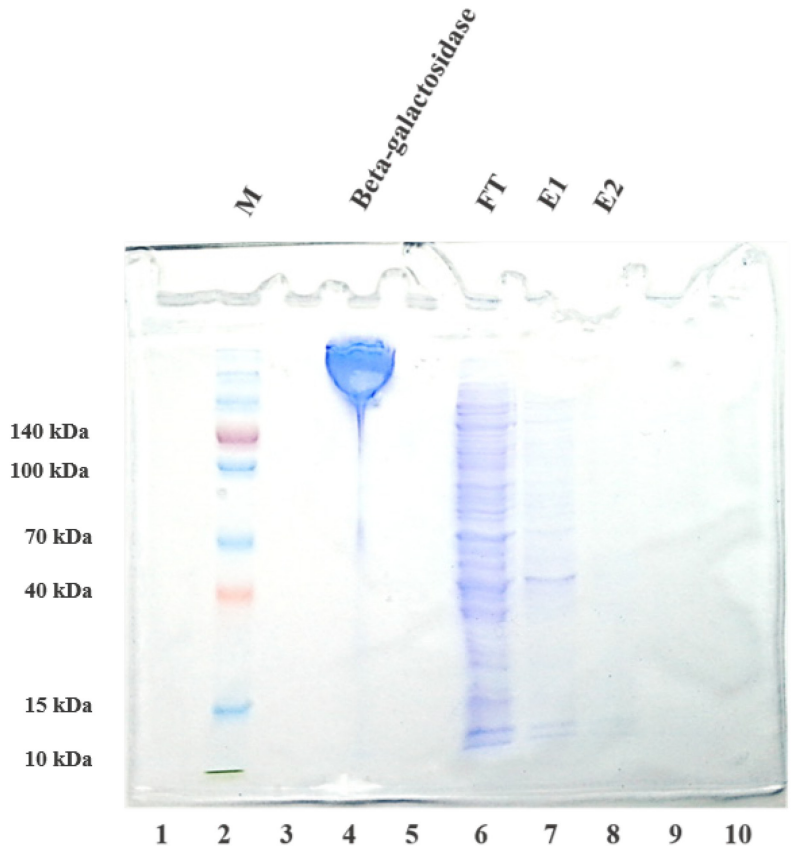


Figure 18: SDS-Page analysis of rCVN (13.4.2016, 2nd culture batch)
All fractions were collected from the purification and resolved using SDS-Page. Lane 1: Buffer Lane 2: protein marker Lane 3: Buffer Lane 4: his-tagged beta-galactosidase c= 2.2 mg/ml Lane 5: Buffer Lane 6: Flow Through Lane 7: First elution fraction Lane 8: Second elution fraction Lane 9: Buffer Lane 10: Buffer

The 7th lane shows the purified recombinant CVN which appeared as a single band (~ 45 kDa). The 4th lane shows an overload of beta-galactosidase. The con-

centration of 4,4 mg/ml was measured with a NanoDrop 2000 spectrophotometer at a later stage of the experiments (AIT).
Repeated experiments showed that IMC72 expression resulted consistently in an observed protein band of 35-40 kDa.

Furthermore, a Western blot to confirm the rCVN findings by detecting its his-tag was performed by the Department of Glycobiology (BOKU).

5.3. Quick Test

Quick Test II

The presence of rCVN was tested by an enzyme-linked immunosorbent assay. Figure 19 shows an ELISA plate illustrating the presumable binding of the HRP-conjugated Anti-SUMO antibody to the purified recombinant Cyanovirin-N. The Quicktest was used as a qualitative method, indicating whether a particular antigen (rCVN) shows a certain response in the samples.

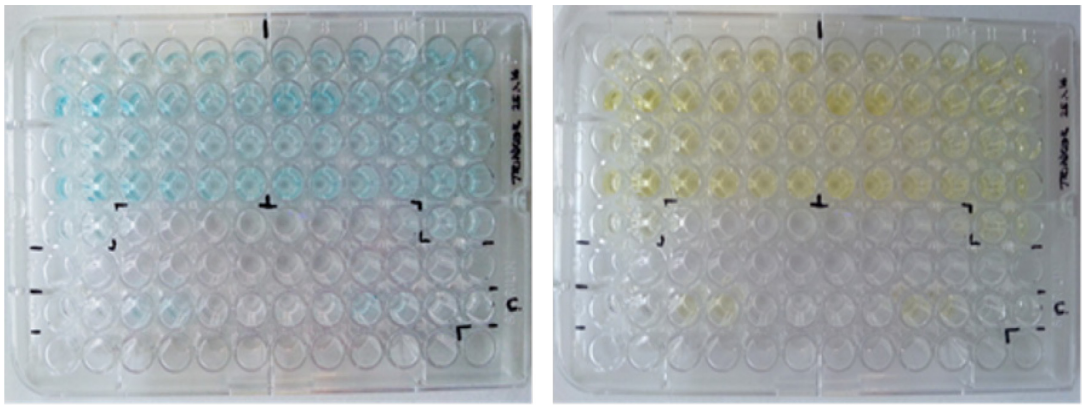
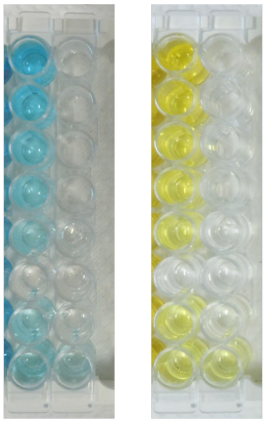


Figure 19: Quick Test I
Left plate: After the addition of TMB solution Right plate: After the addition of stop solution, the colour changes from blue to yellow. Wells were coated with rCVN (samples in triplicate) and tittered with a 1:5.000 dilution of the HRP-conjugated Anti-SUMO antibody.

The basic raster of the mictrotiter plate (Figure 19) is attached in the appendix. This Quicktest shows an overall positive response and allows the assumption that rCVN is present in the samples.
Besides that, a second Quicktest (Quicktest II) was performed to confirm the the strong presumption of the binding of CVN-SUMO to Hemaglutinin (HA H3).

Quick Test II

The following figure (Figure 20) shows the microtiter plate of an Quicktest ELISA illustrating the binding of CVN-SUMO to Hemaglutinin (HA H3).



Left Lane: A-E: Concentration-dependent binding of SUMO-CVN fusion protein to HA.
F: Negative control without antibody readout.
G-H: High background due to unspecific and direct binding of anti-SUMO to HA.

Right Lane: G: Negative control without HA coating and SUMO-CVN, but incubation of antibody solution.
H: Control showing some background binding of SUMO-CVN (25 µg/ml) to blocked wells.

Figure 20: Binding of CVN-SUMO to HA H3 full length protein A) Quick-test ELISA without and B) with addition of stop solution. Wells were coated with 3 µg/ml HA H3, blocked with BSA and titered with 2-fold diluted series of CVN (50 µg/ml). For detection, anti-SUMO-HRP conjugated antibody was used 1:10,000 PBS-T. Figure credited to Irene Maier.

The absorbance values (Figure 20) are given in the appendix. This Quicktest confirms the hypothesis that CVN-SUMO binds to hemagglutinin as a full length protein (HA H3).

5.4. SPR Surface Plasmon Resonance

The first step of the SPR experiments was the measurement of the angle of incidence in order to determine the real thickness of chrome and gold. This plot shows the angle of incidence of the evaporated gold surface.

Figure 21: Angle of incidence of gold surface in air

The thickness of the evaporated chrome and gold layers was controlled with the so-called program Winspall at the specific angle of 26.47°.

Evaporation of chrome according to *Evaporater*: 2.1 nm chrome

Winspall: 2.38 nm chrome

Evaporation of gold according to *Evaporater*: 50 nm gold

Winspall: 56.12 nm gold

Immobilisation via amine coupling

The following two plots show the angle of incidence of the immobilization procedure of beta-galactosidase via amine coupling. A dip shift with about 0.5° is clearly recognizable and hence indicates a successful amine binding of the analyte.

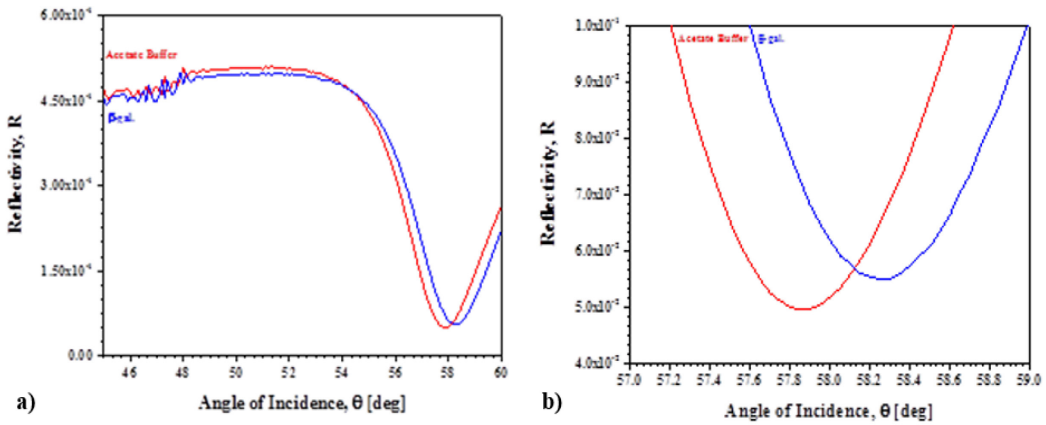


Figure 22: Immobilisation of β-galactosidase (250 µg/ml) via amine coupling
a) Specific angle of beta-galactosidase (250 µg/ml) after amine coupling b) Zoomed in of plot a.

Figure 20 shows a part of a SPR sensorgram (a) as well as the angle of incidence (b) measured from the immobilization procedure of Hemgglutinin via amine coupling.

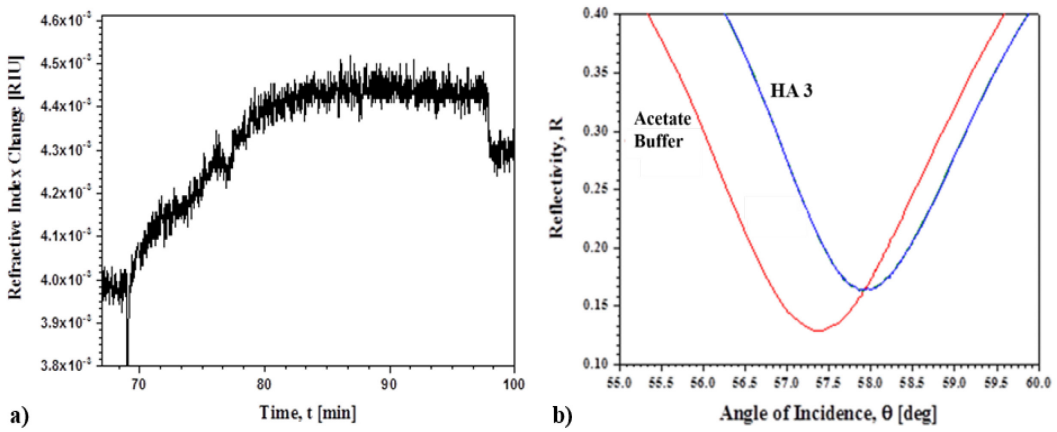


Figure 23: Immobilisation of hemagglutinin (250 µg/ml) via amine coupling
a) Part of SPR sensorgram focusing on Hemagglutinin (250 µg/ml) binding b) Specific angular reflectance spectra of Hemagglutinin (250 µg/ml) after amine binding

The differences in the height of the signal (RIU) before and after the secondary hemagglutinin binding can be considered an effective binding.

6. Discussion

As the research in this field is multidisciplinary, many different methods were carried out at the various institutions. Although, some experiments were already performed in the United States, it is worth mentioning that it was the first time to reproduce these data in Austria.

In spite of the fact that a lot of studies have been performed to gain new details about the interaction between Cyanovirin-N and carbohydrates, the specific interaction had not been explored. To experimentally determine whether the Cyanovirin-N occurs in monomeric or dimeric form, an X-ray crystallography or a mass spectrometer analysis has to be carried out.

Some possible improvements for further research can be mentioned: Especially in the quick "ELISA" presented here, more time may be invested in the assay design. The addition of an anti-his antibody as control and the creation of a standard curve for an additional quantitative evaluation could be optimised. The releasement of the SUMO-tag by cleaving it with sumo protease would allow to distinguish between fusion and untagged protein. Unspecific binding of anti-SUMO antibody to CVN could be specified and SDS-PAGE would estimate the size of each protein part separately.

The next step to finish the development of an appropriate biotinterface for a hemagglutinin to Cyanovirin-N interaction should now be the immobilisation of a freshly prepared purified rCVN to the surface. If a SPR experiment with freshly prepared purified rCVN shows no or low response, the implementation of surface plasmon-enhanced fluorescence spectroscopy (SPFS) may be a better method. The advantages of SPFS-based biosensors are: highly sensitive, enable the detection of small molecules as well as low analyte concentrations.

7. Conclusion

More time is needed to confirm the (unpublished) data on the binding affinity as well as the binding kinetics between Cyanovirin-N and Hemagglutinin.

As the high genetic variability of viruses makes the development of vaccines highly complex, the question remains open whether the research of Cyanovirin-N will lead to the development of a clinical drug candidate in future, or not.

Despite Cyanovirin-N has proved to be an appropriate model system for studying lectin glycan interactions, the detailed mechanism between Cyanovirin-N and enveloped glycoproteins is still elusive.

More detailed pathogenesis studies are needed to answer open questions about the fully understanding of cross reactivities or an unwanted immune response.

Finally, for future consideration, the use of antivirals with different mechanisms of action (multiple inhibitors e.g. HA-inhibitors, Neuraminidase inhibitors) might improve the effectiveness of drugs and become a tool to handle the problem of anti-viral resistance of the influenza virus.

8. Summary

Whenever people come into close contact with each other it is a formidable task to find suitable methods for preventing diseases such as influenza. Influenza is a viral infection of the upper respiratory tract that raises global public health threat and affects millions of people every year.

A certain protection against influenza can be achieved by the administration of antibodies throughout a vaccination. Another approach to reach antiviral binding is based on the analysis of their multi-specific affinity for the monosaccharide or oligosaccharide structures (glycans) present on viral enveloped glycoproteins.

Lectins, such as the prokaryotic Cyanovirin-N (CVN), may have the potential to inhibit Influenza A virus infections by binding glycans such as hemagglutinin (HA).

This thesis aims at developing a suitable surface for biomolecular interaction analyses between lectins and hemagglutinin.

Therefore, purification and characterisation of CVN as a his-SUMO fusion protein were performed. Purification procedure itself was carried out by Ni-NTA affinity chromatography under native conditions. The purity and presence of purified recombinant CVN (rCVN) was checked by using a gel electrophoresis method (SDS-PAGE analysis). Furthermore, a quick test based on Enzyme-linked Immunosorbent Assay (ELISA) designed for estimating the binding properties of rCVN was carried out. Additionally, the development of a suitable assay design and surface modification for a biosensor chip used for SPR analysis was achieved. A sensor surface with immobilized hemagglutinin was designed, which will be ready for further binding of Cyanovirin-N. The thesis was conducted in collaboration with the University of Vienna (Department of Nutritional Sciences), the University of Natural Resources and Life Sciences of Vienna and the Austrian Institute of Technology.

9. Kurzzusammenfassung

Influenza ist eine virale Infektion der oberen Atemwege, welche ein weltweites Gesundheitsproblem darstellt und jährlich Millionen von Menschen betrifft.
Ein gewisser Schutz gegen Influenza kann durch die Verabreichung von Antikörpern erreicht werden (im Zuge einer Impfung). Ein weiterer Ansatz um einen antiviralen Bindungspartner zu entwickeln basiert auf der Erforschung der Affinität dieser Antikörper mit Mono- und Oligosacchariden (Glykanen), der sich an der Virushülle befindlichen Glykoproteine.
Lektine, wie das prokariontische Cyanovirin-N, haben möglicherweise das Potential eine Infektion mit Grippeviren, über die Bindung des Glycans Hemaglutinin (HA) stark abzuschwächen.
Demzufolge beschäftigt sich diese Masterarbeit mit der Entwicklung einer passenden Oberfläche für Bindungsstudien von Lektinen an Hemagglutinin.
Aus diesem Grund, wurde eine Aufreinigung und Charakterisation von CVN als His-SUMO fusions protein ausgeführt. Der Aufreinigungsprozess wurde unter nativen Bedingungen mittels einer Ni-NTA Affinitätschromatographie durchgeführt. Die Reinheit und das Vorkommen des aufgereinigten rekombinanten CVN (rCVN) wurde mittels einer Gelelektrophorese (SDS-Page) überprüft. Desweiteren wurde ein Quicktest, angelehnt an einen Enzym-linked Immunosorbent Assay, durchgeführt um die Bindungseigenschaften des rCVNs abschätzen zu können. Anschließend sollen in dieser Masterarbeit die Entwicklung eines passenden Assaydesigns und die Oberflächenmodifikation eines Biosensor Chips für SPR Analysen erarbeitet werden.
Schließlich wurde eine mit Hemagglutinin immobilisierte Sensor-Oberfläche entworfen, um weitere Analysen mit dem rekombinanten Cyanovirin-N durchzuführen.
Die vorliegende Arbeit wurde in Zusammenarbeit zwischen der Universität Wien (Departement für Ernährungswissenschaften), der Universität für Bodenkultur und dem Austrian Institute of Technology durchgeführt.

References

ABCAM. Direct ELISA protocol. n.d. Available from: http://www.abcam.com/ps/pdf/protocols/Direct%20ELISA%20protocol.pdf [Accessed 12.10.2016]

ABNOVA CORPORATION. ELISA Paar Kits. 2016. Available from: http://www.abnova.com/support/resources.asp?switchfunctionid={70196CA1-59B1-40D0-8394-19F533EB108F}[Accessed 15.5.2016]

BIO-RAD. A Guide to Polyacrylamide Gel Electrophoresis and Detection. Bulletin number: 6040 Rev B.

BIO RAD. Protein Electrophoresis Methods. 2016. Available from: http://www.bio-rad.com/de-at/applications-technologies/protein-electrophoresis-methods [Accessed 12.10.2016]

BIO-RAD. SDS-PAGE Molecular Weight Standards, Broad Range. (n.d.) Available from: http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4006035.pdf [Accessed 10.10.2015]

BIOSENSING INDUSTRY. Surface Plasmon Resonance. n.d. Available from: http://biosensingusa.com/technologies/surface-plasmon-resonance/surface-plasmon-resonance-work/ [Accessed 21.10.2016]

BOYD, M., GUSTAFSON, K., MC MAHON, J., SHOEMAKER, R., O'KEEFE, B., MORI, T., GULAKOWSKI, R., WU, L., RIVERA, M., LAURENCOT, C. Discovery of cyanovirin-N, a novel human immunodeficiency virus-inactivating protein that binds viral surface envelope glycoprotein gp120: Potential applications to microbicide development. Antimicrobial Agents Chemotherapy. 1997; 41: 1521–1530.

BRADFORD, M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Analytical Biochemistry. 1976; 72: 248-254

BROWN, T. Gene Cloning & DNA Analysis. Wiley-Blackwell. Manchester. 2010

CUBE BIOTECH. Purification of His-tagged Proteins Under Native Conditions Using PureCube His Affinity Agarose and PureCube 1-step batch Midi Plus Colums. 2014

DE MOL, N., FISCHER. M. Surface Plasmon Resonance - Methods and Protocols. Humana Press. New York. 2010

DOSTALEK, J., HUANG, C., KNOLL, W. Surface plasmon resonance-based biosensors. Wiley-VCH Verlag. Weinheim. 2009

ECKERT, W., KARTENBECK, J. Proteine: Standardmethoden der Molekular- und Zellbiologie. Springer Labormanual. Heidelberg, 1997

GAO, X., CHEN, W., GUO, C., QIAN, C., LIU, G., GE, F., HUANG, Y., KITAZATO, K., WANG, Y., SHENG, X. Soluble cytoplasmic expression, rapid purification, and characterization of cyanovirin-N as a His-SUMO fusion. Applied Microbiology and Biotechnology. 2009; 85 (4): 1051–1060

GOLD BIOTECHNOLOGY. HIS-TAG METAL AFFINITY CATIONS: WHAT'S THE DIFFERENCE AGAIN?. 2014. Available from: https://www.goldbio.com/blog/28/his-tag-metal-affinity-cations-whats-the-difference-again [Accessed 24.10.2016]

GUO, Z., BOONS, G. Carbohydrate-Based Vaccines and Immunotherapies. Wiley-VCH Verlag. New Jersey. 2009

HUANG, J., DOSTALEK, J., SESSITSCH, A., KNOLL, W. Long-Range Surface Plasmon-Enhanced Fluorescence Spectroscopy Biosensor for ultrasensitve Detection of E. coli O157:H7. Analytical Chemistry. 2011; 83: 647-677

INOUE, H., NOJIMA, H., OKAYAMA, H. High efficiency transformation of Escherichia coli with plasmids. Gene. 1990; 96 (1): 8-23

JOHNSSON, B., LÖFAS, S., LINDQUIST, G. Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. Analytical Biochemistry. 1991; 198: 268-277

KINESIS. Comparison of Iminodiacetic acid (IDA) and Nitriloacetic acid (NTA) as IMAC Media. 2016. Available from: http://kinesis-usa.com/knowledgebase/comparison-of-iminodiacetic-acid-ida-and-nitriloacetic-acid-nta-as-imac-media [Accessed 14.10.2016]

LABOME. Protease Inhibitors. 2015. Available from: https://www.labome.com/method/Protease-Inhibitors.html. [Accessed 11.10.2016]

Liedberg, B., Nylander, C., Lunstrom, I. Surface plasmon resonance for gas detection and biosensing. Sensors and Actuators. 1983; 4: 299-304

NEU, K., DUNUND, C., WILSON, P. Heads, stalks and everything else: how can antibodies indicate influenza as a human disease?. Elsevier. 2016; 42: 48-55

O'KEEFE, R., SMEE, D., TURPIN, J., SAUCEDO, C., GUSTAFSON, M., BLAKESLEE, D., BUCKHEIT, R., BOYD, M. Potent Anti-Influenza Activity of Czanovirin-N and Interactions with Viral Hemagglutinin. Antimicrobial agents and chemotherapy. 2003; 47 (8): 2518–2525

PEZZUTO, A., ULRICHS, T., BURMESTER, G. Taschenatlas der Immunologie. Georg Thieme Verlag. Stuttgart, 2007

PROTEIN DATABANK IN EUROPE. The domain-swapped dimer of CV-N in solution. 2002. Available from: http://www.ebi.ac.uk/pdbe/entry/pdb/1L5E [Accessed 22.11.2016]

QIAGEN. The QIAexpressionist. A handbook for high-level expression and purification of 6xHis-tagged proteins. QUIAGEN, 2003

RAETHER, H. Surface Plasmons on Smooth and Rough Surfaces and on Gratings. Springer Verlag. Berlin. 1988

REHM, H. Der Experimentator – Proteinbiochemie/Proteomics. Spektrum Akademischer Verlag. Bonn. 2006

RICHARD, B., SCHASFOORT, M., TUDOS, A.J. Handbook of Surface Plasmon Resonance. RSC Publishing. UK, 2008

ROSANO, L., CECCARELLI, E. Recombinant protein expression in Escherichia coli: advances and challenges. Frontiers in Microbiology 2014; 5 (172) doi: 10.3389/fmicb.2014.00172

SEZENOV, G., JOSELEAU-PETIT, D. Escherichia coli physiology in Luria-Bertani broth. Journal of bacteriology. 2007; 189 (23): 8746-49

SIGMA-ALDRICH. Introduction to PAGE -Seperation of Proteins Based on Size. 2016. Available from: http://www.sigmaaldrich.com/technical-documents/articles/biology/sds-page.html [Accessed 12.10.2016]

SIGMA-ALDRICH. Bradford Reagent – Technical Bulletin. 2000. Bulletin number: MAM/JDS 6/04

TATE, M., JOB, E., DENG, Y., VITHIAGARAN, G., MAURER-STROH, S., READING, P. Playing Hide and Seek: How Glycosylation of the Influenza Virus Hemagglutinin Can Modulate the Immune Response to Infection. Viruses. 2014; 6 (3): 1294-1316

THE CRANKSHAFT PUBLISHING. Liquid Crystals and Nanostructured Surfaces: A Novel System for Detecting Protein-Binding Events. n.d. Available from: http://what-when-how.com/nanoscience-and-nanotechnology/liquid-crystals-and-nanostructured-surfaces-a-novel-system-for-detecting-protein-binding-events-part-2-nanotechnology/ [Accessed 25.10.2016]

THERMO FISHER SCIENTIFIC. Instructions – HisPur Ni-NTA Resin. 2012

THERMO FISHER SCIENTIFIC. Traditional Methods of Cell Lysis. 2015. Available from: https://www.thermofisher.com/at/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/traditional-methods-cell-lysis.html [Accessed 11.10.2016]

THERMO FISHER SCIENTIFIC. Overview of ELISA methods. 2016. Available from: https://www.thermofisher.com/at/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html [Accessed 13.10.2016]

THERMO FISHER SCIENTIFIC. Overview of Protein Electrophoresis Methods. 2015. Available from: https://www.thermofisher.com/at/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-electrophoresis.html [Accessed 12.10.2016]

THERMO FISHER SCIENTIFIC. Thermo Scientific Pierce Protein Purification Technical Handbook. United States. 2010

UNIVERSITÄT WIEN, FAKULTÄT FÜR CHEMIE. Skriptum – Biochemisches Praktikum.

VIROLOGY. Structure of influenza virus. 2009. Available from: http://www.virology.ws/2009/04/30/structure-of-influenza-virus/ [Accessed 30.12.2016]

WHO, GLOBAL INFLUENZA PROGRAMME. Influenza. 2005. Available from: http://www.who.int/influenza/en/.2005 [Accessed 15.11.2016]

WOLFBEIS, S., HOMOLA, H. Surface Plasmon Resonance Based Sensors. Springer Verlag. Berlin, Heidelberg, 2006

WOLFRAM, T., SPATZ, J., BURGESS, ROBERT. Cell adhesion to agrin presented as a nanopatterned substrate is consistent with an interaction with the extracellular matris and not transmembrane adhesion molecules. BMC Cell biology. 2008; 9 (64) doi: 10.1186/1471-2121-9-64

WHO. Influenza virus infections in humans. 2014; 1-2

WHO. Influenza Update N° 276. 2016; 1-5

XIONG, S., FAN, J., KITAZATO, K. The antiviral protein cyanovirin-N: the current state of its production and applications. Applied microbiology and biotechnology. 2010; 86 (3): 805-812

Appendix

Materials and Equipment

Experiment	Material	Product code	Company
Cell culture	IMC72 SN (Plasmid) E.coli strain: Rosetta		
	Luria-Bertani medium: (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl)		
	SOC outgrowth medium	B9020S	BioLabs
	Ampicillin Ready made solution: c = 50mg/ml		
	IPTG, Stock solution: c = 500 µM	162423	BioLabs
Purification	Lysozyme from chicken egg white	L6876	SIGMA-ALDRICH
	Protease Inhibitor Cocktail	P8849	SIGMA-ALDRICH
	Cell pellet		
	Lysis buffer*		
	HisPur Ni-NTA Resin	88221	Thermo Fisher Scientific
	Cleared lysate samples		
	NaH2PO4	S8282	SIGMA-ALDRICH
	NaCl	S7653	SIGMA-ALDRICH
	Imidazole	I5513	SIGMA-ALDRICH
Bradford Assay	BSA	9048-46-8	SIGMA-ALDRICH
	Lysis buffer*		
	Protein samples		
	Bradford reagent	B6916	SIGMA-ALDRICH
Gelelectrophoresis	Acrylamide/Bis-acrylamide (30% solution) Protein samples	A3574	SIGMA-ALDRICH
	N, N, N', N'-Tetramethylethylenediamine	T9281	SIGMA-ALDRICH
	TRIS base	77-86-1	SIGMA-ALDRICH
	Spectra™ Multicolor Broad Range Protein Ladder		Thermo Fisher Scientific
	Hydrochloric Acid	K025.1	Carl Roth
	Sodium dodecyl sulphate	L3771	SIGMA-ALDRICH
	Ammonium Persulfate (APS)	17874	Thermo Fisher Scientific
	Glycerin	G2289	SIGMA-ALDRICH
	2-Mercaptoethanol	M6250	SIGMA-ALDRICH
Quick Elisa	Pierce™ 96-Well Polystyrene Plates	15041	Thermo Fisher Scientific
	HRP-conjugated Anti-SUMO anti-body	AB7002HRP	Life Sensors
	BSA	A2153	SIGMA-ALDRICH
	Protein samples		
	TMB Liquid Substrate	T0440	SIGMA-ALDRICH
	20 × Wash Buffer Concentration pH: 7.4	ab125966	Abcam

	Stop Solution	ab125966	Abcam
	$NaHCO_3$	71630	Honeywell
	Na_2CO_3	S7795	SIGMA-ALDRICH
	Na_2HPO_4	S7907	SIGMA-ALDRICH
	KCl	P9541	SIGMA-ALDRICH
	KH_2PO_4	KH_2PO_4	Fluka
	NaCl	S9888	Fluka
	H2SO4	339741	SIGMA-ALDRICH
SPR	Carboxy-late/dithiolalkanearomaticPEG6-COOH	SPT 0014A6	SensoPath Technologies
	PEG-dithiol/dithiolalkanearomaticPEG3-OH	SPT 0013 TH 001-m11.n3	SensoPath Technologies Prochimia
	HS-(CH2)11-EG3-NTA	324558 9-307-011-4-507	SIGMA-ALDRICH Hellma Analytics
	Ethylene glycol		SIGMA-ALDRICH
	Hellmanex III	A2153	constructed by BOKU
	BSA		SIGMA-ALDRICH
	His6-Beta-galactosidase		SIGMA-ALDRICH
	rCVN Samples	339350	Abcam
	Copper(II) sulfate pentahydrate	S7907	Thermo Fisher Scientific
	Nickel(II) chloride	ab69751	Fluka
	Influenza A Virus Hemagglutinin H3 protein	22981 24500	Calbiochem
	EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide)	524650-1EA	
	NHS (N-hydroxysuccinimide)		
	PBS, pH: 7.4		

Table 4: Materials

*****Buffer preparation for purification under native conditions preparation see table 6 attached in the Appendix

Experiment	Equipment	Company
Cell culture	Commercial Lab Shaker	Thermo Fisher Scientific
	Thermomixer F	Eppendorf
	Multimode reader, Infinite 200 PRO	Tecan
	Microplate reader program	Tecan i-control
	Benchtop Centrifuge: 5417R	Eppendorf
Cell lysis	Sonicator, Sonorex Super	Bandelin
	Centrifuge, Sorvall RC5C	Thermo Scientific
Purification	Packed Column with Ni-NTA Resin Conditions: T = 25°C	
	ph-Meter, 872 pH-lab	Metrohm
Bradford Assay	UV/VIS Spectrometer UV 4 Conditions: T = 25°C, A260nm and A280nm, Light Path = 1 cm	Unicam
Gelelectrophoresis	Mini-PROTEAN® Tetra Vertical Electrophoresis Cell	BIO-RAD
	PowerPac™ HC High-Current Power Supply	BIO-RAD
	Lab shaker	
	water bath at 95 °C	
	Vortex mixer, Reax top	Heidolph
Quick ELISA	Plate reader, Fluostar Optima Excitation: A-450, Emission: empty Software: FLUOoptima	BMG Labtech
SPR	self-assembled SPR-Setup	by AIT

Table 5: Equipment

Buffers and Solutions

Purification under native conditions

	Lysis buffer*	Wash buffer*	Elution buffer*
NaH_2PO_4	50 mM	50 mM	500 mM
NaCl	300 mM	300 mM	300 mM
Imidazole	10 mM	20 mM	250 mM
	pH adjusted to 8.0 using NaOH		

Table 6: Buffers for purification

* V = 1 L

SDS-Page

Solution A:	Acrylamide/Bis-acrylamide 30% solution
Solution B:	1.5 M TRIS/HCL, pH 8.8; 45.5g TRIS in 200 mL H2O dest., pH: 8.8 (HCl), H2O ad 250 mL
Solution C:	0.5 M TRIS/HCL, pH: 6.8; 15.1 g TRIS in 200 mL H2O dest., pH: 6.8 (HCl), H2O ad 250mL
Solution D:	10% (w/v) sodium dodecyl sulfate (SDS) solution
Solution E:	10% (w/v) ammonium persulfate (APS) solution
Loading dye:	2.5 mL of solution C, 2 mL Glycerin, 0.4 g SDS, 200 µl 2-Mercaptoethanol, 1 mG Bromphenol blue, H2O ad 10 mL (1 mL aliquots in the freezer at -20 °C.)
SDS Running Buffer:	10x stock solution; 15.15 g TRIS, 73 g Glycine, 5 g SDS, H2O ad 0.5 L, pH: 8.3
Staining solution:	0,25% CBB R-250 in 10% Acetic acid, 30 % Methanol; ready to use
Destain solution:	12, 5 % Acetic acid, 30 % Methanol

Quick Test “ELISA”

Coating Buffer 0.05 M, pH: 9.6, 2.93 g/L $NaHCO_3$, 1.59 g/L Na_2CO_3 (Abcam, n.d.)
Blocking Solution: 1% BSA in PBS
PBS 0.01 M: 1.2 g/L Na_2HPO_4, 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 8.0 g/L NaCl (Abcam, n.d.)
Stop solution: 0.5 M H_2SO_4

Surface Plasmon Resonance

HEPES, pH: 7.4 (10 mM HEPES, 150 mM NaCl, 50 µl EDTA, 0,005 % Tween 20)
Sodium acetate buffer, pH: 5.0 (0.1 M acetic acid, 0.1 M sodium acetate)

Growing a bacterial culture

The measured OD600 values of the bacterial growth are listed in table 7 and table 8.

	15-10-2015	5-4-2016
Sample	**OD_{600}***	**OD_{600}***
1	0,3245	0,1787
2	0,4307	0,0973
3	0,3250	0,2134

Table 7: Bacterial growth I

*blank value already abstracted

	15-10-2015	5-4-2016
Sample	**OD_{600}***	**OD_{600}***
1	0,6016	0,7102
2	0,7067	0,6965
3	0,5703	0,8229

Table 8: Bacterial growth II

*blank value already abstracted

	15-10-2015		5-4-2016	
Sample	**OD_{600}* 1:10 dilution**	**OD_{600}* Final**	**OD_{600}* 1:10 dilution**	**OD_{600}* Final**
1	0,1755	1,755	0,1939	1,939
2	0,1897	1,897	0,1673	1,673
3	0,1826	1,826	0,2424	2,424

Table 9: Bacterial growth III

*blank value already abstracted

Quantitative Analysis - BSA Standard curve

	Concentration (mg/ml)	Absorbance* – measurement 1	Absorbance* – measurement 2	Absorbance* – mean value
S1	1.5	1.013	1.030	1.022
S2	1	0.743	0.779	0.761
S3	0.75	0.507	0.511	0.509
S5	0.5	0.287	0.294	0.291
S4	0.25	0.141	0.155	0.148
S6	0.125	0.049	0.073	0.061

Table 10: Absorbance of BSA at 595 nm

*Blank already substracted

Absorbance values – Bradford Assay

SAMPLES, DATE: 3.11.2015

	LZ	FT	W1	W2	E1	E2	E3	E4
A 595 (1)	3.061	2.610	2.432	2.422	1.323	0.425	0.556	0.671
A 595 (2)	3.063	2.614	2.440	2.428	1.317	0.421	0.559	0.683
A 595 (mv)	3.062	2.612	2.430	2.425	1.320	0.423	0.558	0.677

Table 11: Absorbance values I

SAMPLES, DATE: 20.11.2015

	LZ	FT	W1	W2	E1	E2	E3	E4
A 595 (1)	3.003	2.973	2.589	0.523	0.623	0.689	0.678	0.771
A 595 (2)	3.012	2.981	2.577	0.538	0.631	0.692	0.672	0.762
A 595 (mv)	3.008	2.892	2.583	0.531	0.627	0.681	0.675	0.767

Table 12: Absorbance values II

SAMPLES, DATE: 11.12.2015

	LZ	FT	W1	W2	E1	E2	E3	E4
A 595 (1)	2.889	2.612	2.068	0.329	0.563	0.571	0.599	0.631
A 595 (2)	2.881	2.622	2.063	0.334	0.555	0.576	0.587	0.625
A 595 (mv)	2.885	2.617	2.066	0.332	0.559	0.574	0.593	0.578

Table 13: Absorbance values III

SAMPLES, DATE: 20.11.2015

	LZ	FT	W1	W2	E1	E2	E3	E4
c [mg/ml]	2.147	2.064	1.840	0.351	0.421	0.461	0.456	0.523

Table 14: Final concentration of the samples II

SAMPLES, DATE: 2.12.2015

	LZ	FT	W1	W2	E1	E2	E3	E4
A 595 (1)	1.985	1.652	1.001	0.336	0.351	0.128	0.101	0.065
A 595 (2)	1.975	1.658	1.013	0.337	0.351	0.125	0.100	0.063
A 595 (mv)	1.980	1.655	1.007	0.337	0.351	0.127	0.101	0.064

Table 15: Absorbance values IV

SAMPLES, DATE: 2.12.2015

	LZ	FT	W1	W2	E1	E2	E3	E4
c [mg/ml]	1.403	1.167	0.697	0.211	0.221	0.059	0.040	-

Table 16: Final concentration of the samples IV

SAMPLES, DATE: 13.04.2016

	LZ	FT	W1	W2	E1	E2	E3	E4
A 595 (1)	2.676	1.852	1.667	0.271	0.551	0.212	0.198	0.080
A 595 (2)	2.681	1.843	1.666	0.272	0.556	0.210	0.199	0.073
A 595 (mv)	2.679	1.848	1.667	0.272	0.554	0.211	0.199	0.077

Table 17: Absorbance values V

Protein standards

Material	Product code	Company
BSA	A2153	SIGMA-ALDRICH
Pepsin from hog stomach	77160	Fluka Analytical
Lipase	L-3126	SIGMA-ALDRICH
Protease (Proteinase K)	P-0390	SIGMA-ALDRICH
Taka-Diastase from Aspergillus oryzae	86247	Fluka Analytical
Lysozyme from chicken egg white	L6876	SIGMA-ALDRICH

Table 18: Material for preparing protein standards

A gel of BSA dilution standards (0,5 mg/ml – 50 mg/ml) was run in order to figure out the optimal concentration of BSA as a standard (Figure 21).

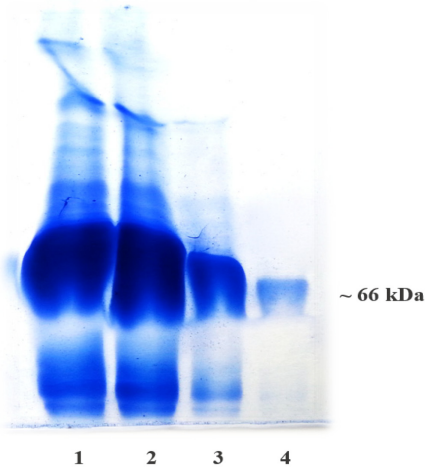


Figure 21: BSA dilutions standards.
Lane 1: BSA 50 mg/ml, Lane 2: 25 mg/ml, Lane 3: 5 mg/ml,
Lane 4: 0,5 mg/ml

Further trials, testing proteins (Pepsin, Lipase, Protease, Taka-Diastase, Lysozym) in different concentrations to figure out a second standard protein for SDS-Page analysis, were performed. The protein Taka-Diastase from Aspergillus oryzae was found to be the best one.

Purification by Batch Method

The same buffers and samples as in the purification by affinity chromatography were used.

Equipment	Company
1-step batch Midi Plus Columns	PureCube
Centrifuge, Heraeus Megafuge 40 Conditions: T = 25°C	Thermo Scientific

Table 19: Equipment for purification by batch method

At first 2 ml (=1 CV) of the resin slurry were pipetted into a batch column and equilibrated with 4 CV lysis buffer; resin was spinned at 300 × g for 5 min (clear spin push cap was used). Several experiment trials suggest a spinning rate of 300 × g for 5 min to pass through about 4 CV of the lysat, without running dry.

Batch incubation step: 4 CV of the cleared lysate were added to the tube and incubated for 1 h on ice (yellow batch incubation cap was screwed).

After the incubation step, the batch was centrifuged at 300 × g for 5 min and the flow through (FT) was collected. Following by two washing steps (W1, W2) with 4 CV washing Buffer each.

At last, the elution step was carried out. The his-tagged protein was eluted by adding 5 CV of elution buffer in total. For a total of 4 elution fractions (~1 CV each; E1-E4), the batch was centrifuged four times at 300 × g for 1 min (Thermo Scientific, n.d. & Cube Biotech, 2014).

The collected samples from each fraction (CL, FT, W1, W2, E1-E4) were subjected to SDS-Page analysis. Furthermore, the protein concentration was determined by a spectrophometric assay.

Quick “ELISA“ I

	1	2	3	4	5	6	7	8	9	10	11	12
A	W1 20.11.15	W1 20.11.15	E1 20.11.15	E1 20.11.15	W1 11.12.15	W1 11.12.15	W1 20.11.15	W1 20.11.15	E1 20.11.15	E1 20.11.15	W1 11.12.15	W1 11.12.15
B	E1 11.12.15	E1 11.12.15	LZ 11.12.15	LZ 11.12.15	LZ (1:10) 11.12.15	LZ (1:10) 11.12.15	E1 11.12.15	E1 11.12.15	LZ 11.12.15	LZ 11.12.15	LZ (1:10) 11.12.15	LZ (1:10) 11.12.15
C	LZ (1:100) 11.12.15	LZ (1:100) 11.12.15	W1 3.11.15	W1 3.11.15	E1 3.11.15	E1 3.11.15	LZ (1:100) 11.12.15	LZ (1:100) 11.12.15	W1 3.11.15	W1 3.11.15	E1 3.11.15	E1 3.11.15
D	LZ 3.11.15	LZ 3.11.15	LZ (1:10) 3.11.15	LZ (1:10) 3.11.15	LZ (1:100) 3.11.15	LZ (1:100) 3.11.15	LZ 3.11.15	LZ 3.11.15	LZ (1:10) 3.11.15	LZ (1:10) 3.11.15	LZ (1:100) 3.11.15	LZ (1:100) 3.11.15
E	E4 20.11.15	E4 20.11.15									E4 20.11.15	E4 20.11.15
F												
G	neg. control (1 % BSA)	neg. control (1 % BSA)	E1 20.11.15	E1 20.11.15	neg. control (without Ab)	neg. control (without Ab)	neg. control (1 % BSA)	neg. control (1 % BSA)	E1 20.11.15	E1 20.11.15	neg. control (without Ab)	neg. control (without Ab)
H												Blank

Table 20: Pipetting scheme of direct ELISA

Quick “ELISA“ II

	1	2
A	1.28	
B	1.246	
C	1.045	
D	0.86	
E	0.615	0.172
F	0.116	0.13
G	0.594	0.179
H	0.576	0.443

Table 21: Absorption data of Quick test

Surface Plasmon Resonance

Ni_2+ mediated immobilisation of his-tagged ligand beta-galactosidase

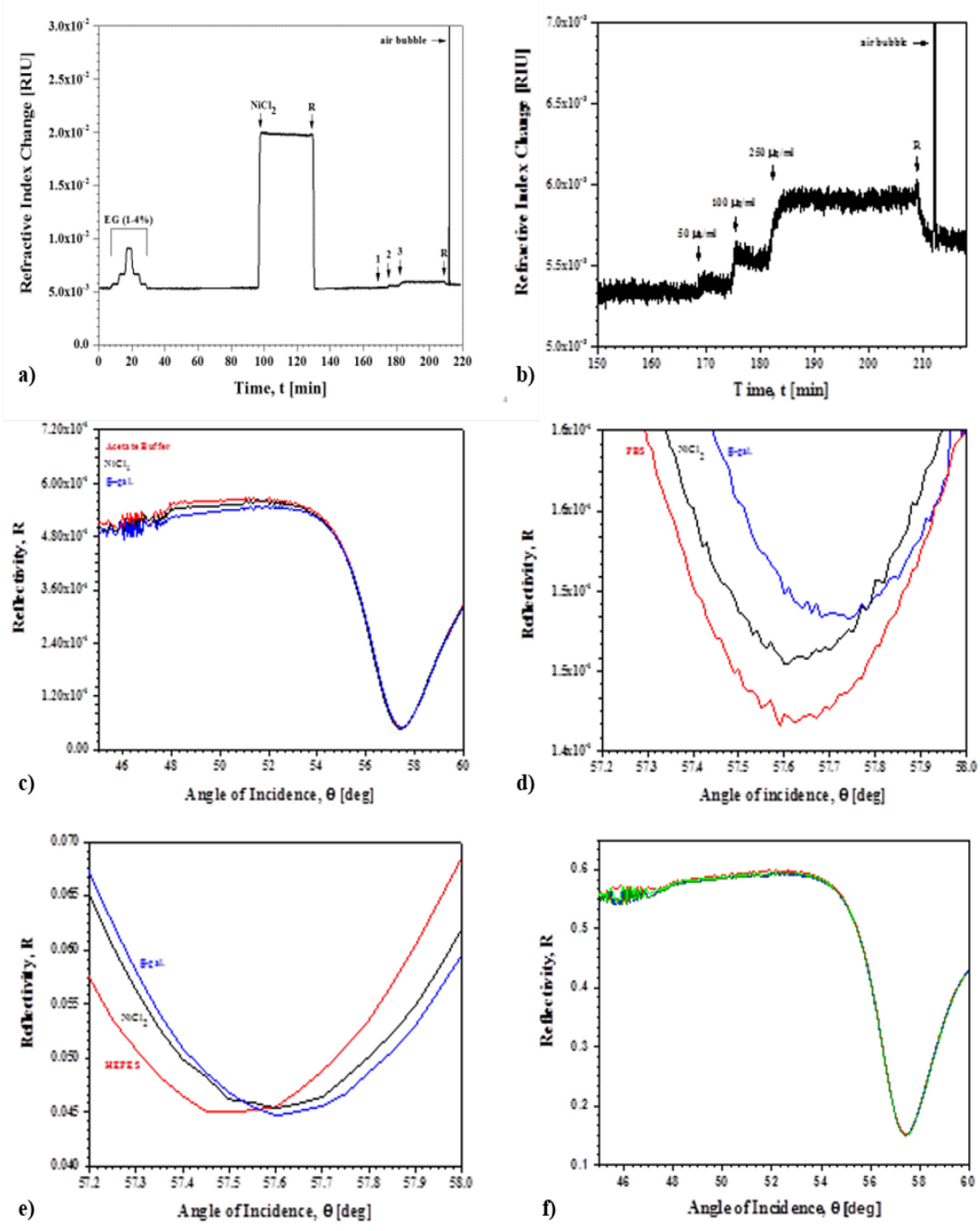


Figure 22: Ni2+ mediated immobilisation of his-tagged betagalactosidase
a) Sensogram. Increasing concentration of the ligand 1:50 µg/ml, 2:100 µg/ml , 3:250 µg/ml) b) Zoomed part of the ligand injection of plot a c) Specific angle of beta-galactosidase after Ni^{2+} mediated immobilisation step (in PBS) d) Zoomed part of plot c e) Zoomed part of the specific angle of beta-galactosidase (250 µg/ml) after Ni2+ mediated immobilisation step (in HEPES) f) Specific angle of BSA (250 µg/ml) after Ni^{2+} mediated immobilisation step (in PBS) as Reference