Emmi A. Mikkola

The Overexpression, Purification and Characterization of FhuA, pb5 and VHH Antibodies, and Complex Formation for Crystallization Screening

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Membrane proteins induce the required communication between the cell and the surrounding environment, therefore they can act as transporter proteins. They can be used by different pathogens, such as *bacteriophages*, among which we find T5, in the process of an infection. Through its RBP, it has the ability to infect *E. coli* with great specificity, using one of the bacteria's transporters to recognize and bind to the cell.

In this study, the proteins FhuA and pb5 were targeted with the purpose of characterizing a membrane protein complex, formed by *a)* the bacterial ferrichrome transporter *'FhuA'*, present in *E. coli* bacterial outer membrane and *b)* the bacteriophage T5's viral tail tip protein *'pb5'.* VHH antibodies were also used in order to optimize the crystallization between FhuA and pb5 by increasing the soluble surface of the complex. A process of overexpression in *E. coli* cells and later purification using different chromatographic methods were performed, followed by crystallization, either by hand in the laboratory or in an HTX platform.

As a result, various steps of the different methods were improved, extra information on the handling of the different proteins was provided and several crystals were obtained successfully. These crystals will be used for X-ray crystallography and if they possess the expected quality, the FhuA-pb5 complex's 3D structure could be obtained. Further studies are still needed to solve this structure, in order to enlighten the phage T5's infection process and interaction with the transporter.

The project was carried out in Grenoble, France, in the Institut Biologie Structurale (IBS) research center in the group of Membranes and Pathogens (M&P).

Key words	Bacteriophage, Membrane proteins, protein complex, Crystallization

Tekijä Otsikko Sivumäärä Päivämäärä	Emmi A. Mikkola FhuA, pb5 ja VHH proteiinien ekspressointi, puhdistus ja karakterisointi, sekä kompleksin muodostus kristallisointia varten 34 sivua 5.11.2017
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Membraaniproteiinit mahdollistavat tarvittavan viestinnän solun ja sen ympäristön välillä, jonka vuoksi ne voivat toimia muun muassa kuljettajaproteiineina. Niitä voi kuitenkin käyttää hyväkseen myös erilaiset patogeenit infektioprosesseissaan, kuten *bakteriofaagit*, joista eräänä myös T5.Se käyttää reseptoriin sitoutuvaa spesifistä proteiiniaan *E.colin* infektoimiseen, käyttäen bakteerin kuljettajaproteiinia solun kiinnittymiseen ja tunnistamiseen.
Kyseisessä tutkimuksessa keskityttiin kahteen proteiiniin, tarkoituksena proteiinikompleksin tutkiminen, muodostuen a*) E. colin* ferrikromikuljettajaproteiini *“FhuA:sta”,* esiintyen bakteerin solukalvolla ja b) bakteriofaagin häntäproteiinin ”*pb5:stä*”: Myös VHH antibodeja käytettiin FhuA:n ja pb5:n kristallisoimisen optimoimiseksi, joiden tarkoituksena oli lisätä kompleksin liukoisuutta. Tekniikkoina käytettiin ekspressiota *E.coli* -soluissa ja erilaisia kromatografisia puhdistusmenetelmiä, jonka jälkeen kristallisaatio suoritettiin, joko käsin pipetoimalla, tai ulkoisen HTX laboratorion avulla.
Tuloksena saavutettiin usean työvaiheen optimointi, lisäinformaation saavuttaminen proteiinien käsittelystä, sekä usean kristallin muodostuminen, joita voidaan jatkossa käyttää röntgensädekristallografiassa, jos niiden laatu täyttää vaatimukset. Tällöin voitaisiin saavuttaa FhuA-pb5 proteiinikompleksin kolmiulotteinen rakenne, mikä avaisi faagin infektioprosessia ja vuorovaikutusta kuljettajaproteiinin kanssa.
Projekti suoritettiin Grenoblessa, Ranskassa, IBS -tutkimuskeskuksessa (Institut Biologie Structurale), tutkimusryhmässä nimeltä Membraanit ja Patogeenit (M&P).

Avainsanat	Bakteriofaagit, Membraaniproteiinit, Proteiinikompleksi, Kristallisaatio

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Abbreviations

AW740	Escherichia coli strain lacking OmpF and OmpC
BL21 (DE3)	Derivative of BL21 E coli strain with T7 RNA polymerase gene
CLAPA	Chymostatin Leupeptin Antipain Pepstatin and aprotinin
CMC	Critical Micelle Concentration
DNase	Deoxyribonuclease
dsDNA	Double-Stranded Deoxyribonucleic Acid
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EM	Electron Microscopy
EMBL	European Molecular Biology Laboratory
FhuA	Ferric Hydroxamate Uptake A
GE	General Electric
HTX	High Throughput Crystallization
IBS	Institut de Biologie Structurale (Structural Biology Institute)
IPTG	Isopropyl β-D-1-Thiogalactopyranoside
LB	Lysogeny Broth
LDAO	Lauryldimethylamine Oxide
LPS	Lipopolysaccharide
M&P	Membrane and Pathogens
MES	2-(N-morpholino) Ethanesulfonic Acid
NiNTA	Nickel-nitrilotriacetic acid
NMR	Nuclear Magnetic Resonance
OPOE	Octyl-polyoxyethylene
PAGE	Polyacrylamide gel electrophoresis
pb5	Protein Binding 5
Phage	Bacteriophage
Psi	pounds per square inch
PTM	Post-Translational Modification
Q HP	Q Sepharose High Performance
RBP	Receptor Binding Protein
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
SP HP	SP Sepharose High Performance
ssDNA	Single-Stranded Deoxyribonucleic Acid
ssRNA	Single-Stranded Ribonucleic Acid
dsRNA	Double-Stranded Ribonucleic Acid
T1	Type 1
T5	Type 5
TB	Terrific Broth
Tris	Tris(hydroxymethyl)aminomethane
UV/Vis	Ultraviolet-Visible Spectrophotometry
VHH	Variable Domain of Heavy-Chain Antibody

1 Introduction

In biology, proteins are the main actors within the cell, where they carry out duties specified by the information encoded in them genetically. Different proteins perform various functions depending on their physicochemical properties and location inside of the cell. The diversity in how they act relies on their ability to bind tightly to other specific molecules, proteins and small-molecule substrates [1]. However, proteins can also bind or be integrated into cell membranes, known as *membrane proteins*. Their binding ability can induce conformational changes in proteins and allow, therefore, the construction of extremely complex signaling networks [2].

Since the membrane surrounds biological cells, it is in charge of protecting its interior against an exterior hostile environment and, consequently, the proteins that reside in this membrane induce the required communication between the cell and the aforementioned environment. These membrane proteins can act as transporters, cell adhesion molecules or even possess enzymatic activity, vital for the normal function of a cell [3]. Because of their presence in the membrane, these proteins have gained increased importance in the field of infectiology, given that they can be used by different pathogens (viruses, bacteria and/or toxins) in the process of an infection. Knowledge of these proteins is essential to understand the functioning of the cell and the pathogenic proteins with which they interact [1].

Pathogens capable of infecting bacteria are *bacteriophages*, bacterial viruses. They possess a receptor binding protein (RBP) located in the tip of the tail in the case of T5, a tailed phage from the *Caudovirales* family [4]. This RBP gives the bacteriophage the ability to infect *E. coli* with great specificity, using one of the bacteria's transporters to recognize and bind to the cell.

The purpose of this project is to study, characterize and crystallize a membrane protein complex, formed by *a)* the bacterial ferrichrome transporter *'FhuA'*, present in *E. coli* bacterial outer membrane and *b)* the bacteriophage T5's viral tail tip protein *'pb5'.*
In vivo, this interaction triggers the infection of the bacteria by the phage, releasing the phage's DNA into the bacterial cell. This complex is an excellent model to study this interaction because at the moment it is the only biochemically available outer membrane

complex with RBP [5]. The interest in this complex relies on the possibility of having different applications, with the use of phages for example in therapy.

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Additionally, to optimize the crystallization between FhuA and pb5, it is possible to use a third protein, *VHH lama antibodies*, to increase the soluble surface of the complex. These lama antibodies are small proteins raised to bind to pb5, which otherwise aggregates easily alone. In this study, four different antibodies were used: VHH_{53}, VHH_{54}, VHH_{64} and VHH_{81}.

In structural biology, crystallization followed by X-ray crystallography is currently the most accurate way to obtain protein structures [6]. Therefore, FhuA-pb5 complex's 3D structure could be obtained with this technique. Determining the structure of this membrane protein complex can be of great importance in understanding in detail how the first step of injection process occurs, enlightening the phage's infection process and interaction with the transporter.

2 Theoretical Background

2.1 Membrane Proteins

Membrane proteins are cell components of great importance. They perform many of the functions that are vital for the survival of cells [7]. They can be bound or integrated into cell membranes. Binding of outside stimuli induces conformational changes in the proteins allowing the construction of extremely complex signaling networks. Membrane proteins reside in highly isolating lipid bilayers, surrounding biological cells, therefore protecting their interior against an external hostile environment. Consequently, these proteins are in charge of the essential communication between the cell and the environment, catalyzing important reactions such as solute transport, charge separation, conversion of energy and signal transduction [3]. Membrane proteins can act as transporters, receptors, cell adhesion molecules or even possess enzymatic activity, vital for the normal function of a cell.

There are three major classes of membrane transport proteins: ATP-powered pumps, channel proteins and transporters (Figure 1). They exhibit a high degree of specificity for the transported substance. Due to differences in their mechanism of transport, the rate of action between them differs considerably [1].

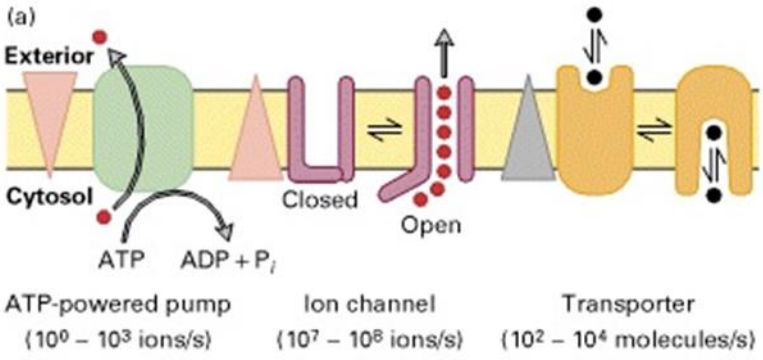


Figure 1. Graphic representation of the three classes of membrane transport proteins [1]

2.1.1 Membrane Transporters

Membrane proteins are capable of transporting a wide variety of ions and molecules across cellular membranes by binding to only one or a few substrate molecules at a time.

After this binding, they undergo a conformational change that impedes more binding to occur, therefore only these molecules are moved across the membrane [8].

Depending on their mode of action, non-ATPase transporters can be sub-classified in uniporters, symporters and antiporters, as shown in Figure 2. Uniporters transport one molecule at a time down a concentration gradient. Antiporters and symporters, however, are in charge of moving one type of ion or molecule against its concentration gradient by coupling it with the movement of a different ion or molecule down its concentration gradient. As in the case of ATP-driven pumps, the latter two transporters mediate coupled reactions, in which a reaction that is energetically unfavorable is coupled to an energetically favorable one. Nonetheless, they do not hydrolyze ATP or any other molecule. These proteins can be referred to as cotransporters, due to their ability to simultaneously transport two different solutes [1].

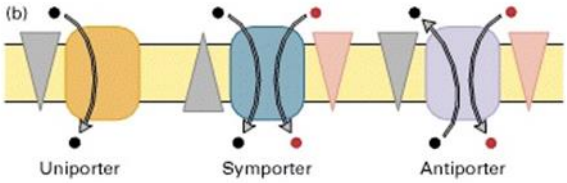


Figure 2. Different mechanisms used by membrane transporters, subclassified depending on whether they transport molecules with or against their concentration gradient [8].

Membrane proteins are present in all living organisms in large numbers, from mammalian to bacterial cells. Because of their presence in the membrane, these proteins have gained increased importance in the infectiology field, since different pathogens make use of them in the process of an infection, as it is the case of bacteriophages that infect bacterial cells.

2.1.1.1 FhuA

A transporter protein present in the outer membrane of *E. coli* is the transporter for ferrichrome-iron, named FhuA (Figure 3). Besides its physiological function to actively transport ferrichrome-iron into the cell, this membrane transporter has been high jacked by different pathogens and toxins; it is the primary receptor for the antibiotic albomycin as a consequence of its resemblance with the structure of its natural ligand for multiple

bacteriophages such as f80, T1, T5 and UC-1, for the bacterial toxin colicin M and lastly for the peptide antibiotic microcin 25 [9].

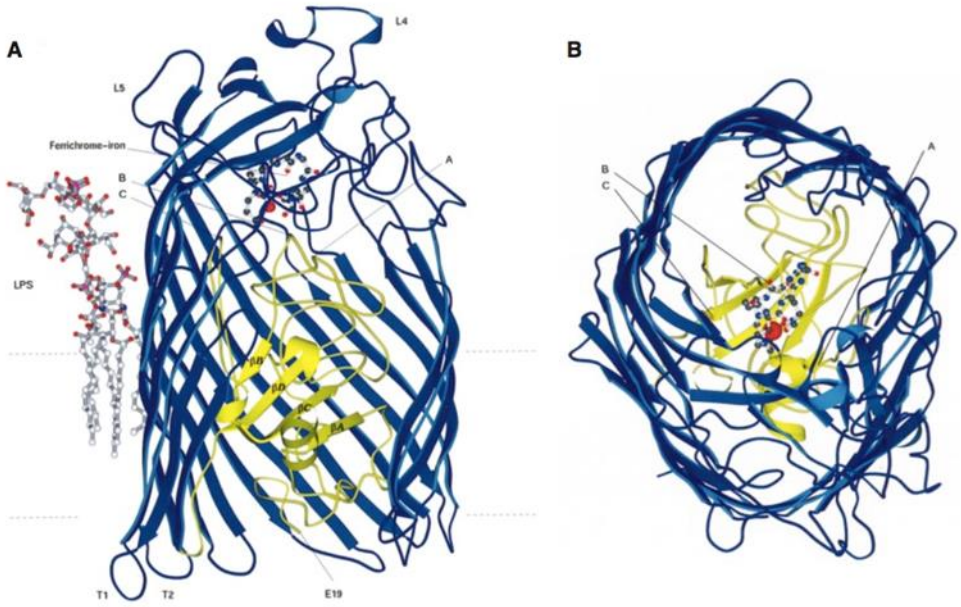


Figure 3. FhuA is represented in a ribbon manner, oriented as it would be found in the outer membrane. The barrel is shown in blue, with a removal of the residues 621 to 723 in order to obtain an unobstructed view of the cork domain, an N-terminal globular domain that folds inside the barrel and occludes it, shown in yellow. Represented as 'ball & stick' models, the LPS and ferrichrome-iron molecules are seen, with a large red sphere indicating the iron atom. As for the dashed lines, they indicate the positions of the upper and lower aromatic girdles. The external pocket is seen as a space above the cork domain and the periplasmic pocket as a space below this domain. On the other hand, panel Figure 3B shows the view of FhuA from the external environment along the barrel axis. [7]

2.1.2 Difficulties in the Study of Membrane Proteins

It is challenging to determine membrane protein structures by any technique [10]. When studying membrane proteins, the main difficulty is obtaining the protein of interest. This is in part due to the low levels the membrane proteins usually present in biological membranes. For this reason, their overexpression is necessary. However, a major problem encountered is the aggregation of the targeted protein in the cytoplasm due to the overcrowding of the membrane-insertion machinery. Consequently, it is difficult to obtain a high yield of stable functional protein. Among the systems used for overexpression, *E. coli* is the most popular one due to its low cost and ease of use. However, mammalian proteins frequently require post-translational modifications (PTMs), which are unobtainable in bacterial hosts [11].

Furthermore, another difficulty is represented in the study of membrane proteins, because of their heterogeneous and complex environment; Membrane proteins are naturally embedded in a lipid bilayer, indicating a large asymmetry and some lateral mobility within the membrane matrix, [12], making it more challenging to use the biophysical techniques known to resolve protein structure and function, including NMR and X-ray crystallography. These, among other techniques, result of restricted application, due to the requirement of an extraction of the protein from its native membrane and the study to be carried out in a detergent or lipid environment *in vitro*. Consequently, sample preparation and spectral contributions becomes difficult [11], representing a major challenge. Also, the insolubility in aqueous solutions of most of the membrane proteins [13] leads to a need to reside in environments that satisfy their high hydrophobicity, hence requiring special synthetic systems for an *in vitro* approach [11]. To help overcome this problem, innovative approaches have been recently developed, by redesigning membrane proteins to obtain hydrophilic variants and adding solubilizing fusion proteins [13].

2.1.2.1 Detergents

Detergents play an essential role when extracting, purifying and manipulating membrane proteins. With an amphiphilic nature that allows them to interact with the hydrophobic domain present in membrane proteins, they manage to keep them water soluble outside of their native bilayer environment.

A golden standard rule for the use of detergents in membrane protein studies and applications does not exist, due to their heterogeneity and complexity. For example, a detergent useful for extraction may not be compatible with purification or biochemical studies [14], or a detergent that works for one membrane protein may not be suitable for another.

Understanding the physicochemical properties associated with different classes of detergents can be useful when deciding which of them may work best for a particular application. Detergents are usually essential in the isolation and purification of the protein and are used in the primary solubilization step. They are also invaluable in membrane protein crystallization [11].

Detergents are amphipathic molecules that consist of a polar head group and a hydrophobic tail (Figure 4A). A few detergents, however, have been described as having a bean-like molecular shape, by containing both polar and nonpolar “faces” (Figure 4B).

How they are constructed results of vital importance because it provides the detergent with the ability to interact with biological structures and alter their specific functions, as in the case of biomembranes [15].

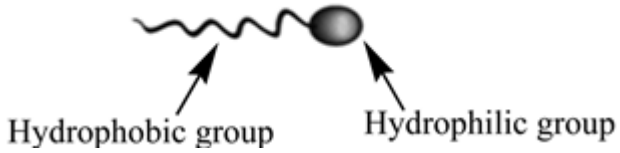


Figure 4. Detergent monomer, representing the most common detergents, with a hydrophilic head group and a hydrophobic tail or chain. [11]

Detergents present unique properties in aqueous solutions, generally forming spherical micellar structures in a spontaneous manner (Figure 5). What detergents do is that they solubilize these membrane proteins by mimicking their natural lipid bilayer environment [11].

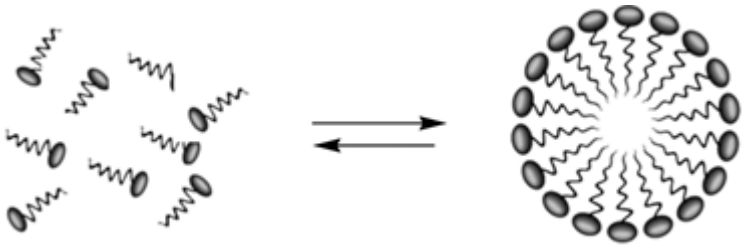


Figure 5. Spontaneous micellization of detergents in hydrophilic solutions, forming spherical micellar structures.[11]

Detergents belong to a class of surface active agents called surfactants, as they reduce the interfacial surface tension of water [15]. In biochemistry, they are used in different applications such as polyacrylamide gel electrophoresis (PAGE, the detergent SDS is used a potent protein denaturant), membrane solubilization and permeabilization, inclusion body solubilization and lipid raft preparation. For the study of membrane proteins, detergents play a major role in their solubilization from the membrane, manipulation in aqueous solution and crystallization.

In the study of membrane proteins, the selection of the detergent to use mainly depends on the type of work to be performed. For this matter, when choosing the most apt detergent, the main characteristic of each detergent is its critical micelle concentration (CMC), which is the minimum concentration of detergent above which monomers assemble together, forming micelles [16]. This leads to a sudden change in some physicochemical

properties like the surface tension [11]. Hence, knowing the CMC is necessary to estimate how much detergent is required in terms of preparation of various protein and membranes. Likewise, when manipulated in detergent solution a membrane protein, the detergent should always be at a concentration higher than its CMC, to insure the correct shielding of the hydrophobic domain of the membrane protein from water.

Since the excess of detergent used to solubilize membrane proteins can complicate the spectra or disrupt further experimental work, it must be often removed once the proteins are solubilized. Various removal methods exist to allow transfer of the membrane protein into a liposome or into a different detergent, which take advantage of the properties of the detergent being used, such as the CMC, the charge or the aggregation number. Among these methods dialysis, hydrophobic adsorption, gel chromatography, ion-exchange chromatography and nickel columns are found [11].

2.2 Bacteriophages

Bacterial viruses, known as bacteriophages or ‘phages’ were found in the beginning of the 20th century, when a British pathologist Frederick William Twort described a glassy transformation of Micrococcus colonies by a transmissible agent in 1915. Among the other proposed explanations, one suggested that the described agent was viral in nature. On the other hand, in 1917 a French Canadian named Félix Hubert d’Hérelle working at the Pasteur Institute (Paris) observed and described the lysis of Shigella cultures. Regardless of the decades Twort dedicated trying to propagate vertebrate viruses on inert media, he did not pursue his discovery, mainly due to scarse funding during war time. Nonetheless, D’Hérelle did acknowledge the viral character of his agent and devoted his life to it: he invented the term bacteriophage, “bacterial eater”, proposed that viruses multiplied intracellularly and introduced phage therapy of infectious diseases. In 1940, the development of the electron microscope enabled the recognition of the viral nature of bacteriophages [17].

Bacteriophages have been the center of many studies since their discovery. In particular, they have allowed the development of modern genetics and of molecular biology [18]. Furthermore, they have been used for over 90 years as an alternative to antibiotics in the Eastern countries due to their capacity of being used for infectious diseases treatment, representing a possible therapy against multi-drug-resistant strains of many bacteria [19].

They are one of the most common and diverse agents in the environment, and phages are indeed found wherever bacteria exist, therefore they have co-evolved with bacteria, turning into virulent pathogens and, thus, have a great impact on their hosts [20].

2.2.1 Classification and the structure of bacteriophages

Phages are classified into 13 families and 30 genera, as seen in Table 1.

Table 1. Classification of bacteriophages based on their shape and properties [15].

Shape	Nucleic acid	Order and families	Genera	Examples	Members	Characteristics
Tailed	DNA, ds, L	*Caudovirales*	15		4950	
		Myoviridae	6	T4	1243	Tail contractile
		Siphoviridae	6	λ	3011	Tail long, noncontractile
		Podoviridae	3	T7	696	Tail short
Polyhedral	DNA, ss, C	*Microviridae*	4	φX174	40	
	ds, C, T	*Corticoviridae*	1	PM2	3?	Complex capsid, lipids
	ds, L	*Tectiviridae*	1	PRD1	18	Internal lipoprotein vesicle
	RNA, ss, L	*Leviviridae*	2	MS2	39	
	ds, L, S	*Cystoviridae*	1	φ6	1	Envelope, lipids
Filamentous	DNA, ss, C	*Inoviridae*	2	fd	57	Filaments or rods
	ds, L	*Lipothrixviridae*	1	TTV1	6?	Envelope, lipids
	ds, L	*Rudiviridae*	1	SIRV1	2	Resembles TMV
Pleomorphic	DNA, ds, C, T	*Plasmaviridae*	1	L2	6	Envelope, lipids, no capsid
	ds, C, T	*Fuselloviridae*	1	SSV1	8?	Spindle-shaped, no capsid

[a]Modified from reference [4]. With permission of John Libbey-Eurotext. Phage numbers are from reference [3]. C, circular; L, linear; S, segmented; T, superhelical; 1, single-stranded; 2, double-stranded.

Bacteriophage virions can be tailed, polyhedral, filamentous or pleomorphic. As shown in the table 1, most of the phages possess dsDNA, but some small phage groups present with ssDNA, ssRNA or dsRNA [17]. More than 95% of the bacteriophages identified belong to the caudovirales order (Figure 6.).

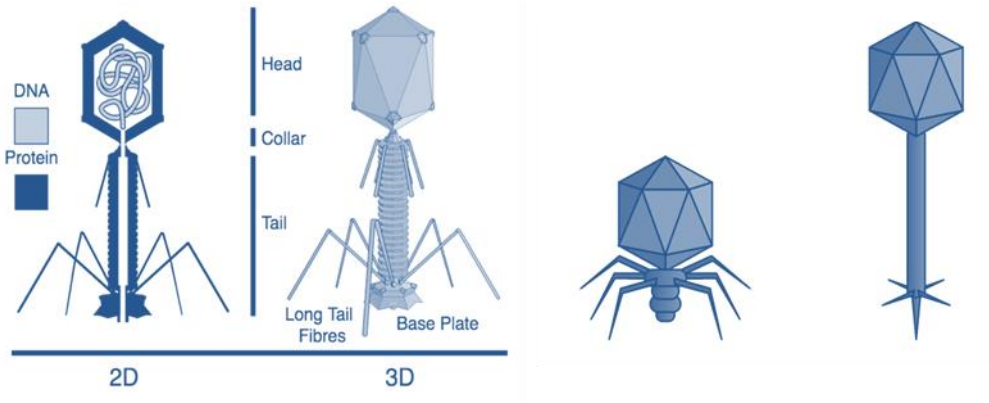


Figure 6. Caudovirales or tailed bacteriophages structures: Myoviridae in 2D and 3D (left), Podoviridae (middle) and Siphoviridae (right).

Morphologically, they possess a head-tail structure that is unique in virology. The head is icosahedral with cubic symmetry, in a capsid enclosing a dsDNA densely packed. The tail is a dynamic multi-proteic assembly that gives them the capacity to recognize the

surface of the bacteria, allowing the delivery of their genome into the host cell [20]. These tailed phages probably represent the most diversified of all the bacterial virus groups. The morphology of their tail has allowed them to be further classified in three families: *Myoviridae* (25% of tailed phages), with a long contracting tail consisting of a sheath and a central tube; *Podoviridae* (14%), with a short non-contractile tail; and *Siphoviridae* (61%), with a long flexible non-contractile tail [17].

Depending on the genome structure, concatemer formation, unusual bases, DNA or RNA polymerase genes and DNA sequence, 15 genera are grouped with vernacular names: groups T4, P1, P2, Mu, Mu, SPO1, H from the *Myoviridae* family; groups, T1, T5, L5, c2, ψM from the *Siphoviridae* and groups T7, P22, φ29 are part of the *Podoviridae* family [17].

2.2.1.1 Coliphage T5

In the study of phage-host interactions, the coliphage T5 from the *Siphoviridae* family (Figure 7.) has proven to be a great model, since its Receptor Binding Protein (RBP) pb5 and its receptor FhuA have both been identified and purified. Additionally, the pb5-FhuA interaction is enough to induce DNA ejection and the purified pb5-FhuA interaction produces a highly stable, stoichiometric complex [5]. This stable complex is presently the only outer membrane receptor - phage RBP complex biochemically available [20].

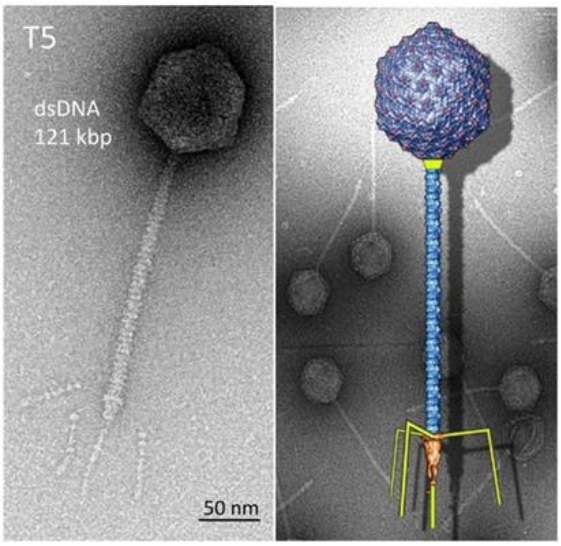


Figure 7. T5 (Type 5) structure. Left: negative stain Electron Microscopy; Right: 3D reconstruction from cryo-EM [18].

It possesses a 250 nm tail ends (light blue) with three L-shaped fibers (yellow) attached to a conical baseplate (orange) and a straight central fiber (yellow protrusion from the orange cone) [21].

The host recognition occurs through reversible binding of the L-shaped fibers to the LPS O-antigen. Irreversible binding of the phage to the iron-ferrichrome transporter FhuA in the outer membrane then follows, through its RBP *pb5*. Interestingly, T5 is able to trigger DNA release *in vitro* in the external medium, only by interacting with the purified FhuA or liposomes containing FhuA [22].

2.2.2 Infection Process of Bacteriophages

When bacteriophages infect a bacterial cell, they can enter two different life cycles: the first mode of reproduction (the lytic cycle) is to control the machineries of the host bacterial cell for replication, transcription and translation to make complete viral particles, while the second one, the lysogenic cycle, is to silently integrate its DNA into the bacterial chromosome and be transmitted to the daughter cells. These “prophages” can wake up under stress conditions and enter a lytic cycle before the host dies. The genes for some toxins are encoded in a prophage, allowing the host bacterium to become pathogenic [23]. Those phages able to undergo this lysogenic cycle are known as temperate phages.

Figure 8. Scheme concerning the infection mechanism of a bacteriophage. *Step 1:* To be able to enter a host cell, bacteriophages attach to specific receptors present on the bacterial surface. The RBP-receptor interaction generates different conformational rearrangements that occur inside of structures such as the tail [20], in the case of *Caudovirales.* This stimulates the capsid to open and the cell wall to be perforated, allowing DNA to be released and transferred via the tail to occur across the bacterial envelope. The host range of different bacteriophages regulates the attachment’s specificity. *Step 2:* Bacterial RNA polymerase starts to transcribe viral DNA tanks to very strong promoters, and ribosomes translate within minutes the viral mRNAs into proteins. *Step 3:* The whole metabolic machinery of the cell is high jacked by the viral DNA, and is turned into a viral factory. *Step 4:* Finally, virions are released via extrusion, cell lysis or budding becoming capable of infecting another bacterium.

Bacteriophage T5 is able to prevent overinfection by other T5 phages by blocking the FhuA receptor protein, by means of a lipoprotein that is expressed early during infection [21]. A few viruses are capable of carrying out both cycles. An example is the phage lambda of *E. coli*.

There are different types of recombination events that can lead to the incorporation of bacterial DNA into the viral DNA, leading to two modes of transduction specialized and generalized. The first one refers to an “excision” event: a lysogenic phage infects the bacterium and viral DNA is incorporated into the bacterial chromosome. When the phage DNA is excised, the flanking bacterial genes may be excised with it.

On the other hand, generalized transduction denotes a packaging event, where the lytic phage cleaves the bacterial DNA. In this process, parts of the bacterial chromosomal DNA may become packaged in viral capsid. When the phage later infects another bacterium, the transfer of these genes occurs. It typically carries only bacterial DNA, instead of viral DNA [23].

As seen in Figure 9., the process of transduction is carried out in several steps: In a first step the phage makes contact with the host bacterial cell, which contains its intact chromosomes, followed by the breakage of the host DNA through phage enzymes. In steps 3 and 4, the cell creates new phages that include the phage and host DNA. After this, the insertion of the donor DNA by the transducing phage is seen in the steps 5 and 6, obtaining a final transduced bacterium with the donor DNA included in the recipient’s chromosome in step 8, through a process known as recombination.

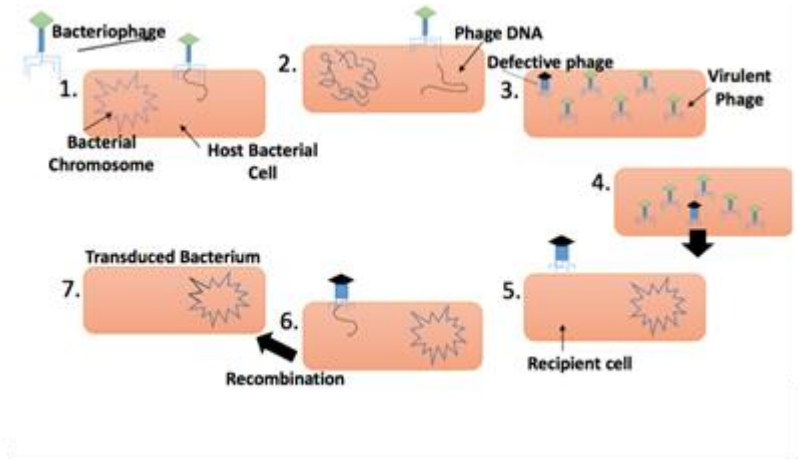


Figure 9. Process of transduction, consisting in the transfer of DNA from one bacterium to another through a virus, known as bacteriophages

Besides this process, DNA exchange can occur through other three mechanisms in prokaryotes: binary fission, transformation and conjugation. In the case of binary fission, it

represents the most common form of reproduction in prokaryotes and occurs in an asexual manner, where a cell splits into two halves, thereby leading to the production of two new cells. As for transformation, it refers to the ability of bacteria to take up naked prokaryotic DNA from the environment, produced by cell lysis for example. In transformation, any DNA can be used. Finally, conjugation refers to the transfer of DNA from one cell to another, occurring after the pilus draws the two bacteria near enough to form a bridge that connects them, therefore allowing their mating [23].

2.2.3 Phage Injection Process

The binding of the phage into the bacteria and the infection mechanism (Figure 10.) is studied with great interest due to many possible future applications, as it is the case of a therapy based on the use of phages.

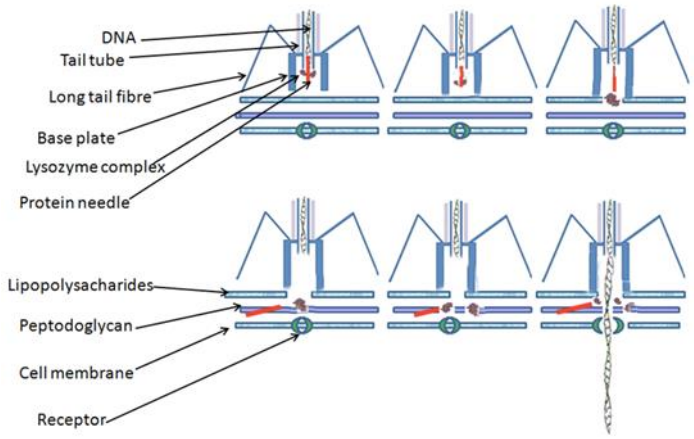


Figure 10. DNA injection process by a bacteriophage during transduction. First, the bacteriophage binds to the host cell surface to specific receptors like LPS, then once it is attached completely, the binding becomes irreversible and the tail contracts. This allows for the genetic material to be injected through the bacterial cell membrane, with the use of a receptor.

Each phage exhibits RBPs (Receptor Binding Proteins), located at the tip of the tail in *Caudovirales*. These determine the host specificity of the phage depending on their interaction with certain receptors present at the surface of the bacteria, either proteins or sugars. The structure of phage tail subcomplexes varies between different families and groups. In the case of the T5 coliphage, irreversible attachment is achieved when the phage tail protein pb5 associates with the outer membrane protein receptor FhuA [24].

In a previous study, the complex formed between these two proteins: pb5 and FhuA, was further characterized: the formation of the complex causes the strength of interaction and upon formation of the complex, conformational changes occur, particularly in pb5. Also pb5 was noticed not to share the common features with other known RBPs [20].

2.3 Lama Antibodies

VHH (Variable Domain of Heavy-Chain Antibody or hcAb) antibodies are antibodies taken from the serum of animals from the *Camelidae* family, specifically lamas. The characteristics of these antibodies is that they lack light chains usually found in mammal antibodies. Their increase use in structural biology is due to these domains, which are easy to clone and express in bacteria, and functionally they are able to bind antigens as effectively as mammal antibodies (Figure 11). VHHs also contain an extra second disulfide bridge that links CDR1 and CDR3, which gives the aforementioned stabilization to the VHH. However, it might also allow the formation of a new loop that could recognize an augmented variety of epitopes [25].

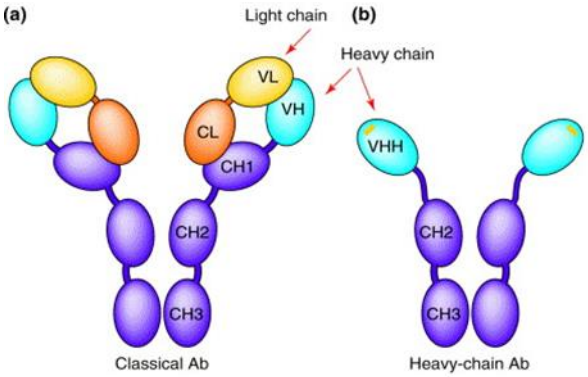


Figure 8. The structure of (a) a classical antibody versus (b) a lama antibody.

During crystallization, it is possible to increase the soluble surface of the complex using these small proteins to optimize the crystallization with easily aggregating proteins, such as pb5.

2.4 Crystallization of Membrane Proteins

The knowledge of the protein structure at a molecular level is necessary for the understanding of membrane functions. In the present, X-ray crystallography following the crystallization of the protein is the most accurate way to obtain structures in the field of structural biology. For this matter, obtaining three-dimensional crystals is necessary in order to perform X-ray crystallography [3].

Even though *in vitro* studies, such as crystallization for membrane proteins, can be performed, they are very dependent on the effective solubilization with detergents and mixed lipid/detergent systems and reconstitution of the membrane proteins [11]. As a result, the study of membrane proteins still represents a big challenge. The problems faced while working outside of their natural lipid environment is one of the major difficulties.

Overall, obtaining structures of membrane proteins, the main obstacle relies on the preparation of crystals of diffraction quality. In the production of proper three-dimensional crystals there is a variety of techniques, based on the use of lipid micelles or detergent and lipid systems [11].

3 Materials and methods

The project was performed by overexpressing, purifying and characterizing three different proteins FhuA, pb5 and VHH antibodies. A detailed scheme is presented in figure 12.

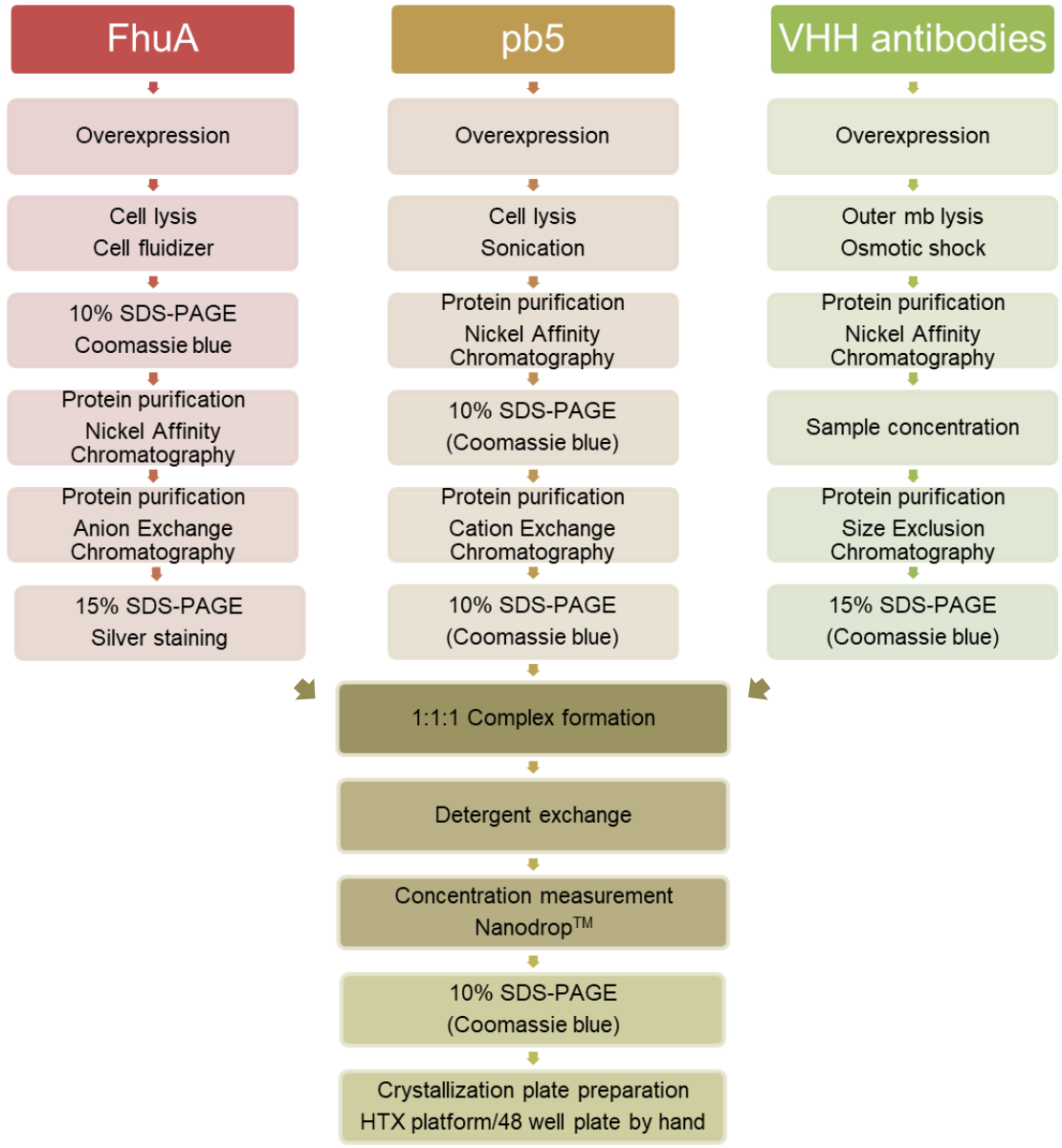


Figure 9. The flow chart of the project.

3.1 Overexpression

The overexpression of the studied proteins was performed in *E. coli*. First, a preculture was prepared in LB broth, using a glycerol stock and the inoculation took place after an overnight growth, until OD_{600} was 0.1, both in 180 rpm agitation. After overnight growth, the cultures were centrifuged at 4°C for 20 min, at 5000 rpm in the JLA8-1000 rotor and the recovered pellets were frozen in liquid nitrogen and stored at -80 ° C. The details of the overexpression are presented in table 2.

Table 2. Overexpression of the studied proteins FhuA, pb5 and VHH antibodies.

Overexpressed protein	Strain	Media	Antibiotic	Tempera-ture	Induction
FhuA	AW740	LB/TB	Ampicillin 125 µg/ml Tetracycline 10 µg/ml	37°C	Dipyridyl 100 µg/ml during inoculation
pb5	BL21(DE3)	LB	Kanamycin 50 µg/ml	20°C	-
VHH	WK6	TB	Ampicillin 100 µg/ml	37°C	1 mM IPTG

The *E.coli* strain AW740 for overexpressing FhuA, lacking two major porines OmpF and OmpC, mutated for endogenous *fhuA* and has resistance to Tetracycline (Tet). This strain is transformed with plasmid pHx405-His-FhuA, which carries the FhuA gene under the control of its own promoter and having a hexahistidine tag inserted into the FhuA gene after amino acid 405 at an exposed loop. Pb5 is overexpressed in the *E. coli* strain. coli BL21 (DE3) transformed with the plasmid pET-28b, in which the *oad* gene was cloned. This construct allows the fusion of a 6-His tag at the C-terminus end of pb5. For VHH antibodies, *E. coli* strain; **WK6** (galE, strA, mutS215::Tn10(Tet)^{R}, Δ (lac-proAB) [F'*traD36 proAB lacI*^{q}ZΔM15]) is used, containing a plasmid vector; **pHEN6** encoding an N-terminal pelB signal sequence in frame with a cassette expression and C-terminal 6xHis detection and purification tag [26].

3.2 Protein Purification

All the different purification steps (table 3.) were performed with Biologic Duoflow™ – system (Bio-Rad), including two pumps, an UV/Vis detector and an automatic sample collector.

Table 3. The summary of the protein purification protocols for the studied proteins.

Protein	Chromatography	Column	Equilibration buffer	Elution buffer	Flow rate	Other buffers	Flow rate	Fraction size
FhuA	Nickel affinity chromatography	5ml HiTrapTM Chelating HP	20 mM Tris pH 8, 5 mM imidazole, 0.1 % LDAO	20 mM Tris pH 8, 200 mM imidazole, 0.1 % LDAO	2 ml/min	Delipidation: 20 mM Tris pH 8, 5 mM imidazole, 1% LDAO	0.2 ml/min	1 ml
	Cation exchange	HiTrapTM Q HP 1 ml	20 mM Tris pH 8, 0.05 % LDAO	7 ml of 20 mM Tris pH 8, 1 M NaCl 0.05 % LDAO	Gradient 0.3 ml/min	-		1 ml
pb5	Nickel affinity chromatography	HiTrapTM 5 ml Chelating HP	50 mM Tris pH 8, 250 mM NaCl, 15 mM imidazole	1. Elution buffer: 25 mM MES pH 6, 200 mM EDTA	5 ml/min	2. Elution buffer: 25 mM MES pH 6, 50 mM EDTA, 250 mM NaCl	0.25 ml/min	1 ml
	Anion exchange	HiTrapTM 1 ml SP HP	25 mM MES pH 6	25 mM MES pH 6, 1 M NaCl	Gradient 0.7 ml/min			0.5 ml
VHH	Nickel affinity chromatography	HiTrapTM 5 ml Chelating HP	50 mM Tris pH 8, 0.5 M NaCl, 20 mM Imidazole	50 mM Tris pH 8, 0.3 M NaCl, 250 mM Imidazole	5 ml/min	-		1 ml
	Sixe exclusion chromatography	SuperdexTM 75 10/300 GL	-	-	-	20 mM Tris pH 8, 250 mM NaCl	0.5 ml/min	1 ml

3.2.1 Purification of FhuA

When the bacterial ferrichrome transporter FhuA was purified, first a frozen cell pellet , resulting from one liter of cell culture, was thawed and lysed with lyse buffer (50 mM Tris pH 8, 150 mM NaCl, 2 mM $MgCl_2$ with DNAse and CLAPA), the cells were passed 10 times through a cell microfluidizer machine at 15 000 psi. To remove the cell debris, the sample was centrifuged for 6 000 rpm, 15 min in 4°C with a JA 25.50 rotor, the pellet

was discarded, and the supernatant was ultracentrifuged for 28 000 rpm, 30 min, 4°C (45Ti rotor).

After the second ultracentrifugation, the pellet containing the membranes of the bacteria was resuspended into Tris 50 mM pH 8, 2 % OPOE and incubated in 37°C in agitation for 30 min.

Then the sample was centrifuged again for 28 000 rpm, 30 min, in 4°C (45Ti rotor) and the pellet, containing the non-solubilized bacterial outer membrane, was suspended into 50 mM Tris pH 8, 1 % LDAO and incubated for an hour in 37°C, followed by an ultracentrifugation with 45Ti rotor (28 000 rpm, 30 min, 4°C).

Finally, the last supernatant was collected and 2 mM $MgCl_2$ and 5 mM Imidazole were added.

During the membrane purification, an aliquot was taken from the supernatants and pellets, which were later loaded into a 10 % SDS-PAGE gel (200 V) stained with Coomassie blue.

a) Affinity Chromatography

The outer membrane solubilization supernatant was loaded into a nickel affinity column (5 ml HiTrap™ Chelating HP, GE Healthcare) which was equilibrated with 20 mM Tris pH 8, 5 mM imidazole, 0.1 % LDAO and after sample injection (5 ml/min) the column was delipidated with a buffer with a higher detergent concentration 20 mM Tris pH 8, 5 mM imidazole, 1% LDAO for 0.2 ml/min, 20 ml. The system was washed with 20 mM Tris pH 8, 5 mM imidazole, 0.1 % LDAO and the protein was eluted in the end with a high concentration of imidazole using 10 ml of 20 mM Tris pH 8, 200 mM imidazole, 0.1 % LDAO 2 ml/min. During the sample injection the flow through and in the elution step, 1 ml fractions were collected and the fractions containing FhuA were pooled together.

b) Anion Exchange Chromatography

The second purification step for FhuA sample, fractions containing the protein from the previous chromatography step, was a cation exchange chromatography (column: HiTrap™ Q HP 1 ml, GE Healthcare). First, the system was equilibrated with 20 mM Tris pH 8, 0.05 % LDAO followed by the sample injection with 2 ml/min flow rate, the flow through was collected as well. The column was washed with the equilibration buffer and then elution was performed with linear gradient, increasing the salt concentration with 7

ml of 20 mM Tris pH 8, 1 M NaCl 0.05 % LDAO, for 0.3 ml/min flow fate. During the elution, 1 ml fractions were collected by the automatic sample collector.
After the purification, the fraction concentrations were determined with UV/Vis spectrophotometer. A 10 % SDS-PAGE, 200 V was performed with silver staining to determine its LPS (lipopolysaccharide) content.

3.2.2 Purification of pb5

When T5 RBP pb5 is purified, the frozen cell sample is first thawed and suspended into lyse buffer [50 mM Tris pH 8, 150 mM NaCl, 2 mM MgCl2 with DNAse and CLAPA (CLAPA: 1 ug each of chymostatin, leupeptin, antipain, and pepstatin per ml and 8 ug of aprotinin per ml)]. After, the lysed cells were sonicated in 2 s cycles with 10 s pause for total 28 min with 70 % power.
To get rid of non-broken cells, the cell debris and membranes in the sample, the cells were ultracentrifuged 30 000 rpm, 20 min, 4°C with a JA 25.50 rotor. After the centrifugation, 100 mM NaCl and 15 mM imidazole were added to the collected supernatant and it was used as a sample in the following steps.

a) Nickel Affinity Chromatography

The affinity column (GE Healthcare HiTrap™ 5 ml Chelating HP) was equilibrated with an equilibration buffer containing 50 mM Tris pH 8, 250 mM NaCl, 15 mM imidazole, which was followed by the sample injection, for 5 ml/min. After, the column was washed with 45 ml equilibration buffer for 5 ml/min. The elution of the protein was performed by adding EDTA to the system using two elution buffers: a) 25 mM MES pH 6, 200 mM EDTA and b) 25 mM MES pH 6, 50 mM EDTA, 250 mM NaCl. During the elution, 1 ml fractions were collected automatically by the fraction collector of the instrument.
Collected fractions were ran in 10 % SDS-PAGE gel, 200 V stained with Coomassie blue.

b) Cation Exchange Chromatography

For the cation exchange chromatography, fractions from affinity chromatography containing pb5 were pooled together and diluted ten times with 25 mM MES pH 6 to decrease the sample salt concentration. The equilibration of the column (GE Healthcare, HiTrap™ 1 ml SP HP) was done with 25 mM MES pH 6. Sample injection was performed with 0.7 ml/min flow rate after the column was fully equilibrated, followed by washing the

column with the equilibration buffer with1 ml 0,70 ml/min. The elution was done using a linear gradient with increasing salt concentration until 1 M NaCl. During the elution, 0.5 ml fractions were collected and the most concentrated ones were diluted with 25 mM MES pH 6, 50 mM NaCl to avoid protein precipitation.
After the purification step, 10 % SDS-PAGE, 200 V, with Coomassie blue staining, was performed with the collected fractions and the flow through of the sample injection. Additionally, the concentrations of the protein containing fractions were measured with UV/Vis spectrophotometer.

3.2.3 Purification of the Lama Antibodies

Because during the overexpression of the VHH antibodies, the proteins are transferred and stored in the periplasmic space, to break the outer membrane of the bacteria, and to release the protein, a frozen bacterial pellet was suspended into a sucrose buffer (100 mM Tris pH 8, 0.5 M saccharose, 20 mM imidazole) and then centrifuged for 13 000 g, 15 min, 4°C (JA 25.50 rotor). This first supernatant was collected, and the pellet was resuspended in water and incubated in ice for 10 min. Before a second ultracentrifugation (13 000 g, 15 min, 4°C, JA 25.50 rotor) 1 mM of $MgCl_2$ was added. After the centrifugation, the obtained, second supernatant was collected as well. To ensure which of the supernatants contains the studied proteins, the aliquots of the both collected supernatants were loaded into a 15 % SDS-PAGE (200 V), stained with Coomassie blue. As a result, the supernatants were pooled together and solutions of Tris 20 mM pH 8 was added as 20 mM imidazole.

a) Nickel Affinity Chromatography

At first, the equilibration of the column (HiTrap™ 5 ml Chelating HP, GE Healthcare) was done with 50 mM Tris pH 8, 0.5 M NaCl, 20 mM Imidazole, followed by the sample injection for 3 ml/min, during this step, the flow through was collected as well. The column was washed with 20 ml of equilibration buffer with 5 ml/min flow rate. During the elution with 50 mM Tris pH 8, 0.3 M NaCl, 250 mM Imidazole, 1 ml fraction were collected, which were later loaded into 15 % SDS-PAGE (200 V), stained with Coomassie blue.

b) Size Exclusion Chromatography

After the affinity chromatography, the NiNTA fractions were concentrated until 500 µl with 3 kDa concentrator (Amicon® Ultra-4 Centrifugal Filter Units, Millipore), and then loaded into Superdex™ 75 10/300 GL (GE healthcare) column to perform Size Exclusion Chromatography (SEC). The used buffer was 20 mM Tris pH 8, 250 mM NaCl with 0.5 ml/min flow rate.
Later the concentrations were measured with UV/Vis spectrofotometer and the obtained, 1 ml fractions from the elution were ran in 15 % SDS-PAGE (200 V) with Coomassie blue staining.

3.3 Complex Formation and the Detergent Exchange

For the formation of the FhuA-pb5-VHH complex, all the three proteins were mixed together in 1:1:1 molar ratio. To perform an exchange of the detergent, the complex was diluted ten times in water and incubated in 4°C over night until aggregation occurred.

After the incubation, the sample was ultracentrifuged 35 000 rpm, 30 min, 4°C and the small pellet was carefully resuspended into 20 mM Tris pH 8, 1.6 % C_{10}DAO buffer.

The success of the complex formation was determined with 10 % SDS-PAGE with 200 V and Coomassie blue staining, by loading heated (reduced protein) and non-heated (non-reduced protein) complex sample on the gel.

3.4 Crystallization

To screen different crystallization environments for the studied protein complex, two different methods were used. To screen small numbers of conditions, previously defined as producing crystals, the crystallization plates were prepared manually (see 3.4.1.) and for larger amounts of conditions to be screened, the samples were sent to HTX platform (see 3.4.2.), where the crystallization plates were done by a crystallization robot Cartesian PixSys 4200.

3.4.1 48-Well Crystallization Plate

When a crystallization plate was prepared by hand; 0.8 µl of protein complex was mixed with 0.8 µl of precipitant reagents with varying salts, pH, and PEG 3350 concentrations. The drop of the reagent-protein mixture was placed as *a hanging drop* in a well containing the used reagents. In that case, the drop is carefully set on a small glass cover slip, that is then placed on top of the well, thus sealing it, the drop hanging on the inside, in an upside-down manner.

The plates were stored in 4°C and they were observed with an inverted microscope daily during the first week and followed less rigorously afterwards, until the last scheduled inspection.

3.4.2 HTX platform

To send samples into the HTX (High Throughput Crystallization) platform in The European Molecular Biology Laboratory (EMBL), Grenoble, the amount of protein should be sufficient (usually 5-10 mg/ml), and the required volume is 15 µl of sample per plate

In the platform, the Olympus CKX53 robot mixes 100 nl of precipitant reagents with 100 nl of the protein sample, placing them on a 96 well plate, three or two drops per well, by an automated high throughput system. 6 classical and complementary screens were used.

The plates were stored in 4°C, and pictured with the following schedule: 1st, 3rd, 7th, 15th, 33rd, 61st and 87th day, by the automated imaging system (RockImager (Formulatrix, Inc., U.S.)). It photographed the drops and sent them via email, when it was possible to access and characterize the drops with an account in the website of the HTX lab.

4 Results and discussion

4.1 Purification

In a first approach, with the use of transformed *E. coli* cells, the protein was overexpressed, with different techniques depending on the protein of interest in each case by following the steps in materials and methods. The cells were later lysed, and the sample was purified by chromatographic methods to obtain the studied protein.

The protein yields are presented in table 4., as seen, not so much protein was obtained per liter of cell culture especially with the tail tip protein pb5 and the antibodies.

Table 4. Yields of the studied proteins FhuA, pb5 and the VHH antibodies.

Protein	Yield (mg/ml)
FhuA	11.6
pb5	2.5
pb5	0.4
pb5	3.6
pb5	5.7
VHH_{53}	0.2
VHH_{64}	0.1
VHH_{81}	0.6

4.1.1 Purification of FhuA

The purification of FhuA was monitored in different steps by taking aliquots from pellets and supernatants, which were loaded onto a 10% SDS-PAGE GEL (200 V). In figure 12 the different steps of the purification are observed, with the FhuA expected to show a band at 80 kDa. Lane a shows the cytosolic proteins, whereas lane b corresponds to the total membranes. Lanes c and d correspond to the supernatant and pellet of the OPOE solubilization of the total membranes. In these conditions, the inner membrane is mainly solubilized, and indeed, FhuA, of the outer membrane, is recovered in the insoluble pellet. Lanes e and f correspond to the supernatant and pellet of the LDAO solubilization of

the OPOE insoluble membranes. In these conditions, FhuA is recovered in the supernatant

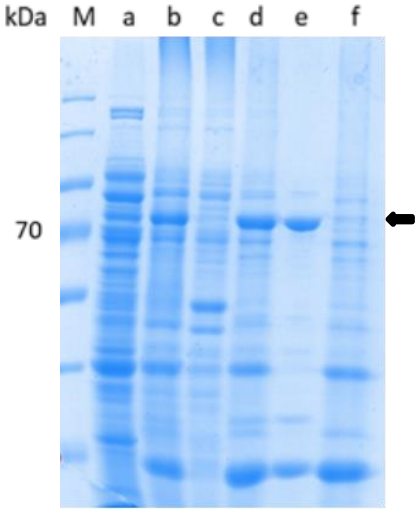


Figure 12. 10 % SDS-PAGE (200 V). The supernatant (a) and the pellet (b) were loaded into the wells after the first ultracentrifugation, whereas the supernatant and the pellet with OPOE 2% were loaded into c and d, respectively. The last obtained supernatant (e) and the pellet (f) correspond to the step where LDAO 1% was used as a detergent. 80 kDa is the molecular weight corresponding to FhuA

This supernatant is further purified on NiNTA that retains proteins with a polyHistidine tag and ion exchange chromatographies. During the chromatographic purification, the elution of the protein is followed by an UV/Vis detector, and the fractions containing protein are visible as an increase of the A_{260} absorption in figure 13., it is possible to see the elution of the protein (green line) as two peaks in Nickel affinity chromatography (left) and in the anion exchange (right). In the case of NiNTA, an elevation preceding the elution peaks can be seen, due to the other proteins that are not interacting with the column but eluted during the sample injection.

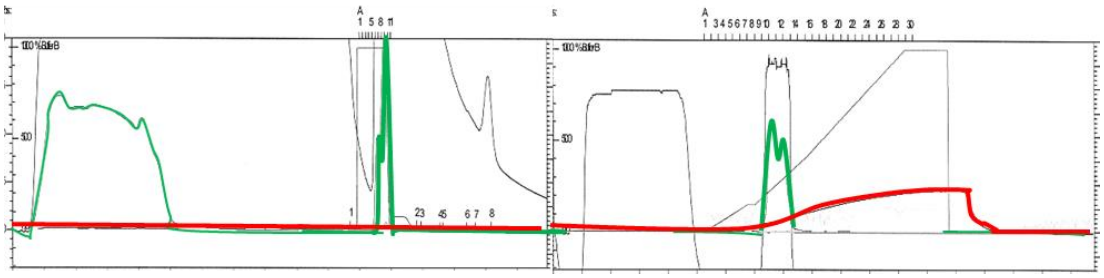


Figure 10. FhuA purification. of FhuA in the Left: NiNTA, right: anion exchange chromatography. The wavelength signal 280 (green) corresponds the protein elution. In the second step, the protein elutes in two peaks; one containing LPS and the other one not containing it. The red shows the conductivity, which means the salt concentration increases during in the column resulting elution of the ion exchange chromatography following the NaCl gradient, inducing protein release from the column.

Also, as a consequence of the linear gradient during the ion exchange chromatography elution, the increasing salt concentration is also visible (red curve). This occurs when the elution buffer, which contains a high salt concentration, flows through the column, leading to a competition for the binding to the column between the negative ions with FhuA, with a final elution of the protein.

In the anion exchange, the two protein peaks seen in the elution profile presumably represent either the presence of the protein alone (first peak) versus the protein bound to LPS (second peak), with the fractions forming the first peak being preferred for forming the protein complex later on, due to the absence of LPS. However, to determine the presence of LPS, the fractions are further analyzed by SDS-PAGE, stained by silver stain to assess the presence and quantity of LPS and another gel stained by Coommassie blue to assess the purity of the obtained protein.

For this reason, after the anion exchange was performed, the fractions expected to contain the FhuA protein bound to LPS were either heated or not, and loaded onto a 15% SDS-PAGE (Figure 14.), as detailed in materials and methods, where 0.5 µg, 1.0 µg and 2.0 µg of LPS were used as control (lanes a to c, respectively). Of each fraction, 2 µg of non-heated FhuA, followed by 2.0 µg of heated FhuA and finally 8.0 µg of non-heated FhuA were loaded into the gel (lanes d-l).

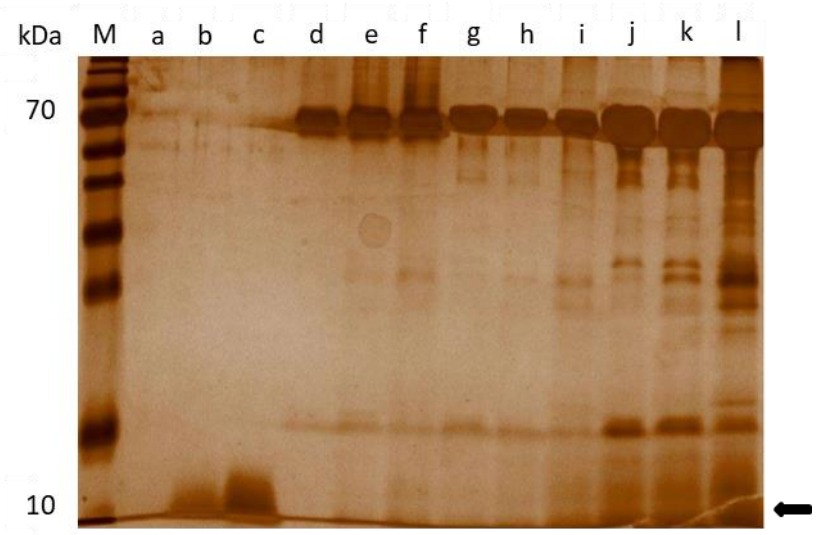


Figure 114. a-c LPS 0.5 µg, 1.0 µg, and 2.0 µg respectively; d-f FhuA 2.0 µg non-heated of fractions 11-13, g-i FhuA 2.0 µg heated, j-l FhuA 8.0 µg non-heated. 15% SDS-PAGE, 200 V, silver staining. The amount position of LPS is shown around 10 kDa. In the loaded fractions there is J-k 1.0 µg and l around 2.0 µg of LPS when compared to the control.

4.1.2 Purification of pb5

The protein purification for the tail tip protein pb5 was followed with a UV-Vis detector during its elution. The supernatant of broken cells was loaded onto a NiNTA column, and eluted by nickel chelation by EDTA and low pH: two peaks are visible (Figure 15 A), therefore the fractions were assessed with a 10% SDS-PAGE. The fractions that resulted in 70 kDa bands (Figure 15 B) corresponded to pb5 and were then pooled and loaded onto a cation exchange chromatography (Figure 15 C). The most concentrated and pure fractions were collected after and pooled, with their concentrations being measured later with UV-Vis spectrophotometer. It is important to be aware of how vital it is to proceed quickly to the next steps during the purification of pb5, since this protein has a tendency to precipitate alone very rapidly.

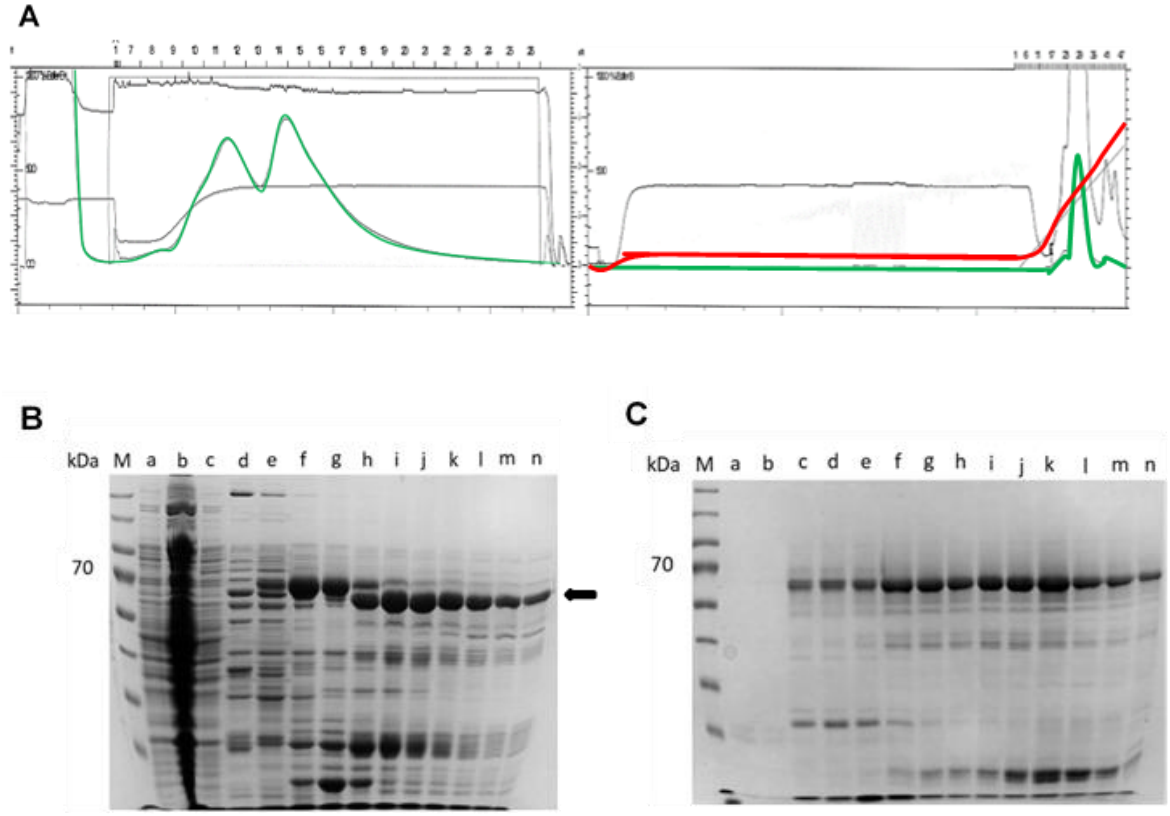


Figure 12. Pb5 purification A) left: chromatogram of the NiNTA elution; right, chromatogram of the cation exchange chromatography, the increased salt concentration causes the elution of the protein (red). In both panels, the green curve corresponds to absorption at 280 nm of the elution. B) 10% acrylamide SDS-PAGE of the elution of the NiNTA column a: before loading to the column, b: flow through, c: wash of the column, d-n: the eluted fractions 13-20. C) The fractions after resulting from the cation exchange chromatography elution., a-b: are the wash, and c-n eluted fractions containing the purified pb5.

4.1.3 Purification of the Lama Antibodies

The analysis for the four VHH antibodies were performed separately. Due to the use of the same protocols and the obtaining of the same results for each antibody, only the VHH_{53} graphs and gels are presented in figures 16 and 17, respectively.

On a first step, the outer membranes were broken using osmotic shock. This step resulted in the obtention of two supernatants: one after centrifugation after incubation in sucrose buffer and the second one after incubation in pure water. Lastly, the pellet was suspended in sucrose buffer and a small aliquot was taken and saved aside, in order to load on a 15% SDS-PAGE gel after all the purification steps.

After lysis, the purification of the four different antibodies was started with an affinity chromatography on a NiNTA column (seen in figure 16, left). Later, Size Exclusion chromatography (SEC) was performed (seen in figure 16, right) to separate the antibodies from possible contaminants by their size.

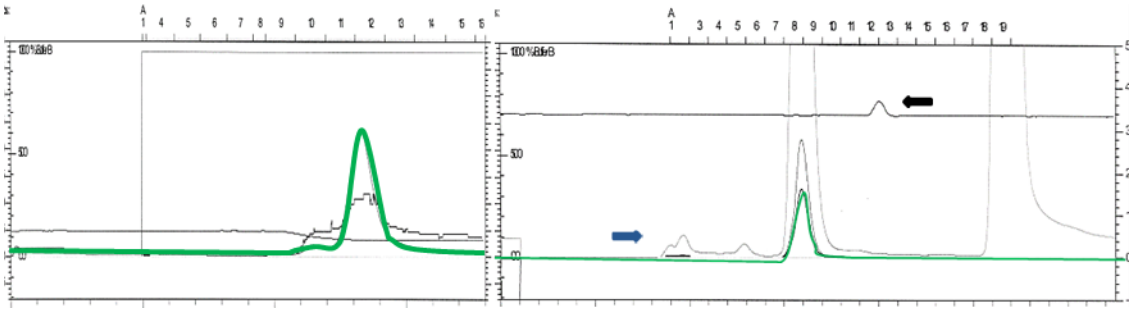


Figure 13. Purification chromatograms for the lama antibodies. Green signal: eluted protein (wavelength 280 nm). Affinity chromatogram, elution (left) and SEC (right) showing one peak corresponding to the VHH antibodies. The blue arrow represents the void volume, and the total volume of the column is marked with a black arrow. The grey line in the background shows the wash of the column and the elution of imidazole (wavelength 214 nm).

Subsequently, the aforementioned aliquots taken before the purification process were loaded onto a 15% SDS-PAGE gel at 200 V (Figure 17, lanes a-c). Normally, if the used protocol for osmotic shock is considered, the lama antibodies should be found in the last supernatant. However, the studied protein was found to show a band in both of the supernatants (lanes a and b), presumably as an effect of either the freezing process or the

age of the cells. Hence, they were pooled together to proceed towards the chromatographic purification. As for the pellet (lane c), it was discarded for not containing the studied protein, as expected.

Next to these three lanes, the eluted protein fractions were also loaded into the same gel electrophoresis (Figure 17, lanes a-c), in order to monitor the success and the purity of the sample. In the same manner, after the second purification step by SEC the fractions are analyzed by SDS-PAGE (Figure 17, lanes j-n). A band can be seen at a molecular weight of around 12 kDa, corresponding to the VHH. showing a concentrated and pure protein.

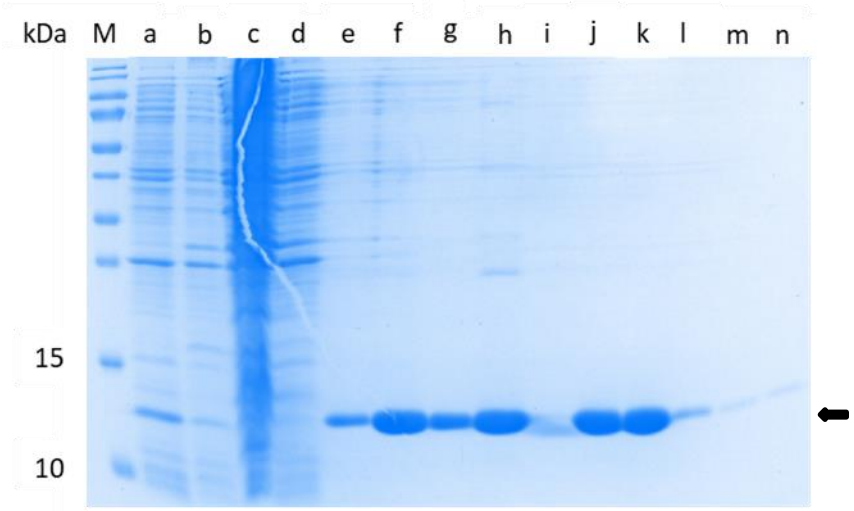


Figure 14. 15% SDS-PAGE, 200 V. a-b: supernatants obtained after cell lysis by osmotic shock showing a faint band for the antibodies at 12 kDa; c: pellet after the cell lysis; d: flow through in the affinity chromatography; d-h: the fractions 10-13, obtained from the affinity chro-matography; i: an aliquot from the concentrating of the sample between the purification steps; j-n: fractions from size exclusion chromatography, where the protein is eluted in two very concentrated and pure fractions seen in lane j and k only (fractions 8-9).

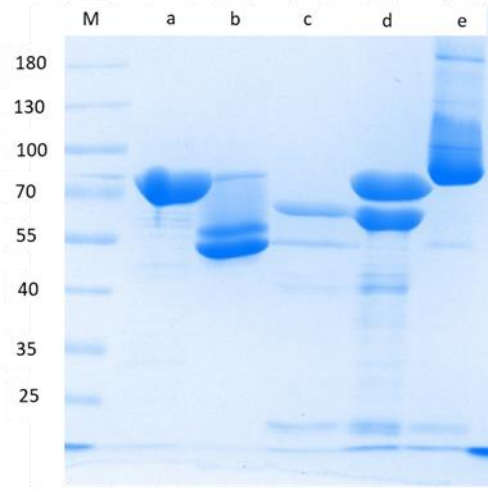
4.2 Complex Formation

Table 5. presents the different complexes formed in the project. The concentrations were measured with the Nanodrop™ instrument. Different trials were attempted that included older protein samples from the research laboratory, as well as newly purified fresh proteins. The complexes were sent to the High Throughput Crystallization (HTX) laboratory whenever a sufficient concentration (around 10 mg/ml) was obtained.

Table 5. Crystallization plates preparation

Complex	Concentration (mg/ml)	Crystallization method	To notice
FhuA- pb5-VHH_{64}	4.45	48-well plate	Old, previously purified FhuA and antibodies were used with freshly purified pb5
FhuA- pb5-VHH_{81}	6.9		
pb5-VHH_{64}	5.4		
FhuA-pb5-VHH_{53}	8.17	HTX	
FhuA-pb5-VHH_{54}	8.0		
pb5-VHH_{81}	1.41		
FhuA-pb5-VHH_{53}	6.7		All freshly purified proteins
FhuA-pb5-VHH_{54}	6.7		
FhuA-pb5-VHH_{64}	6.9		
FhuA-pb5-VHH_{81}	5.7		
pb5-VHH_{53}	5.7		
pb5-VHH_{64}	2.3		
pb5-VHH_{81}	6.5		

The success of the 1:1:1 protein complex formation was assessed with a 10% SDS-PAGE gel (Figure 18.), where the complexes are loaded either after being heated or not. The FhuA-pb5 interaction is very strong: indeed, it is not denatured by SDS, and migrates as a unique band (lane e). The two proteins are separated only when heated to 90°C for 2 min (lane d). The heated sample shows FhuA, pb5 and the VHH, that can be located comparing migration with the individually loaded proteins: FhuA on lane a (80 kDa) and pb5 on lane c (70 kDa), where the faint band results from the low loading concentration. Also, the protein is not extremely pure. In the case of FhuA, it is interesting to see that the non-heated protein does no migrate at the same position as the heated sample. This



is due to the fact that FhuA is not denatured by SDS alone, and thus migrates in the gel as a folded, more compact protein: it migrates faster than the denatured protein. The unique band in the non-heated complex demonstrates that the FhuA-pb5 complex has been successfully formed in a 1:1 ratio, with no visible excess of any of the studied proteins in the complex.

Figure 15. Assessment of the complex formation in a 1:1:1 ratio by gel electrophoresis. 10% SDS-PAGE was ran at 200 V on a: FhuA non-heated, b: FhuA heated, c: pb5, d: FhuA-pb5 heated, e: FhuA-pb5 non-heated. When the complex is heated, the com-plex is denatured and the separated bands are shown in 70 kDa for pb5 and 80 kDa for FhuA. In the non-heated aliquot, the complex stays intact and only one band is visible.

4.3 Crystallization

The crystallization was monitored through the observation of the drops previously placed in wells as detailed in materials and methods, with an inverted optic microscope. Several trials were performed and often only aggregation and precipitation were seen (Figure

19.) and no crystals were formed in the droplets, as obtaining the formation of crystals is a known difficulty in the study of this protein complex.

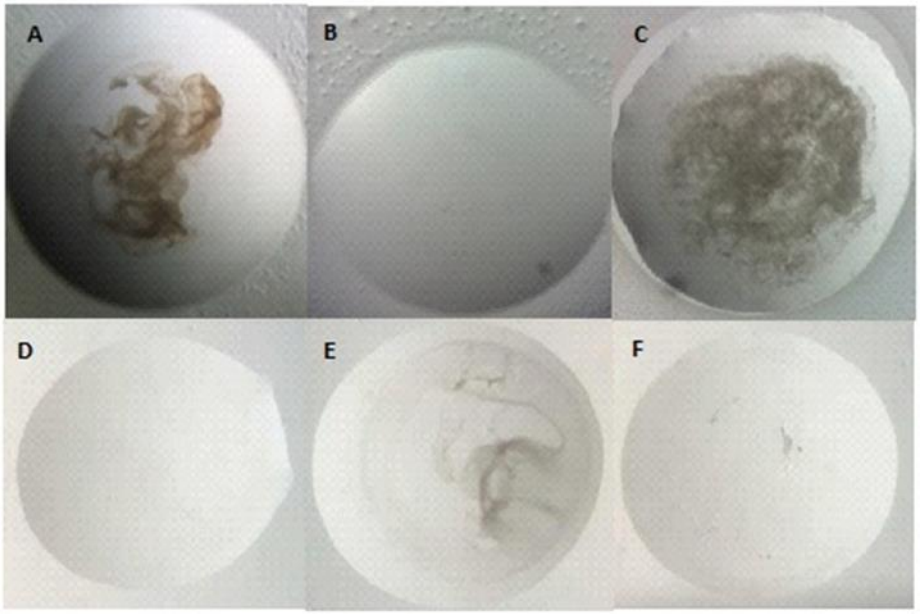


Figure 16. Example of the obtained drops. Made by hand (upper row) where the A and the C drops show protein precipitation, whereas an empty clear drop is seen in the B. In the case of the lower row, they were made in the HTX laboratory, where the drop D is clear with small impurities, the E demonstrates precipitation and the drop F shows small impurities and phase separation.

On the other hand, few crystals were formed in the project, as seen in Figure 20., all of them produced by the HTX platform. Two droplets with the whole FhuA-pb5-VHH formed crystals: Some round crystals of FhuA-pb5-VHH_{64} complex (6.9 mg/ml) with 1 M Potassium Sodium tartrate, as well FhuA-pb5-VHH_{53} (6.7 mg/ml) forming a single crystal with 0,1 M Imidazole pH 8, 0.2 M Sodium chloride, 0.2 M sodium thiocyanate, 20 % w/v PEG 335. Also, four pb5-VHH complexes formed following structures: Beginning of a crystalline formation of pb5-VHH_{53} (5.7 mg/ml) with 0.2 M calcium chloride dihydrate, 0.1 M tris pH 8, 20 % w/v PEG 6000. Two probable crystals of pb5-VHH_{53} (5.7 mg/ml) with 0.2 M calcium chloride dihydrate, 0.1 M tris pH 8, 20 % w/v PEG 6000. Few round crystals of pb5-VHH_{64} (2.3 mg/ml) with 0.2 M calcium chloride dihydrate 0.1 M tris, pH 8, 20 % w/v PEG 6000 and lastly a needle-like crystal of pb5-VHH_{81} (6.5 mg/ml) with 0.1 M citrate pH 5, 20 % w/v PEG 6000.

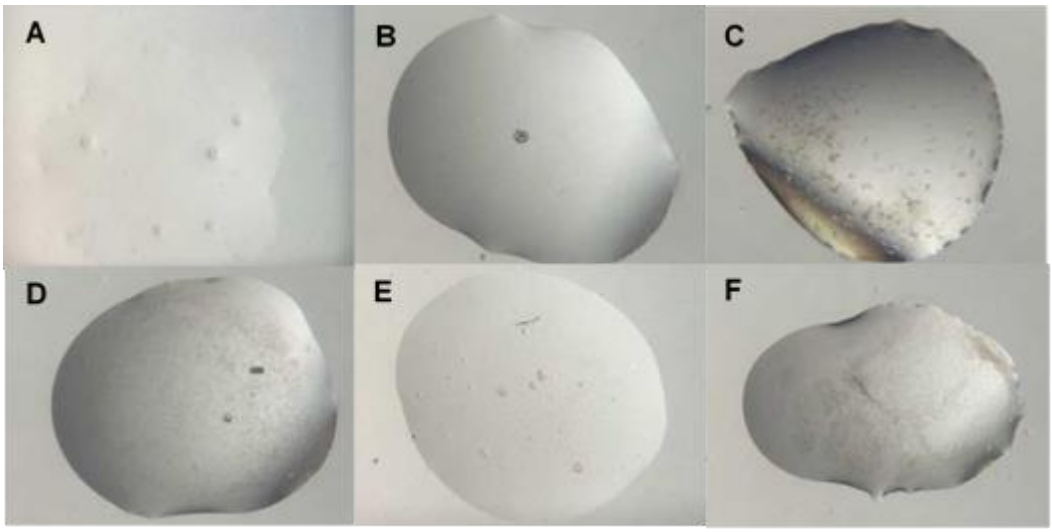


Figure 17. A: Few round crystals of FhuA-pb5-VHH64 complex (6.9 mg/ml) B: FhuA-pb5-VHH_{53} (6.7 mg/ml) forming a single crystal. C: Starting crystalline formation of pb5-VHH_{53} (5.7 mg/ml. D: Two possible crystals of pb5-VHH_{53} (5.7 mg/ml). E: Some round crystals of pb5-VHH_{64} (2.3 mg/ml. F: A needle-like crystal of pb5-VHH_{81} (6.5 mg/ml).

5 Conclusion

Through the process of the elaboration of this project, the handling of the three different types of proteins: FhuA, pb5 and VHH antibodies was successful, concerning the performed expression and purification by the means of using chromatographic methods. Even though small optimizing might be useful, the protocols used in the laboratory are suitable for all the three proteins.

A major difficulty when handling the tail tip protein pb5 was encountered, due to its easy aggregation and resistance to the detergents. It would be of preference to perform a screening of the different environments possible for this protein, in order to find the most suitable buffer and salt concentration, providing the most optimal conditions. In fact, the testing of these different environments was started by employing a Differential Scanning Fluorimetry (DSF) and could be continued further.

As for the crystallization, it has to be taken into account the fact that obtaining crystals with membrane protein complexes represents a major difficulty in the project. Despite of this challenge, some crystals of FhuA-pb5-VHH and pb5-VHH complexes were formed in the HTX laboratory, which is going to lead into X-ray crystallography measurement by the laboratory team.

The present thesis project has provided more information about the expression and purification of the studied proteins, and despite the limited time, it allowed small optimization of the different methods. Overall, the thesis project was favorable towards the improvement of various steps and provided extra information on the handling of the different proteins.

Finally, the research project will be continued in the near future, with the purpose of obtaining optimal crystals of the complex capable of being used for X-ray crystallography. Consequently, the structure of the FhuA-pb5 complex could be solved and the phage's infection process and interaction would be enlightened. This may have future applications in phage therapy, for bacteriophages to treat pathogenic bacterial infections, as bacteriophages are much more specific than antibiotics with a high therapeutic index. This means that phage therapy would be expected to cause fewer side effects.

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