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Bachelor in Biochemistry

Design of a Novel Phage Display Vector to Improve Enrichment of Phages with Antigen-Specific Nanobodies and to Identify the Nanobody Clones of Highest Affinity

Dissertation to obtain the Master's Degree in Biotechnology

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September 2019

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Acknowledgments

This thesis could not be done without the help and support of many. I would like to take a moment to express my gratitude towards these people.

First and foremost, I would like to thank my promoter Prof. Dr. Serge Muyldermans for giving me the opportunity to work on this project, for his patience and guidance and for his comments during the writing process that allowed me to improve this thesis.

Secondly, I would like to thank Ema Romão, for her guidance and support throughout this year. I would like to thank her for adopting me as her student and for teaching me all the experimental techniques. Her dedication and perseverance towards her own experiments and kind words when I needed to hear them were an inspiration to never give up. Not only I gained a mentor, but also a friend.

I would also like to thank Chris and Maxine for answering my countless questions, for always being available to help me and for the companionship throughout this year. I am thankful to all CMIM members for the friendly atmosphere, specially to my fellow master thesis students that always had a word of encouragement when things weren't working as expected.

Finally, but not least important, I would like to thank all my family for all the support. Gostaria de agradecer a toda a família pelo apoio que me deram ao longo destes anos. Aos meus pais, um muito obrigada por me terem proporcionado a oportunidade de tirar um curso superior, apesar de não ter sido fácil, por apoiarem as minhas decisões académicas e por sempre me encorajarem a seguir os meus sonhos. Ao meu irmão e cunhada, um muito obrigada por me terem ajudado a ter esta experiência de Erasmus, por me terem recebido de braços abertos e feito sentir em casa e por me terem apoiado sempre. Ao meu sobrinho, um obrigada pelas distrações e gargalhadas que me proporcionou ao longo deste ano. Um sorriso seu bastava para me fazer sentir melhor.

Thank you all! Obrigada a todos!

Rita

Abstract

Although phage display-based enrichments became a standard procedure, they still suffer from some drawbacks. Nonspecific phage binding limits the enrichment that can be achieved per selection round and therefore, in most cases, at least three or four rounds are required to identify the antigen binding nanobodies (Nbs) from the library. Moreover, the release of the phages captured via their antigen-specific Nb on the immobilized antigen is accomplished most often, by a pH shock that will also release the a-specific absorbed phages, thereby generating an unwanted background. However, the biggest shortcoming is the difficulty to identify the Nb of best binding affinity. We now have to ferment and purify all individual Nb clones to homogeneity and measure their affinity parameters one after the other. This is very tedious, certainly, as the identified Nbs often share a high degree of amino acid sequence identity, which makes it impossible to predict the one having the best affinity. The objective of this thesis aimed to modify the phage display vector so that one round of selection will be sufficient, while the affinity of all antigen-positive Nbs can be compared immediately after ELISA.

Firstly, a vector containing the Calmodulin Binding Peptide (CBP) tag was created, by substituting the hemagglutinin (HA) tag present in a pMECS-GG plasmid by the CBP tag. Moreover, 6 Nbs with well-known and variable kinetic binding rates were also inserted into the vector pMECS-CBP.

Next, the vector was used in a phage display setting, where two mini libraries comprising the 6 Nbs were made. One library had the conventional pMECS vector while the other was made using the pMECS-CBP vector. Although an enrichment of 1000 times was achieved using pMECS, pMECS-CBP failed to show any enrichment, supporting the hypothesis that Nb-CBP encountered serious expression problems. To investigate the validity of this hypothesis, periplasmic expression of Nb-CBP was performed and compared with that of the Nb without the tag. While the Nb yielded almost 4 mg per liter of culture media, the Nb-CBP could only be obtained at 0.63 mg per liter culture. Moreover, the Nb-CBP couldn't be detected on a western blot. Several methods were used, such as adding lysozyme to improve the periplasmic extraction step, expressing 5 different colonies to check if there was a homogeneity of expression between them. In all methods, the amount produced always remained below 1 mg per liter and Nb-CBP protein couldn't be detected in any step of expression by Coomassie stained SDS-PAGE or western blot.

Additionally, a high yield expressing-protein (SIRPα) was cloned into the CBP vector to identify whether the expression problem was caused by the peptide tag itself or by the Nb-CBP combination. Even though SIRPα was obtained at a yield of 8.7 mg/L of culture when expressed from a vector not containing the CBP, its expressed yield using the CBP vector, dropped to 0.7 mg/L of culture, indicating that the CBP tag is incompatible with good periplasmic expression.

Lastly, a nucleotide alignment of our CBP tag with a previously reported one, revealed differences in two Arginine codons, two Alanine codons and one Lysine codon. Moreover, our sequence employed two codons, AGA and CGG, rarely used in *E. coli*, which might be linked to the reduced expression levels that we observed during this thesis.

Keywords: Phage Display, Nanobodies, Periplasmic Protein Expression

Resumo

Apesar dos enriquecimentos através de *phage display* se terem tornado um procedimento habitual, ainda possuem alguns inconvenientes. As ligações inespecíficas dos fagos limitam o enriquecimento que pode ser atingido por ronda de seleção e, dessa forma, pelo menos três a quatro rondas são necessárias para identificar os nanocorpos de uma biblioteca que se ligam ao antigénio. Além disso, a libertação dos fagos capturados através do seu nanocorpo específico para antigénio imobilizado é frequentemente realizada por um choque de pH, que também irá libertar os fagos absorvido inespecificamente, gerando *background* indesejado. No entanto, o maior inconveniente é a dificuldade em identificar o nanocorpo com melhor afinidade. Neste momento, tem que se produzir e purificar todos os clones individualmente e medir os seus parâmetros de afinidade um após o outro. Isto é muito trabalhoso, uma vez que, com frequência, os nanocorpos apresentam sequências de aminoácidos idênticas, o que torna impossível prever qual poderá ter melhor afinidade para o alvo. Esta tese foi criada com o objetivo de modificar o vetor de *phage display*, para que apenas uma ronda de seleção seja necessária, enquanto a afinidade de todos os nanocorpos que se liguem positivamente ao antigénio possa ser comparada imediatamente após ELISA.

Primeiro, um vetor que contivesse uma tag do péptido de ligação à calmodulina (CBP) foi criado, através da substituição da *tag* da hemaglutinina contida no plasmídeo pMECS-GG. Além disso, 6 nanocorpos com constantes de ligação variáveis e bem estabelecidas, foram também inseridos no vetor pMECS-CBP.

De seguida, o novo vetor foi usado em *phage display*, onde duas mini bibliotecas compostas por 6 nanocorpos foram criadas. Uma das bibliotecas continha o vetor pMECS convencional, enquanto a outra foi construída usando o vetor pMECS-CBP. Apesar de um enriquecimento de 1000 vezes ter sido observado usando o pMECS, o vetor pMECS-CBP não apresentou qualquer enriquecimento, sustentando a hipótese de que os nanocorpos conjugados com o CBP teriam problemas de expressão. Para perceber a veracidade desta hipótese, expressão periplasmática dos nanocorpos conjugados com o CBP foi realizada e comparada com a dos nanocorpos sem a *tag*. Enquanto na expressão dos nanocorpos mais de 5 mg por litro de cultura foram produzidos, a expressão dos nanocorpos conjugados com o CBP apenas obteve 0,63 mg por litro de cultura. Para além disso, os nanocorpos conjugados não foram detetados via *western blot*. Outros métodos foram testados, tais como a adição de lisozima para melhorar a fase de extração periplasmática e a expressão de 5 colónias diferentes de cada nanocorpo para verificar se havia uma homogeneidade de expressão entre elas. Em todas as tentativas, a quantidade produzida permaneceu sempre abaixo de 1 mg e os nanocorpos conjugados com o CBP não conseguiam ser detetados em nenhuma fase da expressão usando SDS-PAGE e *western blot*.

Posteriormente, uma proteína com elevada expressão (SIRPα) foi clonada no vetor pMECS-CBP, para perceber se o problema de expressão era causado pela *tag* peptídica ou pela conjugação nanocorpo e CBP. Apesar de o SIRPα ter produzido 8,7 mg/L de cultura quando expresso com o vetor que não continha o CBP, a quantidade produzida usando o vetor pMECS-CBP baixou para 0,7 mg/L de cultura, indicando que a *tag* CBP é incompatível com uma boa expressão periplasmática.

Por fim, um alinhamento de nucleótidos entre o CBP *tag* utlizado e outro previamente descrito, revelou diferenças entre dois codões de Arginina, dois codões de Alanina e um codão de Lisina. Notou-se ainda que a sequência usada no decorrer desta tese continha dois codões, AGA e CGG, raramente usados em *E. coli*, o que pode estar ligado aos reduzidos valores de expressão observados no decorrer da tese.

Palavras-chave: Phage Display, Nanocorpos, Expressão periplasmática de proteína

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List of Abbreviations

%	Percent
µg	Microgram
µL	Microliter
µM	Micromolar
µm	Micrometer
aa	Amino Acids
Ab(s)	Antibody(ies)
Ag(s)	Antigen(s)
Amp	Ampicillin
AP	Alkaline Phosphatase
bp	Base Pairs
Ca^{2+}	Calcium Ions
CaM	Calmodulin
CBP	Calmodulin-Binding Peptide
Chl	Chloramphenicol
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
dsDNA	Double-Stranded DNA
E. coli	*Escherichia coli*
ECC	Electrocompetent Cells
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
Fab	Antigen-Binding Fragment
FACS	Fluorescence-Activated Cell Sorting
$Fe(III)Cl_3.6H_2O$	Iron (III) Chloride Hexahydrate
FWD	Forward
GIII	Gene III
Glu	Glucose

H_2O	Water
H_2O_2	Hydrogen Peroxide
H_3BO_3	Boric Acid
HA	Hemagglutinin
HCAb(s)	Heavy-Chain Only Antibody(ies)
HCl	Hydro Chloridric Acid
HIS	Histidine
HRP	Horse Radish Peroxidase
IF	Infective Form
IgG	Immunoglobulin G
IMAC	Immobilized Metal Ion Affinity Chromatography
IPTG	Isopropyl-β-D-Thiogalacto-Pyranoside
$K_2HPO_4.3H_2O$	Di-Potassium Hydrogen Phosphate Trihydrate
Kan	Kanamycin
KCl	Potassium Chloride
kDa	Kilodalton
KH_2PO_4	Potassium Di-Hydrogen Phosphate
L	Liter
LB	Luria Broth
mAb	Monoclonal Antibody
$MgCl_2$	Magnesium Chloride
min	Minute
mL	Milliliter
mM	Millimolar
MW	Molecular Weight
Na_2CO_3	Sodium Carbonate
Na_2HPO_4	Di-Sodium Hydrogen Phosphate

Na_2SO_4	Sodium Sulphate
NaCl	Sodium Chloride
NaH_2PO_4	Sodium Di-Hydrogen Phosphate
NaN_3	Sodium Azide
NaOH	Sodium Hydroxide
Nb(s)	Nanobody(ies)
ng	Nanogram
NH_4Cl	Ammonium Chloride
nM	Nanomolar
nm	Nanometer
O/N	Over Night
^oC	Degree Celsius
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
REV	Reverse
RF	Replicative Form
RFP	Red Fluorescent Protein
RT	Room Temperature
scFv	Single-Chain Variable Fragment
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
SIRPα	Signal-Regulatory Protein Alpha
ssDNA	Single-Stranded DNA
TB	Terrific Broth
V	Volt
VH	Variable Domain of the Immunoglobulin of the Heavy Chain

VHH	Variable Domain of the Immunoglobulin Heavy Chain Only Antibody
VL	Variable Domain of the Immunoglobulin of the Light Chain
WB	Western Blot

1 Introduction

1.1 Phage Display

Phage display, firstly introduced by G. Smith in 1985 [1], is a well-established molecular screening technique whereby a library of genes with a randomized region is expressed and exposed as peptide or protein on the surface of bacteriophages that are available for selection [2]. Its advantages include being robust, easy to perform, highly versatile and inexpensive [3].

The unique feature of the method relies on the physical linkage that is established between the genotype and the phenotype of the phage particle [2]. A ligand's coding sequence of interest is fused to the sequence of one of the phage coat proteins and, after expression and subsequent incorporation of this new vector on phages, the ligand is presented on its surface, while having the corresponding genetic material inside [4]. This critical linkage allows a rapid analysis and identification (when compared with other techniques), after selection, of the phage particles with the desired binding specificities [5].

Usually, there are 5 key steps involved in phage display (Figure 1.1). It all starts with the construction of a library, which is crucial since the final results will directly depend on the quality of the designed library [6]. Then, the specific ligands of the library will be retrieved, typically via biopanning, which will select them based on their expression level and the capacity to recognize the target [7]. Usually, 3 to 5 cycles of biopanning are required to enrich the clones of highest affinity [8]. The clones with highest affinity are identified after re-amplification and production of their ligand in *Echerichia coli* (*E. coli*). Finally, experiments are performed to confirm the specificity, to measure the affinity parameters and to reveal the sequence of the selected ligand [8].

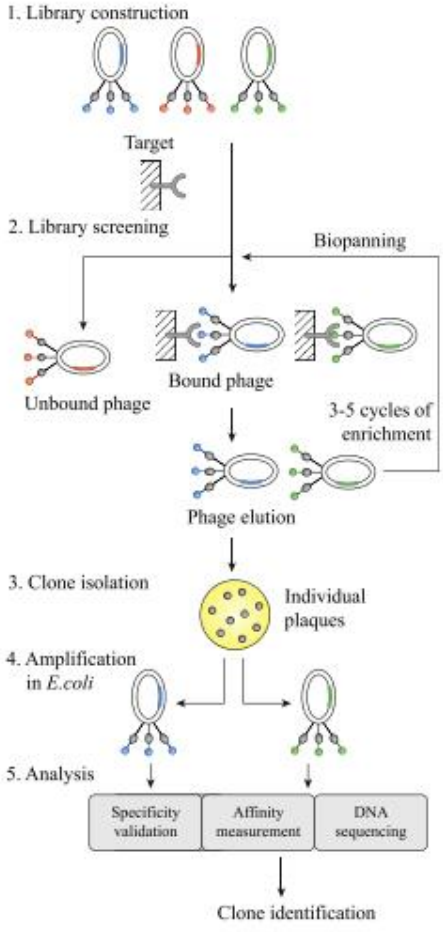


Figure 1.1 - Illustration of the key steps involved in phage display [8].

Phage display can have diverse applications, such as: studies of protein-protein interactions [9], epitope mapping [10], identification of mimic epitopes (mimotopes) [11], discovery and development of new biological drugs [12], *in vivo* imaging of tumors [13] and improvement or modification of affinity of proteins to their binding partners [14]. This allowed major breakthroughs in the field of immunology, cell biology, pharmacology and drug discovery [2]. In recognition of these major achievements, half of the Nobel Prize in Chemistry was awarded in 2018 to George Smith and Sir Greg Winter "for the phage display of peptides and antibodies" [15].

1.1.1 Phage Display Vectors

Bacteriophages, are viruses that can infect a variety of Gram-negative bacteria [6]. Different bacteriophage DNA can be used as phage display vector to transform or infect *E. coli*, such as T4, T7, lambda and filamentous phages, the latter being the preferred one [4].

Among *E.coli*-infective filamentous bacteriophages, the most used and best studied group is the nonlytic F pilus-specific phages (also known as Ff), which include the strains M13, f1 and fd [16]. This group is closely related, being 98.5% identical in their DNA sequence, and infect male *E. coli* after a specific interaction between the phage and the tip of the F-pilus produced by the bacteria [8 17]. Since they are nonlytic phages, the assembly is done in the periplasmic environment and then they are secreted out of the bacterial cell [4]. Although the growth rate of the bacteria decreases significantly, they survive phage infection [4].

There are some unique characteristics that make filamentous bacteriophages suitable vectors for phage display [4]. The infections can be controlled, since these phages infect only *E. coli* strains that express the F-pilus [4]. Moreover, since there is an immediate depolymerization of the F-pilus after infection, a bacterium can only be infected once, thus each bacterium secretes a unique phage with the encoded peptide [4]. Larger foreign sequences inserted in the phage genome simply results in the production of longer phage particles, since DNA replication and the assembly of phages is not limited by the size of the inserted DNA sequence [4 17]. Filamentous phages are stable under a variety of very harsh conditions, such as extreme pH, high and low temperatures, presence of DNase, enzymatic cleavage and nonaqueous solutions [4 6 18].

We will limit ourselves to the description of M13 as it is the most widely used and considered the model of filamentous bacteriophages [8 19].

1.1.1.1 M13 Bacteriophage Structure

M13 has a semi-flexible, extended tube-like shape (Figure 1.2A), with 6.5 nm in diameter and 900 nm long [3 8 19]. The phage contains a genome of single-stranded DNA (ssDNA) with 6407 bp, consisting of 9 genes encoding 11 different proteins (Figure 1.2B) [3]. These proteins are grouped according to their function, five of them are coat proteins, major coat protein (pVIII) and minor coat proteins (pIII, pVI, pVII and pIX), while the other six are involved in replication (pII, pV, and pX) and assembly/secretion of the phage (pI, pIV and pXI) [3 20].

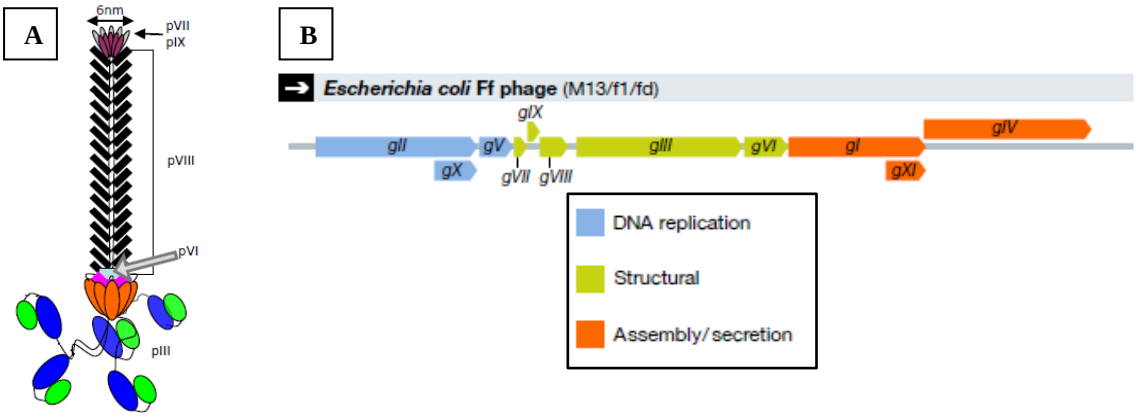


Figure 1.2 – A - Structure of M13 bacteriophage. Adapted from [16]. B – Schematic representation of Ff bacteriophages genome with the function of each gene. Adapted from [21].

M13 consists of 2700 copies of the major coat protein pVIII, a 50 amino acids (aa) residue protein, which polymerizes along the length of the genome [4 8]. The minor coat proteins are located in pairs at each tip of the phage [8]. At one end of the virion, there are 5 molecules of each hydrophobic proteins: pVII, a 33 aa residue protein, and pIX, a 32 aa residue protein [19]. The other end contains 5 copies each of the pIII (406 aa residue protein) and the pVI (112 aa residue protein) [19]. These last two proteins are required for the structural stability, completion of assembly and release of the virion [16].

1.1.1.2 M13 Bacteriophage Life Cycle

Upon infection of F^+ *E. coli* strains, M13 induces a state in which the infected bacteria produce and secrete new phage virions into the growth medium (Figure 1.3) [6].

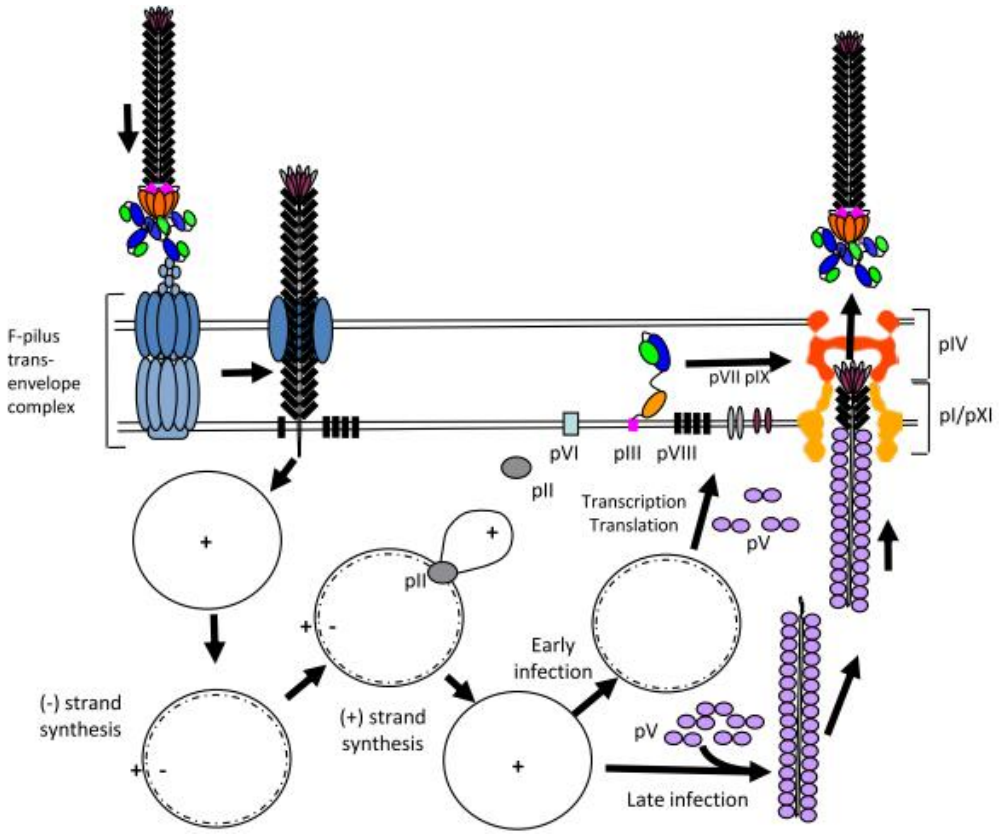


Figure 1.3 - Life Cycle of M13 bacteriophage [16].

Infection is initiated with the adsorption process, occurring by the attachment of the N2 domain of pIII to the tip of the F-pilus [3]. When the phage binds the F-pilus, a depolymerization of the pilus will occur, bringing the virion closer to the surface of the bacterium and allowing the N1 domain of pIII to bind TolA located between the inner and outer membranes of the host cell [3]. Although it is understood that the complex TolQRA present in *E. coli*, containing the proteins TolA, TolR and TolQ, is essential for infection and translocation of the ssDNA into the bacterium, the exact contribution of this complex is still unknown [3 8]. It is only established that this sequence of events, involving pIII binding, contraction of pili and virion passage through the outer membrane, will end up at the injection of the circular ssDNA M13 genome, referred to as infective form (IF), into the host cytoplasm [4 8 21]. Inside the host, the bacterial DNA synthesis machinery will synthetize a DNA strand complementary to the ssDNA to produce circular supercoiled double-stranded DNA (dsDNA), also known as replicative form (RF) [3]. The replication starts when pII cleaves the ori site in the RF (+) strand and binds covalently to its 5' end [3 4]. The exposed 3' end serves as a primer and will be elongated by the DNA polymerase using the (-) strand as a template [3 4]. After the replication completes a full circle, pII nicks and ligates the free ends, resulting in a ssDNA IF and dsDNA RF [21]. In the early stages of infection, the IF is converted in

new RF and the production of RFs, via rolling cycle replication (described above), continues, until pV is expressed in sufficient levels to sequester the IF [4 21]. Late in the infection, the high level of pV inhibits both the RF production and the translation of pII and pX, which leads to the accumulation of the IF [21]. Although the exact mechanism remains unclear, pX plays an essential role in the stable accumulation of IF [3]. Moreover, pV starts to bind to the IF, leading to the change of the circular appearance of the ssDNA to a more rod shaped appearance and initiating the virion assembly [3 8]. The entire IF is covered with pV, except for an exposed hairpin loop, the packing signal, which is required for packing of phage genome [3]. Assembly is initiated by the binding of pVII and pIX to the packing signal, which occurs in the inner membrane of the host [21]. Then, the pV is replaced by pVIII, until the entire phage genome is coated with the major capsid protein and pIII and pVI bind the virion and allow its release from the bacterium [3 21]. This process of phage secretion involves ATP hydrolysis and the complete phages are extruded through the membrane channels created by pII and pIV complex [4].

As mentioned before, M13 assembly is a nonlytic process, therefore newly assembled mature virions will continuously be extruded from the infected host, which continue to grow and divide, even if at a reduced rate (a generation time of around 50% longer than for uninfected bacteria) [17 19]. Moreover, taking into consideration that there is a lack of replication regulation and an ability of the episome to be transferred to daughter cells during cellular division, it is expected that the viral stock reaches a high titer (~10^{13} phages /ml of culture) [8].

1.1.1.3 Phage or Phagemid?

While phage DNA can be used as a cloning vector, sometimes it is advantageous to mix interesting features of phages with that of bacterial plasmids to arrive at chimeric phagemid vectors. Phage display can be performed using phage or phagemid vectors, however since they have different properties they result in different kinds of display [8].

Ligand's genes can be cloned directly into the phage genome that includes the origin of replication and all essential genes required for propagation [8]. As a result, all phage particles will contain only the viral genome and will display the peptide in all coat proteins choosen for the display, which usually results in multivalent display (except when proteolysis degradation results in loss of some of the displayed peptide) [3 22].

In the case of using phagemids, there are three key elements to consider: 1) an antibiotic resistance marker for the selection and propagation of the wanted phages, 2) the gene encoding the displayed ligand, 3) all the M13 genome elements essential for replication and assembly of phages [3]. Phagemids contain the replication origins of both, an *E. coli* plasmid and M13, in conjunction with an antibiotic resistance marker [17]. Moreover, it usually incorporates an amber stop codon between the C-terminus of the cloned gene and the start of the capsid protein gene, allowing for an easy switch between the production of a fusion protein or autonomous soluble protein [17]. A peptide tag is encoded as well, which can be used, at later stage of phage display, for purification methods of the desired ligands [17]. Having these characteristics, the phagemid can produce a large amount of the recombinant displayed ligand, however it is unable to produce phages unless the bacterium is co-infected with a helper phage [22]. The helper phage, such as M13KO7 or VSCM13, carries all the genes necessary for the infection, replication and assembly of phage particles [3]. An antibiotic resistance marker is also encoded and a truncated origin of replication, which ensures a preferential packaging of the phagemid DNA over the helper phage genome [8 23]. Employing a phagemid usually results in monovalent display of the ligand, which is desired when ligands of highest intrinsic affinity are required, without having to worry about avidity effects [8].

Most of the times, phagemids are preferred over phage vectors for library constructions, due to their higher transformation efficiencies that lead to larger and more diverse libraries [3 18]. The variety of restriction enzyme sites present in phagemids facilitates gene manipulation, simplifying the cloning processes of the recombinant peptides [18]. Also, phagemids tend to be genetically more stable after multiple rounds of propagation, due to relative resistance to deletions of extraneous genetic material, when compared with recombinant phages [18 22].

A list of properties that have to be considered when choosing between phage or phagemid vectors to create a phage display library that is suitable for a specific goal is summarized in Table 1.1.

Table 1.1 – Advantages and disadvantages of each vector for GIII phage display [22].

Phage	**Phagemid**	**GIII mutated phage first round, normal subsequent**
Three to five copies of Ab per phage	Only 1-10% of phagemids have one copy of the displayed Ab	Three to five copies of Ab per phage
Difficult to transfect and make DNA	Easy to handle	Easy to handle
Faster to select and easier to use	Slower to select (helper phage needed)	Slower to select (helper phage needed)
Must subclone to make soluble Ab	Soluble Ab made directly	Soluble Ab made directly
Phenotypic and genotypic homogeneity	Phenotypic and genotypic heterogeneity	Phenotypic and genotypic heterogeneity
Genetically less stable (deletions)	More stable genetically	More stable genetically
Greater diversity of Abs selected	Lower diversity of Abs selected	Greater diversity of Abs probably selected
Lower affinity Abs also selected due to avidity effects	Higher affinity Abs selected due to monomeric display	Selection can be directed towards higher affinity Abs
Not suitable for affinity maturation due to avidity effects	Better for affinity maturation	May be suitable for affinity maturation

1.1.2 Phage Display Systems

Since the discovery of phage display, all M13 coat proteins have already been explored as possible display platforms (Figure 1.5) [8]. Depending on the capsid protein, multiple configurations can be achieved on the displayed peptide/protein [8].

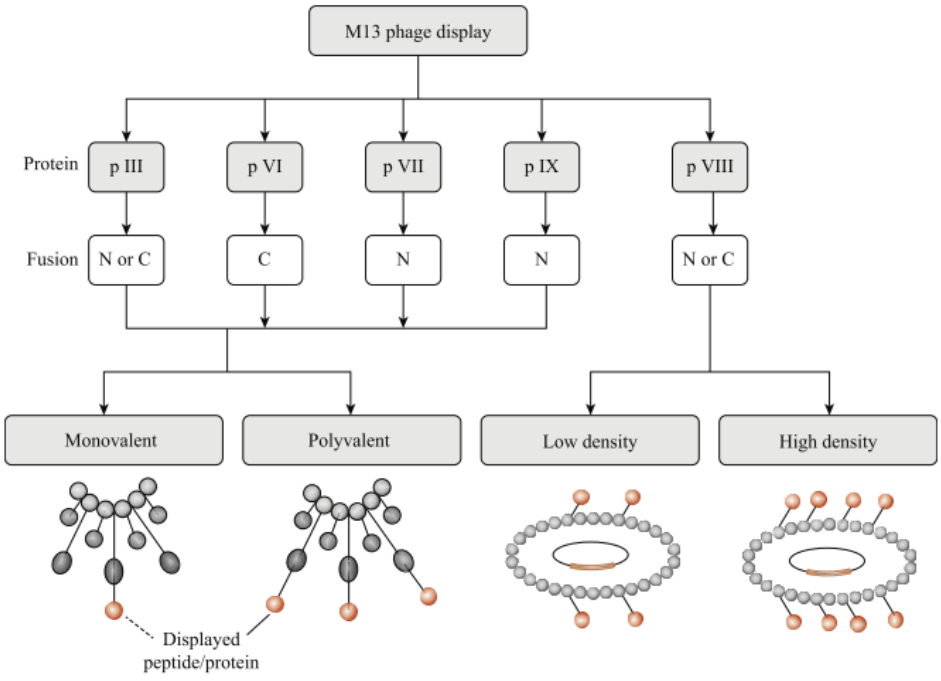


Figure 1.4 - M13 Phage Display Systems [8].

The density of display will depend on which coat protein the ligand is attached [8]. For high-density display, the major coat is used, since it has a high number of copies [8]. However, the size of the ligand will have a major influence on the density level that can be accomplished due to steric hinderance [8]. Usually only peptides with 6-7 residues can guarantee unbiased display in pVIII

systems, so if a peptide is larger, a wild-type pVIII has to be present as well [16]. Furthermore, the density can also be controlled by the availability of wild-type pVIII proteins during virion assembly (more pVIII wild-type, less density) [8]. In the minor coat proteins, valency is also controlled by presence or absence of wild-type coat protein [8]. Moreover, fusions of the encoded recombinant peptides can be done to N-, C- or both termini, nevertheless this will depend on structural and functional constraints [8].

Selection of the system to use will be dependent on the aims that one wants to achieve with the technique. To increase the chances of selecting ligands, usually a high density and polyvalent display is preferred [8]. In cases where avidity has an impact on retrieving specific targets, a polyvalent display is required [17]. However, if highest affinity ligands are required, monovalent displays on the minor coat proteins are preferred [17].

Although all coat proteins can be used as a display platform, most of the times pIII and pVIII are preferred [17]. When comparing both systems, pIII display tolerates larger ligand fusions and has a better performance [17].

1.1.3 Types of Displayed Ligands

Depending on the specific aims of the study undertaken, two types of displayed ligands are widely used – peptides and antibodies (Abs) [24].

Random peptide libraries are generally constructed using $(NNK)_n$ codon degeneracy, where N is an equimolar of all four nucleotides and K is a 1:1 mixture of guanine and thymine [24]. This decreases the number of stop codons that can appear in the randomization, since there is a reduction of the number of stop codons that can be formed from three to one (TAG, the amber stop codon) [24]. These libraries are inserted at the N-terminal of the coat proteins and range between 6 and 43 aa in length, since longer peptides can possibly interfere with the infectious activity or capsid assembly of the virion [25].

Since Abs, such as IgG, are quaternary structures, with a size of 150 kDa, composed of two heavy and two light chains, each with variable and constant domains, it is very challenging for bacteria to express into functional proteins [8 15]. Moreover, their display on the surface of phages, is seriously compromised due to limitations in the bacteria folding machinery [17]. Therefore, Ab libraries created to replace conventional immunization and hybridoma techniques, involve the design of combinatorial libraries of heavy-chain variable domains (VH) and light-chain variable domains (VL) from B lymphocytes, which are ligated as a pair in a phagemid [8 25]. Apparently, these displayed, smaller, monovalent Ab fragments retain the affinity properties of intact Abs [15].

1.1.3.1 Antibody Fragments and Nanobodies

Several Ab formats (Figure 1.5) were already used for the construction of libraries, with each one having advantages and limitations (Table 1.2) [26].

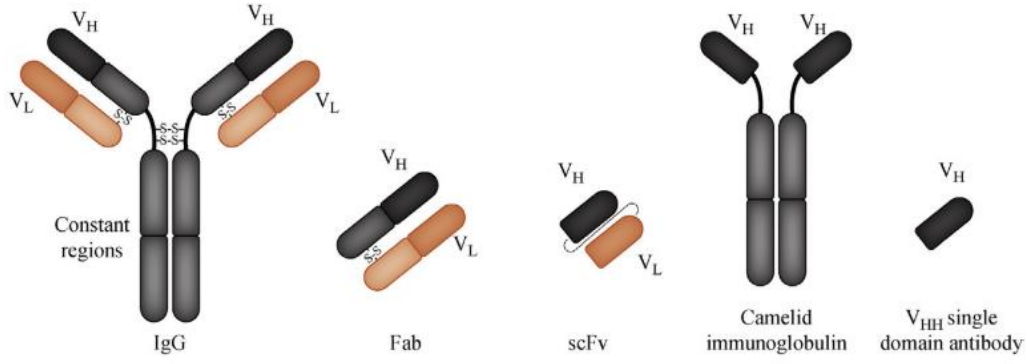


Figure 1.5 - Types of antibody fragments that can be displayed on the surface of bacteriophages [8].

The first two types of Ab fragments successfully displayed on the surface of bacteriophages were antigen-binding fragments (Fab) and single-chain variable fragments (scFv) [17].

Fabs are composed of the full length of variable regions of heavy and light chains (VH and VL), as well as a constant region for each chain (CH1 and CL) linked by a disulfide bond [8 27]. They have a tendency to form homodimers which interfere in finding the fragment with highest affinity [27]. However, if the final goal is to find Abs that bind to a specific target, these fragments are a good solution [24]. Fabs possess better stability, pharmacokinetic and pharmacodynamic properties when compared to scFvs [28]. The main disadvantage of using Fab libraries is their lower expression level in *E.coli* since there is a chance of producing toxic material to the cell (due to the large size of the fragments), in comparison with scFv [24].

An scFv is composed of the variable regions VH and VL, connected by a flexible serine-glycine linker to mimic the binding cavity upon folding [24 27]. Additionally, this linker serves the purpose of increasing the folding, flexibility and stability, as well as the solubility of the fragment [24 27]. These fragments are smaller than Fabs, what make them advantageous in imaging, since they can penetrate tissues much more rapidly and efficiently [26]. Moreover, due to their small size, scFv are easier to clone and can bind to cryptic or sterically restricted epitopes [17 26]. Yet, their main disadvantage is their propensity to aggregate, which can interfere with selection and characterization [17]. Furthermore, scFv's tend to become unstable when stored over prolonged periods of time [17].

These two Ab fragments might also be engineered and converted into other formats with different characteristics that can be used, as well, for libraries (Table 1.2) [26]. Even though scFv and Fab fragments are significantly less complex than conventional Abs, it is still challenging to produce them, due to their requirement of proper domain association [8].

Table 1.2 – Characteristics of different antibody formats [26].

Antibody format	**Size (kDa)**	**Paratope (valency)**	**Fc mediated functions**
IgG	150	2	Yes
scFv	28	1	No
Fab	50	1	No
F(ab)2	110	2	No
scFv-Fc	110	2	Yes
Diabody	55	2	No
Single domain antibody (VHH)	15	1	No

A major breakthrough in the generation of Ab phage display libraries was the discovery of naturally occurring functional heavy chain only antibodies (HCAbs) in sera of Camelids by Hamers and colleagues [8 29]. HCAbs lack light chains, as well as the first constant domain (CH1), so the antigen association occurs solely with the heavy chain variable region (VHH) [30]. The designation VHH was introduced to emphasize the differences between the heavy chain variable regions in camelids and conventional Abs [31]. Even though both contain four conserved framework regions and three CDR regions, their general consensus is somewhat different (Figure 1.6) [32]. Furthermore, key hydrophobic amino acids of framework-2 involved in binding the VL region in conventional Abs are substituted by hydrophilic or smaller residues conferring VHH solubility and explaining the absence of VL association [32]. In comparison with VH, VHH have also an extended CDR1 and longer CDR3, consequently having the ability to adopt novel paratope conformations for antigen recognition [32].

VHH, also known as nanobodies (Nbs), are characterized by their small size (15 kDa), high thermal stability and solubility, resistance to denaturation, ease of manipulation, high affinity and specificity (similar to conventional Abs), higher tissue penetration and low immunogenicity [31 33]. Moreover, they are expressed to a high level in bacterial organisms, routinely with yields of several milligrams per liter of culture [30].

The generation of Nb libraries involves the immunization of camelids with a cocktail of antigens (Ags), the isolation of mRNA from peripheral blood lymphocytes and synthesis of cDNA by

reverse transcription [33]. Nbs, with an encoded gene of around 360bp, can, then, be amplified by polymerase chain reaction (PCR) and ligated into a cloning vector [33].

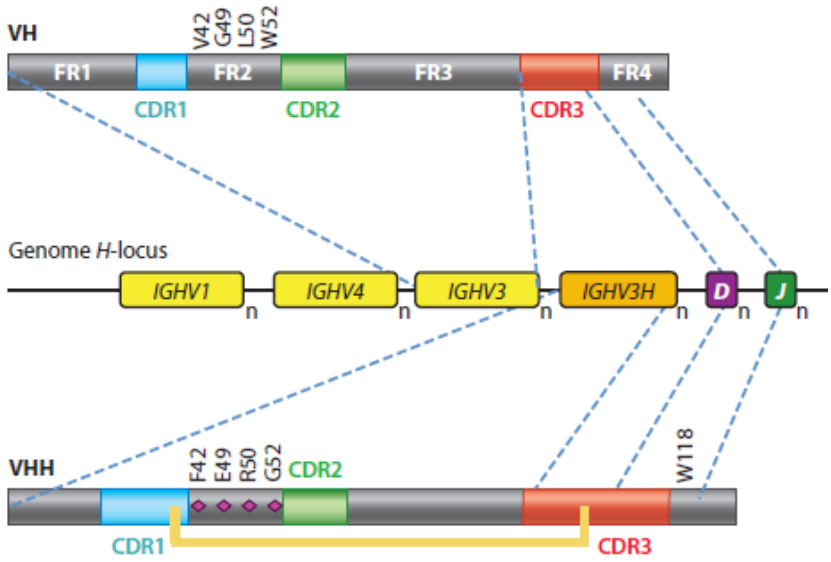


Figure 1.6 – Schematic representation of the sequence organization of the VH versus VHH with frameworks and CDRs. The crucial amino acid substitutions in framework-2 are described. Adapted from [30]

1.1.4 Selection Methods

The selection of phage displayed libraries aims to enrich clones that recognize a target or Ag of interest [34]. Briefly, it involves phage binding to the target, washes to remove nonspecific phages and elution to retrieve specific binding phages [17]. Ideally only one round of selection should be enough, however due to limitations of enrichment and nonspecific phage binding 3 to 5 rounds of selection are required to obtain the best clones from a library [34].

There are some important considerations to keep in mind while planning a selection. To reduce nonspecific phage binding, it is important to use a suitable protein-binding site blocking agent, as well as Tween-20 during the washing step [22]. Moreover, the first selection round should not be too stringent to ensure that all binding phages are recovered for subsequent amplification, including those present in very few copies [22]. Selections on impure Ags are more challenging, since there is a chance of enriching phages specific for non-relevant Ags [34].

Various methods can be envisaged, whereby different strategies can be designed to drive selection into the required direction by controlling specific criteria (Figure 1.7) [17]. These strategies can be separated in two groups: those which attempt to isolate binders against a known Ag and those which use phage binders as a research tool to target unknown Ags [22]. Although the outcome of a selection process depends on the quality of the constructed library, the biopanning strategy influences the ability to select clones not only with the required affinity and specificity, but also physical properties and functionality [17]. In Figure 1.7, the most popular strategies are listed on the top (panels A-E), including biopanning on immobilized Ag coated on solid supports or columns, biopanning in solution with biotinylated Ags and selection on cells [35].

Biopanning on immobilized Ag coated on plastic surfaces by (passive) adsorption is the preferred selection procedure [25]. Biopanning involves following steps [22] [25] (see Figure 1.1, 2. Library screening):

1) Ag immobilization: The Ag can be immobilized by passive adsorption to a modified 96-well polystyrene microtiter plates. The unbound Ag is washed away, and the residual protein binding sites in the well is saturated with a blocking agent, such as BSA or (semi-)skimmed milk powder;

2) Phage binding: The phage display library is added to an Ag coated well. For a better chance of isolating the best binders, it is important to start the first round with a large and highly variable library;

3) Removing unbound phage: In the first round the washes are shorter and less stringent, to recover a high yield of the fittest phage clones over the background. The stringency of selection is increased in subsequent rounds by more extensive and longer washes to isolate phage with higher affinity;

4) Phage elution: The specific elution of the target bound phage can be performed in solution either with free target or a competing ligand. Taking advantage of phage stability, extreme pH, denaturants, ionic strength, limited proteolysis or sonication can be used for non-specific elution of the antigen bound phage.

The eluted phage is amplified and the biopanning process is repeated [25]. During this process, the enrichment achieved per round is monitored to evaluate the efficiency and to understand if a redesign of the strategy might be needed for selection of the better binders [17].

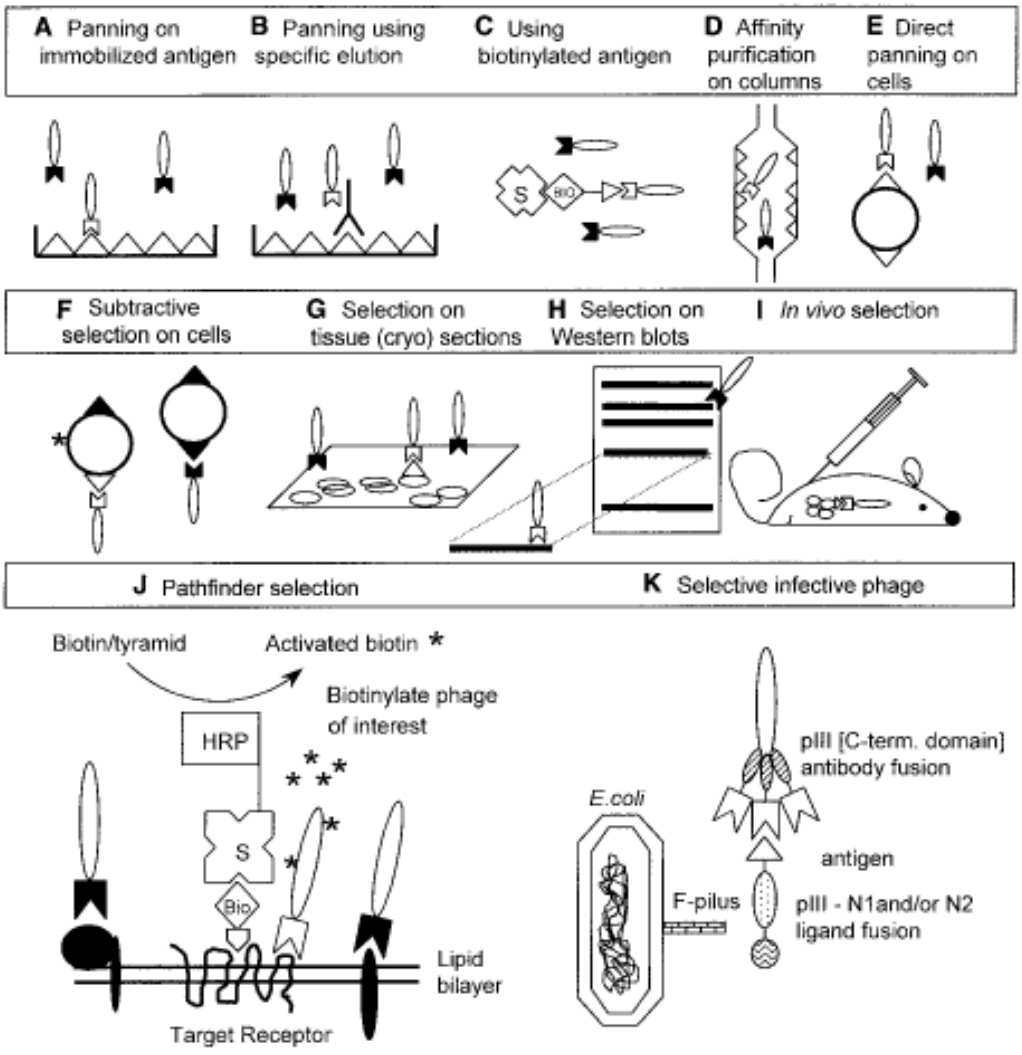


Figure 1.7 - Selection strategies for obtaining specific phage ligands. A – Affinity selection from libraries by biopanning on Ag absorbed onto a solid support, such as wells in microtiter plates. B – After washing, the specific binders can be eluted with acidic or basic solutions, as well as with Ab or excess of Ag. C – to avoid conformational changes during coating, selections on biotinylated Ag captured on coated streptavidin are preferred. D – the Ag can also be immobilized on Sepharose and loaded onto columns for affinity selection. E – Panning directly on cell monolayers or cell suspensions can also be used as a selection strategy. F- In this type of selection the cells of interest are fluorescently labeled and separated from the others by cell sorting. G – Tissue or organ specific binders can be obtained by

selection on tissue slides. H – Non-purifiable or unknown Ags may be separated on SDS-PAGE and blotted onto membranes for selection. I – Selection in vivo is also possible. J – Pathfinder selection. K – Infection-mediated selection [35].

A particular problem of direct adsorption of Ag onto solid surfaces is that the epitope may be partially denatured or hidden, when the adsorption provokes conformational changes in the Ag [17] [22]. This may lead to selection of binders that don't recognize the native Ag, particularly for small-size Ags [17] [22]. Even though this is not a common event, it can be avoided by presenting the Ag in solution, via biotinylated Ag, and by any other indirect coating [17] [22].

1.1.5 Screening Methods

Once the selection procedure is complete, a fast and robust screening assay of individual clones is required, such as Enzyme Linked Immunosorbent Assay (ELISA), Fluorescence-Activated Cell Sorting (FACS), nucleotide sequencing and immunocyto- or histochemistry [22].

For a first screen, ELISA-based assays in combination with PCR and restriction fragment polymorphism of the Ab-encoding DNA are typically performed to narrow the potential candidate clones to the best and different candidates [22] [35]. ELISA is a biochemical assay that uses Abs and an enzyme-mediated color change to detect the presence of either Ag or Ab in a given sample [36]. In this case, usually an anti-M13 phage Ab and either Alkaline Phosphatase (AP) or Horseradish Peroxidase (HRP) are used for the detection [37]. The Ab expression level in *E. coli* is dependent on its primary sequence and can be extremely variable, making it impossible to correlate the affinity parameters with the signal intensity obtained from ELISA [22]. A positive binding event can either be due to a high affinity or a high expression level (or both) [38]. Thus, it is necessary to develop a screening assay that differentiates between clone variants that differ in affinity or in the kinetics of binding [35]. The determination of dissociation rates to differentiate among the clones is performed using sophisticated instruments such as BIAcore or Octet [17].

Additional screening methods might be carried out for a better characterization of the binders regarding their specificity, functionality and stability [17].

After screening assays, it is important to sequence all the positive binders and clone them into vectors suitable for mass production [17] [22].

1.2 Calmodulin

Calmodulin (CaM) is a small (17 kDa, comprised by 148 aa residues), well-conserved protein with a key role in intracellular signal transduction [39] [40]. CaM participates in signaling pathways by interacting with a variety of proteins, including kinases, phosphatases, ion channels and cytoskeleton proteins [40] [41]. It plays important roles in muscle contraction, cell proliferation, inflammation, learning and memory, exo- and endocytoses, immune response and growth [40] [41].

CaM is a dumbbell-shape protein consisting of two lobes, N-lobe and C-lobe, connected by a highly flexible helical linker that allows a variety of conformations when bound to different targets [40] [42]. Each lobe contains a pair of EF-hand motifs which allow the binding of four Ca^{2+} ions, followed by a change of conformation from a hydrophilic structure to a more hydrophobic state that allows CaM to bind amphipathic α-helices in target proteins with very high affinity [39] [40] [41] [42]. Most of the times, the binding is reversible by the addition of calcium chelators, making this system an interesting candidate for biotechnological applications [39].

1.2.1 Calmodulin Binding Peptide

The CaM binding proteins are a large group of proteins that don't show amino acid sequence homology, however can share unique structural features, such as an α-helical propensity, specific distribution of hydrophobic residues (two hydrophobic anchor residues spaced by a certain number of aa) and net positive charge (Figure 1.8) [43].

Among these CaM binding proteins is a peptide residue of 19 aa (RWKKAFIAVSAANRFKKIS) derived from wild type skeletal muscle myosin light chain kinase [39] [44]. This CaM binding peptide

(CBP) has an ultra-high affinity (K_D 2 pM) promoted by a mutation on N5A [44]. This affinity enhancement guarantees that the CBP can resist vigorous washing steps and can be released from the immobilization substrate by addition of a calcium chelator, such as EDTA [44]. Moreover, it has been shown that when fused into a target, it does not affect its expression [44].

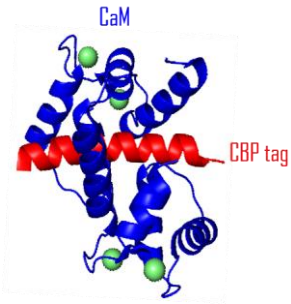


Figure 1.8 - Schematic representation of the interaction between calmodulin and the calmodulin binding peptide. Provided by Prof. Dr. Serge Muyldermans.

1.3 Description of the New Phage Display Selection Approach

Based on the binding properties between CaM and CBP [44], as well as studies with biotinylated Nbs that aim to normalize the captured amount [38], a new approach for phage display was developed (Figure 1.9 and Figure 1.10).

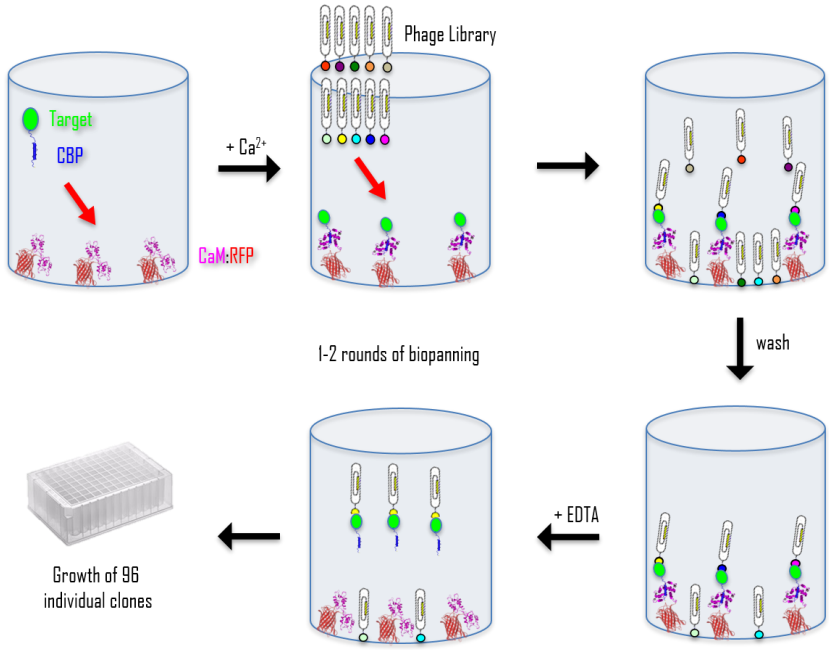


Figure 1.9 - Schematic representation of new biopanning approach.

First a new biopanning method will be developed (Figure 1.9). An Ag-CBP fusion from crude extracts will be captured by CaM indirectly adsorbed into microtiter plate using a buffer containing Ca^{2+} and impurities will be washed away. Then, we will add the phage particles rescued from the pMECS-CBP library carrying Nbs from an immunized camelid. Since all CaM are already loaded with Ag-CBP, it is expected that the phages will not be captured on CaM, but instead via their affinity for the captured Ag. After a short incubation and a few washes, a buffer with EDTA will be added, to release only those phages that were binding to the Ag via their Nb. Consequently, it is expected that the a-specific-bounded phage particles will not be disrupted, remaining attached into the wells and reducing the amount of background generated. For this reason, it is thought that one single round or maximum two consecutive rounds of panning might be sufficient, instead of three to five rounds using classical phage display and pH shock elution. A fraction of the eluted

pMECS-CBP virions will be used to infect WK6 cells, which will be grown individually and induce the expression of Nb-CBP in the periplasm.

After obtaining individual Nb-CBP fusion proteins, a screening modified method of PE-ELISA will be developed (Figure 1.10). The Nb-CBP fusion proteins from a crude periplasmic extract will be captured on a limited and fixed amount of CaM, immobilized on wells of microtiter plates, in presence of Ca^{2+} ions. After washing away the impurities, the Nb-CBP (a fixed amount corresponding to the moles of CaM coated in the wells) will be rescued by a short incubation with a buffer containing EDTA. Then, this Nb-CBP will be added to a well coated with Ag, followed by the addition of alkaline phosphatase fused with CaM in the presence of a calcium-containing buffer. In the presence of AP substrate, the ELISA signal will be monitored. Since a fixed amount of binder is used for ELISA, the discrepancies between expression levels of the different clones are no longer taken into account, so it is expected that the signal from ELISA will directly correlate with the affinity parameters. To verify if this correlation exists, 6 Nbs with well-established and variable association and dissociation kinetic rate constants are going to be used as proof of concept.

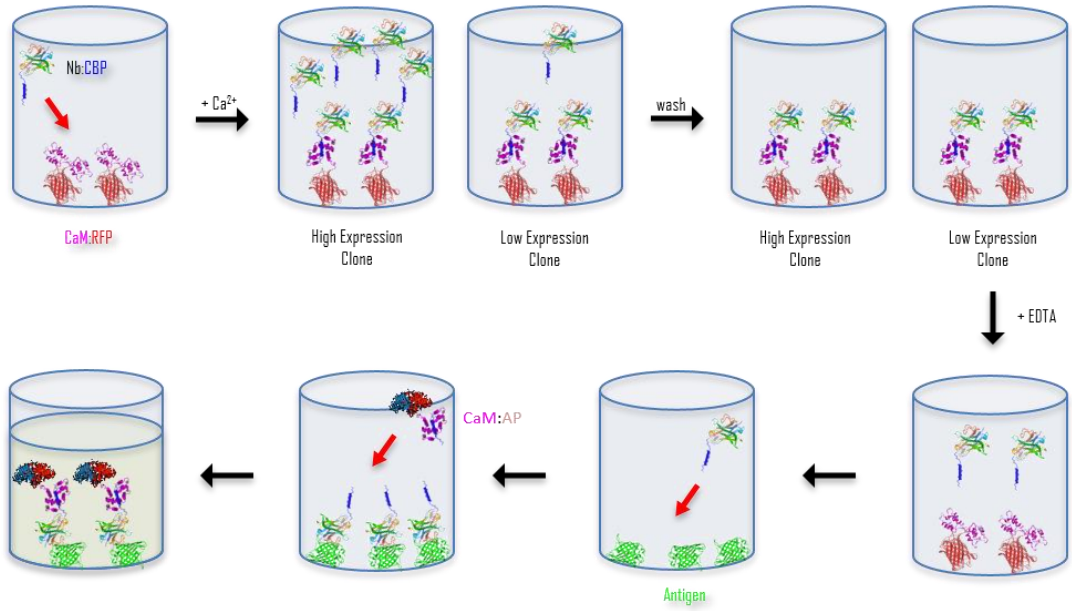


Figure 1.10 - Schematic representation of new PE-ELISA approach.

1.4 Aims of the Project

Although phage display-based enrichments became a standard procedure, they still suffer from some drawbacks. To overcome these drawbacks this thesis was initiated with the purpose of changing a phage display vector so that one round of selection will be enough, while the affinity of all antigen-positive Nbs can be compared immediately after ELISA. More specifically, the main goals of the study are:

- Create a new vector containing the CBP tag that can be used for phage display
- Immobilize CaM on wells and test the effectiveness and efficiency of the interaction between the CBP and the CaM with different buffers, concentrations and incubation times
- Develop a new phage display approach to normalize the amount of expressed Nb before ELISA
- Find the correlation between the ELISA signal and the antigen-binding parameters.

2 Materials and Methods

2.1 Materials

2.1.1 Buffers, Reagents and Culture Media

Table 2.1 - List of all the buffers, reagents and media used throughout the experiments.

Buffer/Media/ Reagent	Composition	Source	City, Country
1% Agarose Gel	4 g Agarose 400 mL TBE buffer Boil until agarose dissolves	Lonza	Basel, Switzerland
10x TBE Buffer (1 L)	108 g Trizma®Base 55 g H_3BO_3 93 g EDTA Adjust final volume to 1 L with milli-Q H_2O	Sigma Aldrich Sigma Aldrich Duchefa Biochemie	St. Louis, USA St. Louis, USA Haarlem, Netherlands
20% D-Glucose (100 mL)	20 g Glucose Adjust final volume to 100 mL with milli-Q H_2O Autoclave	Duchefa Biochemie	Haarlem, Netherlands
20% Ethanol SEC (1 L)	200 mL Ethanol Adjust final volume to 1 L with milli-Q H_2O Filter and degas	Fisher Scientific	Loughborough, UK
20x MES Buffer (1 L)	195.2 g MES Hydrate 121.2 g Trizma®Base 20.0 g SDS 7.44 g EDTA disodium dihydrate Adjust final volume to 1 L with milli-Q H_2O	Sigma Aldrich Sigma Aldrich Duchefa Biochemie Duchefa Biochemie	St. Louis, USA St. Louis, USA Haarlem, Netherlands Haarlem, Netherlands
2x TY Medium (1 L)	16 g Tryptone 10 g Yeast Extract 5 g NaCl Adjust final volume to 1 L with milli-Q H_2O Autoclave	Duchefa Biochemie Duchefa Biochemie Fischer Scientific	Haarlem, Netherlands Haarlem, Netherlands Loughborough, UK
Ampicillin (100 mg/mL)	5 g Ampicillin 50 mL 70% Ethanol Filter with 0.22 µm filter	Sigma Aldrich Fischer Scientific Orange Scientific	St. Louis, USA Loughborough, UK Braine-l'Alleud, Belgium
Chloramphenicol (25 mg/mL)	1.25 g Chloramphenicol 50 mL 100% Ethanol Filter with 0.22 µm filter	Sigma Aldrich Fischer Scientific Orange Scientific	St. Louis, USA Loughborough, UK Braine-l'Alleud, Belgium
Coating Buffer pH 8.2 (1 L)	8.5 mg Na_2CO_3 Adjust pH to 8.2 Adjust final volume to 1 L with milli-Q H_2O	Merck	Darmstadt, Germany
Coomassie Brilliant Blue (1 L)	0.125 g Coomassie® Brilliant Blue R250 50% Methanol 10% Acetic Acid Adjust final volume to 1 L with milli-Q H_2O	Fluka Acros Organics Merk	Buchs, Switzerland Geel, Belgium Darmstadt, Germany
Destaining Solution (1 L)	10% Acetic Acid 40% Methanol Adjust final volume to 1 L with milli-Q H_2O	Merck Acros Organics	Darmstadt, Germany Geel, Belgium

Buffer/Media/ Reagent	Composition	Source	City, Country
Ethidium Bromide (10 mg/mL)	1 g Ethidium Bromide 100 mL of milli-Q H_2O	Merck	Darmstadt, Germany
HisPure™ Ni-NTA Resin	-	Pierce (Thermo Scientific)	Rockford, USA
Imidazole 0.5M in PBS pH 7.5 (100 mL)	3.4 g Imidazole Solubilize in 90 mL PBS Adjust pH to 7.5 Adjust final volume to 100 mL with PBS Store in the dark	Sigma Aldrich	St. Louis, USA
IPTG 1 M (50 mL)	11.92 g IPTG 50 mL milli-Q H_2O Filter with 0.22 µm filter	Duchefa Biochemie Orange Scientific	Haarlem, Netherlands Braine-l'Alleud, Belgium
Kanamycin (70 mg/mL)	3.5 g Kanamycin 50 mL milli-Q H_2O Filter with 0.22 µm filter	Duchefa Biochemie Orange Scientific	Haarlem, Netherlands Braine-l'Alleud, Belgium
LB Agar Amp/Chl/Glu (1 L)	25 g LB Broth high salt 15 g Micro Agar Adjust final volume to 900 mL with milli-Q H_2O Autoclave 1 mL Ampicillin (100 mg/mL) 1 mL Chloramphenicol (25 mg/mL) 100 mL 20% Glucose	Duchefa Biochemie Duchefa Biochemie	Haarlem, Netherlands Haarlem, Netherlands
LB Agar Amp/Glu (1 L)	25 g LB Broth high salt 15 g micro agar Adjust final volume to 900 mL with milli-Q H_2O Autoclave 1 mL Ampicillin 100 mg/mL 100 mL 20% Glucose	Duchefa Biochemie Duchefa Biochemie	Haarlem, Netherlands Haarlem, Netherlands
LB Medium (1 L)	10 g Tryptone 5 g Yeast Extract 10 g NaCl Adjust final volume to 1 L with milli-Q H_2O Autoclave	Duchefa Biochemie Duchefa Biochemie Fischer Scientific	Haarlem, Netherlands Haarlem, Netherlands Loughborough, UK
$MgCl_2$ (2 M)	20.33 g $MgCl_2$ Adjust final volume to 50 mL with milli-Q H_2O Autoclave	Merck	Darmstadt, Germany
NaAc (3 M)	3.69 g NaAc Adjust pH to 5.2 Adjust final volume to 15 mL with milli-Q H_2O	Sigma Aldrich	St. Louis, USA
NaOH-NaCl SEC (1 M)	40 g NaOH 58.44 g NaCl Solubilize in 900 mL milli-Q H_2O 5 mL 10% NaN_3 Adjust final volume to 1 L with milli-Q H_2O Filter and degas	Fischer Scientific Fischer Scientific Duchefa Biochemie	Loughborough, UK Loughborough, UK Haarlem, Netherlands
NuPage® LDS Sample Buffer (4x)	-	Life Technologies	California, USA

Buffer/Media/ Reagent	Composition	Source	City, Country
NuPage® Sample RA (10x)	-	Life Technologies	California, USA
Orange G DNA Loading Dye (6x)	50 mL 100% Glycerol 0.5% orange G powder Adjust final volume to100 mL with milli-Q H_2O Store in the dark	Duchefa Biochemie Sigma Aldrich	Haarlem, Netherlands St. Louis, USA
PBS pH 7.4 (10x)	400 g NaCl 10 g KCl 90 g Na_2HPO_4 12 g NaH_2PO_4 Adjust final volume to 5 L with milli-Q H_2O Autoclave	Fischer Scientific Merck Merck Merck	Loughborough, UK Darmstadt, Germany Darmstadt, Germany Darmstadt, Germany
PBS SEC (1 L)	1 L 1x PBS Filter and degas		
PBS-Milk 2%	1 g Milk powder Adjust final volume to 50 mL with PBS	Nestle	Supermarket
PBS-Tween 0.05%	0.05% Tween-20 Adjust final volume with PBS	Sigma Aldrich	St. Louis; USA
PEG-NaCl (1 L)	20% PEG w/v PEG 6000 145.28 g NaCl Adjust final volume to 1 L with milli-Q H_2O	Duchefa Biochemie Fischer Scientific	Haarlem, Netherlands Loughborough, UK
TB Medium (1 L)	2.3 g KH_2PO_4 16.4 g $K_2HPO_4.3H_2O$ 12 g Tryptone 24 g Yeast Extract 4 mL 100% Glycerol Adjust final volume to 1 L with milli-Q H_2O Autoclave	Merck Merck Duchefa Biochemie Duchefa Biochemie Duchefa Biochemie	Darmstadt, Germany Darmstadt, Germany Haarlem, Netherlands Haarlem, Netherlands Haarlem, Netherlands
TEA pH 10.0	70 µL TEA Adjust final volume to 5 mL with milli-Q H_2O	Sigma Aldrich	St. Louis, USA
TES pH 8.0 (1 L)	24.23 g Trizma®Base 0.15 g EDTA 171.15 g Sucrose Adjust pH to 8 Adjust final volume to 1 L with milli-Q H_2O	Sigma Aldrich Merck Duchefa Biochemie	St. Louis, USA Darmstadt, Germany Haarlem, Netherlands
TES/4 pH 8.0 (1 L)	250 mL TES Adjust final volume to 1 L with milli-Q H_2O		
TPA Buffer	14.63 g NaCl 1.4 g Trizma®Base Adjust pH to 7.5 Adjust final volume to 500 mL with milli-Q H_2O	Fischer Scientific Sigma Aldrich	Loughborough, UK St. Louis, USA
Transfer Buffer	6 g Trizma®Base 28.8 g Glycine 400 mL Methanol Adjust final volume to 2 L with milli-Q H_2O	Sigma Aldrich Sigma Aldrich Acros Organics	St. Louis, USA St. Louis, USA Geel, Belgium

Buffer/Media/ Reagent	Composition	Source	City, Country
Tris-HCl pH 8.0 (1 M)	121.14 g Trizma®Base Solubilize in 500 mL of milli-Q H_2O Adjust pH to 8.0 with 37% HCl Adjust final volume to 1 L with milli-Q H_2O	Sigma Aldrich Merck	St. Louis, USA Darmstadt, Germany
Tris-HCl pH 8.0 SEC (200 mM)	200 mL 1 M Tris-HCl pH 8.0 5 mL 10% NaN_3 Adjust final volume to 1 L with milli-Q H_2O Filter and degas	Duchefa Biochemie	Haarlem, Netherlands
WB Development Solution	18 mg 4-chloro-1-napthol 6 mL Methanol 30 mL PBS 18 µL H_2O_2	Sigma Aldrich Acros Organics Merck	St. Louis, USA Geel, Belgium Darmstadt, Germany

2.1.2 Antibodies, Nanobodies and Antigens

Table 2.2 – List of antibodies, nanobodies and antigens used throughout the experiments.

Antibodies/Nanobodies/Antigens	Source	City, Country
Goat anti-mouse HRP	Sigma Aldrich	St. Louis, USA
Mouse anti-HIS tag	Serotec	Munich, Germany
Nb SH30	In house	Brussels, Belgium
Nb SH61	In house	Brussels, Belgium
Nb SH67	In house	Brussels, Belgium
Nb SH68	In house	Brussels, Belgium
Nb SH69	In house	Brussels, Belgium
Nb Sm75	In house	Brussels, Belgium
SIRPα Human	In house	Brussels, Belgium
SIRPα Mouse	In house	Brussels, Belgium

2.1.3 Plasmids

Table 2.3 – List of plasmids with their main features specified used throughout the experiments.

Plasmid	Main Features	Source	City, Country	See Figure
pMECS	• Plac Promotor • PelB Leader Signal Sequence • Fd GIII • HA Tag • HIS Tag • Ampicillin Resistance	In house	Brussels, Belgium	Figure 2.1
pMECS-GG	• Plac Promotor • PelB Leader Signal Sequence • Fd GIII • HA Tag • HIS Tag • Ampicillin Resistance • Chloramphenicol Resistance • *ccdB* Gene	In house	Brussels, Belgium	Figure 2.2

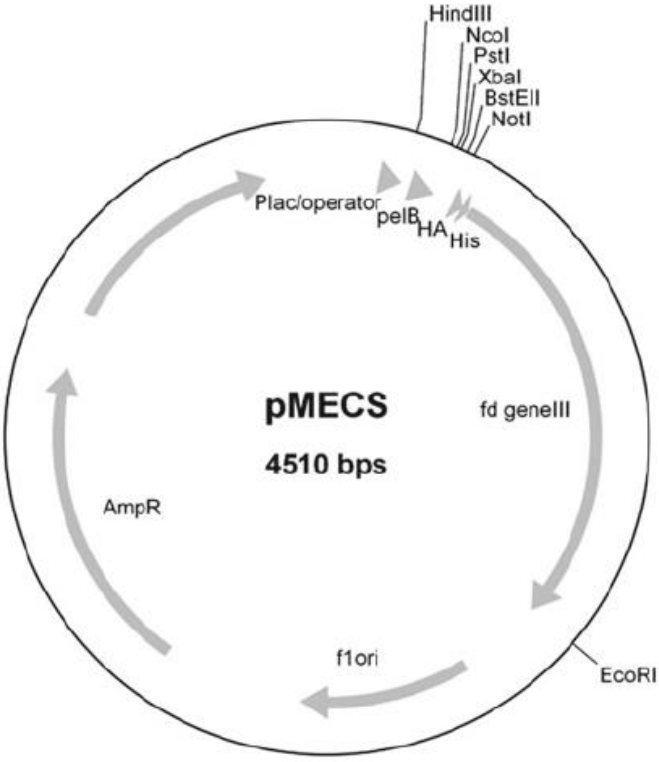


Figure 2.1 – Schematic representation of pMECS vector [45].

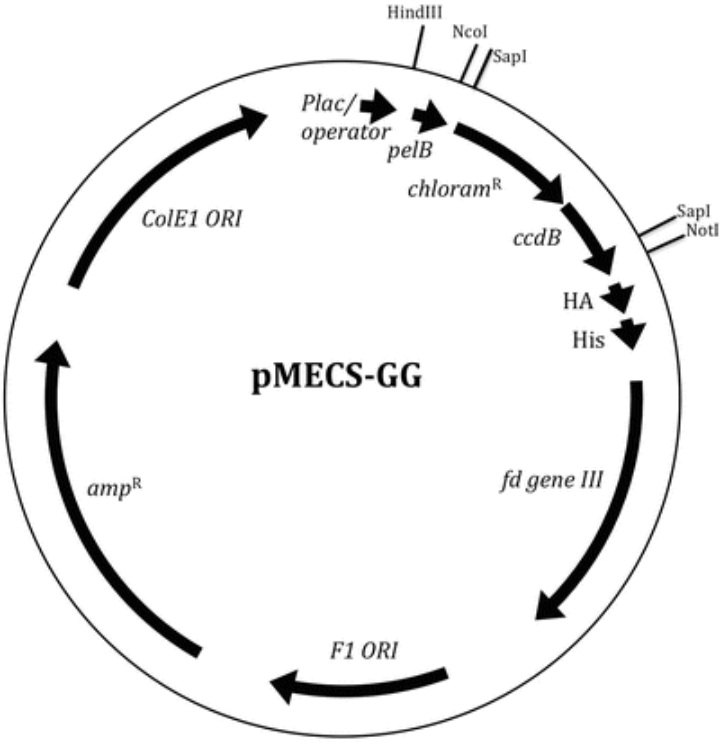


Figure 2.2 - Schematic representation of pMECS-GG vector [46].

2.1.4 DNA Manipulation Reagents and Sequences

Table 2.4 – List of all the DNA manipulation reagents used throughout the experiments.

Reagent	Composition	Source	City, Country
10x O Buffer	-	Thermo Scientific	Vilnius, Lithuania
10x T4 DNA Ligase Buffer	-	Thermo Scientific	Vilnius, Lithuania
5x Colorless GoTaq® Reaction Buffer	-	Promega	Madison, USA
DNase (50 µg/mL)	-	Sigma Aldrich	St. Louis, USA
dNTPs (10 mM)	dATP, 100 mM dCTP, 100 mM dGTP, 100 mM dCTP, 100 mM All diluted 1:10 in dH_2O	Thermo Scientific	Rockford, USA
EcoRI (10 U/µL)	Restriction Site: 5'…G^AATTC…3' 3'…CTTAA^G…5'	Thermo Scientific	Rockford, USA
EXO-I (20 U/µL)	-	Thermo Scientific	Rockford, USA
FastAP Thermosensitive Alkaline Phosphatase (1 U/µL)	-	Thermo Scientific	Rockford, USA
GoTaq® DNA Polymerase (5 U/µL)	-	Promega	Madison, USA
NcoI, 10 U/µL	Restriction Site: 5'…C^CATGG…3' 3'…GGTAC^C…5'	Thermo Scientific	Rockford, USA
NotI, 10 U/µL	Restriction Site: 5'…GC^GGCCGC…3' 3'…CGCCGG^CG…5'	Thermo Scientific	Rockford, USA
Primer CBP-insert	5'-GCTCTTCCAGCGGCCGCAAGATGGAAAAAGGCCTTTATTGCGGTGAGCGCGGCGAACCGGTTTAAAAAAATTAGCGGCAGCGGTTCCCACCACCATCACCATCACTAG-3'	Sigma Aldrich	St. Louis, USA
Primer GIII	5'-CCACAGACAGCCCTCATAG-3'	Sigma Aldrich	St. Louis, USA
Primer M13 FWD (FP)	5'-CGCCAGGGTTTTCCCAGTCACGAC-3'	Sigma Aldrich	St. Louis, USA
Primer MP57	5'-TTATGCTTCCGGCTCGTATG-3'	Sigma Aldrich	St. Louis, USA
T4 DNA Ligase (5 U/µL)	-	Thermo Scientific	Rockford, USA

2.1.5 Kits

Table 2.5 - List of kits used throughout of the experiments.

Kit	Source	City, Country
GenElute™ PCR-Clean-Up	Sigma Aldrich	St. Louis, USA
GenElute™ Plasmid Mini-Prep	Sigma Aldrich	St. Louis, USA
QIAquick Gel Extraction Kit	QIAGEN	Hilden, Germany

2.1.6 Phages

Table 2.6 - List of phages used throughout the experiments.

Phage	Main Features	Source	City, Country
M13KO7	• Lacks the infectivity domains N1 and N2 of pIII • Kanamycin resistance	Invitrogen	California, USA

2.1.7 Bacterial Strains

Table 2.7 – List of bacterial strains used throughout the experiments.

Bacterial Strain	Genotype	Source	City, Country
TG1	*K12, Δ(lac-pro), supE thil, hsD5/F'traD36, lacI^q, lacZΔM15, proA$^+$B$^+$*	In House	Brussels, Belgium
WK6	*K12, Δ(lac-proAB), galE, StrA/F', lacI^q, lacZΔM15, proA$^+$B$^+$*	In House	Brussels, Belgium

2.1.8 Molecular Weight Markers

The Smartladder (Eurogentec, Seraing, Belgium) ranging from 200 bp to 10 kbp was used for molecular weight (MW) estimation of nucleic acids during 1% agarose gel electrophoresis (Figure 2.3A). The PageRuler™ Prestained Protein Ladder (Thermo Scientific, Rockford, USA) ranging from 10 kDa to 180 kDa was used in SDS-PAGE analysis (Figure 2.3B).

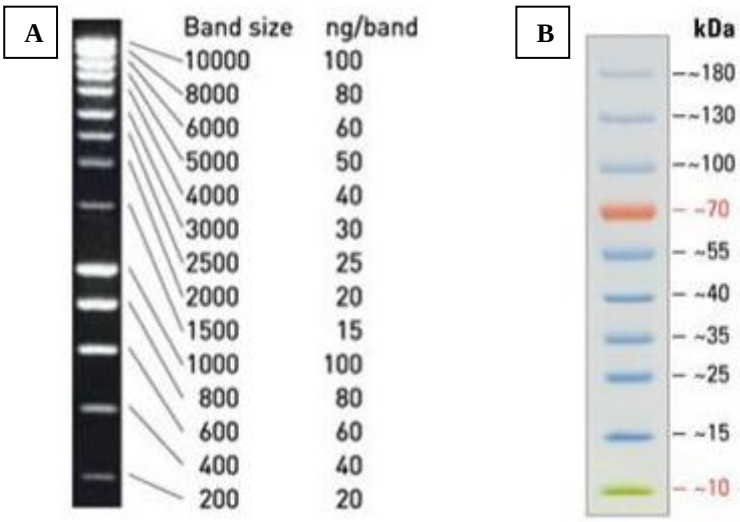


Figure 2.3 - Molecular Weight Markers used throughout the experiments. A – Smartladder (200bp–10kbp) and B – PageRuler™ Prestained Protein Ladder (10-180kDa).

2.2 Methods

2.2.1 Cloning Strategies

2.2.1.1 Amplification of pMECS-GG Using CBP-Insert and FP Primers

The pMECS-GG empty plasmid was amplified using a mutagenic primer (CBP-Insert) with the purpose of adding the CBP tag to the amplified fragment. The primer was designed so that it contained the CBP tag sequence and an overlapping region in the vector (HA tag). The amplification occurred through a polymerase chain reaction (PCR) using the PCR mix (50 µL per reaction) from Table 2.8. Eight reactions were made to ensure that enough material for the subsequent steps was produced.

Table 2.8 – PCR mix used for one reaction for the PCR of pMECS-GG using CBP-Insert and FP primers.

Reagent	Volume (µL)
Sterile H_2O	35.5
5x Colorless Go Taq® Reaction Buffer	10
dNTPs (10mM)	1
CBP-Insert primer (20µM)	1
RP primer (20µM)	1
Go Taq® G2 DNA Polymerase (5U/µL)	0.5
Template	1

The PCR strips were placed in the Westburg T3 thermo cycler from Biometra® and a PCR program started. Since a large fragment should be amplified, the designed program used a longer extension step to have sufficient time to synthesize the full-length amplicon. The program contained an initial denaturation step at 95°C for 5 mins, followed by 28 cycles of 45 seconds at 94°C, 45 seconds at 55°C and 1 min at 72°C, at the end an extension step at 72°C for 10 mins was included.

The amplified product was verified by gel electrophoresis (see section 2.2.1.8) to assess the correct fragment amplification (around 1300 bp) and was purified with the GenElute™ PCR Clean-Up Kit according to the manufacturer's instructions.

2.2.1.2 Amplification of Nbs Using MP57 and GIII Primers

The pMECs plasmids encoding for the SIRPα human Nbs 30, 61, 67, 68, 69 and the SIRPα mouse Nb75 (all provided by Ema Romão, Cellular and Molecular Immunology Laboratory, Vrije Universiteit Brussel) were taken as template for amplification using GIII and MP57 primers. This was a way of obtaining a large amount of Nb fragments without using a lot of template. Moreover, it assured that after digestion, the majority of the product was the fragment containing the Nbs, therefore the purification using the GenElute™ PCR Clean-Up Kit was enough. Amplification via PCR occurred according with Table 2.9. Eight reactions were made to ensure that enough material for the subsequent steps was produced.

Table 2.9 – PCR mix used for one reaction for the PCR of pMECS containing the Nbs using MP57 and GIII primers.

Reagent	Volume (µL)
Sterile H_2O	35.5
5x Colorless Go Taq® Reaction Buffer	10
dNTPs (10mM)	1
GIII primer (20µM)	1
MP57 primer (20µM)	1
Go Taq® G2 DNA Polymerase (5U/µL)	0.5
Template	1

The Westburg T3 thermo cycler from Biometra® was used for the PCR reaction. The PCR strips containing the PCR reaction were placed in the PCR thermocycler and underwent a denaturation

step at 95°C for 3 mins, followed by 28 cycles of 30 seconds at 94°C, 30 seconds at 57°C and 45 seconds at 72°C, finally an extension step at 72°C for 10 mins was included.

The amplified products were verified by gel electrophoresis (see section 2.2.1.8) for the correct fragment amplification (around 600-800 bp) and were purified with the GenElute™ PCR Clean-Up Kit according to the manufacturer's instructions.

2.2.1.3 Digestion of Amplification CBP Fragment and pMECS with Not I and EcoRI

Digestion of amplification fragment: To 5 µg of the CBP amplification fragment, 1 µL NotI (10 U/µL) and 1 µL EcoRI (10 U/µL) were added. 10x buffer O was also added to the mixture to reach a final concentration of 1x and the volume was adjusted with sterile H_2O. The digestion was performed for 3 h at 37°C. The digested product was purified with the GenElute™ PCR Clean-Up Kit according to the manufacturer's instructions.

Digestion of pMECS: To 5 µg of the pMECS vector 1 µL NotI and 1 µL EcoRI were added. 10x buffer O was also added to the mixture to obtain a final concentration of 1x and the volume was adjusted with sterile H_2O. The digestion was performed for 3 h at 37°C. Afterwards, the entire reaction volume was loaded on a 1% agarose gel electrophoresis (see section 2.2.1.8). The band of digested vector was cut out of the agarose gel and purified with the QIAquick Gel Extraction Kit following the manufacturer's protocol.

2.2.1.4 Digestion of pMECS-CBP, pMECS-GG, Amplification Nbs Fragments and pHEN18 containing SIRPα mouse with NotI and NcoI

Digestion of pMECS-CBP: To 5 µg of pMECS-CBP, 4 µL NcoI and 1 µL NotI were added. 10x buffer O was also added to the mixture to reach a final concentration of 1x and the volume was adjusted with sterile H_2O. The digestion was performed for 3 h at 37°C. The digested product was purified with the GenElute™ PCR Clean-Up Kit according to the manufacturer's instructions.

Digestion of pMECS-GG (to insert Golden Gate): To 5 µg of the pMECS-GG vector 4 µL NcoI and 1 µL NotI were added. 10x buffer O was added to the mixture to obtain a final concentration of 1x and the volume was adjusted with sterile H_2O. The digestion was performed for 3 h at 37°C. Afterwards, the entire reaction volume was loaded on a 1% agarose gel electrophoresis (see section 2.2.1.8). The band of digested vector was cut out of the agarose gel and purified with the QIAquick Gel Extraction Kit following the manufacturer's protocol.

Digestion of amplification Nbs fragments: To 5 µg of each Nbs amplification fragments, 4 µL NcoI and 1 µL NotI were added. 10x buffer O was also added to the mixture to reach a final concentration of 1x and the volume was adjusted with sterile H_2O. The digestion was performed for 3 h at 37°C. The digested product was purified with the GenElute™ PCR Clean-Up Kit according to the manufacturer's instructions.

Digestion of pHEN18 containing SIRPα mouse: To 5 µg of the pHEN18 vector containing SIRPα mouse 4 µL NcoI and 1 µL NotI were added. 10x buffer O was also added to the mixture to have a final concentration of 1x and the volume was adjusted with sterile H_2O. The digestion was performed for 3 h at 37°C. Afterwards, the entire reaction volume was loaded on a 1% agarose gel electrophoresis (see section 2.2.1.8). The band of digested vector was cut out of the agarose gel and purified with the QIAquick Gel Extraction Kit following the manufacturer's protocol.

2.2.1.5 Ligation of Fragment to Wanted Vector

The ligation was performed overnight (O/N) at 16°C with 30 ng of the fragment to insert, 100 ng of vector, 1 µL T4 DNA ligase and 2 µL T4 DNA ligase 10x buffer for a total volume of 20 µL. The ligation was followed by purification using phenol-chloroform DNA precipitation. Sterile H_2O (80 µL) was added to the ligation product to make a final volume of 100 µL. Then 100 µL of the lower phase of 25:24:1 phenol/chloroform/isoamyl (Sigma Aldrich, St. Louis, USA) was added and shaken vigorously before centrifugation (Eppendorf Centrifuge 5417R) at 14000 rpm for 5 mins. After centrifugation, the upper phase was transferred to a new Eppendorf tube, 10 µL

3 M NaAc pH 5.2 and 250 µL 100% ethanol were added to the ligation product and it was stored for at least 1 h at -80°C for DNA precipitation. Next, the ligation product was centrifuged for 30 mins at 14000 rpm and 4ºC. Subsequently, the ethanol was removed, the pellet was dried on the heat block (Thermolyne type 16500 Dri Bath) and resuspended in 5 µL of sterile H_2O.

The ligations were made between: amplificon of CBP fragment and pMECS, Golden Gate and pMECS-CBP, amplificon of Nb fragments and pMECS-CBP, as well as SIRPα mouse and pMECS-CBP.

2.2.1.6 Plasmid Transformation in Electrocompetent Bacterial Cells

Transformation of the plasmids in bacterial host cells was required to perform a colony PCR and DNA sequencing for identification of correct constructs. The bacterial cells needed to be made competent so that they were capable to take up extracellular DNA from their surroundings. WK6 *E. coli* cells were made electrocompetent (ECC) as described in [45].

The plasmid transformation was carried out by mixing 50 µL ECC with 2 µL of ligation product, followed by an incubation on ice for 5 mins. After setting the electroporation apparatus *E. coli* Pulser® (Bio-Rad Laboratories, Hercules, California, USA), the cells were loaded into an electroporation cuvette (Bio-Rad Laboratories, Hercules, California, USA). Then, the cuvette was placed into the electroporation chamber and a pulse of 1.8 kV was applied. The cuvette was removed and 500 µL of LB was immediately added and transferred to an Eppendorf tube. The cuvette was rinsed with another 500 µL of LB, that were pooled to the same Eppendorf. The cell suspension was incubated at 37ºC for 1h on a shaker (200 rpm). 100 µL of the obtained culture were plated on a round LB Agar. The remaining culture was centrifuged for 3 mins at 2000 rpm. The pellet was also plated on fresh LB agar and the plates were incubated O/N at 37°C. On vectors where the Golden Gate killer cassette was present, the LB agar plates used were Amp/Chl/Glu, while on the other vectors the plates were only supplemented with Amp/Glu.

2.2.1.7 Colony PCR and DNA Sequencing

For screening of the new vectors created after transformation, some isolated colonies were picked from the round LB agar Amp/Glu plate or LB agar Amp/Chl/Glu (depending on the vector used) with a sterile tip, scratched on a new LB agar plate and submerged in the PCR mix (Table 2.10) in the well of the PCR strip. After the colonies were marked on the LB agar plate, the plate was incubated O/N at 37°C and stored at 4°C subsequently.

Table 2.10 - PCR mix used for one reaction of colony PCR

Reagent	Volume (µL)
Sterile H_2O	37.25
5x Colorless Go Taq® Reaction Buffer	10
dNTPs (10mM)	0.5
GIII primer (20µM)	1
MP57 primer (20µM)	1
Go Taq® G2 DNA Polymerase (5U/µL)	0.25

The PCR strips were placed in the Westburg T3 thermo cycler from Biometra® and a PCR program started. Since the introduction of the Golden Gate generated a large fragment (around 1500 bp) between the primers, the program comprised an initial denaturation step at 95°C for 5 mins, followed by 28 cycles of 45 seconds at 94°C, 45 seconds at 55°C and 1 min at 72°C, a final extension step at 72°C for 10 mins was performed. For the other constructs, the fragment between MP57 and GIII primers was around 400-800 bp, so there was no need for the program to include a long elongation step. The program was an initial denaturation step at 95°C for 3 mins, followed by 28 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 45 seconds at 72°C, a final extension step at 72°C for 10 mins was performed.

Next, the amplicons were verified by gel electrophoresis (see section 2.2.1.8). Constructs with the right size, depending on the construct (sequence length between MP57 and GIII primers),

were selected and purified with the ExoSAP procedure. 1.5 µL of the selected constructs were treated with 0.2 µL of ExoI (20 U/µL) and 0.4 µL of FastAP (1 U/µL), 12.9 µL of sterile H_2O was added as well. The primers and dNTPs degradation occurred by activation of the enzymes at 37°C for 15 mins, followed by an immediate inactivation at 80°C for another 15 mins.

To the cleaned PCR products was added 2 µL of MP57 primer (10 µM) and it was sent for DNA sequencing at Eurofins Genomics. The resulting sequences were analyzed with CLC Main Workbench 7.9.1 software (CLC Bio, Qiagen Bioinformatics).

Using a construct with the correct insertion by picking the corresponding colony from the LB agar plate mentioned before, a preculture of 5 mL supplemented with 1/1000 ampicillin stock was made and incubated O/N at 37ºC and 200 rpm. The preculture was used for plasmid extraction with the GenElute™ Plasmid Mini-Prep kit according to the manufacturer's instructions. The purified plasmids were sequenced again for confirmation of the sequences and stored at -20ºC.

2.2.1.8 Agarose gel Electrophoresis

A 1% agarose gel solution was poured in a gel frame with a well frame and ethidium bromide (0.5 µg/mL) was added and stirred into the gel solution. The gel was solidified for at least 30 mins and then immersed in a tank (Bio-rad Mini-Sub® Cell GT, Bio-Rad Laboratories, Hercules, California, USA) filled with 1x TBE. 10 µL of each sample was supplemented with 2 µL of orange G DNA loading dye 6x and loaded into the wells of the gel. A 5 µL aliquot of the Smartladder MW ladder (Eurogentec, Seraing, Belgium) was also loaded into in one of the wells. The gel was connected to an EC250-90 Electrophoresis Power Supply (E-C Apparatus Corporation) at 115 V for 35 mins. After running, the gels were then visualized on a Vilber Lourmat UV transilluminator (Germany) and photographed with CCD video camera (Rainbow CCTV RMB92) connected to Mitsubishi P91 printer (Mitsubishi electric).

2.2.2 Phage Display of Nanobodies

2.2.2.1 Preparation of the Libraries

The purpose of this phage display was not to select Nbs of highest affinity, but rather understand if the new vector created with the CBP was suitable for this technique. For that reason, the mini-library made was a little bit different, since it was already known that the Nbs used were binders of human SIRPα.

For the creation of these libraries, 100 ng of vectors pMECS and pMECS-CBP containing the Nbs were inserted into TG1 *E. coli* cells, by transformation via electroporation (see section 2.2.1.6). TG1 *E. coli* cells were made ECC as described in [45]. Instead of plating the cell suspensions, these were inoculated into 300 mL 2x TY supplemented with 1/1000 dilution Ampicillin stock (100 mg/mL) and 15 mL glucose stock (20%). Two bottles were used, one had all 6 Nbs without any modification, while the other had all Nb with the CBP tag. The bottles were incubated on a shaker (220 rpm) at 37°C O/N to allow the phagemid vector to grow and were used as precultures.

Two mini libraries comprising the 6 Nbs each were created: "Nbs against SIRPα" and "Nbs-CBP against SIRPα".

2.2.2.2 Inoculation of the Libraries

Medium of 300 mL 2xTY supplemented with 1/1000 dilution Ampicillin stock was prepared, allowing only TG1 *E. coli* cells containing the phagemid vector to grow. Also, 15 mL glucose stock was added to suppress the LacZ promoter, thus preventing leaky expression of the Nb protein, since this could interfere with the infection of TG1 *E. coli* cells with bacteriophages. The medium was inoculated with a 1 mL aliquot of TG1 *E. coli* cells from the mini libraries "Nbs against SIRPα" and "Nbs-CBP against SIRPα" (from section 2.2.2.1), which had been growing O/N (therefore in stationary phase). The bottles were incubated on a shaker (250 rpm) at 37°C until the OD_{600nm} reached 0.6-0.8 (usually 2-3h), meaning they had reached the exponential

growth phase. Following, approximately 10^{12} M13KO7 helper phages preserved in glycerol were added and incubated at 37°C without shaking for 30 mins to allow the phages to attach to the F-pilus of *E. coli*. The cells were centrifuged in 50 mL tubes for 10 mins at 2700 rpm and the supernatant was removed. The cell pellets were resuspended in 300 mL 2xTY supplemented with 1/1000 dilution of Ampicillin stock and 1/1000 dilution of Kanamycin stock (70 mg/mL). Incubation O/N at 37°C on a shaker (225 rpm) allowed the production of recombinant phages containing a Nb at their tip.

2.2.2.3 Preparations for Panning

To prepare for panning, a single well of a Nunc Maxisorp ELISA 96-well plate (Thermo Scientific, Denmark) was coated with 10 µg of SIRPα human in 100 µL of Coating Buffer pH 8.2 and stored at 4°C O/N. TG1 *E. coli* cells were plated on a LB Agar Amp/Glu plate and incubated O/N at 37°C so that a single colonies grown could be used to make a preculture for further steps.

2.2.2.4 Panning

Phage preparation: 300 mL O/N bacterial culture infected with M13KO7 helper phage (from section 2.2.2.2) were centrifuged for 30 mins at 8000 rpm and 8°C. Following, 40 mL supernatant was added to 10 mL PEG/NaCl (20%) until all the supernatant was in contact with the PEG/NaCl solution. After inverting the tubes several times, these were kept on ice for 30 mins and, subsequently, centrifuged for 30 mins at 4000 rpm and at 4°C. The supernatant was discarded and the pellet containing the phages was resuspended in 1 mL sterile PBS. The solution was centrifuged for 2 mins at 14000 rpm and the supernatant was collected. The OD_{260nm} was measured so that approximately 10^{11} phages per well were added, taking into account that OD_{260nm} of 1 = $3x10^{10}$ particles/mL

ELISA-plate preparation: Non-bound antigen of the 96-well plate (from section 2.2.2.3) was removed by inverting the plate. The plate was washed 5x with PBS-Tween (PBST) and the positive well (with antigen), as well as an empty well (negative control), were blocked with 200 µL of PBS-Milk blocking buffer for at least 1h. After the blocked wells have been washed 5x with PBST, 100 µL of phages was added to both wells, followed by incubation of at least 1h at room temperature (RT), to allow the Nb-displayed phages to recognize and bind to the coated antigen. Next, the wells were washed 20x with PBST and the bound phages were eluted after 10 mins of addition of 100 µL TEA solution. Finally, the solution with the phages was transferred into an Eppendorf tube that contained 100 µL Tris-HCl 1 M pH 8.0 to neutralize the solution. All procedures were performed on the positive and negative control wells.

TG1 preculture preparation: At the same time of phage preparation, TG1 colonies were inoculated onto 5 mL LB medium to make a preculture and onto a second 5 mL LB medium to which 1/1000 dilution of Ampicillin stock was added, with the purpose of having a negative control. This was incubated at 37°C on a shaker (200 rpm) until the cells reached the exponential growth phase.

2.2.2.5 Visualization of Enrichment Factor

Two columns (positive and negative) of a 96 round-bottom-well plate (Corning Incorporated, New York, USA) were filled with 90 µL of sterile PBS in each well and two additional columns were filled with 90 µL TG1 cells of the preculture (from section 2.2.2.4). Next, 10 µL of the positive phages (from section 2.2.2.4) were diluted by transferring them to the first well of the PBS column, mixing and adding to the second well, generating a 1/10 serial dilution but leaving the last row as control, meaning without phages.10 µL of all these dilutions was transferred to the corresponding wells of the column with TG1 cells. The same procedure was performed to the negative phages in different columns of the plate.

After an incubation for 30 mins up to 1 h that allowed the phages to infect the TG1 cells, 10 µL of each well were transferred onto a square LB Agar Amp/Glu and the suspension was spread to

obtain two columns of serial dilutions, a positive and a negative control. After an O/N incubation at 37°C, the enrichment was determined.

Enrichment is a representation of the chance to find better Nb binders and can be calculated by counting the colonies of the positive and negative lines where single colonies can be seen and then dividing the number of positive colonies by the number of negative colonies, considering the dilution factor where they can be seen. The larger this enrichment factor, the higher the chances for finding better binders.

2.2.3 Periplasmic Expression and Purification of Nanobodies and SIRP*α* mouse

The expression of the Nbs and Ag (SIRPα mouse) containing or not the CBP tag was done to understand if there was a problem of expression with any of the constructs. All of the procedures mentioned bellow were based on [45].

2.2.3.1 Transformation into Electrocompetent WK6 *E. coli* Cells

For production and purification of Nbs, the vector containing the Nb-insert was introduced into WK6 *E. coli* cells. These cells are a non-suppressor strain that can not to read through the vector's amber stop codon between gene III and the Nb-insert. This will allow the Nb expression as a soluble protein in the periplasm of the bacterial cells.

The introduction of the vectors containing the Nb or Ag into the ECC WK6 *E. coli* cells was performed through an electroshock, as described in section 2.2.1.6., but instead of 2 µL of plasmid, 100 ng were added.

2.2.3.2 Precultures of WK6 *E. coli*

To prepare for Nb expression, a WK6 colony containing the Nb or Ag of interest with and without the CBP tag was used to inoculate 5 mL LB medium supplemented with 1/1000 dilution of Ampicillin stock. This was done three times for each clone to obtain a final volume of 15 mL inoculated medium and ensure that in the next day there was material to proceed. After an O/N incubation at 37°C on a shaker (200 rpm), the precultures of the same clone that shown turbidity were pooled and used for inoculation. Also, 5 mL of this preculture was used for plasmid extraction by GenElute™ Plasmid Mini-prep kit. This was sent for sequencing to Eurofins Genomics to confirm the cloned Nb/Ag insert.

2.2.3.3 Expression in WK6 *E. coli*

To obtain 1 L of WK6 culture, three baffled shaker flasks containing 330 mL TB medium supplemented with 1/1000 Ampicillin stock and 1.5 mL glucose stock. Each flask was also inoculated with 2 mL preculture (from section 2.2.3.2). This was incubated at 37°C on a shaker (220 rpm) for 2-3 h until the OD_{600nm} reached 0.6-0.9, which meant that the cells have reached the exponential growth phase. Then, 1 mM IPTG was added to induce the periplasmic expression of the Nbs or Ag and the flasks were incubated O/N at 28°C on a shaker (220 rpm).

2.2.3.4 Periplasmic Extraction

The next day, the Nbs or Ag were extracted from the periplasm of WK6 cells via osmotic shock. The first flasks of each O/N culture were centrifuged for 8 mins at 8000 rpm. The supernatant was discarded, and this was repeated for the other two flasks whereby the same centrifugation bottle was used per clone in order to obtain one pooled pellet of 1L of media (three pellets). The pellet was dissolved in 12 mL TES and the bottles were incubated for 1 h at 4°C on a shaker (200 rpm). Subsequently, 24 mL TES/4 was added to each bottle to create an osmotic shock, which caused the Nbs and other proteins from the periplasm to exit the cells. When the lysozyme was added to enhance the periplasmic extraction, 300 µL of 2 M $MgCl_2$, DNase (50 µg/mL) and Lysozyme (5 mg/mL) were also added to each bottle. The bottles were further incubated for 2 h at 4°C on a shaker (200 rpm) and centrifuged for 30 mins at 8000 rpm. The supernatant, which

contained the Nbs, was collected in 50 mL tubes and stored at 4°C. The periplasmic extraction was repeated to maximize the yield from each pellet by dissolving it again in TES and doing an osmotic shock with TES/4, however this time the incubation was performed for 4 h and O/N. Again, when the lysozyme was added to enhance the periplasmic extraction, it was on the step of the TES/4, adding 300 mL of 2 M $MgCl_2$, DNase (50 µg/mL) and Lysozyme (5 mg/mL), as well. The next day, a centrifugation for 30 mins at 8000 rpm was done and the supernatant was collected.

2.2.3.5 First Purification Step: IMAC

Immobilized metal affinity chromatography (IMAC) is a purification method that isolates proteins containing a His tag based on the high affinity binding of histidine to metal ions, such as nickel. The Ni-beads needed to be washed, by using 1 mL of HisPure Ni-NTA™ Resin suspension per liter of culture and adding PBS to a final volume of 50 mL. This was centrifuged for 7 mins at 1400 rpm and the excess of PBS was discarded. Subsequently, 500 µL of the washed Ni-beads suspension was added to each 50 mL tube containing the periplasmic extracts (section 2.2.3.4) and the tubes were incubated for 1 h at RT on a shaker (100 rpm) to allow binding of the Nbs to the Ni-beads. The suspension was poured into a PD-10 column (GE Healthcare Life Sciences) containing a filter to retain the Ni-beads conjugated with the Nbs and allow the periplasmic extract to flow through. The tubes were rinsed with 50 mL PBS and then the solution was also added into the column. When the solution had flown through, the column was sealed, and 1 mL of 0.5 M Imidazole in PBS was added to elute the Nbs. Since, imidazole is identical to the R group of Histidine, it will be a competitive binder for Nickel beads. Nevertheless, it is used in such a high concentration that it causes the His tagged Nbs to elute. After 10 mins of incubation with imidazole in PBS, the flow through containing the Nbs was collected into Eppendorf tubes. This step was repeated 5 times to obtain 5 Eppendorf tubes of 1 mL eluted IMAC containing the Nb. The protein concentration of each tube was measured in the Nanodrop spectrophotometer (Isogen Life Sciences ND 1000) at OD_{280nm} considering the theoretical extinction coefficient of each Nb/Ag, calculated by the ProtParam tool (http://www.expasy.ch/tools/protparam.html). Next, the fractions with concentration higher than 0.1 mg/mL were pooled. In the cases where the total concentration of the 5 fractions was lower than 1 mg/mL, all the fractions were pulled and concentrated.

In the cases a concentration of the fractions was needed, the pulled fraction was loaded into a Vivaspin 5000 MW HY (Vivascience – Sartorius) and centrifuged at 2000 g for 20 mins or until reaching the final volume of 500 µL.

2.2.3.6 Second Purification Step: SEC

To further purify the Nbs, size exclusion chromatography (SEC) was done using fast protein liquid chromatography (FPLC), thus the separation was done according to their molecular weight (MW). The samples collected from IMAC (section 2.2.3.5) were loaded onto a Superdex 75 column (16/600) on AktaXpress or a Superdex 75 column (10/300) on AktaXplorer10S (all from GE Healthcare) depending on the concentration of the samples. The column was equilibrated with PBS, which was also the running buffer. The run was carried out following standard operating procedures present in the lab. The OD_{280nm} was measured during the run to determine which of the eluted fractions contained the Nb protein and needed to be pooled. The concentration of these pooled protein fractions was measured in the Nanodrop spectrophotometer (Isogen Life Sciences ND 1000) at OD_{280nm} considering the extinction coefficient of each Nb/Ag, to calculate the final yield of purified Nb.

Between two SEC runs, the column was decontaminated with one column volume of NaOH-NaCl (1 M) SEC buffer, neutralized with two volumes of Tris-HCl (200 mM) SEC buffer, maintained in one volume of 20% ethanol SEC buffer and equilibrated for the next run with one volume of PBS SEC buffer.

2.2.3.7 SDS-PAGE

To check the purity/presence of the obtained Nbs/Ag, a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Therefore, 5 µg of Nb protein and of a positive control (SIRPα human) were mixed with 4x NuPAGE® LDS Sample Buffer and 10x NuPAGE® Sample Reducing Agent. Additionally, several aliquots were taken during the crucial steps of production and purification to evaluate the enrichment. In this case, the aliquots that could have been directly quantified, 5 µg were added, for the samples that couldn't, 13 µL were added. The samples were heated in a heat block for 10 mins at 100°C. Subsequently, a 12 % NuPAGE® Bis-Tris polyacrylamide gel was placed in a Bio-Rad X-cell SureLock™ SDS-PAGE tank that was filled with 300 mL of 1x MES buffer. The samples were loaded onto the gel along with 5 µL of the PageRuler™ Prestained Protein Ladder to determine the MW of the samples. The gel was run at 115 V for at least 1 h to allow the migration of the proteins. Next, it was stained with Coomassie Blue staining solution and placed on a shaker for 1 h to allow coloring of the proteins in the gel. Finally, a destaining solution was added for at least 3 h while shaking to allow visualization of the proteins.

2.2.3.8 Western Blot

For the western blot, the polyacrylamide gel electrophoresis was done the same way as described for the SDS-PAGE (section 2.2.3.7). After obtaining the gel with the separated proteins, it was transferred onto a nitrocellulose blotting membrane (Amersham Protran 0.45 NC, GE Healthcare) and placed into a western blot holder (BioRAD mini protean® II cell) that contained transfer buffer and an ice block to reduce the heating during transfer. The transfer of the proteins within the gel to the membrane was run for 1 h at 100 V. Afterwards, the membrane that contained the transferred proteins needed to be developed the same way as an ELISA. Briefly, protein binding sites on the membrane were blocked with PBS-Milk for at least 1 h while shaking, washed three times with PBS, incubated for at least 1 h with a 1/2000 dilution of α-HIS mAb in PBS while shaking, washed again three times, incubated for at least 1 h with a 1/2000 dilution of α-Mouse IgG - HRP conjugate in PBS, while shaking, and washed three times. The proteins were visualized by addition of the western blot developing solution and incubation of the membrane for 5-10 mins in the dark. The reaction was stopped and rinsed with distilled water.

3 Results and Discussion

3.1 Design of new Vector

This project started with the substitution of the Hemagglutinin (HA) peptide detection tag downstream of the Nb cloning site from pMECS-GG phage display vector with a new CBP tag using a mutagenic primer (CBP-Insert). While designing the cloning strategy, it became clear that this could not be done in only one step. First, the CBP tag had to be cloned into the original pMECS vector. This was achieved by amplifying via PCR a pMECS-GG fragment, using CBP-insert and FP as primers. Then, the amplified fragment and pMECS vector were digested using EcoRI and NotI and, after purification, these fragments were ligated to obtain the vector pMECS-CBP. This construct was then transformed into a non-suppressor WK6 *E. coli* strain. Fourteen bacterial colonies were picked to perform a PCR in order to check which of these contained the CBP insert (Figure 3.1). Analysis of the 1% agarose gel, it seems that not all the colonies had the right insert. Considering that the only difference between the two vectors is the CBP (a 19 aa peptide), the apparent MW is only marginally different. However, it is still possible to see which colonies have the correct insert. For example, the samples 5 and 12 have the pMECS-CBP vector, while the samples 7 and 13 had the self-ligated vector (pMECS). Two or three plasmids with putative correct inserts were sent for sequencing to guarantee that the correct vector was selected, followed by plasmid preparation using the putative correct colonies.

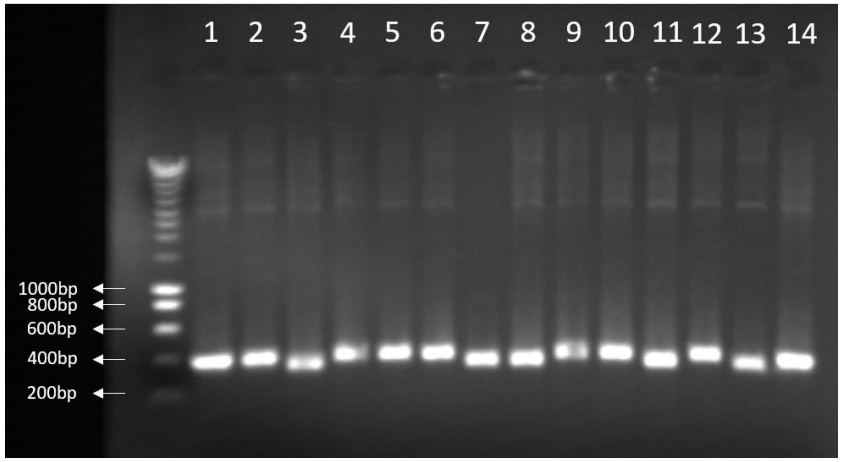


Figure 3.1 – 1% agarose gel after colony PCR with primers MP57 and GIII to examine which have the CBP insert. The first lane contains the DNA SmartLadder. 1-14 Randomly selected WK6 *E. coli* colonies.

A re-cloning of the Golden Gate killer cassette was performed to pMECS-CBP. The restriction enzymes used for this re-cloning were NotI and NcoI. After digestion, a gel extraction to purify the fragment containing the Golden Gate killer cassette was necessary. Since the fragment wanted for the ligation was the smaller (1500 bp), purification only by PCR CleanUp Kit wouldn't remove the larger digested fragment and, consequently, it would promote self-ligation [47], leaving less amplicon available for the wanted ligation. For a better understanding of what is happening during cloning, a digestion profile can be performed (Figure 3.2). The profile reviews all steps of digestion, including before and after purification steps to ensure that fragments used in ligation are pure. Analyzing the contents within lanes 6 and 7 (Figure 3.2), it is observed that the 1500 bp fragment was indeed purified, since the larger fragment disappeared. Most of the times, when a larger fragment disappears is due to gel extraction. A way of understanding if the restriction enzymes are efficient is comparing the digested product with the undigested plasmid (lanes 2 and 3, for example). An undigested plasmid is characterized by three bands on agarose gel that correspond to three different isoforms (supercoiled, linear and open circular) [48]. The electrophoretic migration is dependent on size and conformation, due to differences in hydrodynamic interactions and degree of chain entanglement [48 49]. Thus, the supercoiled conformation is the one that migrates faster, since there are less conformational restrictions, followed by linear conformation and being

open circular the one that migrates less [48]. Analyzing the contents of lanes 2, 5 and 9 it is observed that all isoforms mentioned previously were present in all pure plasmids. If after digestion, a similar result to undigested plasmid is obtained, a problem occurred, and a new digestion must be done. Since all digestion steps seemed acceptable, a ligation was done followed by transformation in bacterial cells for colony PCR with MP57 and GIII as primers. Fourteen bacterial colonies were picked to check which had the vector pMECS-GG-CBP (Figure 3.3).

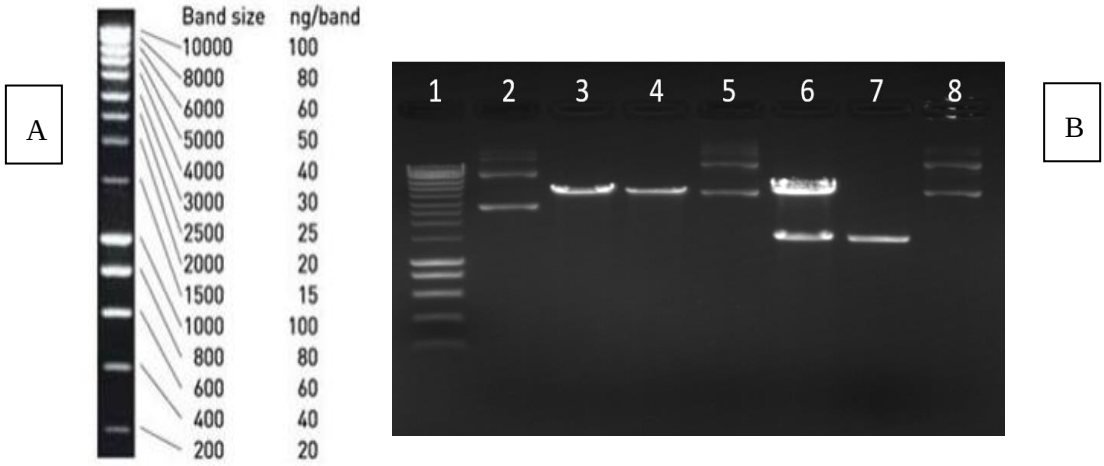


Figure 3.2 – A – DNA SmartLadder used as a marker for the size of the bands (expressed in bp). B – 1% agarose gel of digestion profile of re-cloning Golden Gate killer cassette into pMECS-CBP. 1 – DNA SmartLadder. 2 – Undigested pMECS-CBP. 3 – Digested pMECS-CBP with NotI and NcoI, not purified. 4 - Digested pMECS-CBP with NotI and NcoI, purified. 5 – Undigested pMECS-GG. 6 - Digested pMECS-GG with NotI and NcoI, not purified. 7 - Digested pMECS-CBP with NotI and NcoI, purified. 8 – ligation product pMECS-GG-CBP.

Insertion of Golden Gate killer cassette was expected to be around 1500 bp (lane 3, for example), while pMECS-CBP self-ligation was expected to be around 400 bp (lane 8, for example). Sequencing confirmed the construction of the correct vector and plasmids were prepared using the correspondent colonies.

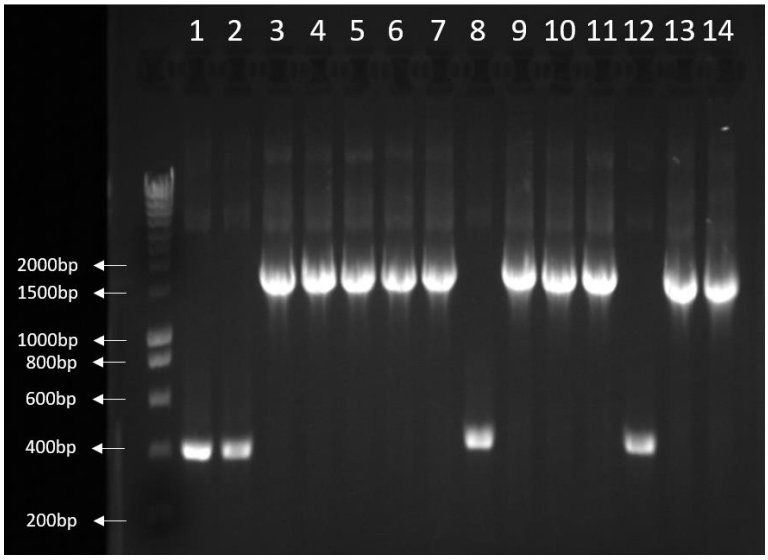


Figure 3.3 – 1% agarose gel after colony PCR with primers MP57 and GIII to examine which have the Golden Gate insert. The first lane is the DNA SmartLadder. 1-14 Randomly selected WK6 *E. coli* colonies.

Also, 6 Nbs with well-established and different association and dissociation kinetic rate constants (provided by Ema Romão) were inserted in the vector pMECS-CBP. NotI and NcoI were the restriction enzymes used for digestion, and, after ligation, the vectors were transformed in WK6 *E. coli* and colony PCR with primers MP57 and GIII was, once more, used to discover the colonies

with the right constructs (Figure 3.4). Vectors with Nbs genes had amplification fragments around 600 bp and were sent for sequencing. Plasmids for each Nb were prepared to be used in further experiments.

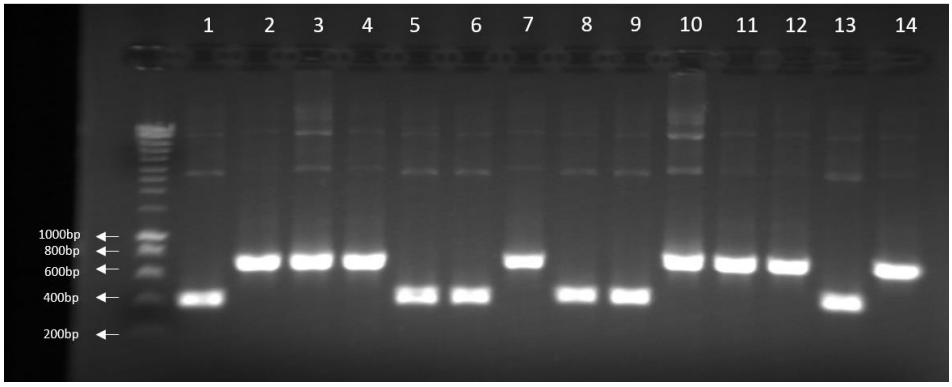


Figure 3.4 - 1% agarose gel after colony PCR with primers MP57 and GIII to examine which have the Nb30 insert. The first lane is the DNA SmartLadder. 1-14 Randomly selected WK6 *E. coli* colonies.

Sequences of all vectors created were analyzed using CLC Main Workbench 7.9.1 software, to guarantee not only the right insertions, but also that all key elements were functional. Figure 3.5 summarizes all modifications made to pMECS, to transform it into all forms of pMECS-CBP, with the red region being a variable sequence depending on the Nb/Golden Gate that were inserted. The vector was designed either for phage display or periplasmic expression of the Nb encoded [45]. The lac promoter/operator is inducible by IPTG and repressed by glucose [50]. The periplasmic transport is provoked by the N-terminal signal peptide pelB, which enables transport through the inner membrane of bacteria [51]. In each Nb there is a CDR3 encoded, which defines a Nb family [52]. Due to deriving from the same B-cell lineage, Nbs from the same family bind to the same epitope on the target [52]. The His tag allow simultaneously immunodetection and purification via IMAC [53]. All these elements can be seen on the vectors created (Figure 3.5), along with the inserted CBP domain, which might indicate that the expression of the Nb-CBP could occur.

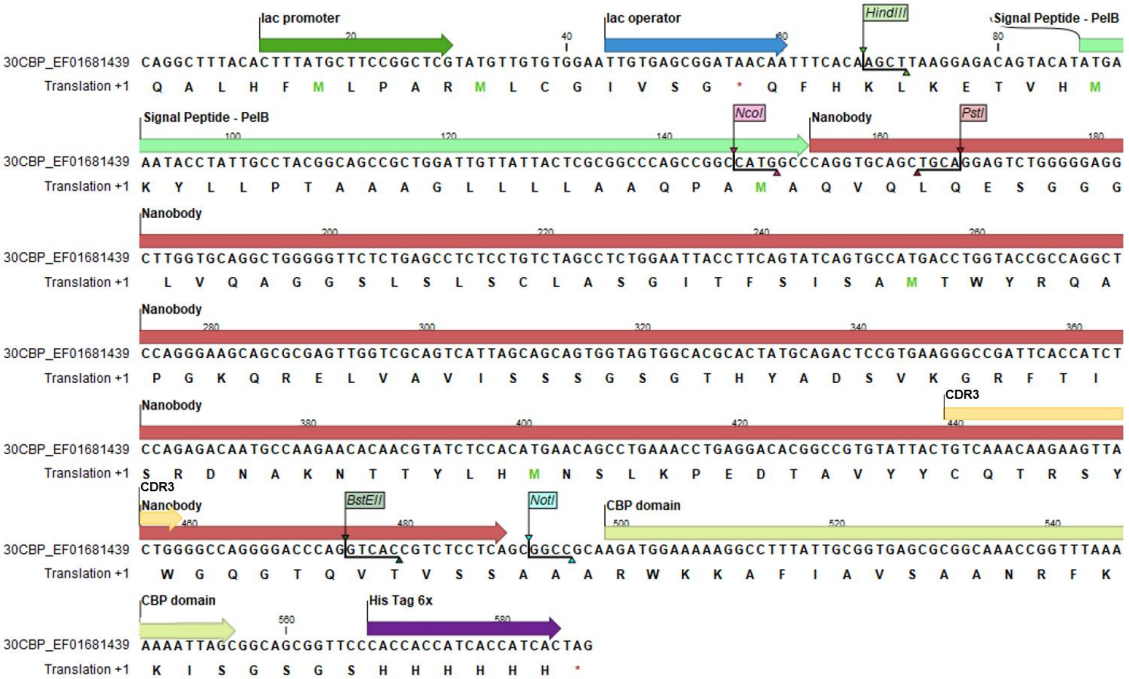


Figure 3.5 - Schematic representation of all modifications made to pMECS to create all pMECS-CBP vectors.

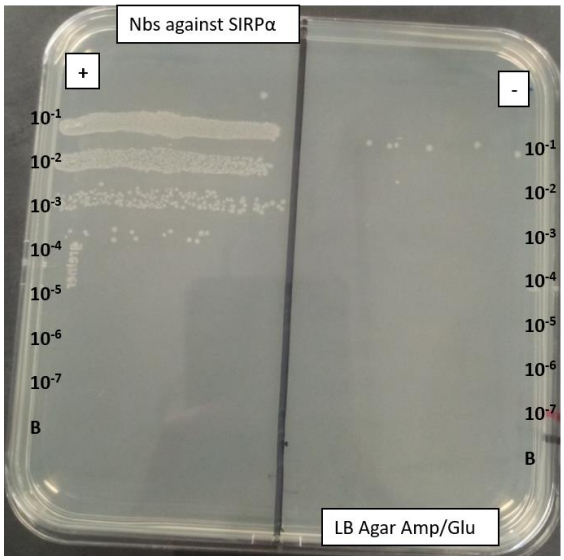
3.2 Phage Display of Nanobodies

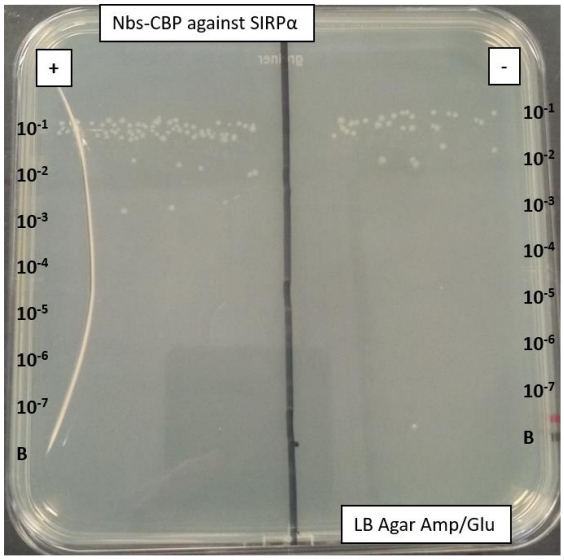
After obtaining the new phage display vector, two mini libraries were created to test the effectiveness of the construct on phage display.

Since the goal wasn't to isolate the Nbs of highest affinity, but rather understand if the new vector was suitable for phage display, a different approach was used. The mini libraries created included 6 Nbs that were already selected in the lab against human SIRPα and the difference between them was the vector used. One library was generated in the pMECS vector, producing the original Nbs discovered by phage display. Thus, even with just one round of panning it was expected that an enrichment occurred. The other library was generated in the new pMECS-CBP vector, to evaluate if a similar enrichment could be achieved. Moreover, the libraries were created by simply transforming the same amount of each Nb in TG1 cells (which suppress the amber stop codon [54]), using electroporation and then allowing all the cells to grow O/N while shaking. The next day, these precultures were used for library inoculation and the cells were infected with M13 helper phages. The next day the phages were purified, and a single round of panning was performed. A comparison between to libraries was done by observing the enrichment on LB Agar Amp/Glu pates (Figure 3.6).

The library in pMECS showed an enrichment by a factor of 1000 or more, whereas the mini library in pMECS-CBP failed to show any enrichment. Since both libraries were comprised by the same Nbs, lack of enrichment on the one using pMECS-CBP might indicate a problem of expression, despite statements on the contrary [44].

A





B

Figure 3.6 – LB Agar Amp/Glu plates that show enrichment after one round of panning with Nbs previously selected for human SIRPα. A – A conventional pMECS vector was used for the construction of the library Nbs against SIRPα. Positive results (Antigen incubated phages) are shown on the left side of the plate, while negative results (phages against empty well) are shown on the right side. B – New vector with a CBP tag was used for the construction of the library Nbs-CBP against SIRPα. Positive results (Antigen incubated phages) are shown on the left side of the plate, while negative results (phages against empty well) are shown on the right side.

3.3 Periplasmic Expression and Purification of Nanobodies

To better understand the lack of enrichment on phage display with Nbs previously selected to the target, expression of Nbs with and without the CBP tag was performed. Moreover, the Nb-CBP tags that might be produced, would later be used to evaluate if the capturing of the tagged Nb on CaM and its subsequent gentle release was efficient. Plasmid DNA of both pMECS and pMECS-CBP was transformed into non-suppressor WK6 *E. coli* strain. In these cells the amber codon downstream of the His tag and upstream the gene for p III is recognized, therefore Nb is expressed as a protein and not fused to gene III protein [45]. This is important because gene III protein can

cause false positives and might interfere with the Nb binding to its target. The Nbs were expressed as periplasmic proteins with the assistance of disulfide-bond catalysts, isomerases and chaperones by induction of the lac promoter with IPTG [53]. The periplasmic extract was collected via an osmotic shock and two purification steps were performed, IMAC followed by SEC. Although the CBP peptide is supposed to enhance the production yield [44], the amount obtained for CBP-tagged Nbs was far below 1 mg/L, while the same Nbs without the CBP could be express without any problems. Moreover, these Nbs could not be detected in any step of expression on western blot. Other attempts were made by adding lysozyme to improve the periplasmic extraction step. Even though the amount after IMAC was higher, when SEC was performed a higher number of contaminants was also revealed (Figure 3.7). Comparing all elution profiles of the different clones, it can be observed that even in well expressed Nbs, contaminants still elute earlier than the Nb, even if at a small scale. This observation is in accordance with the fact that IMAC is not sufficient to avoid the presence of protein contaminants [53]. Moreover, at the time when imidazole is eluted, it causes a change in conductivity, represented by the orange line. In the cases where Nb is expressed with CBP, a seriously contaminated sample is noted and much less material at the elution time of Nb is observed.

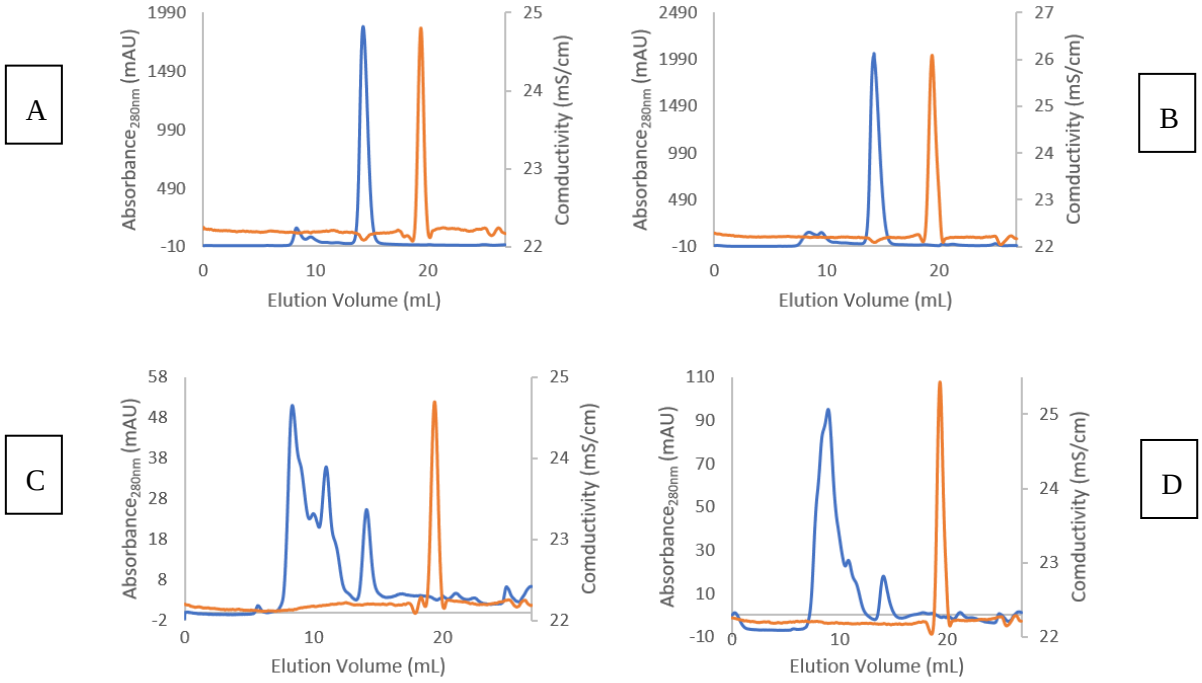


Figure 3.7 – Size Exclusion Chromatography of SH Nb 30 (A); SH Nb 30 extract using lysozyme for the improvement of periplasmic extraction (B); SH Nb 30 CBP (C) and SH Nb 30 CBP with addition of lysozyme during periplasmic extraction (D). The blue line represents the elution profile of the proteins, whereas the orange represent the conductivity.

An overview of the expression can be seen in Figure 3.8 and Figure 3.9. Aliquots of crucial steps during Nb expression and purification steps were loaded onto a polyacrylamide gel and proteins visualized after Coomassie staining or western blot for the detection of the Nb. As seen in Figure 3.8, protein expression was obtained after IPTG induction. The periplasmic extract obtained by osmotic shock released a variety of proteins, which can be noted in the lanes 4 and 5 of the SDS-PAGE. In the subsequent purification, a variety of proteins were being eliminated as noted in lanes 6 and 7, which correspond to the flow-through and wash of the IMAC. After IMAC elution, there were still some contaminating proteins present, as seen in lane 8. A further step of purification was necessary to obtain pure Nbs. After SEC, lane 10, most of contaminants of higher MW were removed and the sample applied on gel in reducing conditions represents a pure Nb protein with MW of 15 kDa. In the western blot it can be seen that the Nb 30 was detected in all steps of expression and purification.

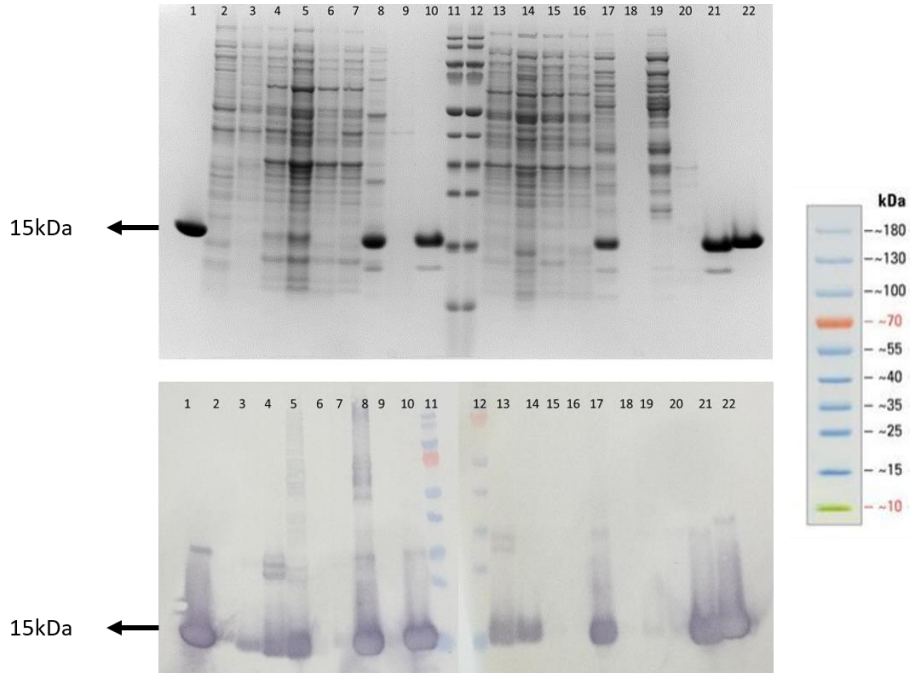


Figure 3.8 - SDS gel and western blot of the expression profile of SH30 and SH30 with addition of lysozyme for periplasmic extraction improvement. 1- positive control (SIRPα), 2- SH30 culture before induction, 3- SH30 culture after induction, 4- SH30 1st extraction, 5- SH30 2nd extraction, 6- SH30 wash PBS, 7-SH30 flow through, 8- SH30 IMAC, 9- SH30 1st peak SEC, 10- SH30 2nd peak SEC, 11- marker, 12- marker, 13– SH30 Lysozyme 1st extraction, 14- SH30 Lysozyme 2nd extraction, 15- SH30 Lysozyme flow through , 16- SH30 Lysozyme wash PBS, 17- SH30 Lysozyme IMAC, 18- SH30 Lysozyme 1st peak SEC, 19- SH30 Lysozyme 2nd peak SEC, 20- SH30 Lysozyme 3rd peak SEC, 21- SH30 Lysozyme 4th peak SEC, 22- positive control (SIRPα).

In the SDS-PAGE of Nb SH30 CBP (Figure 3.9), most of the earlier stages look similar to the Nb SH30, however, a band around the MW of the Nb is never observed. Since a positive control (human SIRPα containing a His tag) was added to the western blot, presence of a clear band indicated that the antibodies, conjugates and substrates we used were functional. Thus, not detecting Nb SH30 CBP could be a sign that this Nb was not being properly expressed.

A comparison between the amounts obtained for the Nb with and without CBP tag are shown in Table 3.1. Note that since nothing could be detected on western blot, measurements for the amount of SH Nb 30 CBP after SEC weren't done. However, analyzing the data of SH Nb 30, there were indications that some Nb material was lost and that impurities were still present after IMAC. Moreover, measurements of concentration in a solution with imidazole are not the most accurate which can lead to overestimating the amounts after IMAC.

Comparing the amounts obtained with and without the addition of lysozyme, as well as the SDS-PAGE bands of 1st and 2nd extractions (5-6 with 13-14 of Figure 3.8), it was shown that lysozyme improved the periplasmic extraction step.

Table 3.1 - Amounts obtained after IMAC and SEC of SH Nb 30, SH Nb 30 with periplasmic extraction enhancement by the addition of lysozyme, SH Nb 30 CBP and SH Nb 30 CBP 30 with periplasmic extraction enhancement by the addition of lysozyme.

Clone	**Amount (mg) obtained after IMAC from 1 L culture**	**Amount (mg) obtained after SEC from 1 L culture**
SH Nb 30	3.96	2.43
SH Nb 30 + Lys	5.05	3.78
SH Nb 30 CBP	0.63	Not applicable
SH Nb 30 CBP + Lys	1.3	Not applicable

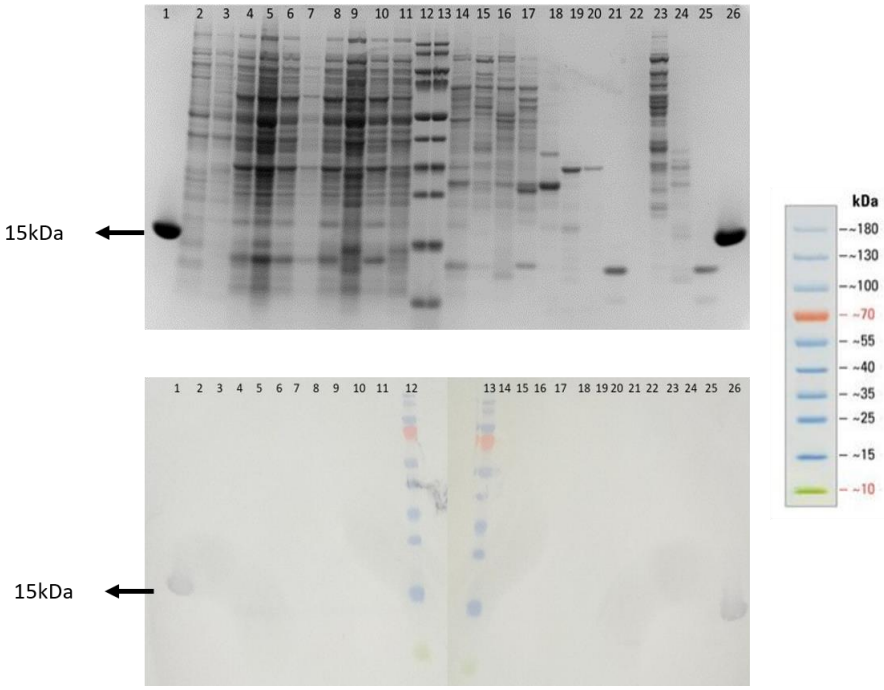


Figure 3.9 - SDS gel and western blot of the expression profile SH30 CBP and SH30 CBP with addition of lysozyme for periplasmic extraction improvement. 1- positive control (SIRPα), 2- SH30CBP before induction, 3- SH30CBP after induction, 4- SH30CBP 1st extraction, 5- SH30CBP 2nd extraction, 6- SH30CBP flow through, 7- SH30CBP wash PBS, 8- SH30CBP Lysozyme 1st extraction, 9- SH30CBP Lysozyme 2nd extraction, 10- SH30CBP Lysozyme flow through, 11- SH30CBP Lysozyme wash PBS, 12- marker, 13- marker, 14- SH30CBP IMAC, 15- SH30CBP Lysozyme IMAC, 16- SH30CBP 1st peak SEC, 17- SH30CBP 2nd peak SEC, 18- SH30CBP 3rd peak SEC, 19- SH30CBP 4th peak SEC, 20- SH30CBP 5th peak SEC, 21 SH30CBP 6th peak SEC, 22- SH30CBP Lysozyme 1st peak SEC, 23- SH30CBP Lysozyme 2nd peak SEC, 24- SH30CBP Lysozyme 3rd peak SEC, 25- SH30CBP Lysozyme 4th peak SEC, 26- positive control (SIRPα).

To ensure that the expression of all Nbs is similar and to exclude that we choose by accident a poor expressing Nb, we tested the expression of 5 additional Nb clones, each of which was tagged with CBP. Table 3.2 summarizes the highest amount achieved of each tagged Nb, while using 330 mL of medium. As observed in all attempts, it was not possible to detect Nbs in any step of the expression.

Table 3.2 – Amount obtained after IMAC for each Nb fused to the CBP as measured by UV absorption at 280 nm. The extinction coefficient of the Nb predicted from the amino acid content was used to calculate those amounts.

Clone	**Max amount (in mg) obtained after IMAC from 330 mL culture**	**Extinction Coefficient (L/g.cm)**
SH Nb 30 CBP	0.43	1.636
SH Nb 61 CBP	0.24	2.155
SH Nb 67 CBP	0.48	1.853
SH Nb 68 CBP	0.43	1.570
SH Nb 69 CBP	0.29	1.922
Sm Nb 75 CBP	0.44	1.636

3.4 Re-Cloning of SIRP*α* mouse

To better understand the expression problem, an Ag with high expression yield (mouse SIRPα) was re-cloned in the pMECS-CBP vector, using NotI and NcoI as restriction enzymes. After ligation, the construct was transformed into *E. coli* bacterial cells and a colony PCR using MP57 and GIII was performed to select colonies with the right insertion (Figure 3.10).

The Ag mouse SIRPα has a size of around 200 bp, meaning that right insertions had an expected size of 600 bp (lane 9, for example), while self-ligation of pMECS-CBP had an expected size of 400 bp (lane 2, for example). From all re-cloning strategies this was the one with lower insertion

percentage. This could be explained by bad quality of DNA obtained during digestion, as shown in Figure 3.11, lane 3. The digested product didn't show two clean bands, but rather a smear between the two bands of the expected size. Even though the percentage was low, sequencing confirmed that colonies with fragments around 600bp contained the right insert.

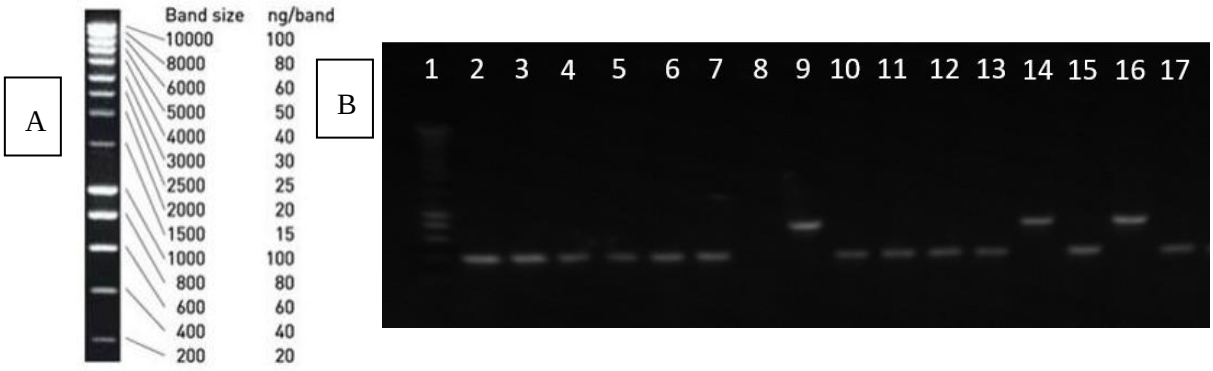


Figure 3.10 – A – DNA SmartLadder used as a marker for the size of the bands (expressed in bp). B – 1% agarose gel after colony PCR with primers MP57 and GIII to examine which have the mouse SIRPα insert. 1 DNA SmartLadder. 2-17 Randomly selected WK6 *E. coli* colonies.

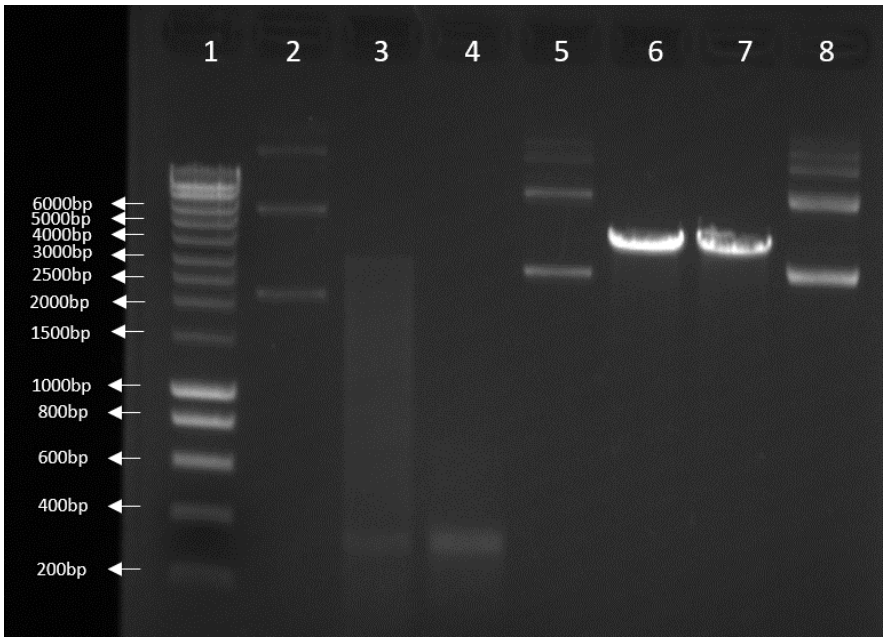


Figure 3.11 – 1% agarose gel of digestion profile of re-cloning mouse SIRPα into pMECS-CBP. 1 – DNA SmartLadder. 2 – Undigested pHEN18 with SIRPα mouse. 3 – Digested pHEN18 with mouse SIRPα cut with NotI and NcoI, not purified. 4 - Digested pHEN18 with mouse SIRPα cut with NotI and NcoI, purified. 5 – Undigested pMECS-CBP. 6 - Digested pMECS-CBP, cut with NotI and NcoI, not purified. 7 - Digested pMECS-CBP cut with NotI and NcoI, purified. 8 – ligation product pMECS- SIRPα-CBP.

3.5 Periplasmic Expression and Purification of SIRPα mouse

The vectors containing the mouse SIRPα were transformed into WK6 *E. coli*. The expression was initiated by the addition of IPTG to the culture, which promoted the production of SIRPα with and without the CBP tag. This antigen was chosen due to its high production yields. A significant drop in yield will be observed if the CBP was impeding the expression level. The SDS-PAGE results showed that SIRPα is present even at early stages of the expression and the SIRPα antigen was pure after IMAC (Fig 3.12). In contrast, a band corresponding to the size of SIRPα-CBP couldn't be revealed, neither in Coomassie stained gels nor after western blot. This is similar to what we observed for Nb-CBP expressions.

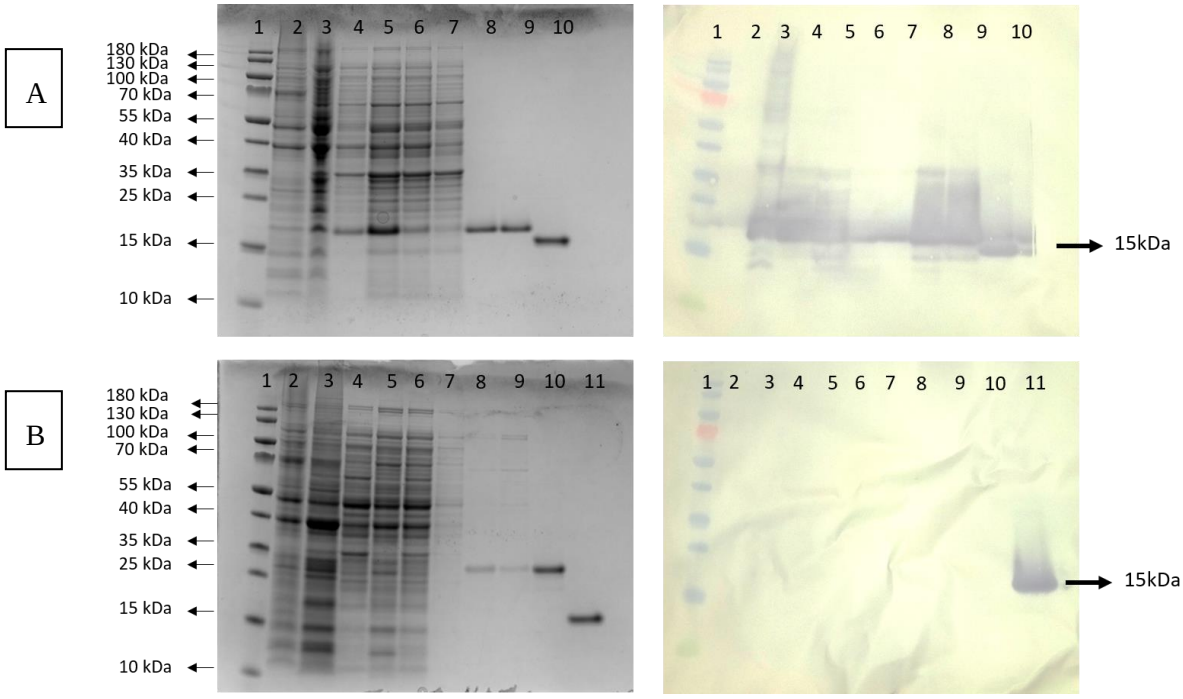


Figure 3.12 – Expression profiles of SIRPα mouse and SIRPα-CBP in bacterial periplasm. A – SDS-PAGE and western blot of SIRPα. 1 - Ladder, 2 – SIRPα before induction, 3 – SIRPα after induction, 4 – 1st extraction, 5 – 2nd extraction, 6 – Flow through, 7 – Wash with PBS, 8 – IMAC, 9 – SEC, 10 – Positive control (SIRPα human). B – SDS-PAGE and western blot of SIRPα-CBP. 1 – Ladder, 2 – SIRPα-CBP before induction, 3 – SIRPα-CBP after induction, 4 – 1st extraction, 5 – 2nd extraction, 6 – Flow through, 7 – Wash with PBS, 8 – IMAC, 9 – SEC 1st peak, 10 – SEC 2nd peak, 11 – positive control (SIRPα human).

Comparing the elution profiles of SEC (Figure 3.13), a high peak corresponding to the Ag was noted, whereas multiple low intensity peaks were observed in case of SIRPα-CBP. A comparison between elution times of the Nbs and SIRPα is also feasible. SIRPα has a slightly lower MW compared to Nbs, thus it was expected to have a slightly longer retention time on the size exclusion column. This was not observed, on the contrary. A faster elution for SIRPα than Nb could be explained if SIRPα does not have a globular shape, but an elongated architecture [55] with a larger hydrodynamic volume than the globular Nb.

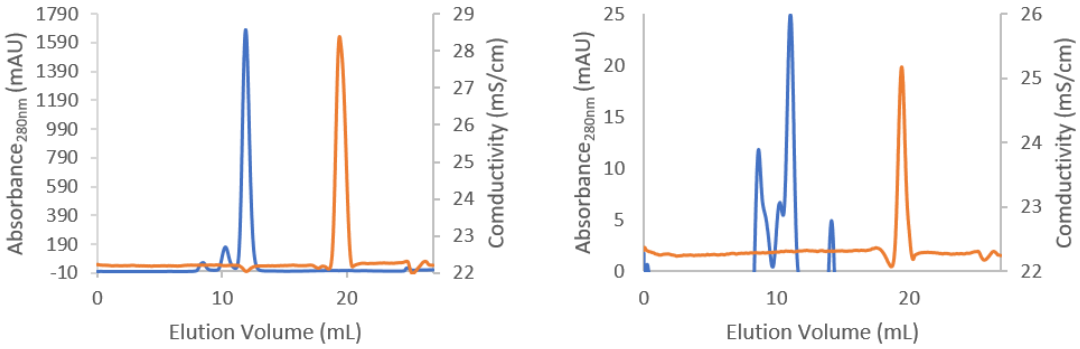


Figure 3.13 - Size Exclusion Chromatography of SIRPα mouse (A) and SIRPα-CBP

The comparison of the amount of SIRP α and SIRPα-CBP, when produced in 1 L of media (Table 3.3), revealed a sharp drop for the latter construct, which might indicate that CBP itself is responsible for the obstruction of the recombinant protein.

Table 3.3 - Amounts obtained after IMAC and SEC of SIRP α and SIRPα-CBP

Clone	Amount (mg) obtained after IMAC from 1 L culture	Amount (mg) obtained after SEC from 1 L culture
SIRPα mouse	8.75	4.56
SIRPα mouse CBP	0.7	Not applicable

3.6 Vectors and CBP Analysis

After suspecting that the problem of expression might be caused by the CBP, further analysis were made. First all the vectors used were analyzed to verify if any other changes occurred while cloning was performed. A comparison between the original pMECS and pMECS-CBP can be seen in Figure 3.14, where it is noted that the only changes that occurred to the vector was the insertion of the CBP sequence (yellow region) comprised in the mutagenic primer used. The rest of the amino acids sequence was maintained, before and after the insertion.

```
                                    20                                  40                                  60
                                     |                                   |                                   |
    pMECS SLRRQYI*NT YCLRQPLDCY YSRPSRPWPR CSCRSLEGTQ VTVSSAAAYP YD-------- 52
pMECS-CBP SLRRQYI*NT YCLRQPLDCY YSRPSRPWPR CSCRSLEGTQ VTVSSAAARW KKAFIAVSAA 60
                                    80                                  100                                 120
                                     |                                   |                                   |
    pMECS -----VPDYG SHHHHHH*TV ESCLAKPHTE NSFTNVWKDD KTLDRYANYE GCLWNATGVV 107
pMECS-CBP NRFKKISGSG SHHHHHH*TV ESCLAKPHTE NSFTNVWKDD KTLDRYANYE GCLWNATGVV 120
                                   140
                                     |
    pMECS VCTGDETQCY GTWVPIGLAI PENEGGGSEG GGSEGGGS 145
pMECS-CBP VCTGDETQCY GTWVPIGLAI PENEGGGSEG GGSEGGGS 158
```

Figure 3.14 – Alignment of original pMECS and pMECS-CBP using CLC Main Workbench 9.9.1 software.

A comparison between all pMECS-CBP vectors was also performed (Figure 3.15). In these, the insertions were made using NcoI and NotI as restrictions enzymes. As it was expected, the sequence differences occurred between those restriction enzymes, while the remaining sequence was conserved, including the CBP domain and the His tag used for purification and immunodetection of the proteins using western blot. Thus, a problem of expression due to incorrect assembly of the vectors is not probable.

```
                                    20          NcoI                    40                                  60
                                     |           |                       |                                   |
  pMECS-30-CBP LKETVHMKYL LPTAAAGLLL LAAQPAMAQV QL---QESGG GLVQAGGSLS LSCLASGITF 57
  pMECS-61-CBP LKETVHMKYL LPTAAAGLLL LAAQPAMAQV QL---QESGG GSVQAGGSLR LSCVDSRYRT 57
  pMECS-67-CBP LKETVHMKYL LPTAAAGLLL LAAQPAMAQV QL---QESGG GSVQAGGSLR LSCAASRYYS 57
  pMECS-68-CBP LKETVHMKYL LPTAAAGLLL LAAQPAMAQV QL---QESGG GLVQPGGSLR LSCAASGFTF 57
  pMECS-69-CBP LKETVHMKYL LPTAAAGLLL LAAQPAMAQV QL---QESGG GSVQTGGSLR LSCAASIYYS 57
  pMECS-75-CBP LKETVHMKYL LPTAAAGLLL LAAQPAMAQV QL---QESGG GLVQPGDSLR LSCAASEFAF 57
pMECS-SIRPa-CBP LKETVHMKYL LPTAAAGLLL LAAQPAMAGK ELKVTQPEKS VSVAAGDSTV LNCTLTSL-L 59
                                    80                                  100                                 120
                                     |                                   |                                   |
  pMECS-30-CBP SISAMTWYRQ APGKQRELVA VISSSGSG-- THYADSVKGR FTISRDNAKN TTYLHMNSLK 115
  pMECS-61-CBP GSNCWGWFRQ VPGKQREAVA GINNSGSSR- TYYADSVKDR FTISQDNAKK VVFLQMNDLK 116
  pMECS-67-CBP -YNCMGWFRQ APGKEREGVA GIY-TGIG-- TYYADSVKGR FTFSRDNAKN TIYLQMNSLK 113
  pMECS-68-CBP SDYFMSWVRQ APGKGLEWVS GIDSDGSN-- TYYASSVKGR FTISRDNAEN TLYLQMNSLK 115
  pMECS-69-CBP -RNCMAWFRQ APGKERERVA GIYNSGAS-- TYYADSVKGR FTISQADAKR TMYLQMNSLK 114
  pMECS-75-CBP SSYFMSWVRQ APGKGLEWVS GINTDGSM-- TYYSDSVKGR FTISRDNAKN TLYLQMNSLK 115
pMECS-SIRPa-CBP PVGPIKWYRG V-GQSRLLIY SFTGEHFPRV TNVSDATK-- ----RNNMDF S--IRISNVT 110
                                    140                                 160          NotI  CBP domain         180
                                     |                                   |            |                       |
  pMECS-30-CBP PEDTAVYYCQ TR-------- ------SYWG QGTQVTV--- SSAAARWKKA FIAVSAANRF 158
  pMECS-61-CBP PGDTAMYYCA SGCVRTW--- --PNGYNFWG QGTQVTV--- SSAAARWKKA FIAVSAANRF 168
  pMECS-67-CBP PEDTAMYYCA AGCGQVMDRT KVPG-FNYWG QGTQVTV--- SSAAARWKKA FIAVSAANRF 169
  pMECS-68-CBP SEDTALYYCA LR-------- GVFSDAALHS QGTQVTV--- SSAAARWKKA FIAVSAANRF 164
  pMECS-69-CBP PEDTAMYYCA AGCAG----- --PGQYNYWG QGTQVTV--- SSAAARWKKA FIAVSAANRF 164
  pMECS-75-CBP SEDTALYYCV LR-------- GVFSDYALQS QGTQVTV--- SSAAARWKKA FIAVSAANRF 164
pMECS-SIRPa-CBP PEDAGTYYCV ----KFQKGP SEPDTEIQSG GGTEVYVLAK PSAAARWKKA FIAVSAANRF 166
                    His tag                 200                                 220
                                             |                                   |
  pMECS-30-CBP KKISGSGSHH HHHH*TVESC LAKPHTENSF TNVWKDDKTL DRYANY 204
  pMECS-61-CBP KKISGSGSHH HHHH*TVESC LAKPHTENSF TNVWKDDKTL DRYANY 214
  pMECS-67-CBP KKISGSGSHH HHHH*TVESC LAKPHTENSF TNVWKDDKTL DRYANY 215
  pMECS-68-CBP KKISGSGSHH HHHH*TVESC LAKPHTENSF TNVWKDDKTL DRYANY 210
  pMECS-69-CBP KKISGSGSHH HHHH*TVESC LAKPHTENSF TNVWKDDKTL DRYANY 210
  pMECS-75-CBP KKISGSGSHH HHHH*TVESC LAKPHTENSF TNVWKDDKTL DRYANY 210
pMECS-SIRPa-CBP KKISGSGSHH HHHH*TVESC LAKPHTENSF TNVWKDDKTL DRYANY 212
```

Figure 3.15 – Alignment of all pMECS-Nb-CBP and pMECS-SIRPa-CBP using CLC Main Workbench 9.9.1 software.

Since the presence of CBP was likely causing the expression drop, we wonder whether it was the protein or the nucleotide sequence that was responsible. We, therefore, compared the coding sequences of our clones with that of a CBP tag that was described previously and that was reported to give good expression levels [44] (Figure 3.16).

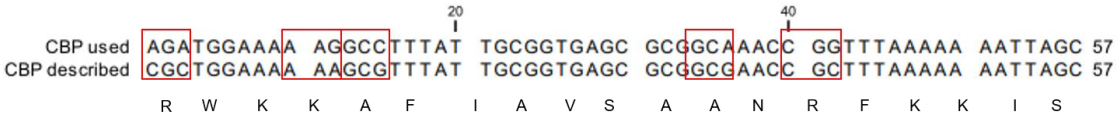


Figure 3.16 – Alignment of CBP used and CBP described in literature [44] using CLC Main Workbench 9.9.1 software.

By comparing the sequence, it became apparent that even though both are encoding the same amino acid sequence, 5 nucleotide mutations were observed, two in Arginine, two in Alanine and one in Lysine codons. These different nucleotide sequences of the tag might cause a different mRNA secondary structure that prevents optimal translation [56]. Alternatively, the preferred codon usage of *E. coli* could be responsible for the reduced expression as well [57]. Among the rare codons are AGA and CGG, two codons that were occurring in our CBP design. Additionally, one of these Arginines is the first codon of the CBP peptide, which could be directly related to the expression problems encountered throughout the experiments. If this hypothesis is correct, then the design of a new mutagenic primer and consequently a new gene for the CBP tag might solve (or remediate) the expression problems we encountered and might allow to proceed with this project.

Although changes on the sequence were found, it is justified to question whether the problem could be related with the exportation of the Nb-CBP to the periplasm through the Sec pathway [58]. If this was correct, a cytoplasmic expression should be performed, using a T7 expression vector without the peptide signal pelB. Even though the expression might be enhanced, another problem will rise using M13 bacteriophages for phage display. The propagation mechanism of M13 requires that all components of the phage particle can be translocated across the bacterial inner membrane before they are assembled into the phage [59]. Thus, if CBP could only be expressed in the cytoplasm, its exportation to the inner membrane would not be possible and enrichment by phage display would not occur and alternatives to this project needed to be found.

4 Conclusions

The aim of this master thesis was to develop a new phage display vector that would improve selection and screening methods of the technique.

The first goal was to create a vector containing the CBP tag that would be compatible for phage display. To this end, the HA tag located downstream of the Nb cloning site present in pMECS-GG was substituted by the CBP tag. Moreover, 6 Nbs with well-known and variable kinetic rates were also inserted into the pMECS-CBP vector.

Next, the vector was used in a phage display setting, with already selected Nbs to verify that it could be used for biopanning. For this purpose, two mini libraries comprising the 6 Nbs were made. One library was made with the conventional pMECS vector while the other was made using the pMECS-CBP vector. Although a 1000-fold enrichment on antigen was achieved using pMECS, the virions from pMECS-CBP failed to show any enrichment. After that failure, it was suspected that an expression problem might have occurred.

Trying to express the Nbs with the CBP tag became a real challenge, especially because expressing them without the CBP tag was so easy and yielded around 3 mg/L of culture were observed. Several approaches were amended, such as adding lysozyme, to improve the periplasmic extraction step and expressing 5 different colonies to check for possible correlations.

All these attempts failed to improve on the Nb expression. We therefore inserted a totally different protein in the CBP containing vector to discriminate between the Nb-CBP fusion and the CBP itself as the core of the expression problems. A high yield expressor (SIRPα) was cloned into the CBP vector. Even though SIRPα expressed at levels above 8 mg/L of culture, the expression from a vector that would attach a CBP at the recombinant protein suffered from a severe reduction in yield to a level below 1 mg per L of culture.

A further analysis of the CBP tag was performed, following an alignment of our CBP sequence with that of a CBP tag, described to be an enhancer of expression. While having the same amino acid sequence, it was found that 5 codons differed, two Arginine codons, two Alanine codons and one Lysine codon. Moreover, among these codons were two codons rarely used in *E. coli*. We surmise that this unfortunate choice of codons might provoke a reduced expression noted in this thesis.

5 Future Perspectives

Phage display is a widely used molecular screening technique for the selection of the Nb binders against a specific target. However, it still suffers from some drawbacks, such as limits of enrichment that can be achieved per round of selection, undesired background created and a time-consuming screening to identify the Nbs with the best binding affinity. To solve these problems this thesis was created, however soon another problem came across us, expressing the CBP tag.

Although the *E. coli* expression system is the most used for the production of proteins, it is still often that researchers find difficulties of expression [60]. Factors that affect the processes of transcription, mRNA processing, translation, post-translational modification, protein folding and assembly and protein expression can influence product yield [56]. Moreover, most of the times, problems with the interference on processes of expression are related with the sequence of the protein expressed [50]. In this case, we found that that could be the reason for the reduced expression noted throughout this thesis. Further steps could be the creation of a new CBP tag that was more *E. coli* friendly so that it could be expressed and used as a phage display vector. Another strategy could be changing the strain used for expression since some *E. coli* strains, such as Rosetta (DE3) from Novagen or BL21-CodonPlus from Stratagene, can supply tRNAs that recognize rare codons in the organism [61]. However, to be used with this pMECS-CBP vector, these strains need to be able to suppress the amber codon present in this vector, while being used for phage display.

After solving the problems of expression, the discovery of the best conditions, such as buffers, concentrations and incubation times, for the effective and efficient reversible binding between CaM and CBP is also crucial for the success of this project, since the only information known is that they bind in the presence of Ca^{2+} ions and are released in the presence of a calcium chelator, such as EDTA [44].

Even though changes to the sequence were found, problems with secretion of the Nb-CBP to the periplasmic space through the Sec pathway could still be occurring. A cytoplasmic expression might solve the problem by using a T7 expression vector [58 62]. In this system, T7 RNA polymerase is under the control of a pLac promoter in the host cells, such as BL21 (DE3), while the Nb-CBP should be under the control of a T7 promoter [61]. After being induced by IPTG, T7 RNA polymerase is expressed, thus the expression of Nb-CBP is also induced to the cytoplasm [61]. If the cytoplasmic expression is efficient, the usage M13 bacteriophages for phage display may not be possible due to the requirement of all components of the phage particle in the inner membrane before assembly [59]. Thus, if CBP could only be expressed in the cytoplasm, phage display using this vector might not be possible and other alternatives to this new phage display system should be found.

Another direction could be using a different system that has the same features as the one presented here between CaM and CBP. A recently presented tag called ALFA-tag could be a good alternative, since it shows a reversible high affinity (nanomolar) with an engineered Nb [63]. This affinity is reversible by the addition of free ALFA peptide [63].

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