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Characterization of anti-Pneumococcal surface protein C affibodies using surface plasmon resonance technology

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Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Preface

The work presented in this thesis was performed at the Department of Protein Science of KTH Royal Institute of Technology (Stockholm, Sweden) during the period of February-December 2020 under the supervision of Prof. Per-Åke Nygren, and within the framework of the Erasmus programme. The thesis was co-supervised at Instituto Superior Técnico by Prof. Ana Azevedo.

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Abstract

Streptococcus pneumoniae is a gram-positive bacterium responsible for a number of both non-invasive and invasive diseases. Pneumococcal surface protein C (PspC), a protein of *S. pneumoniae*, plays an important role in this pathogen's ability to colonize and cause disease through binding human Factor H (FH), a complement control protein. In this thesis surface plasmon resonance (SPR) techniques were employed to measure the affinity of affibodies (small engineered affinity proteins) selected for PspC2 from the BHN418 strain of *S. pneumoniae* to three PspC proteins (PspC1 and PspC2 from BHN418, and PspC from TIGR4). Other main goals involved mapping the affibodies to a specific region of PspC2 and determining if established binding of FH by PspCX (PspC1, PspC2, PspC) could be disrupted by the presence of affibodies. The performed assays show the affibodies, especially in a heterodimer format, display very high affinity and strong binding to PspC2 but do not bind the other tested proteins. In the tested setup the affibodies could not interfere with established FH:PspC2 interaction, however further steps are proposed to better understand the roles affibodies could play in this context. A secondary goal was to determine the crystal structure of PspC2 and pave the way for obtaining structures of affibody:PspC2 complexes. Small needle crystals were obtained, but they didn't have the required quality to be used in X-Ray diffraction and eventually solve the crystal structure. As such, further optimization of the conditions is required.

Keywords

Pneumococcal surface protein C; Affibodies; Surface Plasmon Resonance; Factor H

Resumo

Streptococcus pneumoniae é uma bactéria gram-positiva responsável por múltiplas doenças, tanto invasivas como não invasivas. *Pneumococcal surface protein C* (PspC), uma proteína de *Streptococcus pneumoniae*, tem um papel importante na capacidade colonizadora e patogénica deste organismo, uma vez que se consegue ligar a Factor H (FH), uma proteína do sistema de complemento humano. Nesta tese, técnicas de *surface plasmon resonance* (SPR) foram utilizadas para medir a afinidade de *affibodies* (pequenas proteínas de afinidade) selecionadas para PspC2 da estirpe BHN418 de *Streptococcus pneumoniae* a três proteínas (PspC1 e PspC2 de BHN418 e PspC da estirpe TIGR4). Outros objetivos principais envolveram mapear *affibodies* a uma região específica de PspC2 e determinar se a presença de *affibodies* interferia com ligação pré-estabelecida de PspCX (PspC1, PspC2, PspC) a FH. Os ensaios realizados demonstraram que os *affibodies*, especialmente sob a forma de heterodímeros, mostravam afinidade muito elevada e ligação forte a PspC2 mas não se ligavam às restantes proteínas testadas. No *setup* testado os *affibodies* não interferiram com ligação pré-estabelecida de FH:PspC2. Face a estes resultados sugere-se a realização de experiências futuras para melhor compreensão do papel que estas pequenas moléculas podem assumir neste contexto. Um objetivo secundário foi a determinação da estrutura cristalina de PspC2 e a abertura de caminho para determinação da estrutura de complexos *affibodies*:PspC2. Pequenos cristais foram obtidos mas não possuíam a qualidade necessária para poderem ser utilizados em difração de raios-X e resolver a estrutura. Como tal, mais otimização das condições é necessária.

Palavras Chave

Proteína pneumocócica de superfície C; Affibodies; Ressonância Plasmónica de Superfície; Fator H

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Acronyms

ABD	Albumin Binding Domain
BSA	Bovine Serum Albumin
C3	Complement protein 3
CbpA	Choline binding protein A
CS	Complement system
CTerm	C-Terminal fragment
CV	Column volumes
DTT	Dithiothreitol
FC	Flow Cell
FH	Factor H
FHBD	FH binding domain
HER2	Human epidermal growth factor receptor 2
Hic	Factor H-binding inhibitor of complement
HR	Hormone receptor
IL-17A	Interleukin 17A
IMAC	Immobilized metal affinity chromatography
LRI	Lower respiratory tract infections
PCR	Polymerase chain reaction
PET	Positron emission tomography
PS	Polysaccharide
PspC	Pneumococcal surface protein C
SEC	Size exclusion chromatography
SPA	Staphylococcal protein A
SPR	Surface plasmon resonance
SpsA	*Streptococcus pneumoniae* secretory IgA binding protein

1

Introduction

Contents

1.1 Organization of the document

There are five chapters in this thesis, the first one being this introductory chapter constituted of a state of the art in diagnosis and therapeutic options for infection by *S. pneumoniae*, as well as an aim of studies portion divided in main and secondary goals.
In Chapter 2, an overview of the topic is presented, where data on *Streptococcus pneumoniae* including pathogenicity and mechanisms of infection is detailed, as well as the importance of the protein in study, pneumococcal surface protein C (PspC). There is also a section dedicated to affibody binding proteins including a description of this technology, illustrating how they are currently being adopted and ideas for applications in the context of this thesis and future works on this topic. Finally, the chapter ends with an explanation of surface plasmon resonance (SPR) and crystallography, the main techniques used in this thesis.
Chapter 3 consists of all the materials required for lab work that was conduced, as well as the methods that were followed, for each of the techniques used.
In Chapter 4 results obtained from the purification process of affibodies and PspCX (i.e. PspC2 and PspC1 from the BHN418 strain and PspC from the TIGR4 strain), SPR and crystallography are presented and discussed.
Finally in Chapter 5, the main and secondary goals are revisited with conclusions from the obtained results, and future prospects are discussed.

1.2 State of the art in diagnosis and treatment of *Streptococcus pneumoniae*

Streptococcus pneumoniae is a gram-positive bacterium responsible for a number of both non-invasive diseases, such as pneumonia, sinusitis and otitis media, and invasive ones, such as meningitis and bacteremia. Given *S. pneumoniae's* asymptomatic colonization of the nasopharynx, preventing pathogenicity of this microorganism lies in the host maintaining the equilibrium between *S. pneumoniae* and the nasopharyngeal flora, while the immune system acts to eliminate the pathogen [1]. If the host's defenses are in some way compromised and unable to successfully remove the invader, the presence of *S. pneumoniae* can evolve to lower respiratory tract infections (LRI), i.e. infections that affect the airways below the larynx, or even invasive diseases. Pneumococcal pneumonia caused by *S. pneumoniae* has consistently been the deadliest LRI aetiology, having caused an estimated 1.2 million deaths, globally in 2017 alone [2, Appendix 2].
The three main strategies through which *S. pneumoniae* ensures efficient colonization and pathogenicity are (i) adhesion and invasion of the epithelial tissues, (ii) evasion of the host's immune response,

(iii) biofilm formation [3]. PspC plays a key role in all of these strategies, through its binding of soluble protein Factor H (FH) [4–7].

There are several ways to diagnose infection by *S. pneumoniae* according to surveillance standards from the World Health Organization [8]. Diagnosis for either non-invasive or invasive disease starts by performing physical exams, analysing the patient's medical history and checking for disease symptoms. For suspected pneumonia, a chest X-ray displaying findings consistent with it is a useful diagnosis tool, however *S. pneumoniae* infection can only be confirmed if there is a positive culture from blood or pleural fluid. For invasive diseases such as sepsis and meningitis, cerebrospinal fluid or blood are collected and *S. pneumoniae* infection is confirmed again through either positive culture or methods such as antigen detection, immunochromatography or polymerase chain reaction (PCR). When it comes to treatment, broad-spectrum antibiotics constitute the first line in cases of pneumococcal disease [9]. They are administered while antibiotic sensitivity tests are performed to determine which narrow-spectrum antibiotic is most suitable. It is worth mentioning however that even though the efficacy of the pneumococcal conjugate vaccine coupled with appropriate use of antibiotics contributes to reduction of antibiotic resistance in *S. pneumoniae*, currently in ca. 30% of known cases pneumococcal bacteria are resistant to one or more of the administered antibiotics [9].

It was from this clear need for development of therapeutic alternatives, that interest in the use of affibody molecules stemmed. Affibodies are small engineered affinity proteins with a number of attractive features such as being highly stable, highly soluble and easily coupled to other molecules for many different purposes [10]. Applied to *S. pneumoniae* in a therapeutic or diagnostic setting, there are many ways in which their use could prove interesting, such as:

1. If affibodies are capable of targeting PspC specifically and strongly enough, they could be used to target *S. pneumoniae* for microscopic imaging purposes or even to deliver targeted attacks;
2. If affibodies are capable of blocking adhesion of the bacteria to epithelial cells, they could function as de-colonization agents, potentially having a therapeutic effect;
3. If affibodies are capable of disrupting established FH:PspC binding, or compete with FH, they could theoretically also impede some of the complement system (CS) binding dependent mechanisms used by *S. pneumoniae*.

1.3 Aim of studies

There are three different PspC proteins being studied for this thesis, PspC (from TIGR4), PspC1 and PspC2 (both from BHN418) (see Section 2.1.3 for more details). The affibodies described in this study were selected for binding to PspC2 and the goal for this thesis was to perform domain mapping of the affibodies on PspCX (i.e. PspC, PspC1 and PspC2), through binding studies using SPR. This would

involve:

1. Assessing if the affibodies are specific for PspC2, or if they are cross-reacting with PspC and PspC1;
2. Assessing whether affibodies are binding preferentially to a specific domain in PspC2;
3. Investigating the occurrence of ”super-binding” i.e. two affibodies that bind different epitopes still being able to bind them on the same protein molecule when formatted into a heterodimeric form;
4. Investigating whether established binding of FH by PspCX can be disrupted by the presence of affibodies.

A secondary goal was to obtain the crystal structure of PspC2, and in the long run pave the way for also obtaining structures of complexes between PspC2 and affibodies.

The information obtained from this study would allow for mapping of affibody and FH interactions with PspCX. This would be a very valuable tool in many dimensions given the importance of FH binding by PspC to *S. pneumoniae* for colonization and invasive disease.

2

Literature Review

Contents

2.1 *Streptococcus pneumoniae*

2.1.1 Pathogenicity and mortality rates

S. pneumoniae is a commensal microorganism of the nasopharynx, that colonizes it asymptomatically. Carriage of this microorganism is specially high in children, appearing to be up to a certain point inversely proportional to age [1, 11].

The key to preventing pathogenicity of *S. pneumoniae* resides in the host being able to keep the balance between its nasopharyngeal natural flora and the invader organisms, while the immune system acts to clear them [1]. This can be harder when for instance, an infection with viruses such as influenza is affecting the upper respiratory tract. The resulting inflammation creates an environment that is much more favorable to colonization by *S. pneumoniae* [3]. As such, ensuring that *S. pneumoniae* doesn't become pathogenic and evolve into LRI, or possibly even invasive diseases, is heavily linked to the strength of the immune system. Consequently immunocompromised individuals, children and the elderly are the most susceptible to infections by pathogens such as *S. pneumoniae*: children under the age of 5 don't have a fully developed immune system yet, while after a certain age people's immune system starts deteriorating, hence they can no longer clear pathogens as efficiently [1]. Globally in 2017, pneumonia caused by *S. pneumoniae* lead to the death of an estimated 381 000 children under the age of 5, and of 456 000 adults over the age of 70 [2, Appendix 2].

2.1.2 The role of pneumococcal surface protein C in *S. pneumoniae*'s mechanisms of infection

S. pneumoniae's ability to colonize and cause disease relies heavily on its ability to (i) ensure adhesion and invasion of the epithelial tissues, (ii) evade the host's immune response, (iii) form a biofilm [3]. PspC plays an important role in all three of these strategies. PspC and its many allelic forms have also been referred to as choline binding protein A (CbpA) [12], *Streptococcus pneumoniae* secretory IgA binding protein (SpsA) [13], factor H-binding inhibitor of complement (Hic) [14] and C3-binding protein [15].

Adhesion to the tissues

Since colonization is the initial step of infection, *S. pneumoniae's* ability to adhere to the tissues of the nasopharynx is essential. There are multiple surface adhesins that play a role in the binding process through different mechanisms. PspC is part of the group, achieving this by binding different proteins, including FH [4]. Agarwall et al. [5] proposed and observed a two-step mechanism through which the binding of FH helped ensure pneumococcal adhesion and invasion of epithelial cells. Essential

to this process were the interactions of surface glycosaminoglycans, integrin receptors and signalling molecules from different pathways, all of them triggered by the binding of FH by PspC.

Evasion of the immune response

S. pneumoniae is capable of evading the immune defense mechanisms by interfering with the first line of defense in the innate immune response, the CS. The CS comprises a variety of plasma proteins that interact with each other, giving rise to a cascade of inflammatory processes in response to an infection [16]. The CS helps fight infection through: (i) opsonizing pathogens, i.e. marking them for destruction by phagocytes through covalent attachment of activated complement proteins, (ii) recruiting phagocytes to the infection site and activating them, (iii) lysing pathogens through the action of a membrane-attack complex that will form pores in their membrane [16, 17].

There are three pathways that can lead to the activation of the CS (a) the alternative pathway, (b) the lectin pathway and (c) the classical pathway. Through different mechanisms, all three of them lead to the cleavage and activation of complement protein 3 (C3) into C3a (the smallest fragment, which will act as a chemoattractant for the phagocytes) and C3b (the largest fragment, which will bind to the pathogen and induce opsonization) [16]. It is this activation of C3 that triggers the mechanisms through which the CS fights against pathogens, making it the most important protein of the complement system [17]. It is worth mentioning that while the lectin and classical pathways are only initiated after the recognition of specific molecules takes place, the alternative pathway does not require a trigger for activation [6]. The fact that such a potentially destructive process can be spontaneously activated makes the tight regulation placed on it by the organism that much more important. This regulatory system heavily relies on a number of proteins to stop the inflammatory cascade at different points, among which is FH.

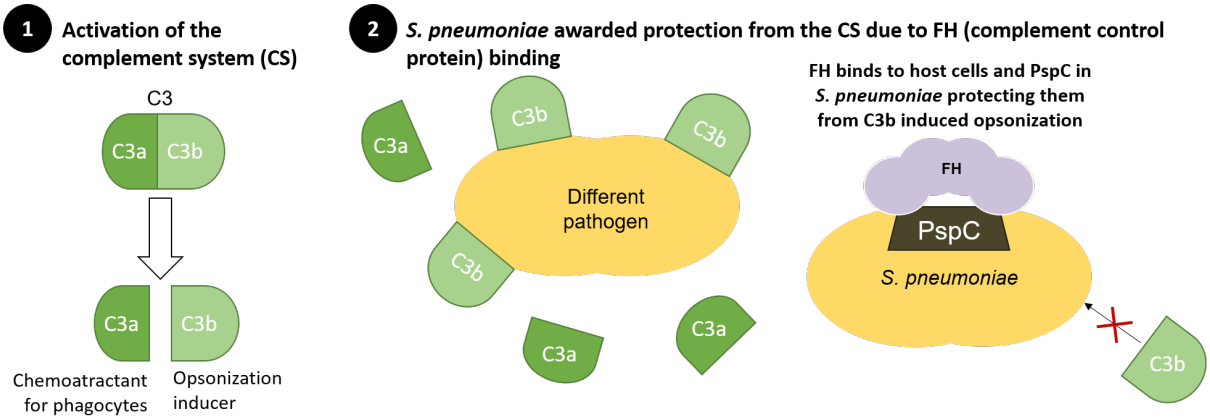


Figure 2.1: Schematic representation of how *S. pneumoniae* evades the complement system binding-dependent mechanisms.

Briefly, C3b can bind pathogens and host cells, which would lead to the destruction of both. FH upon

recognition of host or similar markers in the cell surface, can bind C3b inactivating it and preventing further deposition, leading to de facto protection of the cells it binds [6]. Since PspC binds FH, *S. pneumoniae* is awarded this protection as well (see Figure 2.1).

Biofilm formation

A biofilm was defined as ”matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces” by Costerton et al. [18, p.712]. These populations can be constituted by either a single species or multiple microbial species, and are capable of acting collectively and in a coordinated manner, as a multi-cellular organism would [19].
Existence as a biofilm comes with many advantages to organisms such as increased resistance to environmental stresses, mostly due to the presence of the matrix and the creation of what is effectively a circulatory system in the community to ensure better nutrient availability [19]. Besides these more general advantages, *S. pneumoniae* also benefits from others such as being able to diminish the activation of both the classical and the alternative pathways of the CS, as reported by Domenech et al. in 2013 [7]. The latter is achieved by having much higher levels of PspC at the surface of the biofilm than in just a single organism, which leads to an increase in FH binding. As mentioned before, higher levels of FH binding impair the activation of the alternative pathway, allowing *S. pneumoniae* to bypass the first line of defense that is the CS.

2.1.3 Pneumococcal surface protein C in the different serotypes

As of 2015, 97 different serotypes of *S. pneumoniae* have been identified and divided into 46 different serogroups [20]. This classification is made according to the Danish system, where a serotype is defined as ”pneumococcal strains producing a [capsular] polysaccharide (PS) with unique chemical structure and serologic (immunologic) properties” [20, p.873] and a serogroup as ”[including] serotypes that share many serologic properties (i.e. cross-reactive antibodies)” [20, p.873]. The focus of this thesis was on two different strains of *S. pneumoniae*: TIGR4 (serotype 4) and BHN418 (serotype 6B).
When it comes to PspC, 11 major groups of proteins have been identified. These groups comprise two different variants in terms of anchor sequence: groups 1 to 6 possess a choline-binding domain, whereas groups 7 to 11 rely on an LXPTG motif for anchorage [21]. Additionally, different strains of *S. pneumoniae* can have either a single copy of the gene (like TIGR4) or two tandem copies (like BHN418), which translates into having one or two PspC proteins, respectively. The nomenclature used for the three different PspCs in study, as well as which variant they correspond to is:

- From TIGR4: **PspC** (PspC3.4 variant) [22];
- From BHN418: **PspC1** (PspC6.9 variant, carrying a choline-binding domain); **PspC2** (PspC9.4 variant, carrying an LPSTG motif) [21, 23].

Pathak et al. [24] using peptide mapping and sequence alignments postulated that PspC2 [BHN418] and PspC [TIGR4] belonged to the same family in regards to FH binding, whereas PspC1 [BHN418] belonged to a different one.

2.2 Affibodies

Affibody molecules are small engineered binding proteins. They are constituted by a 58 amino acid scaffold, organized in a three helix-bundle, based on an engineered IgG-binding domain (Z Domain) derived from the B Domain of staphylococcal protein A (SPA) [25]. To create an affibody library, typically 13-14 amino acid positions located at the surfaces of helices 1 and 2 are genetically randomized (see Figure 2.2). ”Naïve” affibody libraries used to be constructed using degenerate codons but more recently defined trinucleotide codon mixtures have been used instead [26]. This change allows for more control over the randomization process, e.g. through the exclusion from the mix of proline codons, since prolines can either break or kink an alpha-helix, and cysteines, for if they are not present in the affibody's sequence they can be introduced later, either at the N-terminal or the C-terminal, for the purposes of conjugation via thiol coupling [27].

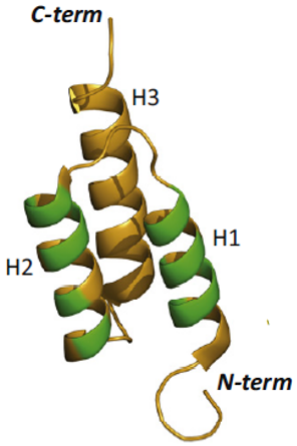


Figure 2.2: Structural model of an affibody molecule. The three-helix bundle Z-domain scaffold is indicated in brown, whereas in green are the genetically randomized 13 to 14 amino acid positions. Helices 1, 2 and 3 are indicated by H1, H2 and H3, respectively. Figure adapted from [26].

From the naïve library, target-binding variants can be selected through multiple display techniques, one of the most popular being phage display. If the affinity of the first-gen affibodies needs to be improved on, they can undergo ”affinity maturation” a process where the knowledge obtained from naïve libraries serves as basis for the development of secondary libraries, where the second-gen affibodies should have higher affinity to the target. This process is usually done either through checking the similarities between a panel of affibodies found to bind the target, or through alanine scanning, where each of

the 13-14 randomized amino acids of a given binder is changed for an alanine in order to identify the importance of the residue to the stability and binding activity of the affibody [26, 28].

2.2.1 Affibody use for different purposes

At the moment antibodies remain the molecule of excellence for affinity purposes in life science applications. However, engineered affinity proteins such as affibodies are gaining ground in both imaging and therapeutic applications [29]. Among the reasons why are their small size (ca. 6.5kDa to an antibody's 150kDa), high stability independent from disulfide bridges, high solubility and ability to be easily coupled to radioactive tracers and optical reporter groups [10, 29]. Currently there are several completed and ongoing clinical trials involving affibodies.

Diagnostic and imaging purposes

The affibody's small size allows for rapid tissue penetration and fast blood clearance [26], both important for *in vivo* imaging purposes. This is an advantage in e.g. radionuclide imaging where the affibody is coupled with a radioactive tracer since it allows for high-contrast tumor imaging to be obtained with lower amounts of exposure to radiation for the patient [30]. One of the ongoing Phase II/III trials concerns the non-invasive quantification of HER2-expression in advanced breast cancer (EudraCT 2017-002115-34). Here, the aim is to assess how the affibody ^{68}Ga-ABY-025 (developed by Affibody AB) used as tracer in a PET scan compares to the gold standard histopathology. ^{68}Ga-ABY-025 PET had already shown promising results in the Phase I/II trial (EudraCT 2012-005228-14), being able to accurately quantify HER2 across the body in patients with metastatic breast cancer [31].
For *in vitro* imaging applications, fusing or coupling affibodies to fluorescent molecules allows for fluorescent -based diagnostic assays such as flow cytometry to be performed [10].

Therapeutic purposes

On the therapeutic front, there are several ways in which affibodies could be of interest. By developing an affibody that e.g. competes with a given ligand of interest for an epitope or binds the ligand itself, it is possible to block protein-ligand binding, which could be useful in disease associated interactions [26]. The affibody ABY-035 (developed by Affibody AB), an interleukin 17A (IL-17A) inhibitor binding both its sub units as well as albumin, is currently being investigated in two Phase II clinical trials. These trials involve assessing the efficacy of different dose regimens of ABY-035 compared to placebo in patients with moderate to severe plaque psoriasis (EudraCT 2017-001615-36) and with active psoriatic arthritis (EudraCT 2019-003405-94). IL-17A was identified as the main effector cytokine driving these diseases, with its inhibition disrupting essential pathways to pathogenesis leading to symptom relief [32].

Another approach could be through payload strategies, in which the affibodies are coupled or fused to toxins or cytotoxic drugs and target the protein they interact with specifically [26].

Affibodies and pneumococcal surface protein C

In the context of this thesis and future works on the topic several of these strategies could be applied. On the blocking protein-protein interactions front, finding an affibody that could effectively disrupt the interaction between PspC and FH could be very useful, given that this interaction is a cornerstone of *S. pneumoniae's* strategy for colonization and causing disease. If an affibody displays sufficient affinity and specificity to a PspC variant, another strategy could be to couple it to a cytotoxic compound and launch a targeted attack on pneumococci.

2.3 Techniques

2.3.1 Surface plasmon resonance

Surface plasmon resonance (SPR) is an optical method that can be employed in a variety of studies for characterization of biomolecules and their interactions. Examples of these include kinetic and thermodynamic studies, as well as determination of analyte concentrations in a given sample [33].
The SPR phenomenon occurs in thin metal films (typically, gold or silver [33, 34]) between two media with different refractive indexes, but only at a specific angle of incident light. In these conditions, the energy provided by the light beam leads to the creation of surface electromagnetic waves in the metal. To extract information from the electromagnetic waves and transform it into useful information for the study being performed, SPR-based instruments employ optical sensors. These sensors are typically composed of (a) a glass slide covered by a thin gold film, (b) a linking layer, to bind the gold film to the immobilization matrix, (c) an immobilization matrix made up of e.g. carboxymethylated dextran, where the ligand is bound, (d) ligand molecules, which should have specific interaction with the analyte [33]. In this setup, the glass slide constitutes one of the media, while the sample solution where the analyte is, constitutes the other.
Essentially, an electromagnetic component of the light beam ("evanescent wave") shone on the glass slide, will propagate to the sensor's gold film, causing excitation of surface plasmons. This translates to a sharp dip in the intensity of reflected light, given that the energy is being absorbed by the evanescent wave field [35]. Given that parameters such as temperature and incident light are kept constant, this wave field is directly dependent on the refractive index of the sample solution. It is also very sensitive to any changes in the environment, e.g. the binding of analyte molecules to ligand molecules, which causes alterations in the refractive index of the sample solution [33]. As such, to obtain the desired

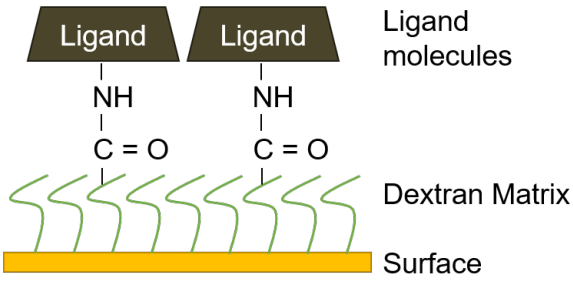


Figure 2.3: Schematics of the sensor chip surface. The ligand molecules are covalently immobilized to the surface through amine coupling, the chosen immobilization method for the purposes of this thesis. Figure adapted from [35].

information the instrument will monitor changes in the refractive index in real-time, transforming them into a so called sensorgram. These changes are proportional to the amount of analyte binding the ligand [33, 35]. By analysing the sensorgram, it's possible to determine the overall affinity and binding kinetics between ligand and analyte, as well as to assess specificity. The main advantages over other techniques include that it doesn't require labels and that only a small amount of sample is necessary (microfluidics system) [33, 34].

By making use of this technique it is possible to determine the affibodies and FH's affinity to PspCX proteins.

2.3.2 Crystallography

Crystallography is an experimental science used to determine the structure and properties of crystals. A very useful application of this science is to proteins and other macromolecules such as nucleic acids. If the crystals are appropriately sized and of high enough quality, they can in turn be used in X-ray crystallography to obtain highly precise structures of the macromolecules in question [36]. This technique consists of analyzing the diffraction patterns produced when X-rays strike crystals. By applying Fourier analysis to the diffraction patterns it's possible to obtain a 3D structure of the molecules that is precise down to the atomic level [37].

Growing crystals

To obtain crystals it is necessary to first supersaturate a solution in the macromolecule in question. In a supersaturated solution the macromolecule is present in concentrations above the solubility limit. For the system to reach equilibrium once again, an outlet in the form of a macromolecule in solid state (e.g. crystals) needs to form. The main challenge in this process is to find the biochemical and physical conditions in which this is possible, with the optimization of e.g. pH, temperature, precipitant type, among others, being a rather time-consuming process. Thus, the first step when dealing with an untested

macro-molecule is commonly a shotgun approach, where commercially available crystallization screening kits are employed [38]. This allows for several conditions to be tested simultaneously, and if any hits are obtained provides a good baseline for further optimization.

There are many methods to grow crystals with the most widely used being vapor diffusion in two setups, the sitting drop (see Figure 2.4) and the hanging drop. In this technique two solutions are involved: the reservoir solution, a solution with high concentration of precipitant, and the protein solution. A drop of crystallization solution (half protein solution and half reservoir solution) either ”sits” on a plastic support or ”hangs” from a glass cover slip, hence the names. In a well, reservoir solution is present at higher volume and higher precipitant concentration. Since in a sealed environment the crystallization droplet and the reservoir solution tend towards osmotic equilibrium, water from the droplet will begin to evaporate as the system inches closer to this state. This promotes supersaturation of the crystallization droplet in both protein and precipitant, a necessary condition for crystals to develop.

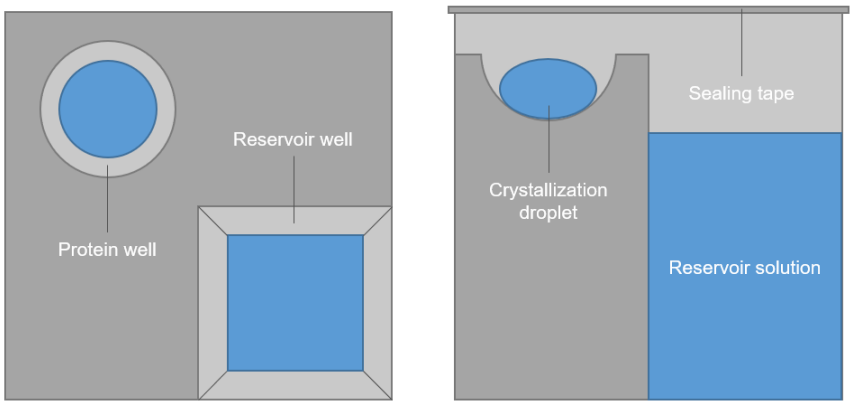


Figure 2.4: Schematics of the crystal wells for the sitting drop method. On the left is a view from above and on the right a side view. In the protein well there is a droplet consisting of half protein solution and half reservoir solution. In the reservoir well reservoir solution is present in larger volume and containing a higher concentration of precipitant than the crystallization droplet.

A variation of this technique involves the addition of a layer of low density oil placed over the reservoir solution, which will slow down the rate of water vapour diffusion to the reservoir leading to less crystals of a larger size developing [39].

The importance of structure

Knowing the structure of a protein can provide crucial information not only about its function but also about the development of potential binders. Throughout the years, structure-guided drug design has lead to many breakthroughs in the healthcare industry, from the development of antiretrovirals targeting HIV that greatly increased life expectancy in carriers of the virus [40] to the CDK4/6 inhibitor drug Kisqali® from Novartis for the treatment of HR+/HER2- advanced or metastatic breast cancer [41, 42], as well as many others.

As such, determining the structure of the PspCX proteins by themselves or even bound to affibodies or FH could be helpful in determining where exactly these are binding, as well as gathering more detailed information into where a binder could disrupt a PspCX:FH interaction.

3

Materials and Methods

Contents

3.1 Protein and affibody constructs

This study is focused on the N-terminal region of PspC from TIGR4, PspC1 and PspC2 from BHN418. These truncated proteins will be indicated by a T in the name from here on out. For PspC2, the FH binding domain (FHBD) as well as the C-Terminal fragment (CTerm) were also tested (see Figure 3.1). The constructs used for the purposes of this study are summarized in Figure 3.2 below.

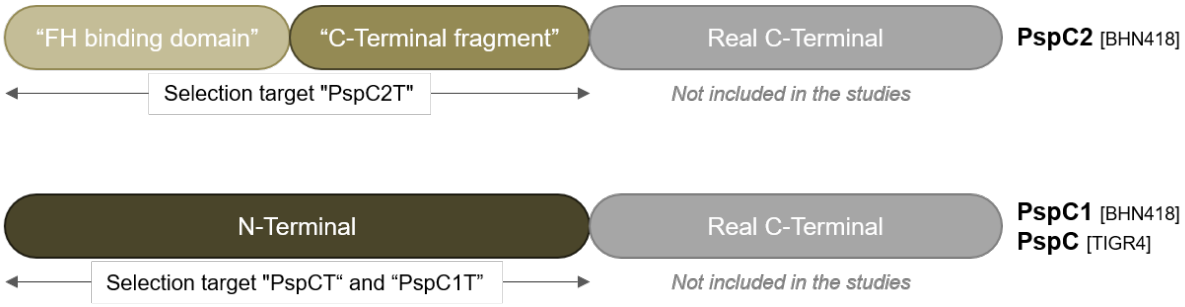


Figure 3.1: Schematic view of the truncated proteins PspCXT (i.e. PspCT, PspC1T and PspC2T). All correspond to the N-terminal region of the full length protein, but for PspC2T the FHBD and CTerm will also be used for the experimental work.

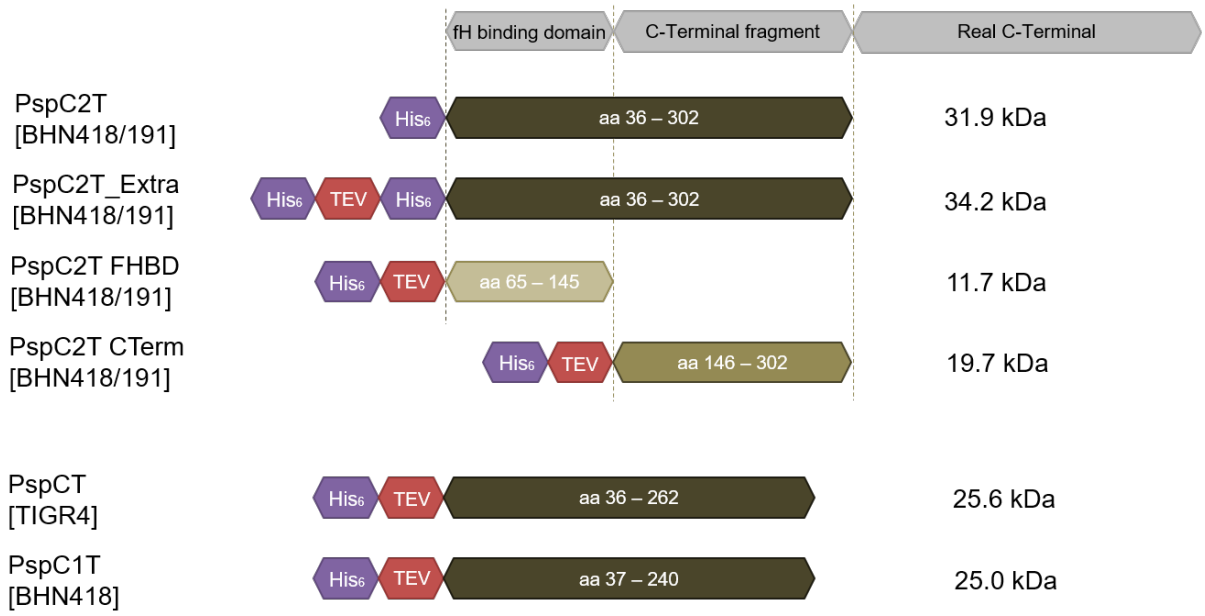


Figure 3.2: Constructs schematics and nomenclature used throughout this thesis. The strain from which they derive is indicated between brackets. Two strains are indicated for PspC2T (BHN418 and BHN191) because the N-Terminals of these two are identical. His6 (in the purple box) is the polyhistidine His-tag, TEV (in the red box) is the TEV cleavage site. The aa. to which the constructs correspond in the full length proteins are indicated in the brown boxes. The size of each construct in kDa is indicated on the right.

The gene encoding for every construct described above was cloned in a pET28 T7 expression vector (resistant to kanamycin) for heterologous expression of the proteins in *E. coli*.

The affibodies used were developed through phage display and selected prior to the beginning of this thesis based on affinity shown to PspC2T in SPR and ELISA assays. A number of affibodies were tested and separated into two non-competitive groups, based on pairwise epitope binning. From each of those groups, the strongest binder was selected. These affibodies were named A4 and D9. Afterwards, dimer constructs of the selected binders were cloned (both homo and heterodimers) in order to test their affinity in this format and see if there had been significant differences. As with the proteins, T7 expression vectors encoding for the required GFP-fused affibody constructs were already available (see Table 3.1 and Figure 3.3).

Table 3.1: Affibody constructs, produced and purified.

Monomers	**Dimers**	
His-tag on N-Terminal	*Homodimers, His-tag on N-Terminal*	*Heterodimers, His-tag on N-Terminal*
A4eGFP D9eGFP	A4A4eGFP D9D9eGFP	A4D9eGFP D9A4eGFP

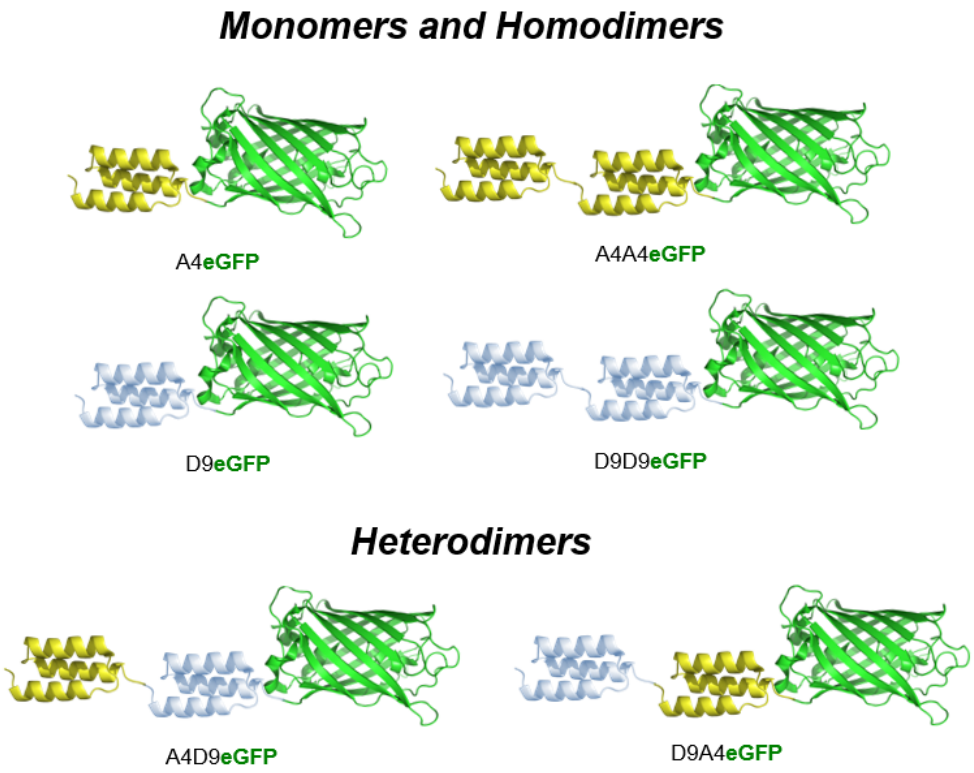


Figure 3.3: Structural models of the affibody constructs.

3.2 Cloning

3.2.1 General cloning procedure

Transformation is the process by which a cell uptakes and incorporates foreign DNA from its surroundings. For this process to take place the cell usually needs to be subjected to some type of stress, e.g. heat shock, lack of nutrients in the environment, among others [43]. For the purposes of cloning, the chosen cells were *E. coli* Top 10 chemically competent cells, available and produced in the lab. The method used for this transformation was heat shock, with the composition of the solution detailed in Table 3.2.

Table 3.2: Cell transformation solution composition.

Component	Volume (µL)
Competent Cells (Top 10)	10
MilliQ®	7
5X KCM	2
Plasmid	1 (50 ng)

First the components were pipetted into an Eppendorf tube, and the solution was incubated for 15-20 min on ice. Afterwards, an incubation for 60 s at 42 °C was performed, followed by a 2 min incubation on ice. 300 µL of TSB medium was added and the resulting solution was incubated with rotation for 1h at 37 °C. 100 µL of the solution were plated in an agar+antibiotic Petri dish under sterile conditions, and the plate was incubated overnight at 37 °C.

To obtain the plasmid DNA, single colonies were picked and incubated in a falcon tube with 5 mL TSB and 100 µg mL^{-1} of appropriate antibiotic, at 37 °C overnight. The next day, after centrifuging the culture at 4000 rpm for 10 min, the QIAprep Spin Miniprep Kit (Qiagen) was used for the plasmid extraction from the pellet. This was done according to the manufacturer's instructions, except for the elution where instead of 50 µL, 40 uL of buffer were used. Plasmid concentration was determined by measuring absorbance at 260 nm using a NanoDrop 1000 (Thermo Scientific, Software version 3.8.1).

3.2.2 Change of plasmid for the protein constructs

The decision was made to add a TEV cleavage site to the constructs, which would allow for an easier removal of the His-tag if necessary for say crystallography purposes, where it's presence could prove problematic. Since new cloning had to be performed, the choice was made to change from pET28 to another T7 expression vector, pET-45b(+). This was due to the fact that while pET28 is kanamycin resistant, pET-45b(+) is carbenicillin/ampicillin resistant, with these two being much more easily degraded, namely through the use of virkon.

The initial pET28 constructs were used as template for a PCR (see Table 3.3 for the composition of the solution and Table 3.4 for the PCR program used). All PCR were performed in a GeneAmp® PCR System 9700 running firmware version 3.12 (Applied Biosystems). The diagram for the used primers can be seen in Figure 3.4. From the constructs used PspC2T_Extra (with an extra His-tag at the N-Terminal), PspC2T FHBD, PspC2T CTerm and PspCT are in pET-45b(+) and PspC1T and PspC2T are in pET28.

Figure 3.4: Schematics of the primers used to clone from pET28 to pET-45b(+). "Added" are three overhanging bases at the beginning of the primer, "KpnI" and "XhoI" are the recognition sequences for the two namesake restriction enzymes for double digestion, "TEV" is the TEV cleavage site and "Stop" is the stop codon.

Table 3.3: PCR solution composition to amplify the protein/region of interest from pET28.

Component	Volume (µL)
Template	1
MilliQ®	28.5
5X Phusion™ HF Buffer (Thermo Scientific, F-518)	10
dNTP mix (2 mM)	5
Forward Primer (10 mM)	2.5
Reverse Primer (10 mM)	2.5
Phusion™ DNA Polymerase (2 U/µL, Thermo Scientific, F-530L)	0.5

Table 3.4: PCR program to amplify the protein/region of interest from pET28.

Temperature (°C)	Time	
98	30 s	
98	10 s	35 cycles
72	30 s	
72	30 s	
72	10 min	
4	Forever (hold)	

To obtain the purified PCR product the QIAquick PCR Purification Kit (Qiagen) was used, according to the manufacturer's instructions, except for the elution, where instead of 50 µL, 40 µL of buffer were used. For the plasmid DNA from pET-45b(+), the procedure was the one described in Section 3.2.1.

Once the PCR product and the plasmid DNA were obtained, both underwent separate overnight double digestions with the restriction enzymes KpnI-HF (New England Biolabs (NEB), R3142) and XhoI (NEB, R0146). The protocol used was obtained from the NEBcloner® (v1.4.1) tool available online in the manufacturer's website (see Table 3.5). Afterwards, given that KpnI-HF can not be heat inactivated, a purification using the QIAquick PCR Purification Kit took place, in order to successfully remove the enzymes, as indicated by the manufacturer. For PspCT a gel extraction was performed instead using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

After this, a ligation step took place. For this purpose, the vector (pET-45b(+)) and the insert were present in a ratio of 1 to 3. To that solution, water was added in order for the total volume to be 17 µL

Table 3.5: Overnight double digestion solution composition.

Component	50 µL reaction
DNA	1 µg
10x Cutsmart® Buffer (NEB, B7204S)	5 µL
KpnI-HF (20 U/µL)	1 µL
XhoI (20 U/µL)	1 µL
MilliQ®	To 50 µL

Table 3.6: Post-ligation transformation solution composition.

Component	Volume (µL)
Competent Cells (Top 10)	25
Solution from ligation	20
KCM (5X)	5

(18 µL for the control solution, where no ligase was added), before proceeding to a 10 min incubation at 50 °C. After this incubation 2 µL of T4 DNA Ligase Reaction Buffer (NEB, B0202) and 1 µL of T4 DNA Ligase (400 U/µL, NEB, M0202) were added, with the Eppendorf tubes being incubated at room temperature for 20-40 min, followed by 10 min at 65 °C. Once the ligation was finalized, a transformation in *E. Coli* Top 10 competent cells was performed. The composition of the solution used in this step can be found in Table 3.6. For the transformation, the method followed was very similar to the one described in Section 3.2.1, with the differences being that only 200 µL of medium were added instead of 300 µL, and all the solution volume was plated, instead of only 100 µL.

To ensure the insertion had taken place correctly a colony PCR was performed. For this purpose, colonies from the plate were individually picked and collected in a PCR plate/tube in 20 µL sterile water, undergoing incubation at 95 °C for 10 min for an initial lysing step. Then, 1 µL from each template (colony) was added to a solution (see Table 3.7 for composition and Table 3.8 for the PCR program).

Table 3.7: Colony PCR solution composition. Primer sequences can be found in Appendix A.

Component	Volume (µL)
Template	1
MilliQ®	17.3
10X DreamTaq Buffer (Thermo Scientific, B65)	2.5
dNTP mix (2 mM)	1.5
Lama27 Forward Primer (10 mM)	1.25
Lama14 Reverse Primer (10 mM)	1.25
DreamTaq DNA Polymerase (5 U/µL, Thermo Scientific, EP0702)	0.2

Table 3.8: PCR program to check for the presence of the correct insertion.

Temperature (°C)	Time	
95	1 min	
95	30 s	25 cycles
55	30 s	
72	1 min	
72	5 min	
4	Forever (hold)	

Afterwards 5 µL of the PCR product were mixed with 1 µL of either Gel Loading Dye (6X) (NEB, B7024S) or DNA Gel Loading Dye (6X) (Thermo Scientific, R0611) and loaded onto a 1% agarose gel along with the GeneRuler DNA Ladder Mix (Thermo Scientific, SM0333). The gel ran for 30 min at 160 V in 1X TAE buffer. If the size obtained from gel electrophoresis was correct, the construct was sent to Microsynth

Seqlab GmbH (Goettingen, Germany) for Sanger Cycle Sequencing/Capillary Electrophoresis.

3.3 Expression

3.3.1 The T7 expression system

There are several bacterial-cell based protein expression systems, but the one chosen for this study was the T7 expression system. This system is based on bacteriophage T7 RNA polymerase that has several advantages over other known RNA polymerases, namely (i) ability to handle long transcripts, (ii) very high transcription speed, (iii) being highly selective for its own promoters (not naturally present in *E. coli*) [44]. For this system, a T7 expression host is required, e.g. DE3 strains that carry the gene for T7 RNA polymerase under control of the lacUV5 promoter [45]. Finally, once the appropriate conditions are met, it is necessary to add isopropyl β-D-1-thiogalactopyranoside (IPTG) in order to induce expression of T7 RNA polymerase, which will then direct the efforts of the cell machinery towards the transcription of the gene of interest.

3.3.2 General expression protocol

The selected strain for protein expression was BL21 (DE3) pLys, available and produced at the lab. For the transformation of the different plasmids with the genes of interest, the protocol followed was the same as described in Section 3.2.1. After transformation, in order to prepare the inoculum for the larger culture, single colonies grown in the petri dish were transferred under sterile conditions to a falcon tube with 10 mL TSB (or TB) and 100 µg mL^{-1} antibiotic and grown with agitation at 220 rpm at 37 °C overnight. For the proteins, the antibiotic used was ampicillin or carbenicillin, while for the affibodies, it was kanamycin.

Afterwards, the contents of the falcon tube were transferred to a larger culture flask (previously sterilized at 121 °C in the autoclave), with 1000 mL TB and 100 µg mL^{-1} antibiotic. Those were then incubated with agitation at 160 rpm at 37 °C in an New Brunswick Scientific innova® 44 incubator, until $OD_{600\ nm} \approx 0.7\text{-}1$. When this was reached, the culture was induced by adding IPTG to a 1 mM final concentration, with the temperature being then lowered to 20 °C for overnight incubation with agitation. The following day, cells were harvested through centrifuging at 6000 rpm for 20 min at 4 °C, in 500 mL GSA-tubes using an Fiberlite™ F12-6 x 500 LEX Fixed Angle Rotor (Thermo Scientific) in a Sorvall LYNX 6000 Superspeed Centrifuge (Thermo Scientific). If purification wasn't done immediately after the harvest, the cells were frozen at –20 °C in a 50 mL falcon tube.

3.4 Purification

The purification process started with an immobilized metal affinity chromatography (IMAC) step. The basis of separation through IMAC is the affinity of specific amino acids to transition metal ions immobilized in an insoluble matrix [46]. Histidine in particular shows very high affinity to immobilized metal ion matrices, especially when the metal employed is cobalt or nickel, which is what makes this the method of choice for purification of his-tagged proteins. For the proteins of interest, this was achieved using a HisTrap™ HP 1 mL column (Cytiva, formerly GE Healthcare Life Sciences), while for the affibodies a HisTrap™ HP 5 mL column (Cytiva) was used. The HisTrap™ HP columns were stored in ethanol and had to be equilibrated prior to being used. For that purpose, 4 column volumes (CV) of water were injected in the column, followed by 4 CV of buffer A (see Table 3.9).

The sample preparation and elution process started with resuspension by vortex of the cell pellet (between 10-15 g) in 40 mL buffer A with the addition of 100 µL Halt™ Protease Inhibitor Cocktail, EDTA-free (100X) (Thermo Scientific, 78425). Afterwards the sample was sonicated for 30-40 min (Pulse: 3 s active + 7 s inactive; amplitude: 37%) in a Vibra-Cell™ VCX 750 Ultrasonic Processor (Sonics). The lysate was then transferred to SS34-tubes and centrifuged at 20 000 rpm for 30 min at 4 °C using the A27-8 x 50 Fixed Angle Rotor (Thermo Scientific) in the Sorvall LYNX 6000 Superspeed Centrifuge. Afterwards the supernatant was filtered using a 0.45 µm (VWR, 514-0065) and injected in the HisTrap™ HP column. A wash step with 4 CV of buffer A was then performed, followed by elution with either 10 mL or 5 mL of buffer B (see Table 3.9), for the 5 mL or the 1 mL HisTrap™ HP column, respectively. Dithiothreitol (DTT) was added to buffer B to a final concentration of 1 µM for the elution of affibodies, in order to prevent disulfide bonds from forming between cysteine residues present in GFP. The 10 mL sample was then concentrated down to 5 mL, using an Amicon® Ultra-15 centrifugal filter unit (depending on the molecular weight Ultracel-3 (Merck, UFC9003) or Ultracel-10 (Merck, UFC9010)) and spinning at 3500 rpm at 4 °C for approximately 40 min in a Multifuge® 1 S-R centrifuge (Heraeus).

Table 3.9: Composition of buffer A (lysis and wash buffer), buffer B (elution buffer) and buffer C (crystallization buffer) used in the purification process. All three buffers' pH was adjusted to 8.0 with an appropriate amount of concentrated HCl and then filtered with a PES 0.22 µm filter (TPP, 99505).

Components	*Buffer A (mM)*	*Buffer B (mM)*	*Buffer C (mM)*
Sodium Chloride (PanReac AppliChem, A2942)	500	500	150
Tris (PanReac AppliChem, A1379)	50	50	20
Imidazole (Merck, 104716)	20	500	-

The IMAC purification is then followed by a size exclusion chromatography (SEC) using a HiLoad 16/600 Superdex 200 prep grade (Cytiva), to ensure high levels of sample purity. The running buffers were:

- PBS (Karolinska Universitetslaboratoriet, MIK2010) pH = 7.4 filtered with a PES 0.22 µm filter for

D9D9eGFP, PspC1T, PspCT, PspC2T_Extra, PspC2T FHBD and PspC2T CTerm;

- Buffer B for all affibodies except D9D9eGFP;
- Buffer C for PspC2T.

The SEC was performed using an Äkta Purifier™ (Cytiva) running Unicorn 5.11 for better control of the chromatogram over the process. The affibodies that had been eluted with buffer B were buffer exchanged into PBS using PD-10 desalting columns packed with Sephadex G-25 resin (Cytiva, 17085101). The PspC2T produced and purified was used only for crystallization purposes. For the SPR experiments the PspC2T used was already purified and available at the lab.

After the SEC, an SDS-PAGE was performed to check if the purity level of samples from selected peaks (proteins detected as blue bands on a clear background). The samples were mixed with gel loading dye, in a 4:1 ratio and then 8-20 µL were loaded onto a Mini-PROTEAN® Precast Mini PAGE gel (Biorad). 5 µL of PageRuler™ Plus Prestained Protein Ladder (Thermo Fischer Scientific, 26619) were loaded as well and the buffer used was 1X SDS. The gel was run at 200 V for 30 min at room temperature and subsequently stained with Commassie Brillant Blue (40% Ethanol, 10% Acetic acid, 0.01% Commassie) for another 30 min. The excess dye incorporated into the gel was removed with MilliQ®.

The oligomeric state of the samples used in the Biacore 8k, i.e. PspC2T_Extra and all the affibodies, assays was evaluated through an analytical SEC using a Superdex 200 Increase 10/300 GL (Cytiva), with PBS pH = 7.4 as running buffer. The purpose of this analysis was to ensure the samples were not dimerizing post-purification.

3.5 Surface plasmon resonance

Prior to starting an SPR assay, the proteins need to be immobilized on a sensor chip. The method used for covalent immobilization of the ligand to the surface was amine coupling (see Figure 3.5). The SPR experiments were performed on a Biacore 3000 (Cytiva) running version 4.1.2 of the control software and on a Biacore 8K (Cytiva) running version 2.0.15.12933 of the control software.

3.5.1 Biacore 3000

Sensor chip preparation

Two different Biacore Sensor Chip CM5 (Cytiva, 29149604) were used, one for single-injection runs, where the immobilization of the three PspCXT proteins had to be performed, and one for co-injection runs, which had been previously prepared (see Figure 3.6).

After starting out with a PBST flow of 50 µL min^{-1} for ca.10 min to establish a stable baseline, immobilization was performed. This process consists of (1) pre-concentration, (2) activation, (3) ligand injection

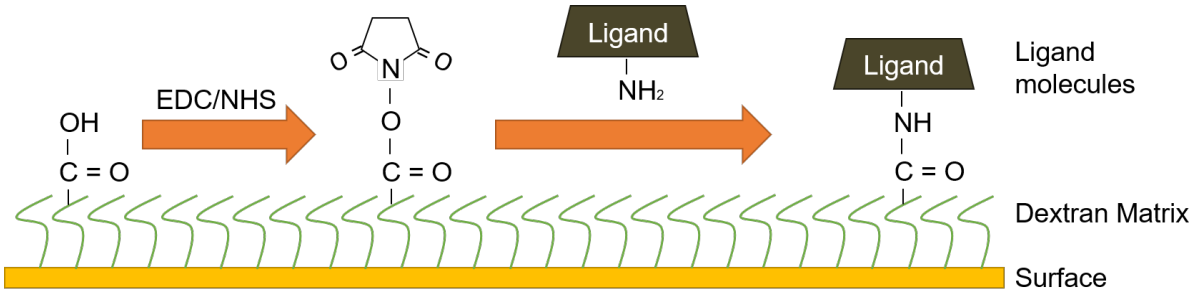


Figure 3.5: Schematic summary of the chemistry involved in the amine coupling of ligand molecules to the sensor surface. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) are injected first to activate the surface, i.e. modify the carboxymethyl groups to N-Hydroxysuccinimide esters. When the ligand is then injected over the surface at a pH value lower than its isoelectric point, it is attracted to the matrix. The amines on the ligand spontaneously react with the N-Hydroxysuccinimide esters on the matrix, forming covalent links [47]. Figure adapted from [35].

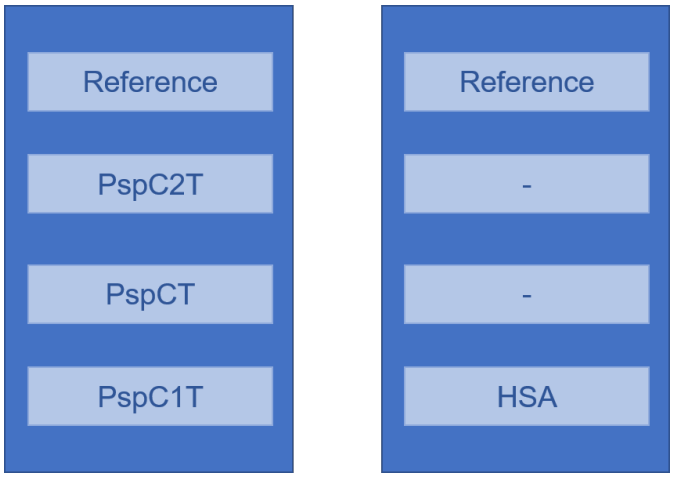


Figure 3.6: Biacore 3000 CM5 sensor chips used for single-injection runs (left) and for co-injection runs (right). The reference (blank) flow cell is FC1 for both chips. The protein immobilization levels on the flow cells of the single-injection chip were ca. 2000 RU. For the chip used in the co-injection, only FC4 (Human Serum Albumin (HSA)) is considered which has a level of immobilization of ca. 7000 RU.

and (4) deactivation, all of these steps performed at 5 µL min^{-1}. The first step wasn't performed when dealing with the blank flow cell (FC1), since its purpose is assessing the amount of ligand to inject, and in FC1 no ligand was injected. For this step, 5-10 µL at 5-10 µg mL^{-1} of ligand in 10 mM NaOAc pH 4.5 were injected in order to assess whether higher concentration of ligand was required to achieve the desired level of immobilization (ca. 2000 RU for this experiment). If a steep positive slope was seen during injection, the next step could be performed, otherwise the concentration needed to be adjusted until this happened. For the activation step, 85 µL of NHS and 85 µL of EDC were mixed together immediately before being used after thawing on ice, and 50 µL of this mix was injected. For the ligand injection, 10 µL of 10 mM NaOAc pH 4.5 were injected with ligand at an appropriate concentration (for FC1 no ligand is present). Even though this concentration had been determined in the pre-concentration step several

pulses could be required to achieve the desired immobilization level. Finally, 50 µL of ethanolamine are injected to deactivate unreacted NHS-esters. There usually is a drop in the response level after this step, since the ethanolamine injection washes away every non-covalently attached ligand.

Single-injection runs

The single-injection runs consist of either analytes run by themselves, to establish a baseline, or pre-incubated with other analytes. The injections and their purposes are detailed in Table 3.10. The running buffer was PBST pH = 7.4 degassed and filtered with a 0.22 µm filter.

Table 3.10: Single-injection runs and their purpose. The fact that the three PspCXT proteins are both immobilized and injected serves as control for the effect of protein immobilization in binding to affibodies and FH, since sometimes the process of immobilization leads to some loss of function in the protein. Injecting all three PspCXT proteins as well as PspC2T FHBD and PspC2T CTerm, allows for determination of which of them the affibodies and FH are binding to.

Injection	Purpose
Affibodies (200 nM) + PspC2T FHBD (10 µM) Affibodies (200 nM) + PspC2T CTerm (10 µM) Affibodies (50 nM) + PspC2T (910 nM) Affibodies (50 nM) + PspCT (910 nM) Affibodies (50 nM) + PspC1T (910 nM)	Domain mapping for the affibodies Control for the effect of protein immobilization on the binding to affibodies
FH (50 nM) + PspC2T FHBD (10 µM) FH (50 nM) + PspC2T CTerm (10 µM) FH (50 nM) + PspC2T (910 nM) FH (50 nM) + PspCT (910 nM) FH (50 nM) + PspC1T (910 nM)	Domain mapping for FH Control for the effect of protein immobilization on the binding to FH
FH (50 nM) + PspC2T_Extra (1 µM) FH (50 nM) + Bovine Serum Albumin (BSA) (1 µM)	Assessing whether FH is promiscuous

Co-injection runs

For the co-injection runs a different Biacore sensor chip that was already prepared was used (see Figure 3.6). The running buffer was PBST pH = 7.4 degassed and filtered with a 0.22 µm filter. In this capture assay setup (see Figure 3.7), two injections are included in the same run: monomer affibody (A4 or D9) with a fused Albumin Binding Domain (ABD) was first injected so that it would bind non-covalently to the HSA immobilized on the chip surface. The second injections and aims of the assay are summarized in Table 3.11.

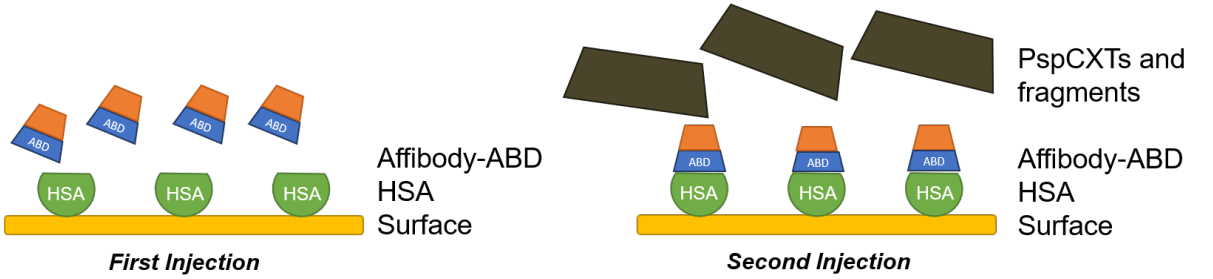


Figure 3.7: A4-ABD or D9-ABD are injected over the surface to bind to immobilized HSA. Afterwards, analytes are injected to determine their binding activity to the non-covalently captured affibody-ABD.

Table 3.11: Co-injection runs and their purposes.

1st Injection	2nd Injection	Purpose
A4-ABD (1 µM)	PspC2T_Extra (50 nM) + FH (400 nM)	Determining if immobilized affibodies can destabilize established FH:PspC2T interaction
or	PspC2T FHBD (100 nM) + A4eGFP (1 µM)	
	PspC2T FHBD (100 nM) + D9eGFP (1 µM)	Confirming domain mapping for the affibodies to PspC2T regions (PspC2T FHBD and PspC2T CTerm)
D9-ABD (1 µM)	PspC2T CTerm (100 nM) + A4eGFP (1 µM)	
	PspC2T CTerm (100 nM) + D9eGFP (1 µM)	

3.5.2 Biacore 8k

For the runs performed on the Biacore 8k a Biacore Series S Sensor Chip CM5 (Cytiva, 29149603) was prepared. The immobilization protocol used was the one indicated by the control software, but the components used and immobilization chemistry were the same as for the Biacore 3000. This chip includes 8 channels, each of them with 2 flow cells, a reference flow cell (FC1) and an ”active” one (FC2). For these assays the 6 affibodies (A4eGFP, D9eGFP, A4A4eGFP, D9D9eGFP, A4D9eGFP and D9A4eGFP) were immobilized on FC2 of each channel (see Figure 3.8). The running buffer was PBST pH = 7.4 filtered with a 0.45 µm filter.

Table 3.12: Biacore 8k runs and their purpose. Incubation with a monomer (e.g. A4eGFP) is expected to block binding to the surface in flow cells/channels where the same monomer or homodimer is immobilized (e.g. A4eGFP (Channel 1) and A4A4eGFP (Channel 3)) because the epitope on PspC2T is blocked. However when a heterodimer is immobilized (A4D9eGFP and D9A4eGFP (Channels 5 and 6)), signal is expected to be reduced to half, because even though one of the epitopes is blocked (by A4eGFP) the other can still bind (epitope for D9eGFP) and the heterodimers possess both.

Injections	Purpose
PspC2T_Extra (167 nM) + A4eGFP (1667 nM)	Determining the effect of blocking one of
PspC2T_Extra (167 nM) + D9eGFP (1667 nM)	PspC2Ts epitopes on surface binding

Figure 3.8: Biacore 8k Series S CM5 sensor chip used. The levels of immobilization of the affibodies on the respective flow cells are A4eGFP (333 RU), D9eGFP (650 RU), A4A4eGFP (668 RU); D9D9eGFP (843 RU), A4D9eGFP (327 RU) and D9A4eGFP (1659 RU).

3.6 Crystallography

The chosen method for crystallization was the sitting drop method. Crystallization was attempted for two concentrations of PspC2T (12 mg mL^{-1} and 24 mg mL^{-1}) using four different crystallization screens: PEG/Ion HT™ (Hampton Research, HR2-139), Crystal Screen HT™ (Hampton Research, HR2-130), JCSG+ (Qiagen) and Morpheus® (Molecular Dimensions, MD1-46).

50 µL of solution from the screen were dispensed onto the reservoir wells of the 96 well crystallography plates (Corning, 3550) using the Crystal Phoenix dispenser (ARI - Art Robins Instruments, Phoenix 1.9 software). The mosquito® LCP dispenser (SPT Labtech) was used for mixing PspC2T and reservoir solution in a 1:1 ratio, and then dispensing 0.5 µL drops onto the protein wells. After the plates were sealed, they were checked in to the Rock Imager® 1000 (Formulatrix®). The software used to design and manage the crystallography experiments was the Rock Maker® Version 3.16.3.1 (Formulatrix®) and to monitor the experiment's progression the Rock Imager® Version 3.8.5.1-x64 (Formulatrix®).

4

Results and Discussion

Contents

4.1 Cloning results

Different PspCX encoding gene fragments were cloned from pET28 to another T7 expression vector, pET-45b(+). For this a ligation and transformation steps were required. To determine whether the correct insertion had taken place, a colony PCR was performed on sample colonies of the constructs that had undergone vector exchange followed by an agarose gel electrophoresis (see Figure 4.1).

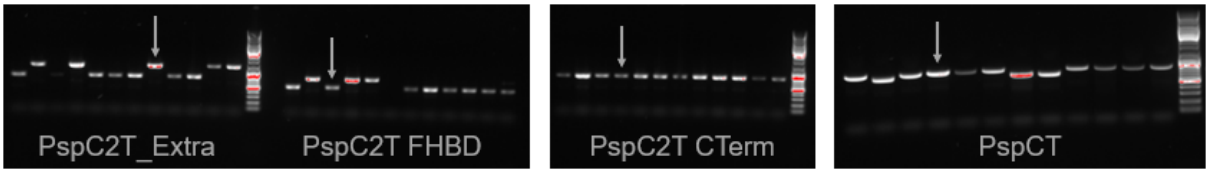


Figure 4.1: Results from gel electrophoresis performed pre-sequencing for the constructs where vector exchange was performed. The arrows indicate the colonies chosen for transformation in BL21 and subsequently for protein expression and purification.

A number of the colonies with the expected size were sent for sequencing and the sequences obtained are summarized in Figure A.1.

4.2 Purification results

Given that SPR and crystallography techniques require high levels of purity, after overnight expression in BL21 (DE3) pLys the proteins and affibodies underwent purification, first by IMAC and then by SEC. This was done to isolate homogeneous monomeric forms of the used constructs. The obtained chromatograms showed that both the proteins and affibodies were expressed in monomeric and oligomeric forms with well defined peaks (see Figures A.2 and A.3). Since in SEC the order of elution depends on the size of the molecule, the first peaks were interpreted as the oligomeric forms of the constructs. To confirm that the appropriate form had been isolated an SDS-PAGE was performed. The results (see Figure 4.2) show that all of the constructs are approximately of the expected size.

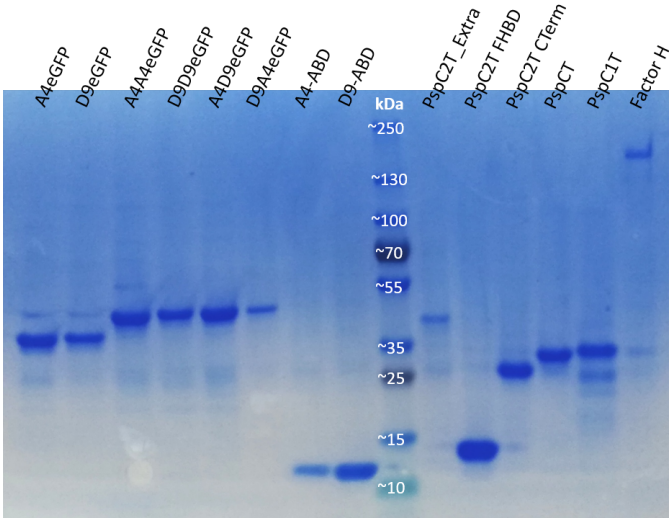


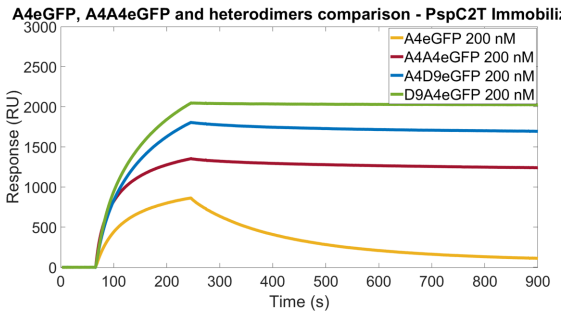
Figure 4.2: Results obtained from SDS-PAGE.

4.3 Surface plasmon resonance

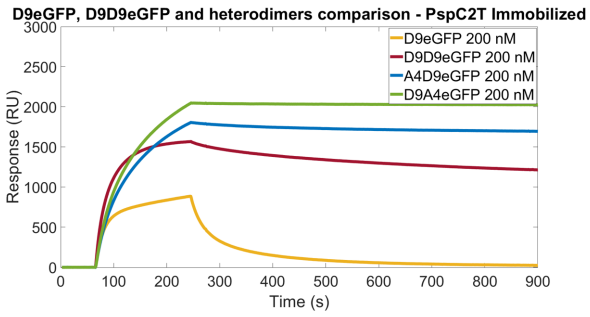
Double referencing was performed on all data from the Biacore 3000 and Biacore 8k runs. This means that the signal from the reference surface (i.e. FC1) and from a buffer injection (i.e. PBST) were both subtracted from the signal of surfaces with immobilized ligand. For data from the single-injection runs performed on the Biacore 3000, only the referenced sensorgram from FC2 will be displayed and analysed. This is due to the fact that no binding was detected in FC3 and FC4, i.e. immobilized PspCT and PspC1T. For data from the co-injection runs performed on the Biacore 3000, the double referencing was performed by subtracting a buffer+affibody-ABD injection, to better showcase the detected binding to the non-covalently captured protein. For data from the Biacore 8k, given that the amount of immobilized affibodies on the surface varied significantly between flow cells, the data shown was also normalized. Normalization consisted in dividing the sensorgrams obtained for each of the flow cells by the corresponding value of immobilized RUs, since the response level is proportional to the amount of immobilized protein on the surface.

Affibodies bind PspC2T better in dimer format

To start, we compared the affibodies affinity to PspC2T (immobilized) by injecting them all at the same concentration. From Figure 4.3 it is obvious that monomers, homodimers and heterodimers have a rising level of affinity to PspC2T, with D9A4 being the best binder. This can be proved by analysing the curve shapes and comparing the response levels. For both monomers, the response level is about the same with the dissociation being fast when compared to the homodimers and heterodimers. The homodimers show better results, with higher response level for the same concentration and much slower dissociation rates, evidenced by the much flatter curves after the end of the injection.



(a) Comparison of A4eGFP (yellow), A4A4eGFP (red), A4D9eGFP (blue) and D9A4eGFP (green).



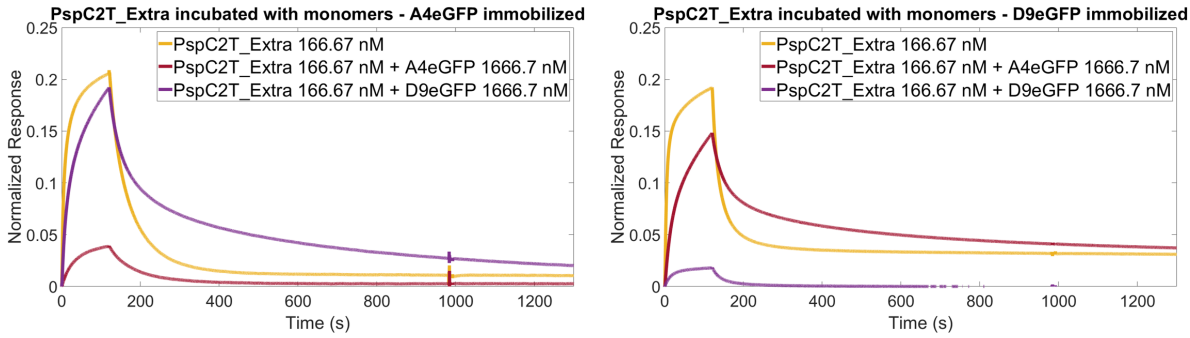
(b) Comparison of D9eGFP (yellow), D9D9eGFP (red), A4D9eGFP (blue) and D9A4eGFP (green).

Figure 4.3: Sensorgrams depicting the injection of monomers, homodimers and heterodimers. This setup allows us to show the increasing affinity of monomers, homodimers and heterodimers to PspC2T. Performed on the Biacore 3000.

Finally, we have the heterodimers. The fact that we can see binding means that both A4D9 and D9A4 are super-binders, i.e. still capable of binding two different epitopes when in dimer form. Out of the two, D9A4 with a response level of almost 3 times that of the monomers and a practically flat curve post-injection appears to be the best candidate for studies involving PspC2.

Affibodies bind non-overlapping epitopes of PspC2T [BHN418/191]

Afterwards there was a need to ascertain that A4 and D9 were binding non-overlapping epitopes. This was done by running PspC2T_Extra alone, as well as incubated with excess A4eGFP or D9eGFP over both channels where A4eGFP and D9eGFP were immobilized (see Figure 4.4).



(a) PspC2T_Extra injected by itself (yellow) and pre-incubated with 10X molar excess of A4eGFP (red) and D9eGFP (purple), over immobilized A4eGFP.

(b) PspC2T_Extra injected by itself (yellow) and pre-incubated with 10X molar excess of A4eGFP (red) and D9eGFP (purple), over immobilized D9eGFP.

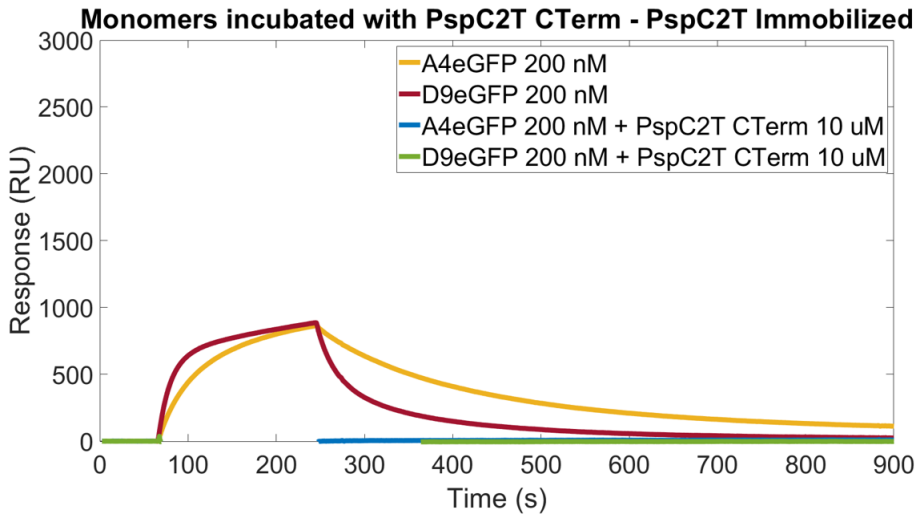
Figure 4.4: Sensorgrams depicting the injection of PspC2T_Extra alone or pre-incubated with either the same monomer in solution and immobilized (e.g. A4eGFP immobilized and in solution), or a different monomer in solution and immobilized (e.g. A4eGFP immobilized and D9eGFP in solution). This setup allows for determination of whether the affibodies bind overlapping epitopes of PspC2. Performed on the Biacore 8k.

When PspC2T_Extra was pre-incubated with the same monomer as the one on the surface, the signal was blocked. This is because the epitope was already taken up due to binding that occurred pre-injection. By comparing the curves of PspC2T_Extra injected by itself and PspC2T_Extra with excess of the affibody that is not immobilized on the surface, we can see that the signal is not blocked. This indicates that A4 and D9 bind non-overlapping epitopes (see Figure 4.12(a)).

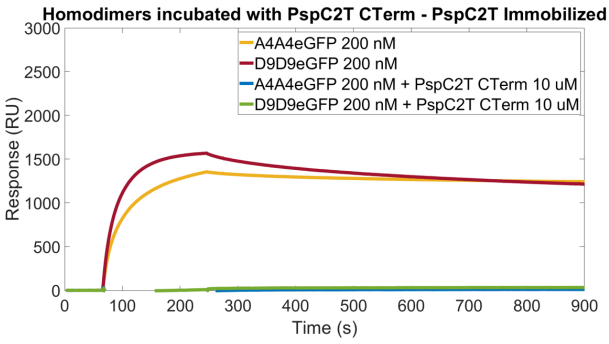
Affibodies bind the C-Terminal fragment of PspC2T not the FH binding domain

To determine to which of the regions of PspC2T the affibodies were binding, they were pre-incubated with excess of both the fragments separately and then injected over the surface. Since surface binding

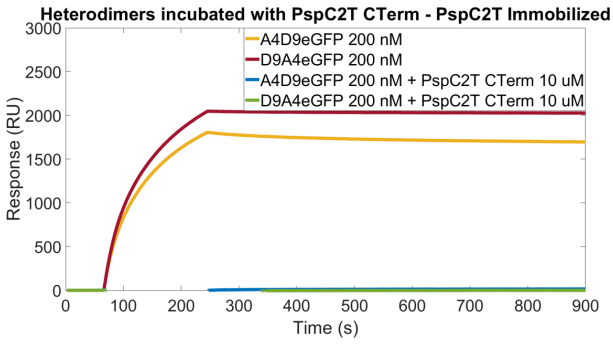
was detected when the affibodies were injected by themselves, if the signal was blocked when they were pre-incubated with either fragment this would mean they were binding to the fragments in solution.



(a) Monomers injected by themselves (yellow and red) and pre-incubated with 50X PspC2T CTerm (blue and green), over immobilized PspC2T.



(b) Homodimers injected by themselves (yellow and red) and pre-incubated with 50X PspC2T CTerm (blue and green), over immobilized PspC2T.



(c) Heterodimers injected by themselves (yellow and red) and pre-incubated with 50X PspC2T CTerm (blue and green), over immobilized PspC2T.

Figure 4.5: Sensorgrams depicting the injection of affibodies alone and pre-incubated with PspC2T CTerm. These runs allow for determination of whether the affibodies bind PspC2T CTerm. Performed on the Biacore 3000.

When the affibodies were injected by themselves, binding was detected. This contrasts with the results of their pre-incubation with PspC2T CTerm, where the signal was blocked. These results indicate the affibodies are binding to PspC2T CTerm in solution (see Figure 4.12(b)).

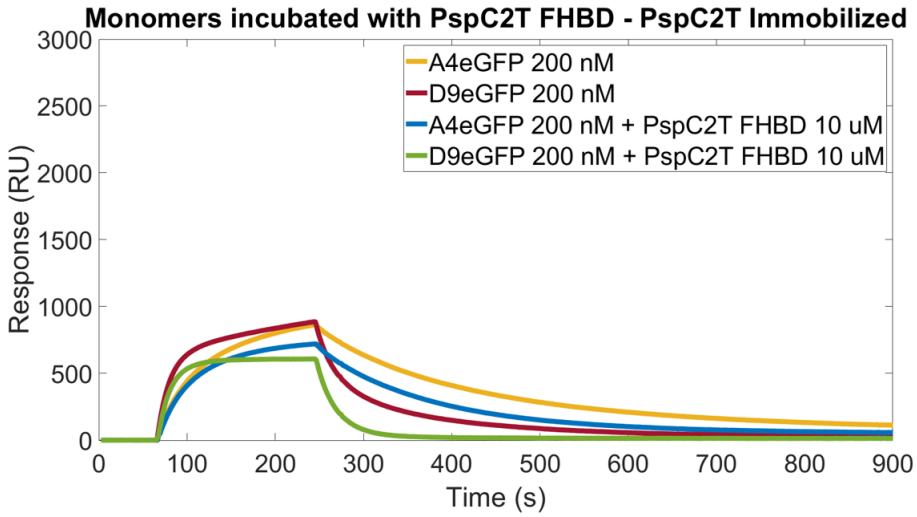
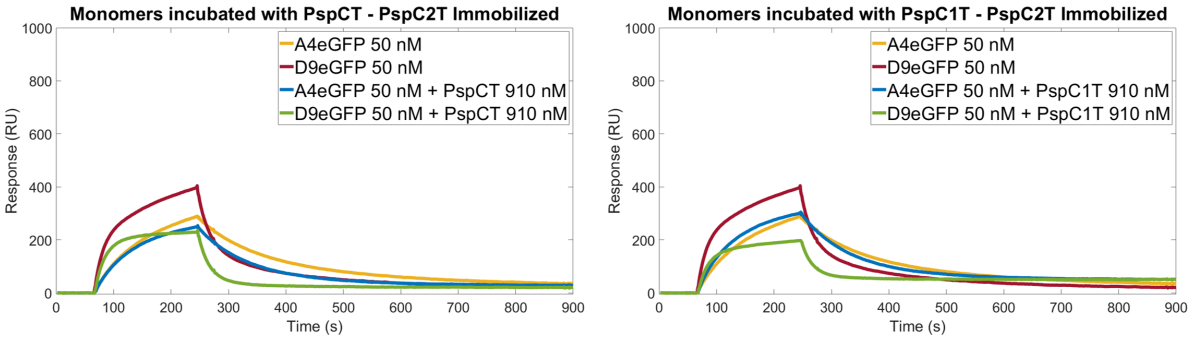


Figure 4.6: Sensorgram depicting the injection of monomers alone (yellow and red) and pre-incubated with PspC2T FHBD (blue and green). These runs allow for determination of whether the affibodies bind PspC2T FHBD. The same behaviour was observed for homodimers and heterodimers (not pictured). Performed on the Biacore 3000.

When the affibodies were pre-incubated with PspC2T FHBD (see Figure 4.6) however, the signal was not blocked. This means the affibodies are not binding PspC2T FHBD (see Figure 4.12(c)).

Affibodies don't bind PspCT [TIGR4] or PspC1T [BHN418]

Afterwards, the specificity of the affibodies to PspC2T was investigated, testing their possible interaction with PspCT and/or PspC1T. This was performed in the same pre-incubation setup as the other studies since no binding was detected for FC3 and FC4 (immobilized PspCT and PspC1T, respectively).



(a) Monomers injected alone (yellow and red) and pre-incubated with PspCT (blue and green).

(b) Monomers injected alone (yellow and red) and pre-incubated with PspC1T (blue and green).

Figure 4.7: Sensorgrams depicting the injection of affibodies alone and pre-incubated with PspCT or PspC1T, over immobilized PspC2T. These runs allow for determination of whether the affibodies bind either of these proteins. Performed on the Biacore 3000.

When affibodies were pre-incubated with PspCT or PspC1T no signal blocking was detected, similarly to what happened with PspC2T FHBD. The same behaviour was observed for homodimers and heterodimers (results not shown). This demonstrates that the affibodies do not bind PspC1T or PspCT in solution (see Figure 4.12(d)), from which we can conclude they are specific to PspC2T.

Factor H binds all tested proteins and fragments

Since it was known from the literature review that FH bound all PspCX proteins [24], FH was first injected to determine whether there was binding to the surface or not and then it was pre-incubated with PspCXT, PspC2T FHBD and PspC2T CTerm to determine if it bound them in solution.

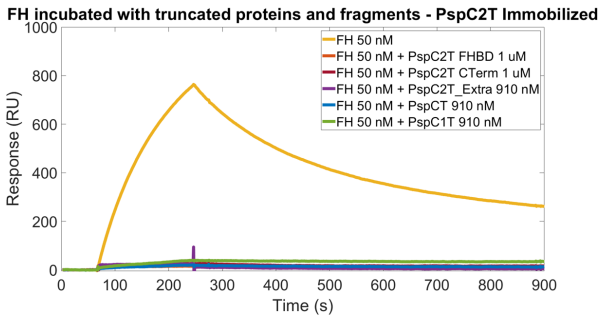


Figure 4.8: Sensorgram depicting the injection of FH alone (yellow) and pre-incubated with PspCXT/PspC2T FHBD/PspC2T CTerm. These runs allows for determination of which tested proteins or fragments FH is binding to. Performed on the Biacore 3000.

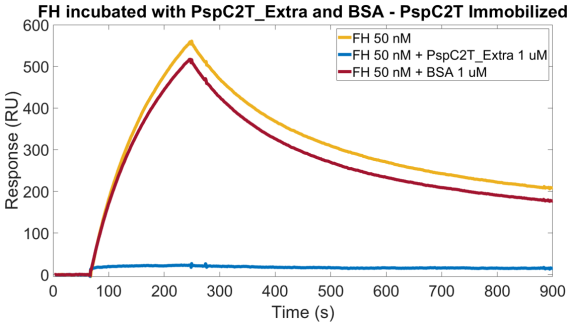


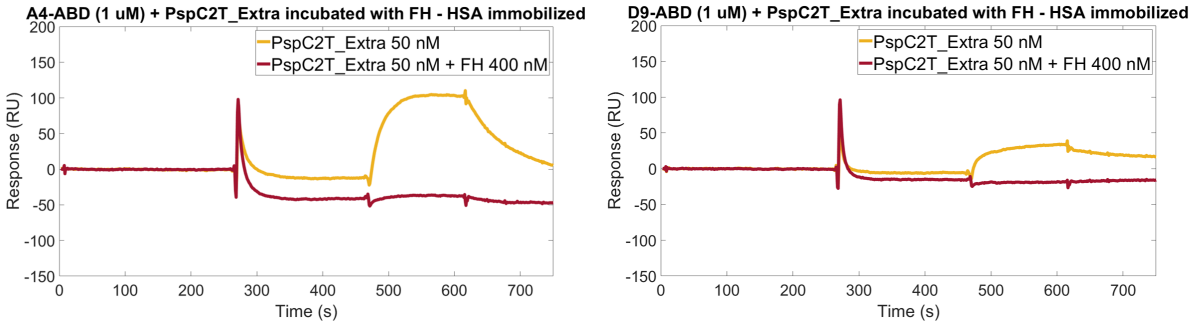
Figure 4.9: Sensorgrams depicting the injection of FH alone (yellow) and incubated with 20x molar excess of PspC2T_Extra (blue) or BSA (red). This assay allows to test for possible promiscuity of FH. Performed on the Biacore 3000.

By analysing the sensorgrams (Figure 4.8) it appears that FH is capable of binding all proteins and fragments in solution when pre-incubated with them, since the signal detected when FH was injected by itself disappeared on the other injections (see Figure 4.12(e)). This allows us to say that PspC1T and PspCT lost some degree of function when immobilized and that this could be the reason for no signal to be detected on those FCs. The fact that FH bound PspC2T CTerm in solution came as a surprise, given previous work developed by Pathak et al. [24]. According to their findings, the FH binding motifs in PspC2 were located only in what corresponds to PspC2T FHBD.

Since FH bound all tested proteins and fragments, a promiscuity assay was performed to ensure the binding was not just indiscriminate. For this purpose, FH was pre-incubated with either PspC2T_Extra, a protein to which we'd shown there was binding, or BSA, a protein to which FH wouldn't theoretically bind. When comparing the curves on Figure 4.9, we can see that there is virtually no difference between FH injected by itself and FH incubated with excess BSA. However, when FH is incubated with excess PspC2T_Extra the signal disappeared indicating that there was binding in solution rather than to the surface. As such, we can say that FH doesn't appear to be promiscuous (see Figure 4.12(f)).

Pre-incubation of PspC2 with Factor H inhibits binding to affibodies

To determine whether the affibodies could disrupt established FH:PspC2 binding, the capture assay setup was used (see Figure 4.11). On the first injection, A4-ABD or D9-ABD bind non-covalently to immobilized HSA, becoming ”immobilized”. Afterwards a second injection where PspC2T_Extra was pre-incubated with 8x molar excess of FH was administered.



(a) Co-injection runs of A4-ABD followed by injection of PspC2T_Extra either by itself (yellow) or pre-incubated with molar excess of FH (red).

(b) Co-injection runs of D9-ABD followed by injection of PspC2T_Extra either by itself (yellow) or pre-incubated with molar excess of FH (red).

Figure 4.10: Sensorgrams depicting the injection of PspC2T_Extra by itself or pre-incubated with 8x molar excess of FH, after the first injection of either A4-ABD (on the left) or D9-ABD (on the right). These runs allow for testing whether the immobilized affibodies can interact with established FH:PspC2T interaction. Performed on the Biacore 3000.

When PspC2T_Extra is injected by itself, binding occurs. When it is pre-incubated with 8x molar excess of FH however, the signal is blocked. This means the immobilized affibody can not interfere with established FH:PspC2T interaction.

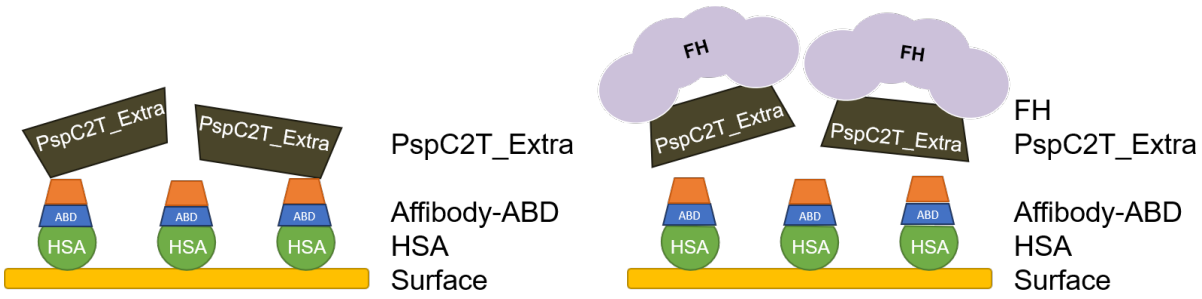
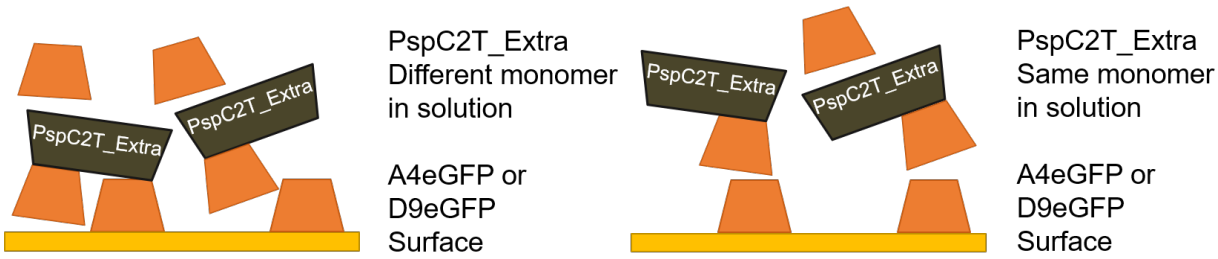
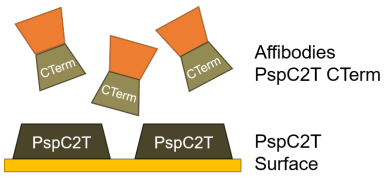


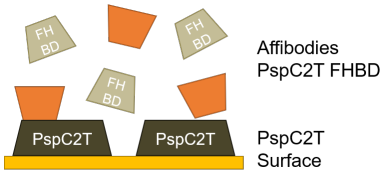
Figure 4.11: Visual representation of the performed co-injection runs.



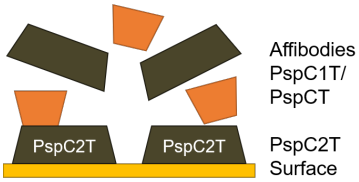
(a) Diagram of the runs to determine whether A4 and D9 bound non-overlapping epitopes in PspC2.



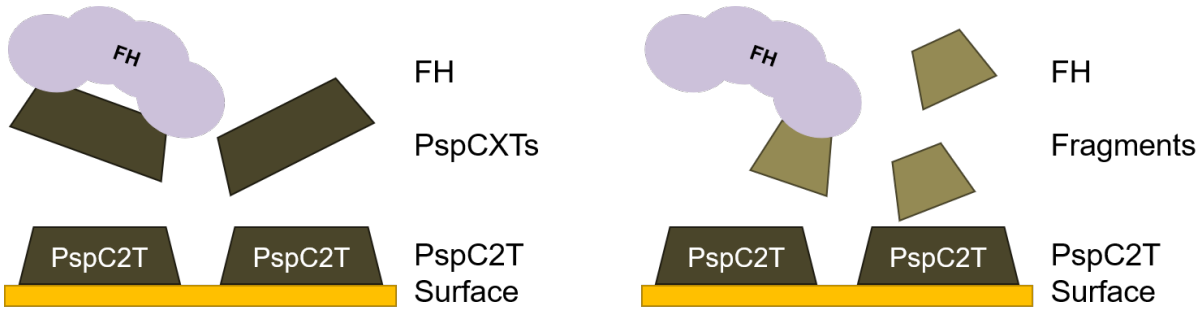
(b) Diagram of the run where affibodies were pre-incubated with PspC2T CTerm.



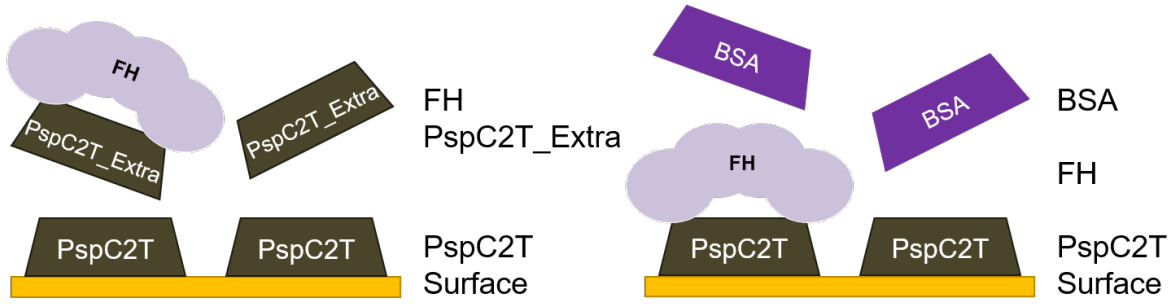
(c) Diagram of the run where affibodies were pre-incubated with PspC2T FHBD.



(d) Diagram of the runs where affibodies were pre-incubated with PspCT or PspC1T.



(e) Diagram of the runs to determine what FH bound in solution.



(f) Diagram of the runs to determine whether or not FH was promiscuous.

Figure 4.12: Visual summaries of the performed single injection runs.

4.4 Crystallography

We tried to identify suitable conditions for the crystallization of PspC2 at different protein concentrations by screening pre-made buffer kits. Mainly small needle crystals appeared after 52 days of incubation (see Figure 4.13). Crystals were obtained at both 12 mg mL^{-1} and 24 mg mL^{-1} concentration of PspC2T. Even though hits were present in six different conditions, none of the observed crystals had enough quality for X-Ray diffraction. As such further optimization screens need to be designed around the initial hit conditions.

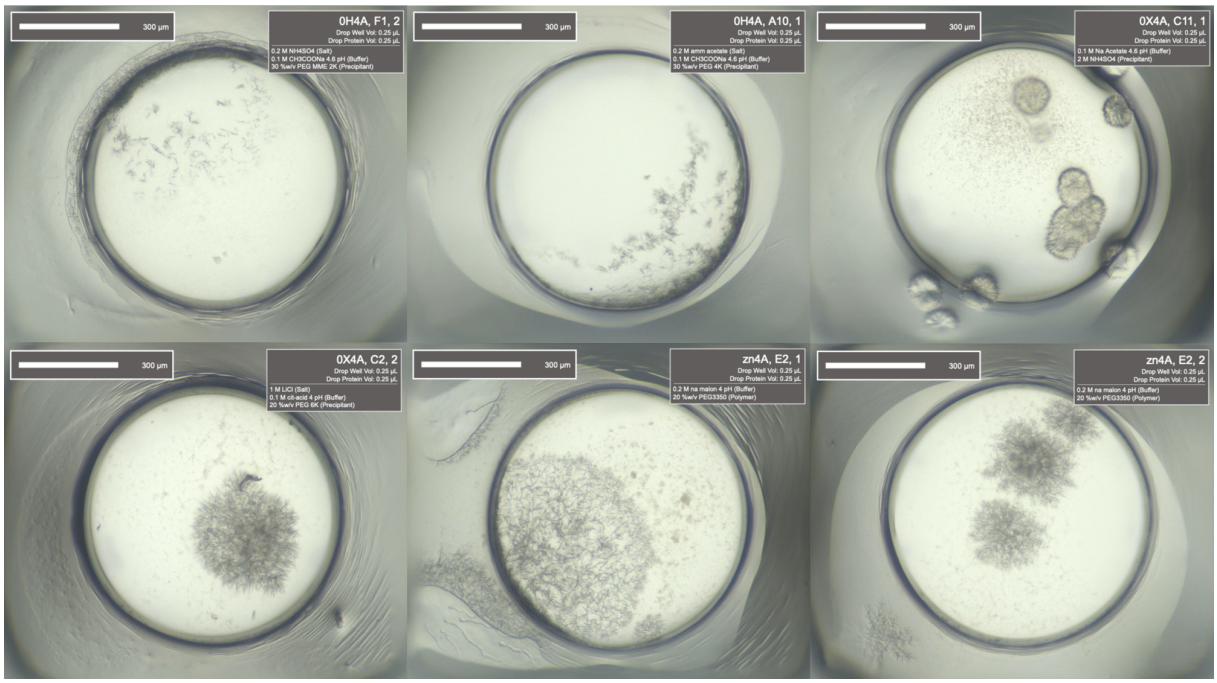


Figure 4.13: Results from crystallography. Each of the wells corresponds to a different condition after 52 days in the Rock Imager® 1000. Across the 384 tested conditions only these 6 showed hits.

5

Conclusions

Contents

5.1 Conclusions

5.1.1 Main and secondary goals

The main goals of this thesis were:

1. Assessing if the affibodies were binding to PspC and PspC1 in addition to PspC2;
2. Assessing whether affibodies were binding preferentially to a specific domain in PspC2;
3. Investigating the occurrence of ”super-binding”;
4. Investigating whether established binding of FH by PspCX can be disrupted by affibodies.

For the first two points, through the mapping experiments we found that the affibodies were binding only to PspC2, more specifically to the CTerm fragment. With the knowledge that both monomers bound different non-overlapping epitopes and that in the heterodimeric forms (A4D9 and D9A4) they were still able to bind immobilized PspC2, we can state that the heterodimers are super-binders. As for the last point, we have proved that when the affibodies are immobilized they are not capable of disturbing established FH:PspC2 binding or of binding to this complex. For the crystallization portion, i.e. the secondary goals, even though small needle crystals were obtained, they didn't have the required quality to be used in X-Ray diffraction and eventually solve the crystal structure.

5.1.2 Work summary and future prospects

This thesis illustrates that functional super-binder heterodimers specific for PspC2 have been developed. The work developed also allowed for mapping of FH to the CTerm fragment of PspC2T, an unexpected binding location given previous work developed in the field. It is important to point out that blocking just one of the FH binding places in PspC2 (one located in the CTerm fragment the other in the FHBD fragment of PspC2T) appears to prevent binding from occurring. This discovery could eventually have therapeutic value since binding FH is a cornerstone of *S. pneumoniae*'s invasive strategy.

Future works on the SPR dimension could involve performing competition assays between FH and the affibodies, to determine if the affibodies can stop FH from binding PspC2 in solution, or with affibodies and other proteins PspCX binds (e.g. vibronectin [48], secretory IgA [49], secretory component portion of human polymeric Ig receptor [50]), for the same purpose.

Another point of interest would be to test the affibodies in an *in vivo* setting, and perform a cell adhesion assay to determine whether affibodies can act as de-colonization agents for *S. pneumoniae*.

For the crystallography portion, further optimization of the conditions is needed to obtain appropriate crystals for X-Ray diffraction. This could involve moving the His-tag from the N-Terminal to the C-Terminal or even removing it altogether. It would be interesting to attempt co-crystallization of affibodies and PspC2, to obtain a crystal structure that shows where exactly the affibodies are binding to PspC2.

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Appendix A

Sequencing results for the used constructs

PspC2T

MGSSHHHHHHSSGLVPRGSHMTEKEGSTQAATSFNRGNGSQAEQRGELDLERDKAMKAVSEYVGKMVRDAYVKSDRK
RHKNTVALVNQLGNIKNRYLNEIVHSTSKSQLQELMMKSQSEVDEAVSKFEKDSFSSSSSGSSTKPETPQPENPEHQ
KPTTPSPDTKPSPQPEGKKPSVPDINQEKEKAXLAVVTYMSKILDDIQKHHLQKEKHRQIVALIKELDELKKQALSE
IDNVNTKVEIENTVHKIFADMDAVVTKFKKGLNSGHTKRTR

PspC2T_Extra

MAHHHHHHVGTGENLYFQGSMGSSHHHHHHSSGLVPRGSHMTEKEGSTQAATSFNRGNGSQAEQRGELDLERDKAMK
AVSEYVGKMVRDAYVKSDRKRHKNTVALVNQLGNIKNRYLNEIVHSTSKSQLQELMMKSQSEVDEAVSKFEKDSFSS
SSSGSSTKPETPQPENPEHQKPTTPSPDTKPSPQPEGKKPSVPDINQEKEKAKLAVVTYMSKILDDIQKHHLQKEKH
RQIVALIKELDELKKQALSEIDNVNTKVEIENTVHKIFADMDAVVTKFKKGLTQDTPKEPGNKKPSAPKPGMQPS

PspC2T FHBD

MAHHHHHHVGTGENLYFQGSDLERDKAMKAVSEYVGKMVRDAYVKSDRKRHKNTVALVNQLGNIKNRYLNEIVHSTS
KSQLQELMMKSQSEVDEAVSKFEK

PspC2T CTerm

MAHHHHHHVGTGENLYFQGSDSFSSSSSGSSTKPETPQPENPEHQKPTTPSPDTKPSPQPEGKKPSVPDINQEKEKA
KLAVVTYMSKILDDIQKHHLQKEKHRQIVALIKELDELKKQALSEIDNVNTKVEIENTVHKIFADMDAVVTKFKKGL
TQDTPKEPGNKKPSAPKPGMQPS

PspCT

MAHHHHHHVGTGENLYFQGSHATENEGATQVPTSSNRANESQAEQGEQPKKLDSERDKARKEVEEYVKKIVGESYAK
STKKRHTITVALVNELNNIKNEYLNKIVESTSESQLQILMMESRSKVDEAVSKFEKDSSSSSSSDSSTKPEASDTAK
PNKPTEPGEKVAEAKKKVEEAEKKAKDQKEEDRRNYPTITYKTLELEIAESDVEVKKAELELVKVKANEPRVW

PspC1T

MHHHHHHENLYFQGEEVGGRNTPTVTSSGQDISKKYADEVESHLKKILSEIQTQLDRKRHTKTVALINELQDIKKTY
LYNLNVLKEKSELPSKIKAKLEVAFDQFKKDTLKPGEKVAEAEKKVAEAKKKAEDQKEEDRRNYPTNTYKTLELEIA
ESDVKVKEAELELVNEEAKPGNEEKIKKAKAKVESKKAEATRLEKIKTDRKKAEEAKRKAAEE

Figure A.1: Sequences of the used protein constructs.

Sequences of the primers used for the colony PCR

Lama27 (Forward primer): ATCCCGCGAAATTAATACGACTCAC

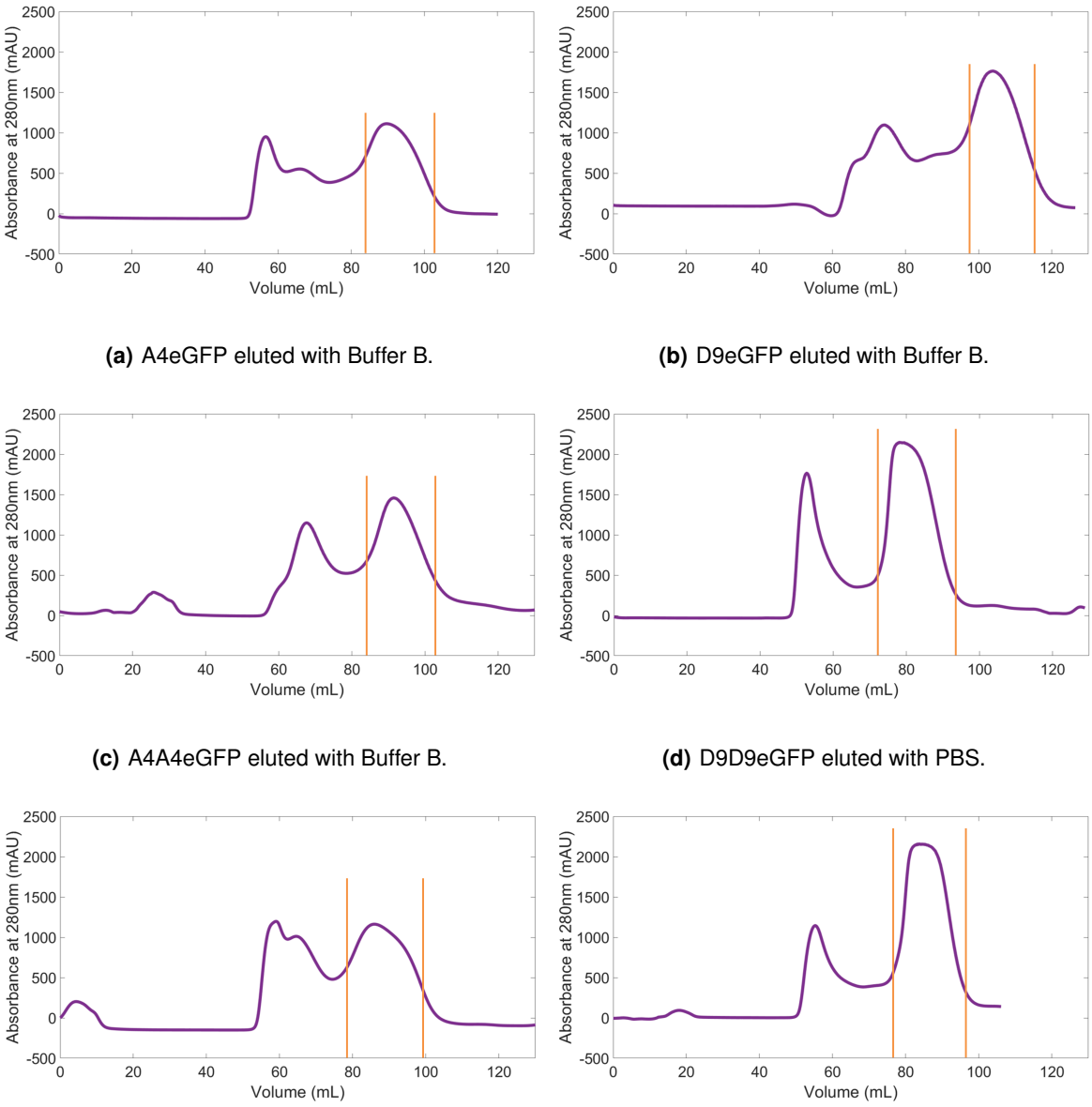
Lama14 (Reverse primer): ATGCTAGTTATTGCTCAGCGGTGG

Parameters used to determine the concentration of used proteins and affibodies in solution

Table A.1: Molecular weight and extinction coefficient for each of the proteins and affibodies used. Parameters computed using Expasy's ProtParam online tool.

Proteins	**MW** (kDa)	**E** (M^{-1} cm^{-1})	*Affibodies*	**MW** (kDa)	**E** (M^{-1} cm^{-1})
PspC2T	31.9	5960	A4eGFP	35.8	37423
PspC2T_Extra	34.2	7450	D9eGFP	35.8	33413
PspC2T FHBD	11.7	5960	A4A4eGFP	43.3	48423
PspC2T CTerm	19.7	2980	D9D9eGFP	43.3	40403
PspCT	25.7	14440	A4D9eGFP	43.3	44413
PspC1T	25.0	8940	D9A4eGFP	43.3	44413
Factor H	137	244300	A4-ABD	13.7	19940
BSA	66.4	41863	D9-ABD	13.8	22920

Chromatograms obtained after SEC



(a) A4eGFP eluted with Buffer B.

(b) D9eGFP eluted with Buffer B.

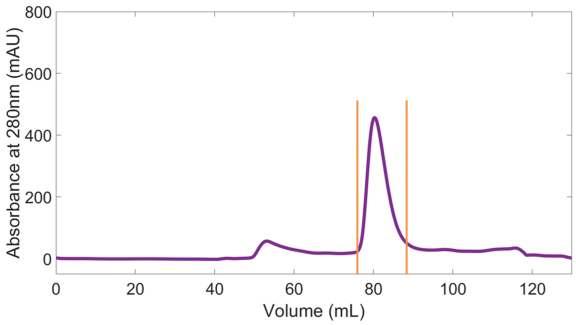
(c) A4A4eGFP eluted with Buffer B.

(d) D9D9eGFP eluted with PBS.

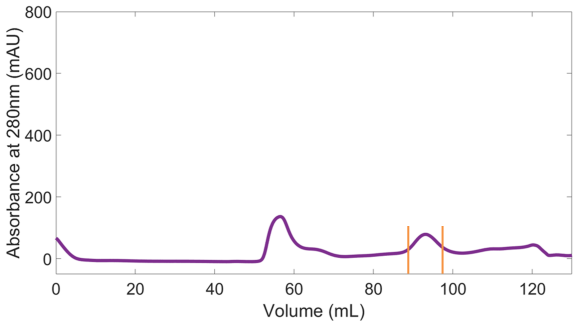
(e) A4D9eGFP eluted with Buffer B.

(f) D9A4eGFP eluted with Buffer B.

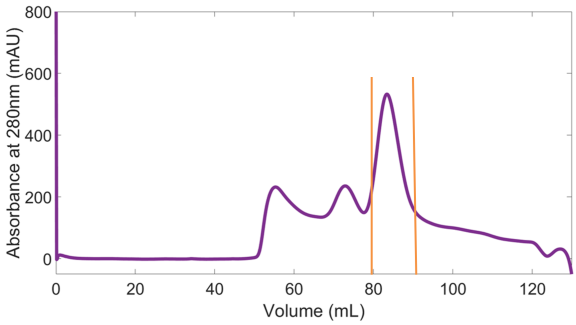
Figure A.3: Chromatograms for the elution of the affibodies in the first SEC. The orange vertical lines approximately delimit the peaks analysed through SDS-PAGE (see Figure 4.2).



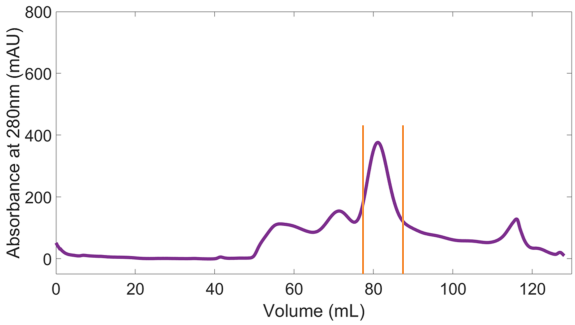
(a) PspCT eluted with PBS pH = 7.4.



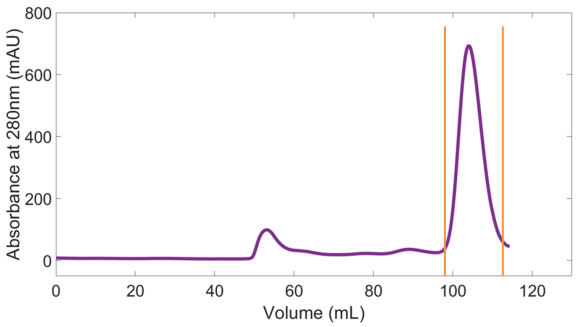
(b) PspC1T eluted with PBS pH = 7.4.



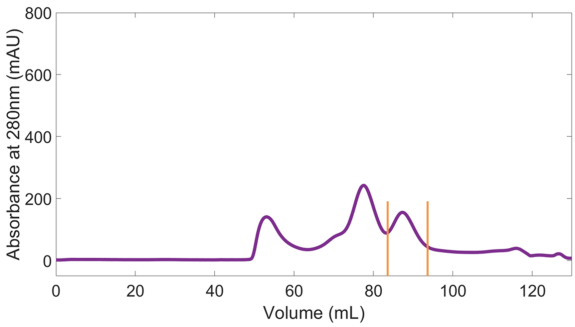
(c) PspC2T eluted with Buffer C.



(d) PspC2T_Extra eluted with PBS pH = 7.4.



(e) PspC2T FHBD eluted with PBS pH = 7.4.



(f) PspC2T CTerm eluted with PBS pH = 7.4.

Figure A.2: Chromatograms for the elution of the proteins in the first SEC. The orange vertical lines approximately delimit the peaks analysed through SDS-PAGE (see Figure 4.2).