The University Of Sheffield.

Secreted neisserial proteins as potential vaccines or tools for biotechnology

MPhil Thesis

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Contents

List of Figures

Abbreviations

°C	degrees celcius	fHBP	factor H binding protein
(v/v)	volume per volume	FITC	fluorescein isothiocyanate
(w/v)	weight per volume	FSC	forward scatter
µg	microgram	ɣ	gamma protein from *Neisseria meningitidis* serogroup B
µl	microlitre	g	gram
µM	micromolar	HA	hyaluronic acid
5-IAF	5-iodoacetamido fluorescein	Hbp	haemoglobin protease
Å	angstrom	HpuA/B	Hemoglobin-haptoglobin utilization protein A/B
aa	amino acids	HRP	horse radish peroxidase
APS	ammonium peroxidosulphate	hrs	hours
ATP	adenosine triphosphate	HSPG	heparan sulphate proteoglycans
BCA	bicinchoninic acid	IgA	immunoglobulin A
bp	base pairs	IgA1/2	Immunoglobulin A type 1/2
BrkA	*Bordatella* resistance to killing A	IgA1P	Immunoglobulin A type 1 protease
BSA	bovine serum albumin	IgG	Immunoglobulin G
CEACAM	carcinoembryonic antigen-related cell adhesion molecules	IL-6/8	interleukin 6/8
cm	centimetres	IαI	Inter-α-inhibitor
CSD	cleavage specificity determinant	IαI	Inter-alpha-inhibitor
cv	column volumes	Kb	Kilo bases
c-ɣ-α	cysteine-gamma-alpha (NMB *N. Meningitidis*)	kDa	kilo Daltons
dH_20	Distilled water	KPB	potassium phosphate buffer
DMF	dimethylformamide	L	litre
DMSO	dimethyl sulphoxide	L/min	litres per minute
DNA	deoxyribonucleic acid	LAL	limulus amebocyte lysate
dNTP	deoxyribonucleotide triphosphate	LAMP1/2	lysosomal associated membrane protein 1/2
DTBP	dimethyl 3,3'-dithiobispropionimidate	LB	lysogeny broth
DTT	dithiothreitol	LPS	lipidpolysaccharide
eDNA	extracellular DNA	M	molar
EDTA	ethylenediaminetetraacetic acid	mA	milliamps
ELISA	enzyme linked immunoabsorbant assay	MenB	*Neisseria meningitidis* serogroup B
EspP	Extracellular serine protease plasmid-encoded	MES	2-(*N*-morpholino)ethanesulfonic acid
FACS	fluorescence activated cell sorting	mg	milligram
FEN	flap endonuclease	MH para	*Mannheimia haemolytica* Gly1 paralogue

MH0579	*Mannheimia haemolytica* Gly1 homologue MH0579	PDB	protein database
MH1205	*Mannheimia haemolytica* Gly1 homologue MH1205	PEI	polyethyleneamine
min	minutes	pI	Isoelectric point
ml	millilitre	PMSF	phenylmethylsulfonyl fluoride
mM	millimolar	PorA	porin A
mRNA	messenger ribonucleic acid	RBC	red blood cells
MWCO	molecular weight cut off	rpm	rotations per minute
NadA	neisserial adhesin A	RT	room temperature
NHBA	*Nesseira* heparin binding protein	SATP	N-succinimidyl-S-acetylthiopropionate
nHBP	neisserial heparin biding protein	SDS	sodium dodecyl sulphate
NLS	nuclear localisation signal	sec	*seconds*
nm	nanometer	Sec A	Protein translocase subunit SecA
NMB	*Neisseria meningitidis* serogroup B	Skp	Seventeen kilodalton protein (OmpH)
O/N	overnight	SMCC	sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate
OD	optical density	SSC	side scatter
Omp85	outer membrane protein 85	TAE	Tris-acetate-EDTA
OMV	outer membrane vesicles	TEMED	tetramethylethylenediamine
Opa / Opc	opacity protein	TNFRII	tumour necrosis factor receptor type II
PAGE	polyacrylamide gel electrophoresis	TNFα	tumour necrosis factor α
PBS	phosphate buffered saline	UV	ultra violet light
PBS-T	phosphate buffered saline with 0.1% Tween-20	V	Volts
PCR	polymerase chain reaction	WBC	white blood cells

x *g*	acceleration due to gravity
α	alpha protein from *Neisseria meningitidis* serogroup B
Δβ	beta core deletion

Chapter 1- General Introduction

Summary

***N. meningitidis* is a Gram negative, human specific pathogen which is one of the leading causes of bacterial meningitis worldwide. Despite the severity of the disease the current available vaccines do not provide complete protection against infection therefore prompting research into other proteins which have potential as vaccine targets. A variety of possible virulence factors have been found for this pathogen including IgA1 protease which has been attributed with immune evasion and causing severe inflammation. It has also been noted that natural antibodies to IgA1P persist in the body for five years or longer post infection or carriage of *N. meningitidis*. All of these factors suggest a role in virulence determination and the potential for the use of IgA1P and its co-secreted peptides as potential vaccine targets. This report outlines how the α and c-γ-α proteins, which are co-secreted alongside neisserial IgA1P, underwent several trials in order to elucidate structural data and cross-linking experiments which have determined a potential binding partner in the circulation system. Further the potential of α as a vaccine carrier protein has been evaluated, showing that it boosts immune response to other proteins when inoculated alongside them. In parallel with this work neisserial IgA1P protein has undergone multiple mutagenesis reactions creating inactive and easier to purify versions of the protein which can be used in future structural and functional trials.**

1. Introduction

1.1 Meningitis overview

Meningitis is a life threatening disease affecting between 1/100000 and 1/1000000 people worldwide with significantly higher incidences in developing countries, in particular the sub-Saharan Africa "meningitis belt" [1]. Upon infection patients present with inflammation of the meninges which surround the brain and the spinal cord, at a later stage septicaemia can also develop. The inflammation of the meninges causes pressure to be exerted on the brain which can lead to a number of symptoms including vomiting, purpuric rash and septicaemia. Ultimately this pressure on the brain and septicaemia, if left untreated is fatal [1]. Despite the fact that meningitis can be treated simply if caught early it still is associated with around a 10% mortality rate in developed countries, this mortality rate rises significantly in those

patients who develop septicaemia or in less developed countries [2]. Patients who survive meningitis infection are often left with a number of permanent after effects such as deafness, brain damage or the loss of limbs due to prolonged pressure on the brain or septicaemia [3]. These effects often leave patients needing ongoing support post-infection which can decrease their quality of life and burden the healthcare system. As a result work into developing a protective vaccine for meningitis, specifically bacterial meningitis as will be explained later, is ongoing.

1.2 Meningitis causes

Meningitis can be brought about by a number of disease states such as fungal infection, cancer, viral infection or bacterial infection [4]. Generally however, meningitis in developed countries is associated most commonly with viral or bacterial infection (**Figure 1**.)[4,5].

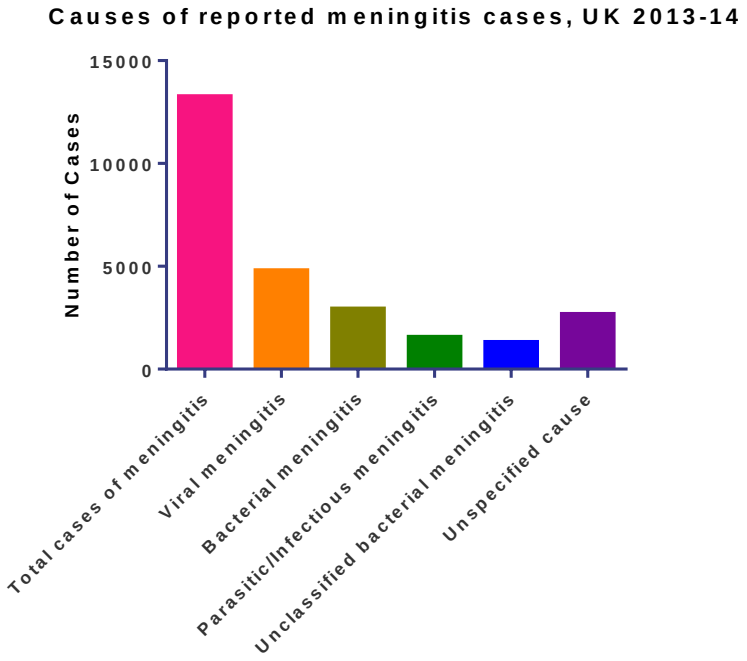

Figure 1. A breakdown of the number of reported cases of meningitis in the UK between 2013-2014. This graph shows the relative prevalence of different causes of meningitis, highlighting how viral and bacterial meningitis are the leading causes of infectious meningitis in the UK [5].

Across all of these causes meningitis incidence peaks in children below 1 year old and in adults between 15-39 years old (**Figure 2**.) [5,6] . The main reasons for these peaks is that children under 1 tend to have weaker, developing immune systems, whilst people between 15-39 tend to live in more close knit communities with more social contact [7–11] . This increased social contact leads to easier transmission of meningitis causing pathogens and thus a higher carriage and infection rate, as will be discussed in more detail later.

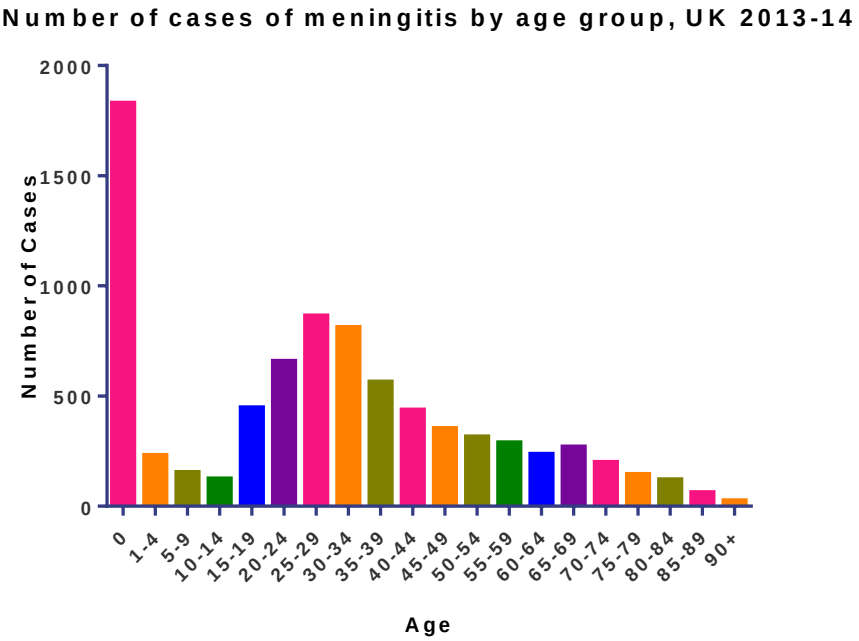

Figure 2. A breakdown of the number of cases on meningitis by age group. The graph shows a clear two peak incidence trend at the ages of 0-1 and 15-39 [5] .

Bacterial meningitis in the UK and other developed countries is most commonly caused by infection of meningococcal bacteria such as *Neisseria meningitidis, Haemophilus influenza, Myobacterium tuberculosis* and *Streptococcus pnuemoniae* (**Figure 3**.) [5] .

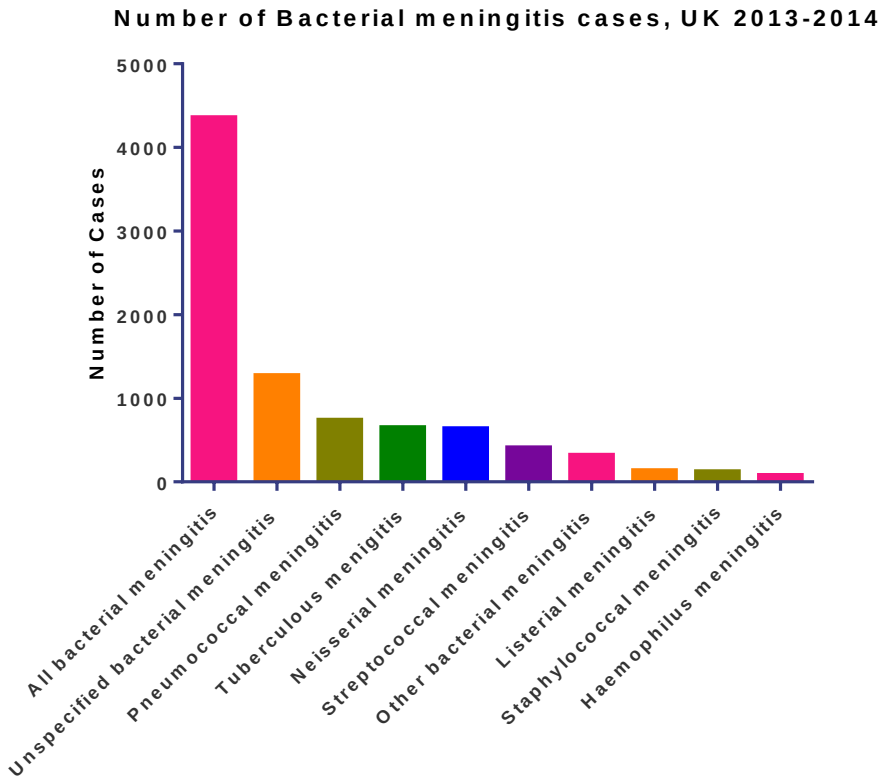

Figure 3. A breakdown of the causes of bacterial meningitis cases. The main causes of meningitis in the UK and the developed world are pneumococcal, *Neisserial* and streptococcal pathogens [5].

Although over the last few decades the infection and associated mortality rate of meningococcal disease has been falling there are still some major challenges to be overcome, particularly with regards to meningitis caused by *Neisseria meningitidis.*

1.3 *Neisseria meningitidis*

N. meningitidis is the second or third leading cause of meningococcal disease in the UK [5] and has been associated worldwide with both meningitis epidemics [12,13] and some of the most invasive strains of menigococci. *N. meningitidis* is a Gram negative pathogenic bacteria which is part of the *Neisseriae* genus [1,14], this genus also includes *N. lactamica* and *N. gonorrhoeae* which both exclusively colonise humans. *N. meningitidis* has been shown to exclusively colonise the nasopharynx of humans [15–17] in a similar way to *N. lactamica*. In order for infectious disease to occur *N. meningitidis* must be able to spread from the hosts

nasopharynx into the circulatory system allowing for systemic infection to occur, the virulence factors associated with this switch from commensal to invasive will be discussed in detail later. The systemic infection of the pathogen leads to the onset of septicaemia and the inflammation of the meninges classically associated with meningococcal disease [9,14]. Despite *N. meningitidis'* association with infectious disease it is most commonly found as a commensal bacteria which is carried asymptomatically by between 10-25% of the population at a time [1,7,14]. Carriage rates have been show to exceed this figure in groups living in close communities such as students, military recruits and young children, in these groups carriage can rise as high as 50% [8,9,11]. As a result of these increased carriage rates in young adults and the weak immune systems of young children, meningococcal disease shows two clear incidence peaks in children under 1 and people between the ages of 15-24 (Fig 4) [5].

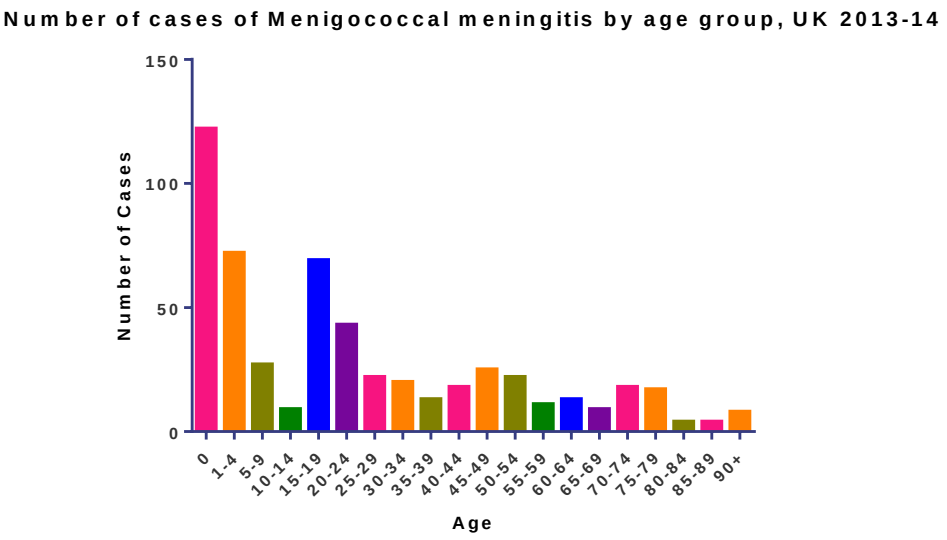

Figure 4. Meningococcal meningitis has two clear incidence between the ages of 0-4 and 15-24 and a lesser peak at 70-79 [5]. This is thought to be due to the weakened immune systems of infants and the elderly and increased social contact at ages 15-24.

N. meningitidis is not one homogenous species and is instead further subdivided into 12 separate serogroups [14,18]. These are distinguished from one another by differences in the capsular polysaccharide structure, with mortality rate of the infection often varying by

serogroup. Serogroups A, B, C, Y and W-135 have been shown to account for most of the life threatening outbreaks of neisserial meningitis worldwide [14,18]. Generally serogroup B and C are the most common cause of life threatening neisserial meningitis in the UK (Fig 5.) and other developed countries, whilst serogroup A is accountable for the epidemic meningococcal disease found in the African meningitis belt (Fig 6.) [12,13].

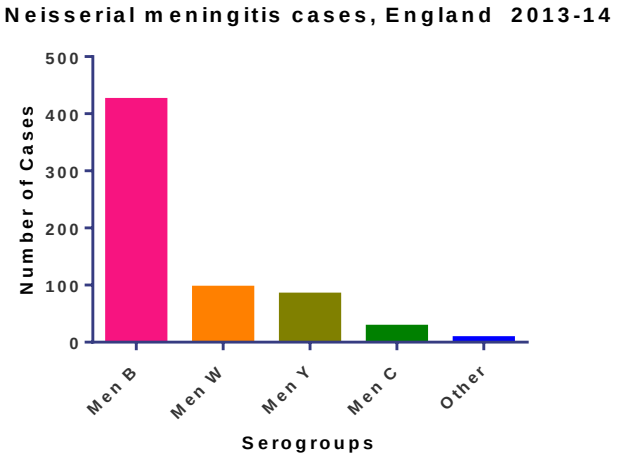

Figure 5. Neisserial meningitis is most often caused by serogroup B *Neisseria meningitidis* strains in the UK and the developed world [5]. This incidence changes drastically globally, for instance serogroup A is the most prevalent disease causing serogroup in sub-Saharan Africa [12].

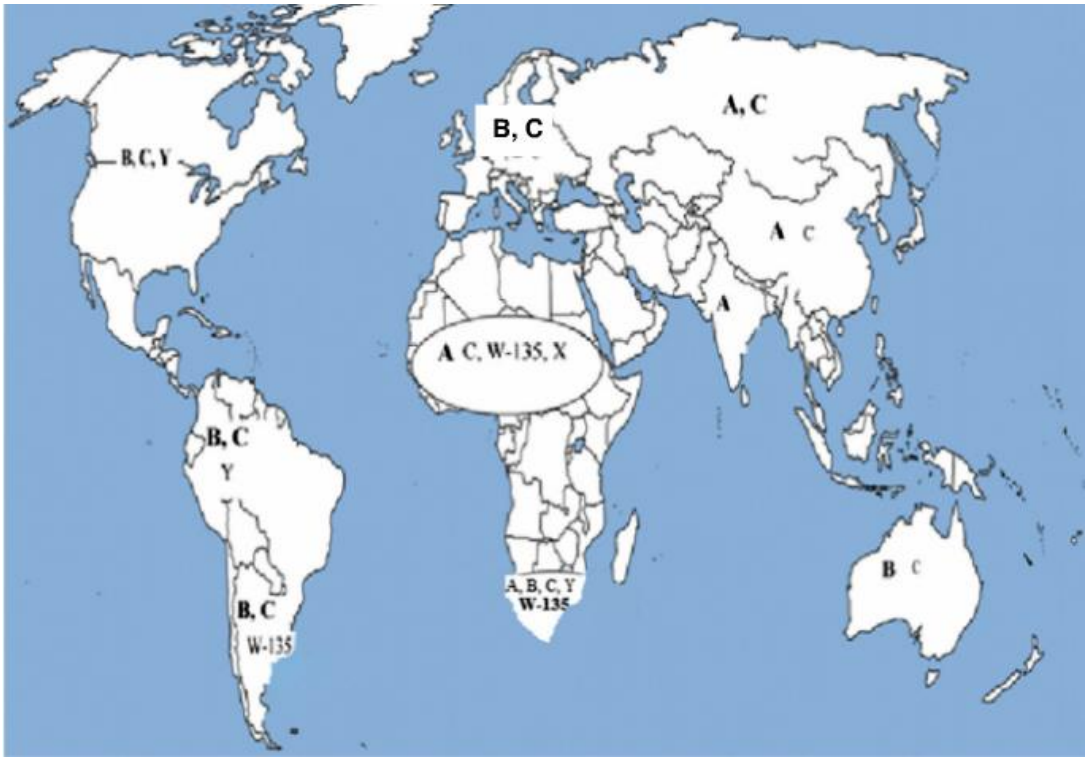

Figure 6. The global epidemiology of invasive meningococcal disease, this figure shows how the major disease causing serogroup of *Neisseria meningitidis* varied globally. Generally more developed countries have more prevalent invasive disease outbreaks from serogroup B isolates, whilst less developed countries have more outbreaks caused by serogroup A isolates. (Figure taken from [13])

N. meningitidis is becoming a growing target for vaccine development due to its high incidence in young children coupled with its high mortality rate and associated long term after effects.

1.4 Vaccines

Invasive meningococcal disease is now routinely treated with β lactam antibiotics such as third generation cephalosporin [1], however there is now a growing arsenal of meningococcal vaccines aimed at providing protection against most major serogroups [11,13,19–22]. The aim of these vaccines is to prevent invasive disease from ever occurring and therefore lowering invasive disease rates and the morbidity rates associated with invasive meningococcal disease.

Vaccines were first famously invented by Edward Jenner in 1798 as a method of preventing the spread of smallpox using the related cow pox virus. Since then vaccine development has rapidly expanded and given rise to three major subclasses of vaccine. These are vaccines containing live attenuated bacteria, such as in the BCG vaccine, vaccines containing purified bacterial matter or vaccines containing carrier proteins conjugated to polysaccharides[126].

Lately this search for vaccine targets has been aided by the use of reverse vaccinology. Reverse vaccinology was coined in 2000 by Rappuoli[127] after whole genome sequence allowed the mining of sequences for surface displayed antigens which could be potential targets for vaccine development. Reverse vaccinology allows a user to mine a data sequence for proteins of interest using computer algorithms, this identifies DNA fragments which encode proteins (ORFs) and excludes those which have homology to known cytoplasmic proteins. These ORFs are then screened for features typical of surface displayed proteins[126]. Another version of this reverse vaccinology approach highlights any potential proteins which show homology to known or putative virulence factors[128]. These selected regions are then cloned and subjected to high-throughput expression before being tested for immunogenicity in a mouse model. In order to test the immunogenicity, the serum from the mice is used in a western blot against the whole bacterium and the protein of interest, this confirms the specificity and immunogenicity of the protein. This is then further tested in a series of ELISAs and flow cytometry experiments using whole live bacteria[126]. More recently the first commercially available vaccine developed through reverse vaccinology was meningitis B vaccine[129].

Most modern vaccines against meningitis serogroup B have made use of outer membrane vesicle (OMV) technology. Initially these vaccines were used in strain specific cases using wild type non-recombinant OMVs giving monovalent coverage, for example in the Cuban meningitis B vaccine[125]. Modern vaccine development has made use of two different

methods of OMV generation and purification. Spontaneous OMVs (S-OMVs) can be purified from culture supernatant, this gives rise to natural OMVs containing most lipoproteins associated with the membrane but high levels of LPS inclusion. Detergent extracted OMVs (D-OMVs) are formed by treating cells with detergent, this reduces the LPS load of the OMVs and therefore lowers unwanted immunogenic responses but can also lower the level of other important lipoproteins which are less tightly associated with the OM[130].

As mentioned above most modern vaccines targeting *Neisseria meningitidis* are based on outer membrane proteins, these can then be conjugated to a carrier protein such a tetanus toxoid [2] . The carrier protein is a non-toxic protein which increases the immune response elicited by the vaccine by providing T cell helper epitopes [2,23] . These T cell helper epitopes are recognised by specific T cells causing proliferation and differentiation of B cells [24,25] . T cell helper epitopes are short amino acid sequences consisting of 7-10 amino acids which are able to bind to histocompatibility complex proteins found on the surface of T cells [24,25] . The binding of these carrier proteins cause a change in the immune response to the capsular proteins in the vaccine from a B cell dependent response to a T cell dependent response. This is achieved by activating CD4+ T helper cells which are required for memory B cell formation, therefore this T helper cell activation allows a long lasting immune system memory to be formed against the vaccine [24,25] . This increases the overall effectiveness of the vaccine in both children and adults whilst simultaneously increasing the length of the time for which the vaccine provides protection from meningococcal disease.

Vaccines currently on the market tend to be multi-valent polysaccharide vaccines which are either specific, bivalent or tetravalent in order to confer immunity to multiple serogroups [26,27] . The vaccines provide meningococcal protection in two main ways; by directly protecting the vaccinated individual and also by providing a “herd” immunity protection [2,21,26 , 28] . The “herd” immunity effect is due to the fact that in populations with some vaccinated

individuals the overall carriage rate of the pathogen are lower, this therefore lowers the risk of colonisation and disease in non-vaccinated individuals [28]. As seen previously in **Figure 5**, *N. meningitidis* serogroup B is currently the leading cause of meningococcal disease in the UK and the western world, however there is currently only 1 vaccine (Bexsero, GlaxoSmithKline) that has a protective effect against serogroup B meningococcal disease [19,20,26,29,30]. This vaccine is a very recent development and so its protective effects are as yet unknown, however it has been estimated that it will be protective against around 78-88% of serogroup B *N. meningitidis* strains found in Europe and the UK [31,32]. The main problem with developing a protective serogroup B vaccine has been that the capsular proteins in serogroup B strains mimic NCAM (neural cell adhesion molecule) [14]. This prevents the vaccine from eliciting a strong immune response, therefore rendering the vaccine useless or at best very weakly protective. As a result, the Bexsero (4CMenB) vaccine contains 3 proteins which have been found to be crucial for *N. meningitidis* serogroup Bs survival and invasive pathogenesis. The 3 remaining proteins are factor H binding protein (fHbp), neisserial adhesin A (NadA) and *Neisseria* heparin binding antigen (NHBA) these 3 proteins are also combined with outer membrane vesicles derived from the NZ98/254 strain of MenB which contain porin A (PorA) [20,33]. Porin A has been shown to exhibit high sequence variability therefore, In order to account for this vaccines must include a large variety of PorA subtypes in order to gain sufficient strain coverage[125]. As a result in Bexsero many types of outer membrane vesicles have been included, such as OMVs derived from NZ98/254 are included as they were previously used in a local vaccine which was shown to have a strong protective effect against certain MenB strains involved in a meningitis outbreak in New Zealand [33].

Despite the release of Bexsero there is still room for further Men B vaccine development and improvement due to the ability of *Neisseria meningitidis* to undergo capsule switching via horizontal gene transfer [18]. This phenomenon allows bacteria within the same genotype to express different capsules therefore rendering vaccines based upon capsular proteins less

effective. This has led to the frequent use of tetravalent vaccines which target the 4 serogroups most commonly associated with invasive meningococcal disease, therefore limiting the effect of capsule switching. However it has been observed that invasive serogroup Y and C bacteria were able to acquire a serogroup B capsule via horizontal transfer, thus making them more difficult to target with a vaccine [18]. This raises the possibility of hyperinvasive strains developing a resistance to vaccines by switching to capsule types that are not covered by currently available vaccines. As such a new target for global meningitis vaccine development is required, the new targets will likely be ubiquitous virulence factors expressed by the pathogen rather than capsular proteins.

1.5 Virulence factors

The virulence of *N. meningitidis* has been attributed to a broad range of factors such as: capsule type, adhesins, pili, iron acquisition proteins and IgA1P production however, none of these factors alone have proven to be sufficient for invasive disease [1,34–36]. It therefore seems reasonable that a variety of these factors must interplay in order to allow the bacteria to switch from commensal to an invasive disease causing strain, although interplay between these factors has not been well studied and is not well understood.

As previously mentioned *N. meningitidis* is split into 12 serogroups based upon the capsular structure [18,37]. These capsules are distinguished from one another by differences in the polysaccharide structure, with serogroup B, C, Y and W-135 all having a capsule formed from sialic acid linked to neuraminic acid [37]. In comparison Serogroup A has a capsule formed from N-acetyl mannosamine-1-phosphate [37]. The use of sialic acid in the capsules of serogroup B, C, Y and W-135 mediates the hosts immune response to the bacteria as sialic acid is commonly found on human cell surfaces. The most notable mimicry is that of the serogroup B capsule which contains the same sialic acid-neuraminic acid polymer as is found in the human neural cell adhesion molecule[18,37]. As mentioned earlier this lowers its

immunogenicity greatly, therefore allowing it to evade clearance by the immune system to a much greater degree than other serogroups. As previously mentioned *N. meningitidis* is also capable of capsule witching via horizontal gene transfer, further aiding the pathogens ability to evade the immune system.

The neisserial capsules not only help to lower the immunogenicity of the bacteria they also help to prevent the desiccation of the bacteria and thus increase the survival rate of the bacteria when compared with acapsular strains [38] . Capsules achieve this protection by protecting the encapsulated bacteria from environmental factors, allowing it to survive for several days *ex vivo* [38] . The protection from desiccation can most clearly be highlighted by comparing the transmission of encapsulated *N. meningitidis* to the acapsular *N. gonorrhoeae*. Both of these pathogens are obligate human pathogens in the same family however, *N. meningitidis* can be transferred from person to person relatively easily via close contact with respiratory secretions (eg. Sneezing) or saliva whereas, *N. gonorrhoeae* requires intimate contact for transmission [14] . These differences in the ease of transmission are primarily due to the lack of a capsule in *N. gonorrhoeae* and therefore its susceptibility to drying out and becoming non-viable. The protection from environmental factors provided by the capsule also increases the invasive capacity and survival rates of the *N. meningitidis* strains, with acapsular strains tending to be less invasive and having poor survival rates. One of the main causes for increased invasive capacity in invasive strains is that the capsule often contains other virulence factors such as pili or adhesins [39] .

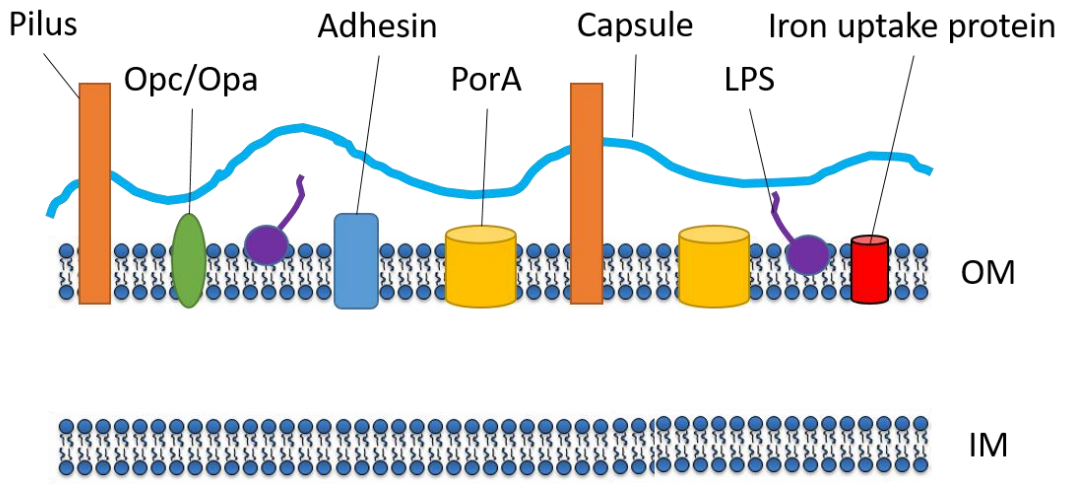

Figure 7. The major surface antigens of *N. meningitidis. N. meningitidis* has a number of major surface antigens which contribute towards its ability to colonise the human nasopharynx and cause invasive disease. Factors such as the pili and adhesins allow the bacteria to adhere to the epithelial cells of the nasopharynx, whilst factors such as the iron uptake proteins and the capsule increase its survivability within a hostile environment.

The adhesion of *N. meningitidis* to host epithelial cells in the nasopharynx has been shown to be a vital part of colonisation and as a result is sternly linked to virulence and invasive disease progression. The adhesion of N. meningitidis to epithelial cells is mediated by a number of factors such as pili, NadA, Opa and Opc **Fig 7**., as such most of these can be classed as essential for bacterial survival and in some cases as potential virulence factors.

Opa and Opc are a pair of related membrane proteins both expressed by *Neisseria meningitidis* which are composed of 8 and 10 transmembrane domains respectively which form a β barrel with 4 surface exposed loops [40,41].

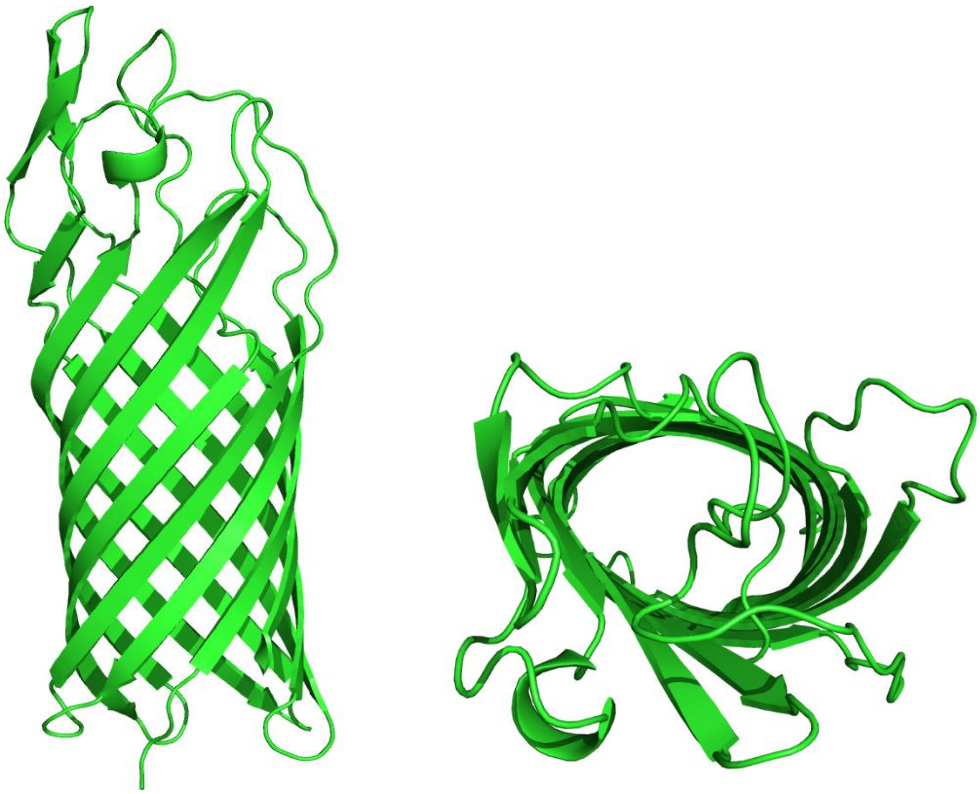

Figure 8. The crystal structure of OPCa from *Neisseria meningitidis* [42] . This structure highlights the classic β barrel shape formed from 8 transmembrane anti-parallel β sheets. (Structure downloaded from EMBL-EBI PDB entry no. 1K24 and modified in PyMOL)

The Opa proteins expressed by *Neisseriae* are around 28 kDa and are crucial to the adherence of bacteria to epithelial cells, as well as the invasion of host cells[40,41]. As a result of their function they are thought to play a vital role in determining the virulence of the pathogen due to their ability to promote colonisation of the mucosal membranes of the nasopharynx as well as facilitating invasion of host cells in order to gain access to required nutrients such as iron [41] . This invasion of host cells may also allow the bacteria to traverse the mucosal membrane and invade the circulatory system giving rise to systemic disease [43] . Opa proteins are also capable of very rapid genetic mutation due to a conserved repeat which allows recombination between related Opa proteins from other neisserial strains via horizontal gene transfer [44] . This genetic and protein mutation leads to surface antigenic variation which

helps to protect the pathogen from host immune responses, as well as allowing neisserial species to acquire Opa genes which may increase the pathogens virulence [45].

Opa and Opc both recognise and bind to cell surface receptor proteins found on host epithelial cells. Opa recognises members of the CEACAM protein family [44] which are found on epithelial cells as well as some immune system cells, such as neutrophils and T and B lymphocytes, whilst Opc recognises HSPGs [43]. The ability to bind either of these families of proteins therefore mediates adherence to host epithelial cells, allowing bacterial colonisation and the adherence that is crucial for invasive disease progression. The binding of CEACAMs by Opa and causes the *Neisseria* to be engulfed and allows access to the sub-epithelial spaces through polarised epithelia whereas, the binding of HSPGs allows uptake of the bacteria into non-polarised epithelia [46,47]. This reaction helps to prevent clearing of the pathogen from the mucosal membranes via agglutination by IgA or any sweeping mechanisms by causing it to adhere strongly to cell surfaces or allowing it to colonise sub-epithelial layers that are not prone to these sorts of clearing methods.

Binding to CEACAMs is also notable as CEACAMs are upregulated by inflammatory cytokines such as interferon ɣ which is released during bacterial colonisation and infection [48]. This response can increase the density of CEACAMs on the mucosal cell surface by between two and six fold, therefore facilitating adherence and invasion of Opa expressing meningococcal strains [48].

The binding of Opa to CEACAMs has also been shown to inhibit the activation and proliferation of CD4+ T lymphocytes [49]. During infection *N. meningitidis* have been shown to produce outer membrane vesicles (OMVs) or “blebs”, these OMVs often contain Opa proteins which retain their binding capacity for the CEACAMs found on immune cells or epithelial cells [49]. It has therefore been speculated that Opa in OMVs could bind CEACAM1 proteins found on CD4+ T lymphocytes and inhibit their action by activating the tyrosine

phosphatases SHP-1 and SHP-2 which prevent kinase dependent activation signals from being triggered [49]. This inhibition of T lymphocytes could then create an immunosuppressed zone around the site of infection allowing immune system evasion and further colonisation of the epithelial cells to take place [49]. Interestingly in contrast to this neutrophils have been shown to express cell surface CEACAM 1, 3 and 6 to which Opa positive *Neisseria* have been shown to bind. This allows the neutrophils to use the CEACAMs as a method of binding and phogocytosing the bacteria, preventing further colonisation [50,51]. In particular binding of CEACAM 3 on neutrophils by Opa causes phagocytosis and stimulation of the oxidative burst response used to kill invading pathogens [44, 50]. One interesting thing to note is that Opa containing OMVs may activate the neutrophils and subvert their effects from the bacteria, therefore mitigating this effect [44]. Finally the binding of CEACAM 1 on B lymphocytes by Opa has been shown to initiate the cell death pathway in B cells, leading to the reduction in antibody production by B cells [52]. This allows for sustained colonisation by the pathogen and allows repeat infections of the host.

A further adhesion mediating factor are the pili, these hair like structures are made from multiple subunits of pilins which assemble into long filaments [36]. Type IV pili have been found on the bacterial surface of a wide range of bacterial species including *E. coli*, *Vibrio cholera* and *N. meningitidis* and carry out a broad range of functions including allowing adhesion of bacteria to host epithelial cells [53]. Type IV pili have a core role in adhesion of the bacteria to host cells and as such they have been identified as key virulence factors as they allow colonisation and host cell invasion [54]. Not only do pili mediate adherence of the bacteria to epithelial cells, they also promote auto aggregation of the bacteria into microcolonies or bacterial aggregates [54]. Within these microcolonies it has been shown that *N. meningitidis* proliferates readily and is capable of surviving not only within the hosts nasopharynx but also resisting the pressure exerted by blood flow [55,56]. Further to their role as adhesins pili also

confer a number of other advantages to the pathogen such as motility and the ability to uptake DNA readily from its environment [36].

The motility given to *N. meningitidis* by pili is known as twitching motility, this achieved by the rapid withdrawal and extension of the pili using energy from ATP synthase [57]. This motility allows the bacteria to migrate across the hosts' cells, which is key to the spread of infection and the colonisation of the mucosal membranes [57 36 3636].

Furthermore type IV pili are also involved in genomic variation of *N. meningitidis* by permitting the bacteria to uptake DNA from extracellular space [53]. This DNA can then be integrated into the genome of *N. meningitidis* due to its natural competency. This natural competency along with a method of obtaining and importing DNA allows for a large degree of both genomic and antigenic variation within a colony of *N. meningitidis* [53]. This variation can lead to increased virulence of the pathogen or better survivability of the bacteria due to better host immune evasion.

Another prominent virulence factor being used in vaccine development is neisserial adhesin A (NadA) [58]. NadA has been shown to be present in a higher proportion of invasive strains, around 30%, compared to commensal strains, around 16%, and crucially has been detected in nearly all hyperinvasive strains detected clinically [59,60]. NadA is a trimeric autotransporter protein involved in adhesion of host epithelial cells as well as interactions with host immune cells during colonisation and infection [59]. NadA has been shown to be transported to the outer membrane of *N. meningtidis* where it forms large, highly stable oligomers [59, 61]. These large oligomers have been shown to mediate adherence to epithelial cells *in vitro*, therefore it is theorised that they perform a similar role to other bacterial adhesins *in vivo* [61]. In a similar set of experiments it has also been found that, in a similar way to Opc, NadA is ably to promote invasion of epithelial cells [61]. This invasion promotes survival of the bacteria and protection from killing by host immune responses by allowing the bacteria to colonise sub-

epithelial layers. What is important to note is that it appears that Opc and NadA operate independently of one another as strains containing only one of the proteins still retains invasive properties, whereas strains lacking both proteins exhibit a 10 fold reduction in invasive capacity compared with strains expressing both [61].

Bacteria, like many other forms of life, require iron in order to survive and proliferate and as such iron acquisition is a key regulator of bacterial pathogenicity [62,63]. Due to irons ability to undergo redox reactions it plays an essential role in many catalytic reactions performed within cells and is also required for electron transport and therefore respiration. However due to irons ability to create reactive oxygen species that would cause damage to nearby cells around 95% of iron in mammalian hosts is sequestered by heme, cytochromes or other enzymes therefore rendering it non-bioavailable for pathogens [64]. As a direct result many bacteria have evolved ways of obtaining iron from the hosts cells in order to survive, it has also been shown that the ability of a pathogen to obtain iron from its host directly relates to its pathogenicity. This is because iron allows a pathogen to survive the oxidative burst of phagocytosis used to kill invading bacteria whilst also allowing it to survive and replicate more efficiently[63].

In Gram negative bacteria such as *Neisseriae* 3 types of iron acquisition systems have been identified; direct uptake, bipartite haem receptors and hemophore mediated uptake systems [65]. Whilst these all vary in their methods of iron acquisition, they all allow bacteria to acquire and uptake iron before transporting it across the cell membranes and into the cell.

The bipartite haem receptor system was first described in *Neisseria* [63] and uses a two part ton B dependent outer membrane receptor and lipoprotein, known as HpuAB to acquire haem [66, 67]. More recently homologs for HpuA have been discovered in other bacterial species, suggesting that this system is not just confined to *Neisseriae*. The two parts of the bipartite system are HpuA, a surface exposed lipoprotein, and HpuB which is a

transmembrane protein [66]. Together they are capable of binding haemoglobin, releasing the haem group and then transporting the haem into the cell, utilising the TonB pathway [66, 67]. In order to move haem across the cell membranes TonB harnesses the proton motive force in an energy dependent manner, this allows the bacteria to intake the haem allowing cellular enzymes to degrade the haem and release the iron [66].

More recently two proteins, Gly1-ORF1 and Gly1-ORF2, have been identified as possible conserved iron scavenging and acquiring proteins. It has been shown that neisserial strains expressing Gly1-ORF1 show haemolytic activity *in vitro* and that Gly1-ORF2 shows homology to HemD which is involved in the haem biosynthesis pathway in converting linear tetrapyrole molecules into uroporphyrinogen III synthase therefore strengthening the case for the 2 Gly1 genes being involved in iron acquisition and release.

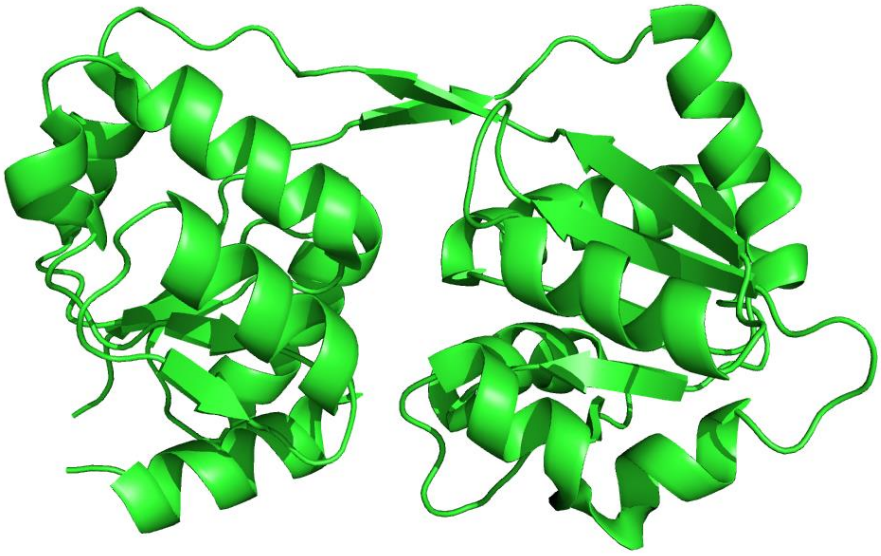

Figure 9. Crystal structure of HemD from *Pseudomonas aeruginosa* [68]. The HemD protein has been shown to have homology to Gly1-ORF2, this may indicate that they fulfil similar roles within the bactera. Structure downloaded from EMBL-EBI PDB entry no. 4ES6 and modified in PyMOL.

The neisserial heparin binding protein (nHBP) is a surface exposed protein first detected during a reverse vaccinology screen to identify potential *N. meningitidis* serogroup B vaccine targets [69]. During this study it was shown that immunisation of mice with this protein created antibodies capable of eliciting complement mediated killing of the bacteria, as a result it is now one of the key components in Bexsero (4CMenB) [20,69]. Due to its discovery route initially very little was known about the structure or function of the protein, despite the fact it was thought to be a possible vaccine candidate [69]. Further study by Serruto et al. showed that nHBP was capable of binding both heparin and heparin sulphate [69], this is particularly interesting as the binding of heparin is associated with an increase in resistance to complement mediated killing [70,71]. What is unclear is how the binding of heparin by nHBP elicits this response, although it has been theorised that the bound heparin could be interacting with complement regulatory proteins found in serum and preventing complement mediated killing [70,71]. Furthermore the binding of heparin by nHBP could facilitate attachment of the pathogen to host epithelial cells in a similar way to other adhesins by binding surface exposed heparin sulphate on the hosts epithelial cells [69].

Another potential key virulence factor, and the focus of this study, is IgA1 protease (IgA1P). IgA1P, as the name suggests, was initially discovered in 1793 due to its ability to cleave IgA1 which is secreted at the mucosal membrane [72]. As a result, it was thought that IgA1P may have a role in host immune evasion, which would allow the bacteria to protect itself from host immune response and effectively colonise and infect the host. What makes IgA1P look particularly promising as a virulence factor is the fact that clinical studies of meningitis have shown that invasive strains of both *N. meningitidis* and *H. influenzae* have up to a fivefold elevation in IgA1P production when compared to carriage isolates of the same bacteria [34,35,73]. This therefore seems to indicate a strong link between the virulence of a strain and the level of IgA1P production [34,35,74,75]. It has also been reported that ~98% of invasive isolates produce IgA1P compared with around 76% of carriage isolates [34]. This suggests that although IgA1P

expression may not be required for invasive disease and may not initiate invasive disease, it could be a strong factor in bacterial survival on the mucosal membrane allowing further colonisation and infection to take place.

1.6 IgA1P

1.6.1 Background

As previously mentioned IgA1P was discovered by Mehta et al. and was named due to its apparent specificity for human IgA1 molecules and its inability to cleave other immunoglobulins [72, 73]. Since its initial discovery in 1973 IgA1P has been found to be conserved in many of the major infectious disease causing Gram negative bacteria such as *N. meningitidis*, *H. influenzae* and *S. pneumoniae* [34,35,75,76]. Interestingly it appears to be particularly highly conserved in those bacteria that colonise host mucosal surfaces [73]. It was later isolated from *Neisseria gonorrhoeae* and used to discover the type V autotransporter system, of which it became the archetypal and founding member [77].

IgA1P is formed of 4 domains (Fig 9) which are common to all type Va autotransporters these are; the leader, the passenger, the linker and the β core [78,79]. The β core has been found to have a fairly diverse N terminus region between organisms however, it has a key conserved region of 300 amino acids in the C terminus which plays a vital role in the protein export process [74].

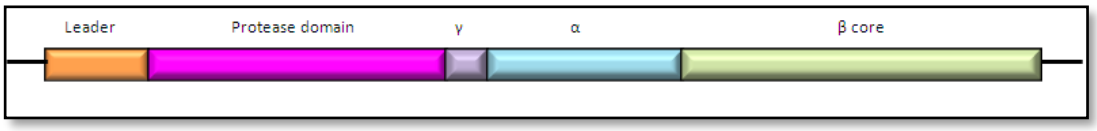

Figure 10. A simplified view (not to scale) of the IgA1P pre-protein. IgA1P is translated as a large pre-protein prior to translocation and self export with several domains which allow for efficient IgA1P extracellular transport. The leader sequence is coloured orange, the protease passenger domain in pink, the γ peptide in purple, the α protein in blue and the β core domain in green. Together the γ peptide in purple, the α protein in blue form the linker region of the protein

Mature IgA1P secreted by Gram negative bacteria is divided into 3 classes based upon the active site of the protease, these 3 types are; Serine protease, as found in *N. meningitidis, N. gonorrhoeae* and *H. influenza*, Metalloproteases as found in *S. pneumoniae* and Cysteine proteases as found in *Prevotella melaninogenica* [74,79]. The three types of IgA1P all have

similar structures as well as very similar functions and have been found to cut the hinge region of the heavy chain of IgA1 but not IgA2[79]. This is inability to cleave IgA2 is due to a deletion mutation in IgA2 which causes loss of the protease recognition site [79,80] .

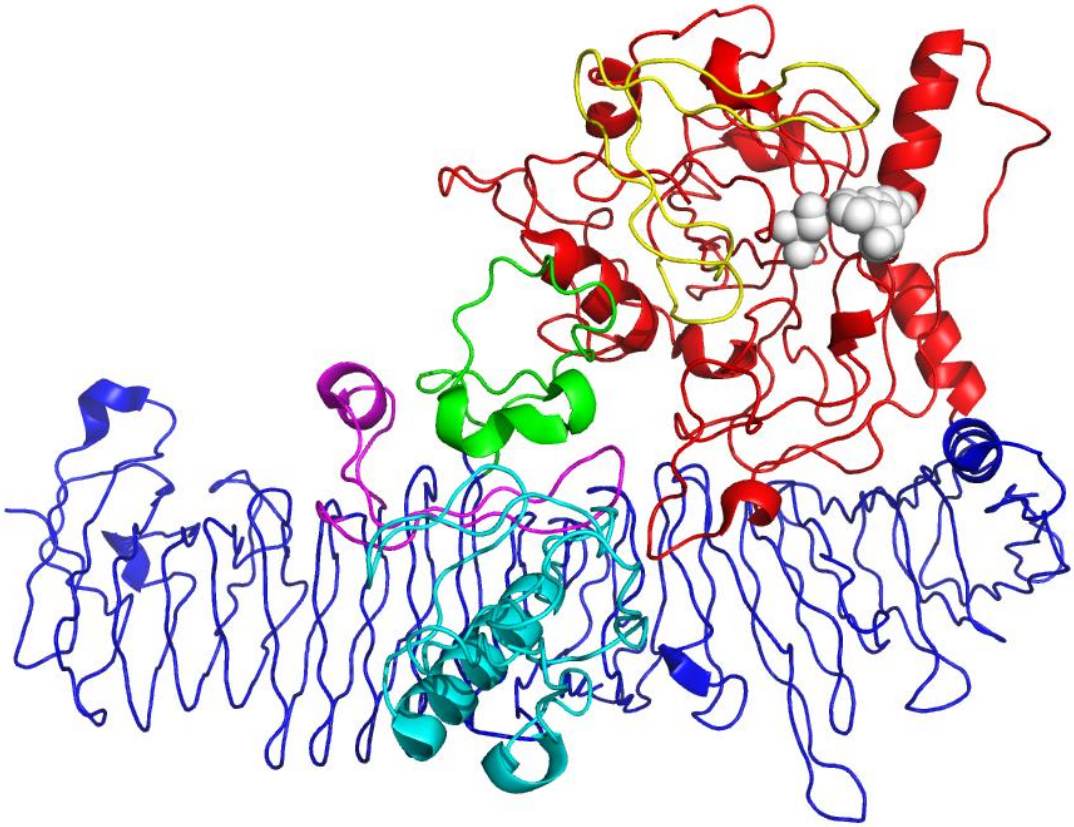

Figure 11. The crystal structure of IgA1P from *H. influenzae* [75] . The red ribbon show the protease domain (residues 26-337) with loop D (205-243) of this domain highlighted in yellow, the light blue ribbon shows domain 2 (residues 564-657), the green ribbon shows domain 3 (residues 710-743), the magenta ribbon shows domain 4 (residues 786-819), the dark blue ribbon shows the rest of structure, including the long β spine (residues 338-369, 388-563, 658-709, 744-785 and 820-989). The catalytic triad (residues H100, D164 and S288) are rendered as light blue ball and stick models and circled in green (Structure downloaded from EMBL-EBI PDB entry no. 3H09 and modified in PyMOL)

The mature and exported IgA1P itself consists of five domains (**Fig 11**) with the most important of these being the large protease domain which contains the active site [75] . There is also a large β-helix "spine", which may facilitate substrate binding and orientation, as well as 3 smaller domains known as domain 2,3 and 4 [75] . The overall sequence and structure of IgA1P has been shown to be highly similar to haemoglobin protease (Hbp) from *E. coli*, which is the only other serine protease autotransporter with a solved crystal structure [75] . Interestingly IgA1P also has high structural homology to both the trypsin and chymotrypsin

serine proteases, although the solved *Haemophilus influenzae* IgA1P has unique insertion in a region known as loop D of the protease domain(**Fig 12**) [75] .

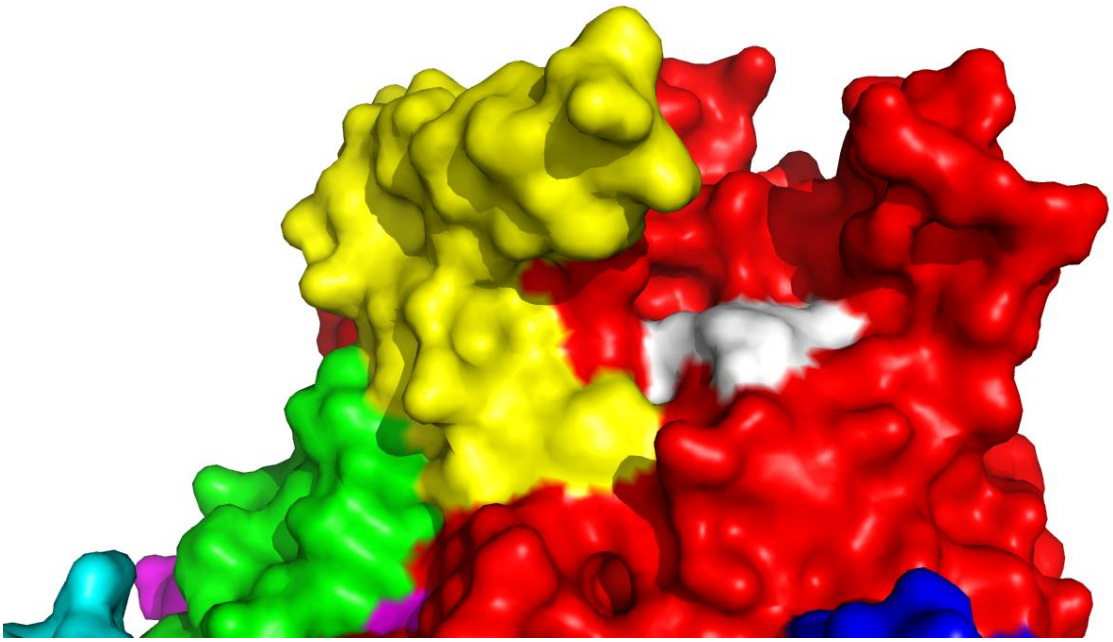

Figure 12. A close up of the protease domain of IgA1P from *H. influenzae* shows how the unique loop D insertion (yellow) forms a lid over the active site of the protease (light grey). This lid prevents the protease from cleaving non-specifically by covering the active site unless certain specific interactions between itself and the substrate are made (Structure downloaded from EMBL-EBI PDB entry no. 3H09 [75] and modified in PyMOL)

This unique loop insertion is thought to act like a lid, covering the active site of the protease and preventing non-specific cleavage from occurring [75] . The theory is that the loop D insertion is able to interact with the β barrel section of the Fc fragment of intact IgA1, which stabilises loop D in an open conformation therefore uncovering the active site and allowing cleavage to occur [73] . What is particularly interesting is that modelling of the *Neisseria meningitidis* IgA1P using the Phyre2 protein fold recognition server [81] suggests that the unique loop D insertion is absent in this protein (**Fig 13**). This may account for the propensity of IgA1P from *N. meningitidis* to cleave molecules other than IgA1, as will be discussed in detail later in this chapter.

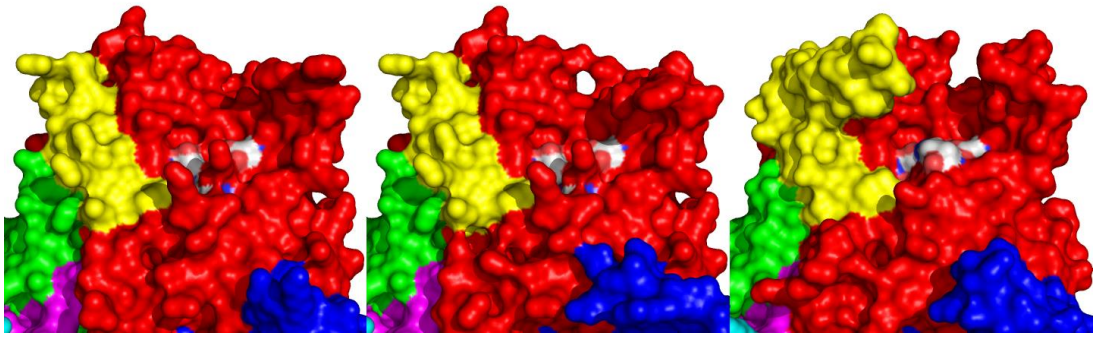

Figure 13. A comparison of the IgA1P loop D insertions. The predicted structure of the *N. meningitidis* IgA1P is shown on the left and in the centre (The left hand structure was predicted using the Phyre2 protein fold recognition server [81] whilst the centre structure is based on a threading model using the *N. meningitidis* sequence and the solved *H. influenza*e crystal structure), the structure on the right shows the solved *H. influenzae* structure (PDB entry 3H09 [75]). The red surface shows the protease domain (residues 26-337) with loop D (205-243) of this domain highlighted in yellow, the light blue surface shows domain 2 (residues 564-657), the green surface shows domain 3 (residues 710-743), the magenta surface shows domain 4 (residues 786-819), the dark blue ribbon shows the rest of structure, including the long β spine (residues 338-369, 388-563, 658-709, 744-785 and 820-989). The catalytic triad (residues H100, D164 and S288) are rendered in light grey. These images show that the unique loop D insertion found in the *H. influenzae* structure is likely absent in the neisserial structure, this might explain its ability to cleave multiple substrates, as will be explained later.

The largest domain within IgA1P is the β helical spine which is a 100 Å long domain formed from β helices, with a hydrophobic core made of stacked leucine and isoleucine residues and a hydrophilic external face [75] . The β spine forms the backbone of the molecule to which the other domains extend and are attached [75] . The spine also forms stabilising disulphide binds with the cysteine residues present in domain 4 [75] . These bonds have been useful in determining the size and shape of the protease as it is exported, allowing the pore diameter and export mechanism to be calculated [75] .

As well as the β spine and the protease domains there are 3 smaller domains known as domains 2, 3 and 4 [75] . Despite the fact that these domains are relatively small it is thought that they play a crucial role in substrate orientation and specificity (**Fig 14**). Domain 2 is a small domain formed off the stalk of the β helix which has been shown in crystal packing studies to have independent movement from the β helical spine [75] . This allows it to interact with the unique loop D of another IgA1P molecule placing itself over the active site of the other molecule, therefore acting as a lid and preventing random cleavage [73] . As previously mentioned this unique loop D insertion is most likely absent in neisserial IgA1P and as such

domain 2 alone may be acting as a lid, it is also possible that this is just an artefact that has arisen due to crystal packing effects. Domains 3 and 4 are smaller domains than domain 2 and also are β strand insertions which extend away from the β helical spine and lie between the β helical spine and the protease domain at the base of domain 2. These smaller domains create a unique topography which is most likely involved in substrate recognition and specificity [75].

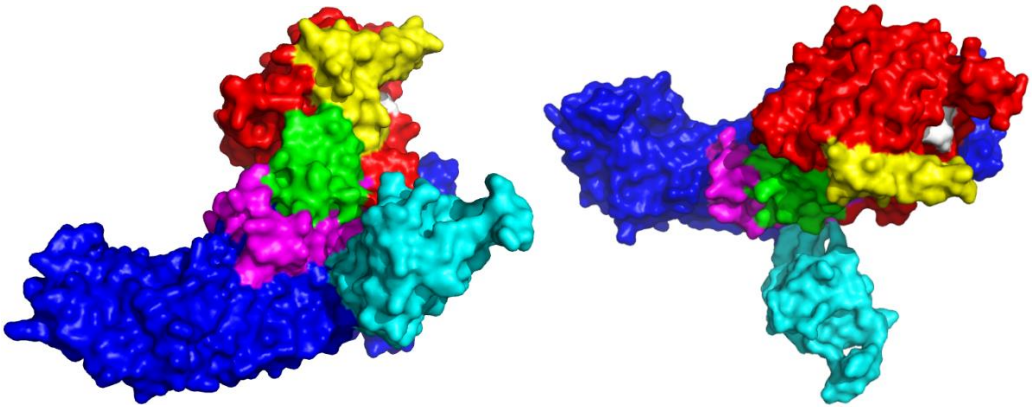

Figure 14. The crystal structure of *H. influenzae* as viewed from above, this shows how domain 2 (light blue) extends away from the β spine (dark blue) as well as the other domains (domain 3-green, domain 4-magenta). Domain 2 can be seen to extend away from the rest of the protein on a thin stalk which allows it independent movement, whilst domain 3 and 4 create a uniquely structured cleft for substrate binding. (Structure downloaded from EMBL-EBI PDB entry no. 3H09 [75] and modified in PyMOL)

The final domain is the protease domain, as the name suggests this domain contains the active site of the protein and is required for its enzymatic function(**Fig 15**) [75]. It has been shown that *N. meningitidis, N. gonorrhoeae* and *H. influenzae* all contain a consensus sequence around the serine active site of GDSGSPLF [82], with the serine involved in the enzymatic reaction highlighted in red. This consensus sequence is also found in other related serine proteases such as trypsin and chymotrypsin [82] which also have been shown to have similar structures to IgA1P [75].

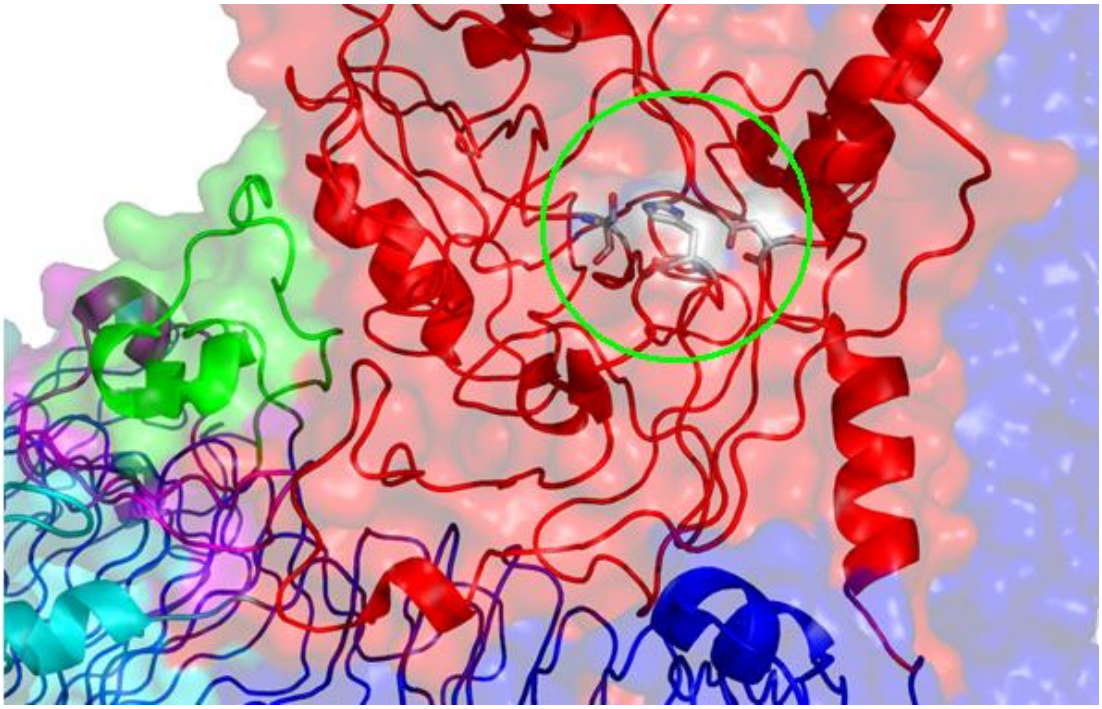

Figure 15. Close up of the catalytic triad which makes up the active site of IgA1P. The residues H100, D164 and S288 which form the catalytic triad have been rendered as ball and stick models and circled in green (Structure downloaded from EMBL-EBI PDB entry no. 3H09 [82] and modified in PyMOL).

1.6.2 Link to virulence

Although IgA1P is predicted to be a virulence factor due to its up-regulation in invasive strains and also its role in the cleavage of IgA as of yet researchers have only been able to predict IgA1Ps role in virulence. This is due to the fact that any attempt at animal modelling has failed due to the specificity of IgA1P for the human IgA [74] . Further reasoning behind IgA1P being a virulence factor is that it has been found to be constitutively expressed during the bacterial growth phase [83] , although its exact role in virulence is unclear at the present time. Most research seems to conclude that IgA1P is most likely involved in host immune response evasion prior to the secretion of specific antibodies as well as protecting the bacteria from TNFα mediated apoptosis [84,85] .

Since its initial discovery it has been postulated that IgA1P would play a vital role in immune evasion and host colonisation in pathogenic organisms which colonise the mucosal membranes. This theory stems from the proteins ability to cut IgA1 and also from studies which show up to a fivefold up-regulation of IgA1P expression in invasive isolates [34,35] . It has been shown that this fivefold upregulation in expression of IgA1P is not due to co-expression with another virulence factor, therefore showing that elevated IgA1P levels play an active

part in virulence determination and are not just a by-product of the expression of a linked factor. This was confirmed by monitoring IgA1P expression levels in different isolates of the pathogen when allowed to undergo random mating [86]. This random mating led to a random assortment of alleles being present within the different pathogens, however despite the random alleles present IgA1P levels were nearly consistently detectable in all of the invasive isolates that were tested.

1.6.2.1 The structure and function of IgA

Immunoglobulin A is the most abundantly secreted immunoglobulin at the mucosal surfaces within the body and these mucosal surfaces are estimated to have a combined area of up to 400m^2 [2, 87,88]. As a result IgA is the most secreted immunoglobulin in the body and any bacteria hoping to colonise the nasopharynx (such as *Neisseria meningitidis*), gut or urogenital tract must be able to evade its effects in order to colonise and survive within a host. The role of IgA at the mucosal membrane is not generally to kill the bacteria directly but rather to aid expulsion of the bacteria or signal phagocytic ingestion [87,89]. This is generally achieved via the ability of secretory IgA to cause opsonisisation/agglutination of pathogens on the mucosal membranes; this then targets them for clearance by the cilia. This agglutination prevents colonisation of bacteria upon the mucosal membranes and also limits the exposure of the epithelial cells to the pathogenic effects of the bacteria [87,89].

The IgA protein itself is formed from 2 light and 2 heavy chains, similar to the other immunoglobulins. What differentiates IgA from other immunoglobulins is its ability to function as a monomer, dimer or trimer although secretory IgA at mucosal surfaces is most commonly found as a dimer[86]. These dimers are comprised of two IgA molecules with a small J chain that links them together, along with an associated glycoprotein call the secretory component[,88].

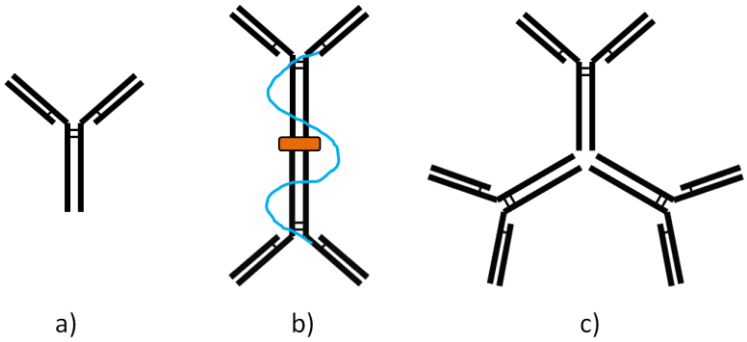

Figure 16. IgA is most commonly found in 3 forms at the mucosal surface a) IgA monomer b) Iga dimer with J chain rendered in orange and the secretory component rendered in blue c) IgA trimer. Of these forms the Iga dimer is most commonly secreted at the mucosal surface and is capable of agglutinating bacteria for removal by ciliated cells. It has also been found to be weakly capable of activating the complement system for direct pathogen killing, however this appears to be a minor role as other immunoglobulins are much more effective at activating this system[86].

1.6.2.2 Cleavage of IgA and other targets by IgA1P

In humans there are 2 subclasses of IgA known as IgA1 and IgA2, these have subtle structural differences with a key difference being that IgA2 has a deletion mutation within the hinge region of the heavy chain [87,89] . This deletion mutation removes the IgA1P recognition site, making IgA2 resistant to cleavage by the protease [79,80] . This deletion mutation is found within a duplicated octapeptide repeat in the heavy chain which contains both the recognition and the cleavage sites for the IgA1P enzyme. Although IgA1P is unable to cleave IgA2 it can cleave IgA1, which makes up around 50% of all secreted IgA at the mucosal surface[86]. Cleavage of IgA1 by IgA1P is possible at two different consensus sequences, this gives rise to two subclasses of IgA1P enzymes. Type 1 IgA1P has been shown to cleave between Pro-Ser residues, whilst type 2 IgA1P is known to cleave between Pro-Thr residues [73,87,90] .

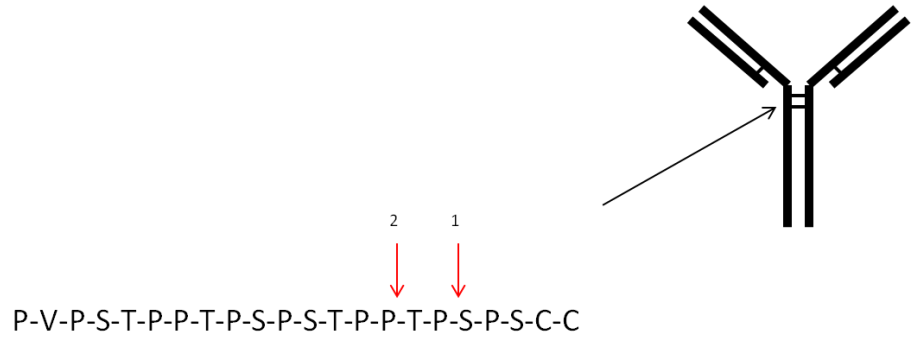

Figure 17. Diagram to show the duplicated octapeptide within the heavy chain hinge region of IgA1 along with the cleavage sites of *N. meningitidis*. Arrow 1 shows the site for type 1 cleavage of IgA1 by IgA1P and arrow 2 shows the type 2 cleavage site of IgA1 by IgA1P. As can be seen, cleavage products produced by IgA1P subtypes vary in length therefore allowing classification of the enzyme type [91].

Interestingly study of *Haemophilus influenzae* has shown that it is possible for a single strain to encode and express both type 1 and type 2 IgA1P proteins [92,93]. It has been speculated that having both subclasses of IgA1P may make the bacteria more invasive and by providing the bacteria with more host immune evasion. The N terminal region of IgA1P has been shown to alter the specificity of the enzyme and is instrumental in determining whether the enzyme exhibits type 1 or type 2 cleavage, this N terminal region is known as the cleavage specificity determinant (CSD) region [94]. Both types of cleavage result in production of an intact Fab and an Fc fragment, which vary in length depending on the subtype of IgA1P that carried out the cleavage [94]. This cleavage of the IgA1 molecule therefore renders it unable to carry out its function within the host as the IgA molecule can no longer crosslink with other Fc regions in order to agglutinate and neutralise the pathogen [87,90,91]. This therefore confers an increased survival chance to the pathogen allowing for prolonged survival and colonisation of the mucosal membrane.

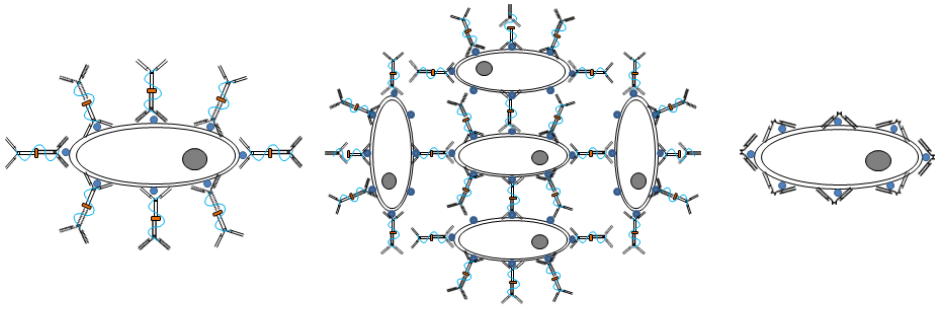

Figure 18. IgA binds to pathogenic antigens causing agglutination, however IgA1P can lessen this effect and by causing fabulation. a) shows the normal binding of IgA to bacterial antigens, during this process the Fab region of the IgA molecule binds the antigen leaving the free Fc tail capable of making interactions with other Fc regions for agglutination to occur or white blood cells in order to elicit a direct immune response, b) shows the process of agglutination and how interaction between Fc regions allows the cross-linking of bacteria into a large group capable of being expelled by the cilia, c) shows how, after exposure to IgA1P, the hinge region of IgA has been cleaved. This leaves an intact Fab region which is still capable of binding and masking the bacterial antigen in a process called fabulation while removing the Fc region therefore preventing an immune response and agglutination of the pathogen [91,95].

Although IgA1P was initially thought to only cleave IgA1 it has more recently been shown to be able to cleave a wide range of substrates. This observation is linked to the more recent discovery that IgA1P is able to tolerate changes in its consensus cleavage sequence, allowing it to interact with and cleave proteins other than IgA1 [85,96]. In addition to the hinge region of IgA1 IgA1P has also been found to cleave the type II tumour necrosis factor receptor (TNFRII), synaptobrevin and lysosomal associated membrane protein (LAMP) 1 and 2 [85,97, 98, 99]. Cleavage of all of these targets may be linked to an increase in virulence and invasiveness of the pathogen [85,100].

The cleavage of LAMP1 is a perfect example of how IgA1Ps off target effects can increase the pathogens virulence by prolonging colonisation and preventing clearance of the bacteria. LAMP1 is a key glycoprotein in lysosomes which is involved in the stabilisation of lysosomal membranes which contains a region with a high degree of similarity to the IgA1 hinge region [85,97, 98]. It has been shown *in vitro* that it is possible for IgA1P to cleave this region causing a drop in active LAMP1 levels by between 20-50% [85,97, 98]. It is hypothesised that in vivo this would lead to destabilisation of the lysosomal membranes therefore promoting survival of the bacteria within the epithelial cells [85,97, 98].

Cleavage of LAMP2 is further evidence of how the cleavage of other targets by IgA1P can increase the virulence of the pathogen. LAMP2 is another lysosomal membrane protein that

has been hypothesised to be involved in intracellular signalling effects within phagocytic cells. Therefore the cleavage of LAMP2 would affect lysosomal structure and function within phagocytes, preventing pathogen neutralisation from being carried out [85]. This increases the pathogens ability to survive within a host and allows it to evade clearance by the immune system.

The cleavage of TNFRII is also interesting as TNFRII normally controls apoptotic events in monocytes by binding tumour necrosis factor α (TNFα) and hence acts as a key regulator of host immune response. Therefore interference by IgA1P with the effects of TNFRII would allow pathogenic bacteria such as *N. meningitidis* to prevent apoptosis of the niche required by the pathogen induced by TNFα and therefore survive for a longer period of time [84,100]. This cleavage of TNFRII is particularly interesting as it has been shown that secretion of IgA1P is linked to increased release of pro-inflammatory cytokines such as IL-6, IL-8 and TNFα from monocytes [84]. These pro-inflammatory cytokines have been shown to be key to the ability of *N. meningitidis* to cause disease and are elevated in the cerebrospinal fluid of patients suffering with meningitis, therefore the release of IgA1P is simultaneously contributing to virulence whilst protecting the organism from the effects of the inflammatory cytokines [84]. There is also growing evidence that IgA1P produces a very immunogenic response *in vivo* and that people vaccinated against IgA1P have gained a long lasting immune response which in some cases has actually proven to become stronger overtime due to asymptomatic carriage [25,101].

The strong immune response to IgA1P is thought to be due to the stimulation of T cells by both invasive and carriage strains, this then leads to high circulating levels of anti-IgA1P antibodies [102]. However due to the heterogeneity of the IgA1P sequence any antibodies that are produced by the host due to a prior infection will not necessarily prevent the IgA1P secreted in a subsequent infection from being effective. Despite this fact antibodies against IgA1P have been shown to be long lasting *in vivo* and therefore possibly capable of helping to prevent meningococcal invasion. It should also be noted that antibodies against type 1 IgA1P are not effective against type 2 IgA1P and vice versa.

The strong immunogenic response provided by both IgA1P and the co-secreted α protein, as will be described later, however may prove useful for vaccine production, although the heterogeneity of IgA1P itself will serve as a challenge if a universal vaccine is to be made in the future. There is however the possibility of fusing the α protein to IgA1P or other secreted

Neisserial proteins in an attempt to create a vaccine that is highly immunogenic and also effective against two separate secreted factors involved in colonisation and invasion.

1.6.3 Export pathway

In Gram negative bacteria there are 6 major families of secreted proteins; I,II,III,IV,V and VI all of which are highly conserved and are functionally independent in protein export [79]. The type V autotransporter family to which IgA1P belongs to can be divided into 3 subtypes; Va- autotransporter proteins. Vb- two partner secretion system and Vc [78,79]. Since its discovery in 1973 IgA1P has become the archetypal type Va autotransporter protein and all other type Va proteins have been shown to have homologous structures to IgA1P [79]. The type Va autotransporter proteins encode many of the necessary domains required for the protein to be export competent however, despite the family name, it is has been found that proteins such as Skp, Sec and Omp85 are all essential for export and processing of the proteins once translated [103,104]. All of the type Va autotransporters studied to date have been found to have similar structures and biogenesis pathways but have shown slight variation in the methods of cellular export [79]. As mentioned in section 1.6.1 type Va autotransporters are comprised of 4 domains which are all crucial for export; the leader sequence, the passenger domain, the linker domain and the beta core. Together these domains all carry out specific roles during export from the cell, the leader targets the protein through the inner membrane, the passenger domain is the enzyme for export, the linker is a helical flexible region and the beta core forms a pore in the outer membrane allowing export of the passenger domain [105].

The leader domain of IgA1P has been described as an unusually long sec dependent signal sequence, for which Sec B seems to act as the designated chaperone [75,76]. During transit through the IM Sec B binds to the IgA1P pre-protein and targets it to Sec A for transport through the inner membrane [79]. The leader sequence itself consists of a positively charged N-domain, an H-domain containing hydrophobic amino acids and a C-domain with a signal

peptidase recognition site as well as a C-terminal cleavage region [78]. All of these regions are key to allowing the leader sequence to target the protein for transport across the inner membrane [79]. A further role of the leader sequence is to slow the cleavage of the protein in the periplasm. This slow cleavage of sec prevents the protein from folding fully in the periplasm by keeping the protein tethered to the inner membrane, therefore keeping it in a translocation competent state [78].

After the pre-protein has undergone inner membrane translocation the passenger and linker domains remain in an unfolded or partially folded state within the periplasm. During this time the β-core interacts with the outer membrane and forms a β barrel structure which inserts into the outer membrane and acts as a pore for the passenger and linker domains [79]. It was recently shown that the β domain alone is not capable of folding and inserting itself into the outer membrane properly and that in *Neisseria* a protein called Omp85 is absolutely essential for IgA1P export and processing. Omp85 is thought to act as a chaperone for the β core by facilitating protein assembly within the outer membrane [103,106,107]. As mentioned previously the IgA1P protein is kept in an unfolded state in the periplasm, this is due to the fact that a fully folded IgA1P protein would not be able to fit through the pore made by the β core. Although it has been shown that the leader sequence helps to keep the protein in this unfolded state it is unclear how the protein is protected from proteolytic degradation in the periplasm. This is because partially folded or unfolded proteins are routinely degraded by periplasmic proteases in order to clear the periplasm of unwanted or unneeded proteins. The β barrel structure which is formed by the β core is made up of 15 β sheets aligned in an anti-parallel fashion, as is typical for a classic β barrel [76,108]. Within the family of type Va autotransporters the β domain structures are all homologous but have extremely diverse sequences, as a result the conserved structure of the β domain is believed to be key in its ability to form an outer membrane pore capable of translocating the protease domain [78].

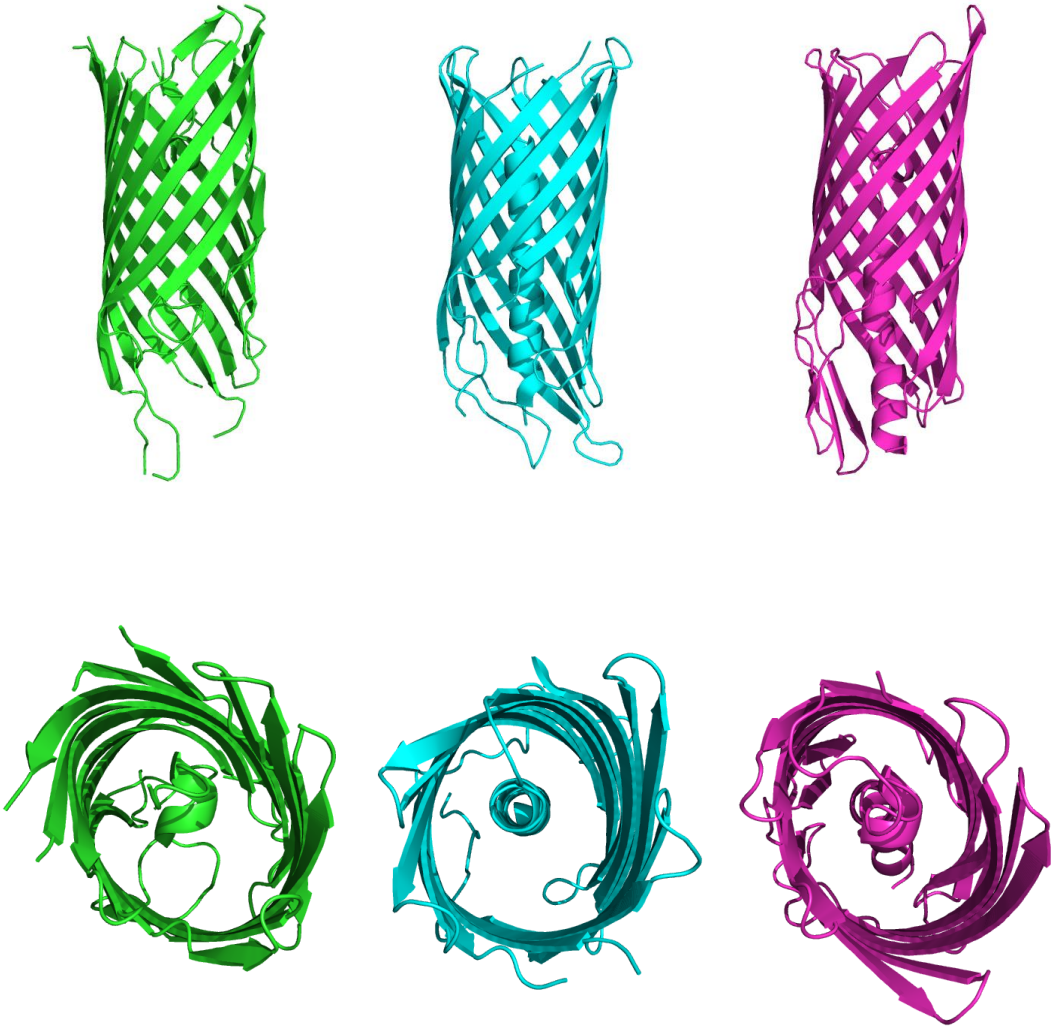

Figure 19. Comparison of the β barrel structures formed by various type Va autotransporters proteins. *E. coli* EspP translocation domain (green), *Neisseria meningitidis* NalP translocation domain (blue) and the predicted structure of the *Neisseria meningitidis* IgA1P βcore (pink, predicted using Phyre 2 protein fold recognition server [81]). Although the proteins have very different sequences the proteins all form highly similar β barrel structures with a short internal α helix. (Structures downloaded from EMBL-EBI PDB entry no. 2QOM [109] and 1UYN [107] and modified in PyMOL)

The β domains also contain a consensus amino acid sequence at the very end of the C terminus which has alternating hydrophilic and hydrophobic residues with a terminal F or W residue [79]. This sequence is also found in other Gram negative outer membrane proteins and is believed to have a role in stabilisation, localisation and folding of the protein [79]. Recently it has been shown that in order to efficiently insert into the outer membrane the β barrel requires a molecular chaperone, such as Skp. This chaperone has been shown to bind to the

unfolded β core allowing it to interact with a number of LPS molecules, this makes the β core folding competent and allows efficient outer membrane insertion [104]. There is also recent experimental evidence of an auto chaperone domain within the protease domain of IgAP. This region was found by carrying out a position specific iterated-BLAST search (NCBI) of a region found in the passenger domain of BrkA from *Bordetella Pertussis,* which was shown to have a chaperone role in exporting the domains through the β pore [110]. This domain tends to be β helical in shape in order to contribute to the energy independent translocation that takes place through the outer membrane and has been found to lie in the C terminus of the beta spine in IgA1P [110].

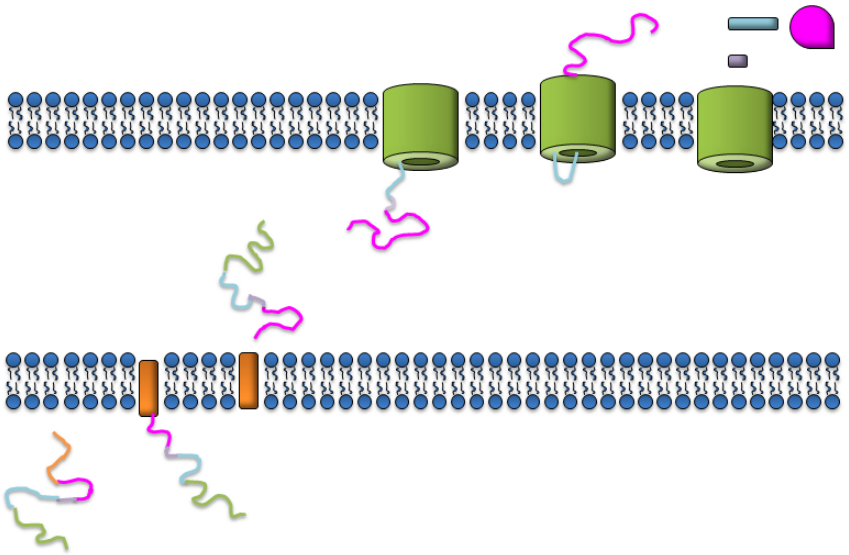

Figure 20. A diaGram showing the type Va autotransporter pathway. The leader sequence is coloured orange, the protease domain pink, the γ peptide purple, the α protein in light blue and the β domain in green. This diaGram show how the leader sequence first inserts into the inner membrane in a sec dependent manner, this rotates allowing the rest of the pre-protein to be cleaved into periplasmic space. This cleavage happens very slowly therefore preventing any degradation of the protein within the periplasmic space. During this slow cleavage the β domain forms a β barrel structure in the outer membrane where the interior acts as a pore so that the protein can translocate to extracellular space. After this translocation has occurred an auto processing event occurs whereby the IgA1P folds and cleaves itself from the pre protein and also cleaves the linker domain in order to release a γ peptide and the α protein. (Adapted from [74])

In the case of IgA1P after translocation across the outer membrane an autoprocessing event occurs where the integral serine protease cuts a cleavage site in the passenger domain releasing itself into the extracellular space [105]. This autoprocessing event can occur as the β helix of the protease that requires cleaving for mature protein release mimics the IgA1 hinge

region allowing proteolysis and self-cleavage to occur. The fully folded IgA1P can also then cut the linker region in 2 distinct places, this cleavage event release 2 discreet proteins known as α and ɣ [25,76].

1.7 Alpha

As its name suggests the α protein is a long α helical peptide which can vary in length from 400 aa in its long form to around 200 aa in its shorter form. The long and short forms also differ slightly in composition with the longer form, which is expressed by more invasive strains such as MC58, containing 4 nuclear localisation signals (NLS) and the short form, which is expressed by carriage strains such as NMB, only containing 1 NLS which appears to be inactive [111]. These NLS are short positively charged runs of 5-6 aa which help to target the protein to the nuclei of surrounding eukaryotic cells [111].

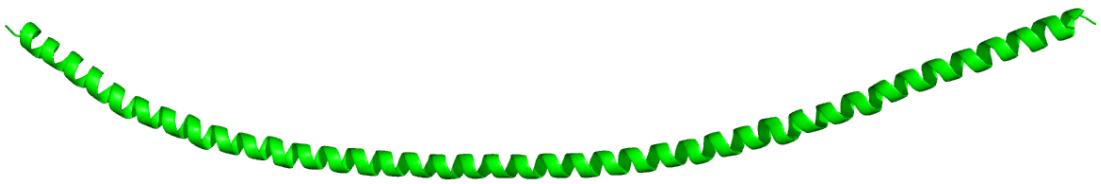

Figure 21. The crystal structure of the α monomer. The α protein is composed of one long, slightly curved alpha helix. (Unpublished crystal structure provided by Prof PJ Artymiuk of the University of Sheffield UK).

Figure 22. Unpublished crystal structure of the α protein derived from *Neisseria meningitidis* IgA1P showing how under crystal packing conditions large multimers of up to 24 proteins are formed (structure supplied by Prof PJ Artymiuk of the University of Sheffield UK).

These NLS have recently been shown to allow α protein bound to IgA1P to invade host cells and deposit IgA1P inside [111]. This then allows the IgA1P to cleave many host specific targets such as LAMP or TNRFRII, which would otherwise be inaccessible, in order to prevent apoptosis mediated destruction of the *N. meningitidis* niche or to prolong the survival of the pathogen by creating lysosomal instability [85,97, 98, 99].

Structural data on the α peptide shows a long continuous alpha helical protein with amphipathic helices capable of dimerising to form multimers [111]. This is a structure that is typical of DNA binding proteins, with a recent paper showing that α may be involved in binding extracellular DNA (eDNA) facilitating biofilm formation within the host [112]. The α protein also has a pI of around 10 which results in a strong positive charge at neutral pHs, as found within the host, which may facilitate its eDNA binding ability [112]. The α proteins high pI is due to the fact that 80% of the protein is comprised of arginine, glutamic acid, alanine,

glutamine and lysine residues which are highly charged. Also it does not contain any cysteine, tryptophan or phenylalanine residues, therefore making protein quantification difficult.

As was mentioned previously the α protein is released along with the γ peptide via an autoproteolytic event where IgA1P cleaves the linker region in order to release itself and the α and ɣ peptides from the β barrel [25]. This is autoproteolytic cleavages is possible due to the fact that α is flanked by 2 autoproteolytic cleavage site sites, PPSP and PPEL in NMB, which are used to release alpha from the β barrel and also potentially IgA1P. These are recognised in the same way that the cleavage sites are recognised in intact IgA1 allowing cleavage after the proline residue.

Despite the α protein being conserved in *Neisseria meningitidis, Neisseria gonorrhoea, Haemophilus influenzae and possibly Streptococcus pneumoniae* the α protein has only been shown to be secreted during IgA1P export from neisserial species and not in other meningococcal bacteria such as *H. influenzae*. As yet there is no clear evidence for why this is, but it is possible that α stays attached to the outer membrane of these bacteria via the β domain and performing some other function.

A series of studies have shown that IgA1P is capable of giving rise to long lasting immune responses with antibodies to IgA1P being detectable up to 5 years after meningococcal carriage. It is possible that this long lasting protection may be partially due to the α protein which is often linked to IgA1P during export. The alpha protein is a highly repetitive protein which has been show to contain a number of T cell helper epitopes [25], which are short runs of 7-10 amino acids. These epitopes activate CD4+ helper T cells which then stimulate maturation of B cells into memory B cells which provide long lasting immunity [24, 25].

It is hypothesised that the ability of alpha to create memory B cells could be exploited and used in vaccine development, with alpha acting as a carrier protein in vaccine formulations. As such it could increase the immunogenic response to other proteins in the vaccine and give rise to long lasting vaccine coverage. Furthermore, alphas propensity to form large multimers could also be utilised. By fusing a target protein to α, it may be possible to create a large multimer of both target and α. This would effectively increase the size of the target protein, therefore lowering the rate of renal clearing whilst also increasing the immunogenicity of the protein.

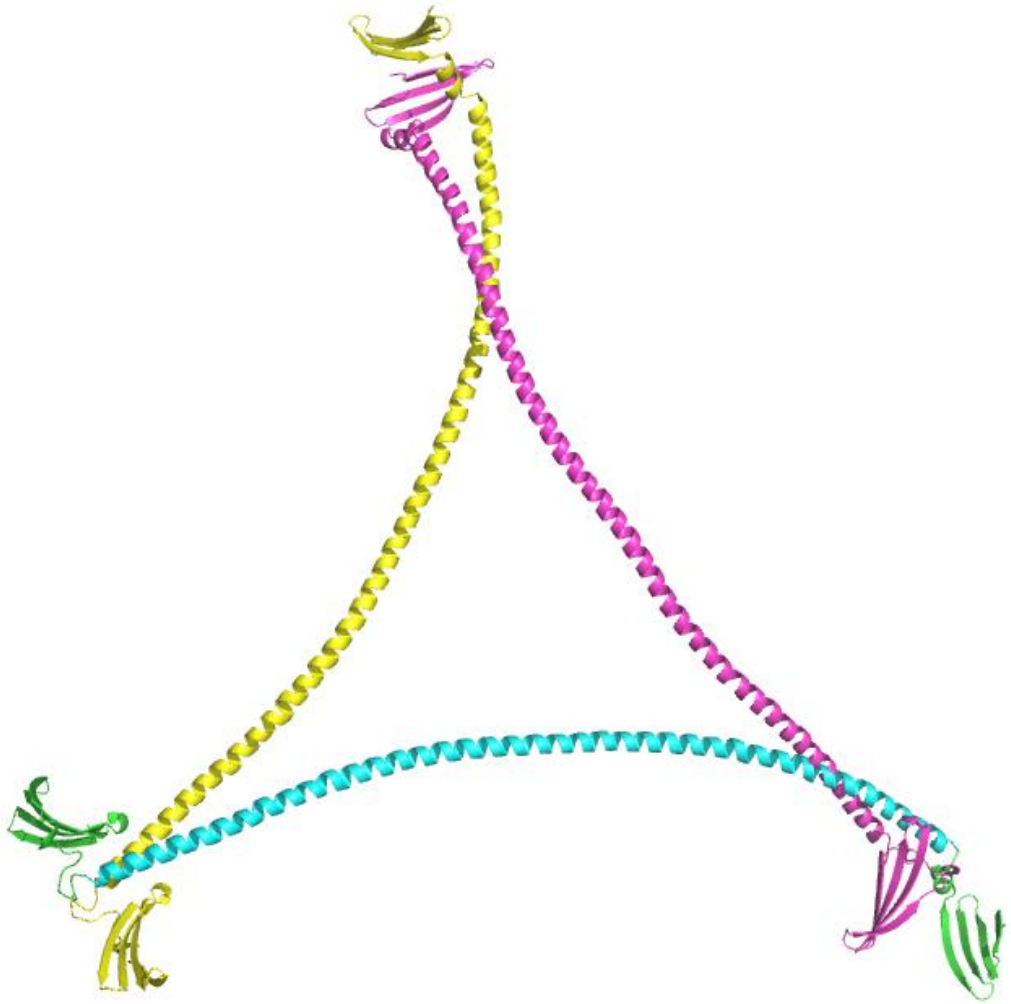

Figure 23. A predicted structure of an α-Gly1-ORF1 fusion, showing how theoretically the α protein could retain its ability to form multimers. This would increase the complex's size, therefore increasing its immunogenicity and renal clearing time. (image created in PyMOL using structures provided by Prof JR Sayers and Prof PJ Artymiuk of the University of Sheffield, UK)

This could possibly increase the effectiveness of the vaccine by keeping the protein in the circulation system for a longer period of time whilst simultaneously increasing the production of memory B cells against it.

1.8 Gamma

The gamma (γ) peptide makes up a small section of the linker region in IgA1P secreted by *Neisseria* along with the much longer α protein. It is released during the same autoproteolytic event that releases α and IgA1P from the β barrel. As previously stated it is a very short

peptide consisting of just 25 aa residues: PATNTASQAQTDSAQIAKPQNIVVA, with a predicted weight of just 2.5 kDa.

Structure prediction shows that it may form an alpha helix although an unstructured loop may be more likely.

Despite very little being known about the ɣ peptide it appears to be conserved in *Neisseria meningitidis, Neisseria gonorrhoea and Haemophilus influenzae*. As such it is likely to have some function in these bacteria and was therefore investigated over the course of this project.

1.9 Hypothesis

The hypothesis of this study was fourfold;

Firstly, that the *Neisseria meningitidis* IgA1P would show a high structural homology to the solved *Haemophilus influenzae* IgAP protein. This would be tested using mutant IgA1P proteins derived from a plasmid containing *E. coli* M72 cell line.

Secondly that the α protein derived from neisserial IgA1P would show binding of serum or blood based factors which would account for its high level of immunogenicity.

Thirdly that the α protein may exhibit potential as a carrier protein due to its multiple T cell helper epitopes.

Finally that the ɣ peptide will have a functional role within the bacteria due to its conserved nature.

Chapter 2- Materials and methods

2. Materials and Methods

All chemicals were analytical grade and supplied by Fisher Scientific unless otherwise stated.

2.1 Bacterial Culturing

2.1.1 Culture media

Lysogeny broth (LB) was used to culture small volumes of *E. coli* cells, this contained 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) sodium chloride. For larger cultures of *E. coli* 5YT growth medium was used as this provides a higher concentration of nutrients which can sustain bacterial growth for longer. The 5YT medium used contained 4% (w/v) tryptone, 2.5% (w/v) yeast extract and 0.5% (w/v) sodium chloride. For isolating single colonies of *E. coli* LB agar plates were used, these contained 1% tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride and 15 g/L bacto agar. All of these media were also used with a plasmid appropriate antibiotic for positive selection. The two antibiotics used were ampicillin and carbenicillin at 100 µg/ml, which are both degraded by β lactamases. Ampicillin was used in small scale cultures and carbenicillin was used for larger cultures such as the fermenter as the breakdown products are less toxic than that of ampicillin.

2.1.2 Bacteria

For all work carried out *E. coli M72* cells were used either alone or transformed with one of the plasmids listed in section 2.1.3. *E. coli* M72 cells encode a heat sensitive version of the cI protein known as cI857, this specifies that M72 cells use a λP_L repressor system on the plasmid vector [113].

2.1.3 Plasmids

All of the genes of interest had previously been cloned into the pJONEX4 plasmid which is based upon pBR322 with a pUC19 multiple cloning site and a λP_L promoter [114] .The λP_L is a heat inducible promoter composed of 3 operator regions, one of these regions is the CI857 heat sensitive repressor protein which is supplied on the M72 cells. The variants of this plasmid used were; pJONEX4_NMB_alpha containing the region encoding the alpha protein of IgA1P from the NMB strain of *N. meningitidis*, pJONEX4_NMB_cys_γ_α containing the region encoding the γ and α proteins of IgA1P from the NMB strain of *N. meningitidis* which has been modified with a C terminal cysteine residue, pJONEX4_neisserial_Gly1_ORF1his

containing the gene for Gly1-ORF1 from *N. meningitidis* and pJONEX4_ IgA1P containing the IgA1P gene isolated from *N. meningitides* (for plasmid sequences See Appendix II*).*

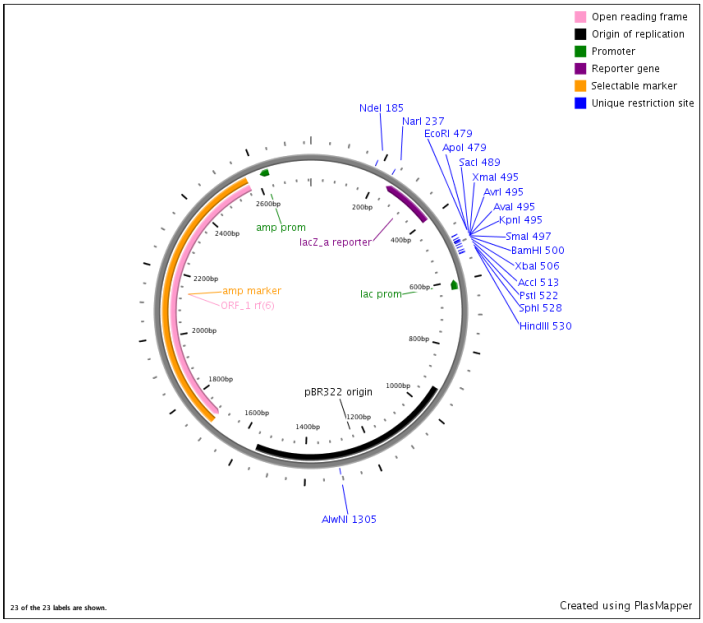

Figure 24. Map of the pJONEX4 expression plasmid. All inserted genes were cloned in using the multiple cloning site of pUC19 so as to be under the control of the λP_L promoter with the lac promoter as an antisense promoter. Image generated using Plasmapper [115] .

2.1.4 SDS PAGE reagents

The reagents used for SDS PAGE were; loading dye consisting of 2 ml 10% SDS, 4 ml glycerol, 3 ml 250 mM Tris.HCl pH8, 1 ml 0.5% (w/v) bromophenol blue and 100 µl 500 mM ethylenediaminetetraacetic acid (EDTA) pH 7, SDS-PAGE running buffer comprised of 121.4 g Tris, 163.2 g Bicine, 100 ml 10% SDS, 7900 ml dH_20 and 4 ml 500 mM EDTA.

The reagents used to stain and de-stain the acyrlamide gel were; Coomassie stain which consisted of 400 ml methanol, 100 ml acetic acid, 2 mg/ml Coomassie blue, 500 ml dH_20 water and Coomassie de-stain which was composed of 200 ml methanol, 100 ml acetic acid and 700 ml dH_20.

2.1.5 Culturing

An LB agar plate containing 100 µg/ml of ampicillin was streaked with bacteria from a glycerol stock of bacteria stored at -80°C, this was then incubated at 30°C overnight. After incubation a single colony was picked and used to inoculate 6 ml of LB containing 100 µg/ml of ampicillin and incubated whilst shaking at RT until mid-log phase was reached. The 6 ml

culture was then either used in order to elucidate optimal protein expression conditions or 1 ml was then used to inoculate 100 ml of LB containing 100 µg/ml of ampicillin which was then incubated with shaking at RT O/N.

2.1.6 Monitoring protein expression

Cells were cultured in 6ml of LB media as described in section 2.1.5 using E. coli M72 cells containing either the pJONEX4_NMB_cys_γ_α or the pJONEX4_NMB_α plasmid. A 1ml sample was taken from the culture and centrifuged at 14000 *x g* in order to separate the cells from the supernatant, these were then stored at -20°C, the remaining culture was then induced at 42°C and sampled at 2hrs, 4hrs, 8hrs and 24 hrs post induction as before.

2.1.7 Fermentation

A 5 L fermenter was filled with 5 L of 5YT then autoclaved and carbenicillin added to give a final concentration of 100 µg/ml. The temperature of the media was then maintained at a 30°C and aerated with sterile air at 5 L/min. Next 400 ml of overnight culture (cultured as in section 2.1.6) was used to inoculate the fermenter prior to sealing it. After sealing the fermenter a stirrer was set to 300 rpm in order to ensure mixing of the culture allowing even growth. The culture was then incubated at 30°C until mid-log phase was reached; the bacteria were then induced overnight by increasing the fermenter temperature to 42°C. After induction the cells were centrifuged in an ultracentrifuge at 3500 x *g* at 4°C for 20 min. The supernatant was then removed and the pellet resuspended by shaking in 200 ml of 25 mM Tris pH8, 2 mM EDTA and 100 mM NaCl. Resuspended cells were then transferred to a single bottle and centrifuged again as before. The supernatant was again removed and the cells made into ~10 g aliquots wrapped in cling film then foil and stored at -80°C.

2.1.8 SDS PAGE gel

An SDS PAGE resolving gel was made using; 3.3 ml of 30% acrylamide (National Diagnostics), 4.4 ml of dH_20, 2 ml of 500 mM pH 8.3 Tris-bicine, 100 µl 10% SDS, 200 µl 25 mg/ml APS and 15 µl tetramethylethylenediamine (TEMED) this was allowed to set in a gel tank ,made in house. Once set the ethanol was then removed before a 3ml stacking gel was made as follows; 0.5 ml 30% acrylamide, 1 ml dH_20, 1.5 ml pH 6.9 Tris, 30 µl of 10% SDS, 60 µl 25 mg/ml APS and 7 µl TEMED this was then poured on top of the resolving gel and allowed to set with a 20 µl well comb inserted.

2.1.9 SDS PAGE

Culture samples of interest had 1ml removed and were centrifuged at 13000 x *g* for 1 min, the pellet was then resuspended in 200 µl of 25 mM Tris pH 6.9, 12.5 mM EDTA and vortexed thoroughly before 10 µl of 10% SDS was added. Samples were heated at 95°C for 3 min, 15 µl of the sample was then mixed with 10 µl of loading dye containing fresh dithiothreitol (DTT) before being heated again at 95°C for 3 min. Supernatant samples were only mixed with loading dye as above and boiled once at 95°C.

A gel rig was then filled with 1x SDS PAGE running buffer. The samples were then loaded into separate wells as was an appropriate ladder. A constant current of 20 mA was applied until the samples reached the bottom of the stacking gel, the current was then increased to 30 mA until the dye front reached the bottom of the gel. The gel was then removed from the rig and stained using Coomassie stain whilst the container was gently heated in a hot water bath. This was done for 5 min then the gel was then gently shaken with Coomassie stain for 5 min. The stain was then removed and the staining process was then repeated with Coomassie de-stain and an image was taken on a camera capable of capturing Coomassie blue stained images.

2.1.10 Preparation of competent cells

A 100 ml overnight culture of *E. coli* M72 cells were cultured as section 2.1.5 without the addition of antibiotics. This was then chilled on ice for 10 min then centrifuged at 250 x *g* for 10 min at 4°C, the supernatant was then removed and the cells resuspended in 10 ml of 100 mM $CaCl_2$ and incubated on ice for 20 min. This was then centrifuged as before, the supernatant removed and then resuspended in 85 mM $CaCl_2$ with 15% glycerol and stored at -80°C in 250 µl aliquots.

2.2 Protein purification

2.2.1 Buffers

For protein purification 3 buffers were used. Cell lysis buffer consisting of 50 mM Tris pH8, 5 mM EDTA, 150 mM NaCl and 5% glycerol (v/v) and was used for the initial cell lysis. Potassium phosphate buffer (KPB) was used for ion exchange chromatography when using a Hi-trap Heparin HP column (GE Healthcare) and was comprised of 20 mM potassium phosphate which was made from a mixture of K_2HPO_4 and KH_2PO_4 salts dependent on final pH, 1 mM EDTA, 1 mM DTT and 5% glycerol (v/v). For ion exchange on the Hi-trap Q HP

column (GE Healthcare) Q buffer was used and was made of 20 mM Tris pH8, 1 mM EDTA, 1 mM DTT and 5% glycerol (v/v).

2.2.2 Purification of cys-gamma-alpha and alpha

A 10 g frozen cell pellet (prepared as in section 2.1.6) was resuspended in cell lysis buffer using 5ml per Gram, then treated with lysozyme to give a final concentration of 200 µg/ml and stirred on ice until viscous. The solution was then treated with sodium deoxycholate and DTT to give final concentrations of 500 µg/ml and 1 mM respectively. Phenylmethylsulfonyl fluoride (PMSF) was then added to give a final concentration of 2.3 µg/ml, the solution was then mixed for 5 min.

The resulting solution was then sonicated at 20% amplitude using a microtip for 4 x 30 s, with a 1 min rest on ice between sonications. The sonication allowed full lysis of the cells and the release of cell bound protein into solution. This mix was then centrifuged at 40000 x *g* for 30 min at 4^{o}C and the resulting supernatant diluted with an equal volume of sterile deionised water.

Polyethylenimine (PEI) was then added to this diluted supernatant in order to precipitate out the DNA released from the lysed cells, the amount of PEI needed was first calculated by slowly adding PEI to a 1ml Sample of the lysate. This volume of PEI was then scaled up inline with the volume of lysate present. This step insures a clean protein preparation allowing easier purification. It was then centrifuged at 48000 x *g* for 20 min and ammonium sulphate added to create a 4 M solution, this was mixed and then left for 1 hour at 4^{o}C. This allows the precipitation of protein from solution allowing easy separation of the proteins from any unwanted cellular material still in solution. The resulting slurry was centrifuged at 15000 x *g* for 20 min and the pellet resuspended in pH 5.5 KPB this was then dialysed at 4^{o}C against 300 ml of pH 5.5 KPB 3 times for 5 hours or longer between buffer changes, in order to remove ammonium sulphate.

Ion exchange chromatography was then carried out on the sample. For the purification of both NMB alpha protein and the cys-γ-α protein a 5 ml Hi-trap Heparin HP column (GE Healthcare) was used according to the manufacturer's instructions with pH 5.5 KPB as the column running buffer. This was then followed by further ion exchange chromatography using a 1 ml Hi-trap Q HP column (GE Healthcare), used according to the manufacturer's instructions with Q buffer as the column running buffer, followed by another 5 ml Hi-trap Heparin HP column which was run identically to the first. For all of the columns the dead volume of the system was collected along with the flowthrough, wash fraction and the eluted

fractions. In all cases 20 fractions were eluted from the column using a 0.1 M-1 M sodium chloride gradient based upon the column running buffer. Between each column the column dead volume, flowthrough, wash and collected fractions were analysed by SDS-PAGE (section 2.1.9) in order to locate the protein of interest. The fractions containing the protein of interest then underwent buffer exchange dialysis prior to application to the subsequent column.

Pure protein was then quantified by the far UV protein assay (section 2.2.3) and then concentrated and stored in 50% glycerol at -20^{o}C.

2.2.3 Purification of IgA1P

IgA1P protein was purified either using size exclusion chromatography, section 2.7.4 or nickel affinity resin. Fresh nickel beads were loaded into a column and washed with 2 column volumes of dH_2O, cells were then lysed as detailed in section 2.2.2 without PEI precipitation. Ammonium sulphate precipitated protein was resuspended in Ni load buffer (25 mM Tris.hcl, 0.5 M NaCl, 20 mM imidazole and 5% glycerol pH8) and dialysed against 400 ml of the same buffer. The nickel column was prepared for sample loading as per the manufacturer's instructions before the cell lysate was loaded on to the beads, the flowthrough was collected as the lysate was loaded on to the column. The column was then washed with Ni load buffer and a series of stepwise elutions were performed using 100, 200 and then 500 mM imidazole, these elutions were collected and stored separately prior to SDS PAGE analysis.

2.2.4 Far UV protein assay

Samples of protein underwent buffer exchange dialysis against pH7 20 mM KPB, buffer exchanged samples were then diluted fourfold with distilled water to pH7 5 mM KPB, this was also done with buffer outside the dialysis bag as a blank.

Absorbance of the samples at 220 nm was measured using a NanoDrop ND-1000 spectrophotometer (Thermoscientific) with a 10 µl load. The sample was then diluted until an absorbance reading at 220 nm (A_{220}) of between 0.2-0.5 A_{220} was reached. At 220 nm a mg of protein gives an absorbance reading of 11, therefore the concentration of the sample was calculated by using the following equation:

((A_{220}x dilution factor) x(1000/loadsize)) / (1/L) = A_{220}/mL

(A_{220}/mL) / 11 = Sample concentration in mg/mL

Where L = light pathlength (cm) and load size = load on to NanoDrop ND-1000 spectrophotometer (µl)

2.2.5 BCA assay

Protein concentrations were measured using the BCA protein assay kit (Thermoscientific) by following the small volume protocol for low concentration solutions. BSA standards were made at 250, 125, 50, 25, 5 and 0 µg/mL by diluting 2mg/mL BSA in the same buffer as the protein. BCA working reagent was made by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B in order to give sufficient volume to add 200 µL to every sample or standard. The standards or sample (25 µL) were pipetted in to a microplate well, samples and standards were all measured in triplicate, and 200 µL or working reagent was then added to every well and mixed using a shaker. The plate was then covered and incubated at 37°C for 30 minutes. The absorbance of the wells was then measured using a plate reader at 562nm.

2.3 Protein structural studies

2.3.1 Crystallography buffers

All crystallography screens were set up using protein in pH7 20 mM potassium phosphate, made from a mix of K_2HPO_4 and KH_2PO_4, 1 mM EDTA and 1 mM DTT.

2.3.2 X-ray crystallography screens

The following 96 well crystallography screens were used in order to assess the crystallisation conditions required for both the α protein and the c-γ-α protein; PACT suite, JCSG+, HR2-137, MPD, pH clear, ammonium sulphate suite, classic suite and PEG suite (All supplied by Qiagen except HR2-137 which was supplied by Hampton Research) . The use of multiple screens per protein allowed many different crystallisation conditions to be tested, therefore allowing more efficient crystallisation condition optimisation.

2.3.3 X-ray crystallography

Crystallography screens were set up using c-γ-α and α stocks buffer exchanged via dialysis into pH7 20 mM potassium phosphate, 1 mM DTT and 1 mM EDTA. Screens were set up using a Matrix Hydra II (Thermoscientific) set to take up 100 µl of buffer and precipitant mix and 0.2 µl of protein sample. Both α and c-γ-α were screened against all of the screens listed in section 2.3.2 prior to optimisation of crystal growth conditions.

2.3.4 Circular dichroism

Protein stocks were buffer exchanged in to 1xPBS using a Vivaspin 6 MWCO 5000 column (Sartorius) and diluted to around 0.1mg/mL, 100 µL of the sample was then placed in a quartz cuvette and loaded in to a Jasco J-10 Spectropolarimeter with the following settings;

Parameter	**Value**
Sensitivity	100 mdeg
Start	300 nM
End	190 nM
Data pitch	1 nM
Scanning mode	Continuous
Scanning speed	20 nM/min
Response	8 Sec
Band width	1 nM
Slit width	1 nM
Accumulations	5

Raw data was then exported into GraphPad Prism for analysis.

2.4 Protein interactions with blood

2.4.1 Buffers

In order to measure protein interactions with blood the proteins first had to be resuspended in carbonate buffer consisting of 25 mM sodium hydrogen carbonate, 1 mM DTT and 5% glycerol buffer adjusted to pH 8.5 which allowed FITC labelling. In order to purify leukocytes, erythrocyte lysis buffer was used to preferentially lyse the erythrocytes in human whole blood samples obtained via the Clinical Research Facility (The Royal Hallamshire Hospital, Sheffield). This lysis buffer was composed of 168 mM ammonium chloride, 10 mM potassium hydrogen carbonate and 126 µM EDTA adjusted to pH 7.3. For washing blood cells prior to incubation with labelled proteins PBS was used and contained 137 mM sodium chloride, 2.5 mM potassium chloride, 1 mM sodium hydrogen phosphate and 2 mM monopotassium phosphate adjusted to pH 7.4. Prior to analysis on the FACScalibur cells were resuspended in FACS buffer made of PBS with 1% BSA (w/v). Western blotting was also performed on SDS PAGE gels, for transferring the protein on to Amersham Hybond-ECL blotting membrane (GE Healthcare) transfer buffer consisting of 20% methanol, 48 mM Tris and 27 mM glycine all in

dH_2O. For the washing of the membrane washing buffer was used which consisted of 1 x PBS with 0.05% (v/v) tween was used and for the blocking of non-specific antibody binding on the membrane blocking buffer was used which consisted of 1 x PBS with 0.05% (v/v) tween and 1% (w/v) milk powder.

2.4.2 FITC Labelling

FITC labelling was carried out in order to allow the measurement of protein interactions with blood cells via flow cytommetry as discussed in section 2.4.4. α, c-γ-α and BSA at 0.4 mg/ml in carbonate buffer were incubated with a 5 fold molar excess of FITC dissolved in DMF, this was increased to a 10 fold molar excess in the case of BSA, in the dark for 1 hour at RT. Excess FITC was removed by dialysing at 4°C overnight against 400 ml of 250 mM pH 8.5 sodium carbonate buffer.

2.4.3 Flow cytometry analysis of protein interactions with blood

2.4.3.1 Interactions with whole blood

A 100 µl aliquot of whole blood was mixed with the 100 µl of the 3 FITC labelled proteins from section 2.4.2 diluted to varying concentrations in order to give a final concentration of the proteins of 100 µg/ml, 40 µg/ml, 200 µg/ml, 5 µg/ml and 0 µg/ml whilst keeping the buffer concentrations equal. The resulting mix was incubated at RT for 20 min and then centrifuged at 300 x *g* and the pellet washed twice with cold PBS, the washed pellet was then resuspended in 0.5 ml of FACS buffer before protein-cell interactions were measured and analysed using a FACScalibur (BD Biosciences) and Cellquest software (BD Biosciences). The obtained measurements for forward scatter (FSC) and side scatter (SSC) allowed the cells to be sorted by size and granularity. White blood cells were then excluded from analysis by size and granularity measurements in order to focus on interactions between the labelled proteins and erythrocytes in the presence of serum based factors. This was followed by taking SSC and fluorescence measurements in order to quantify protein-cell interactions.

2.4.3.2 Interactions with white blood cells

A 100 µl aliquot of whole blood was mixed with 100 µl of the 3 FITC labelled proteins from section 2.4.2 in order to give final concentrations of 100 µg/ml, 40 µg/ml, 20 µg/ml, 5 µg/ml and 0 µg/ml whilst keeping the buffer concentrations equal. This mixture was incubated at RT for 20 min then incubated for 5 min after the addition of 1.3 ml of erythrocyte lysing solution. The solution was then centrifuged at 300 x *g* and the pellet washed twice with cold PBS, the washed pellet was then resuspended in 0.5 ml of FACS buffer before protein-cell

interactions were measured and analysed using a FACScalibur (BD Biosciences) and Cellquest software (BD Biosciences). Any remaining erythrocytes were excluded from analysis by size using the measured SSC and FSC readings this also allowed white blood cell gating according to size and granularity, prior to quantifying protein-cell interactions by measuring SSC and fluorescence.

2.4.4 Electrophoretic mobility shift analysis of interactions with blood

An electrophoretic mobility shift assay was used to analyse interactions between α and c-γ-α and whole blood and serum. This assay uses a band shift analysis technique that monitors the change in migration between a control sample of the protein and a sample of the protein incubated with a prospective interaction partner. If a positive interaction is detected the protein will migrate slower through the SDS PAGE gel due to the increase in molecular weight caused by the interaction of the two proteins. In this assay α and c-γ-α were incubated with either; whole human, ovine or equine blood or human serum as well as a cross linking agent. These samples were then analysed by western blotting in order to measure any shift in protein position on the acrylamide gel caused by the binding of blood or serum proteins by α or c-γ-α and its resultant larger size and therefore slower migration.

2.4.4.1 Western blotting

Western blotting was carried out using a 13% (w/v) acrylamide SDS PAGE gel made up of 4.3 ml 30% acrylamide, 3.5 ml dH_2O, 2 ml 500 mM Tris-Bicine pH 8.3, 100 µl 10% (w/v) SDS, 50 µl 25 mg/ml APS and 16 µl TEMED with a stacking gel made as in section 2.1.7 which was allowed to set with a 15 µl well comb inserted. The SDS PAGE gel was then loaded and run at a constant current of 40 mA until the dye front reaches the bottom of the gel as in section 2.1.9. This gel was then soaked in transfer buffer along with the membrane and 2 blotting paper pads for 10 minutes. The protein was then transferred from the gel to Amersham Hybond-ECL blotting membrane (GE Healthsciences) using a Biorad semi-dry transfer cell at 10 V for 1 hour. This membrane was then blocked overnight using 10 ml of blocking buffer at 4^{o}C whilst the tube was rolled, it was subsequently washed in 5 ml of washing buffer at RT for 30 min whilst rolling. Primary antibody was diluted to a 1:2500 dilution in 10 ml of blocking buffer, the membrane was then incubated in 5 ml of this for 1 hour at RT whilst rolling, after an hour the membrane was again washed with 5ml of washing buffer for 30 minutes at RT whilst rolling. The membrane was then incubated with 5 ml of 1:10000 diluted secondary horse radish peroxidase conjugated antibody for 2 hours at RT whilst rolling then washed with 5 ml of washing buffer for 20 minutes at RT whilst rolling. The membrane was

then imaged by incubating it for 2 minutes with Pierce ECL western blotting substrate (Pierce) before exposing Kodak BioMax chemiluminescense film (Kodak) to the membrane for 1 min in a developing cassette in the dark room before developing and fixing the film.

2.4.4.2 Cross linking of protein to serum and whole blood

De-fibrinated blood samples, in this case equine and sheep, were centrifuged at 500 x *g* for 5 minutes before the cells were washed in PBS twice by resuspending the cells in 1 ml of PBS and centrifuging as before. These cells were then diluted to a 1:10 dilution using PBS, human serum was also used and diluted 1:10 with PBS, 35 µl of the diluted serum or cell samples was the mixed with 25 µl of 100 µg/ml α or c-γ-α protein then made up to 100 µl using PBS. This mixture was then incubated on ice for 1 hour. After 1 hour 5 µl of 2.5% (w/v) glutaraldehyde was added to each reaction and the resulting mix incubated on ice for 2 hours, the reaction was subsequently stopped by the addition of 10 µl of 1 M Tris pH 8.3 with a 15 minute incubation on ice. The reaction was then analysed by western blotting as described in 2.4.4.1 in order to identify any band shift caused by protein interaction.

2.5 Analysis of protein interactions with blood

2.5.1 Biotinylation of alpha

Alpha protein was biotinylated using EZ-link NHS-biotin (Thermoscientific). Alpha was dialysed into 1x PBS buffer and then labelled with 10 mM NHS-biotin dissolved in DMF at either a 5 times molar excess or a 1:1 ratio of Alpha to NHS-biotin. The reaction was incubated at RT for 30 mins and then excess biotin was removed by dialysis against 1x PBS.

2.5.2 IαI purification

A 450 mL sample of pig blood was collected directly into a sterile container containing 63 mL of sterile filtered 15 mM citric acid, 100 mM sodium citrate, 16 mM sodium phosphate buffer at pH 5.6, 160 mM glucose and 2 mM adenine hydrochloride. Serum was isolated from the blood by centrifugation at 1500 *x g* for 20 minutes and then stored in 100 mL aliquots at -20^{o}C.

A 100mL aliquot of serum was thawed at RT before being mixed with a buffer containing 20 mM Tris.HCl, 4 mM EDTA and 0.3 M NaCl (Buffer A) to give a final volume of 1 L. Diluted serum was then gravity fed through a Q column containing 20 mL of Q HP resin (GE lifesciences) at 4^{o}C. The loaded column was then washed with 300 mL of buffer A, this wash was then stored at 4^{o}C. IαI was then eluted using 150 mL of buffer A with 0.6 M NaCl followed

by 150 mL of buffer A with 1 M NaCl, both elutions were stored separately at 4°C for analysis by SDS PAGE.

2.5.3 Protein-Protein Interaction Analysis by Biolayer Interferometry

Protein-protein interactions were analysed using a BLItz system (FortéBio) fitted with streptavidin coated Dip and Read biosensors (FortéBio). Interactions were analysed using a multistep kinetics protocol. A baseline was established using PBS, afterwards 4 µL of biotinylated alpha was added to the probe and incubated for 120 seconds. The probe was then washed for 30 seconds with fresh PBS, following this 4 µL of a potential binding partner was added to the probe, either IαI or whole serum, and incubated for a further 120 seconds. This step was followed by another 30 second wash in PBS and then a 120 second dissociation step to analyse the dissociation rate.

2.5.3.1 Conditions used for Biolayer interferometry analysis of alpha and IαI interactions

Biolayer interferometry analysis of the interaction between α and purified IαI was carried out as described in section 2.5.3. In order to test possible protein-protein interactions the following experimental conditions were used;

Run 1- 1xPBS baseline (30 sec), 4 µl of Biotinylated α (20 µg/ml) bound to a streptavidin probe (120 sec), 1xPBS wash (30 sec), 4 µl of undiluted pig serum incubated with the probe (120 sec), dissociation step in 1xPBS (120 sec).

Run 2- 1xPBS baseline (30 sec), 4 µl of Biotinylated α (2 0µg/ml) bound to a streptavidin probe (120 sec), 1xPBS wash (30 sec), 4 µl of 1.33 fold diluted pig serum incubated with the probe (120 sec), dissociation step in 1xPBS (120 sec).

Run 3- 1xPBS baseline (30 sec), 4 µl of Biotinylated α (20 µg/ml) bound to a streptavidin probe (120 sec), 1xPBS wash (30 sec), 4 µl of 2 fold diluted pig serum incubated with the probe (120 sec), dissociation step in 1xPBS (120 sec).

Run 4- 1xPBS baseline (30 sec), 4 µl of Biotinylated α (20 µg/ml) bound to a streptavidin probe (120 sec), 1xPBS wash (30 sec), 4 µl of 4 fold diluted pig serum incubated with the probe (120 sec), dissociation step in 1xPBS (120 sec).

Run 5- 1xPBS baseline (30 sec), 4 µl of Biotinylated α (20 µg/ml) bound to a streptavidin probe (120 sec), 1xPBS wash (30 sec), 4 µl of 10 fold diluted pig serum incubated with the probe (120 sec), dissociation step in 1xPBS (120 sec).

Run 6- 1xPBS baseline (150 sec), 4 µl of 2 fold diluted pig serum incubated with the probe (120 sec), dissociation step in 1xPBS (120 sec).

2.5.3.2 Conditions used for biolayer interferometry analysis of pre-incubated alpha and Iαl interactions

Further to section 3.2.2 several reactions were set up to measure protein-protein interactions using pre-incubated protein mixtures. It was hoped that by mixing the α and IαI proteins together and incubating them for 1 hour at RT it would eliminate any possible effects of steric hindrance caused by α annealing to the probe. In order to test this the following conditions were used;

Run 1- 1xPBS baseline (30 sec), 4 µl of 0.2x biotinylated α protein (120 sec)

Run 2- 1xPBS baseline (30 sec), 4 µl of 1x biotinylated α protein (120 sec)

Run 3- 1xPBS baseline (30 sec), 4 µl of 0.2x biotinylated α protein which had been incubated with 100 µl of purified IαI at RT for 1 hour (120 sec)

Run 4- 1xPBS baseline (30 sec), 4 µl of 1x biotinylated α protein which had been incubated with 100 µl of purified IαI at RT for 1 hour (120 sec)

Run 5- 1xPBS baseline (30 sec), 4 µl of 0.2x biotinylated α protein which had been incubated with 100 µl of purified 1.5 fold diluted IαI at RT for 1 hour (120 sec)

Run 6- 1xPBS baseline (30 sec), 4 µl of 1x biotinylated α protein which had been incubated with 100 µl of purified 1.5 fold diluted IαI at RT for 1 hour (120 sec)

Run 7- 1xPBS baseline (30 sec), 4 µl of purified IαI (120 sec)

2.5.4 Alpha-WBC and serum pulldowns

White blood cells were isolated from whole blood using erythrocyte lysis buffer as in section 2.4.3.2. Serum was isolated by centrifuging citrated whole blood at 20000 *x g* in a benchtop centrifuge for 2 minutes. Pulldown reactions were set up in triplicate in 0.5 mL eppendorfs containing 100µl of avadin agarose slurry (ThermoScientific Pierce) as follows;

W- 50 µL Purified white blood cells, avidin beads, 100 µL 1xPBS, 50 µL 1x biotinylated NMB α, 20 µL of dimethyl 3,3'-dithiobispropionimidate (DTBP)

S- 50 µL Serum, avidin beads, 100 µL 1xPBS, 50 µL 1x biotinylated NMB α, 20 µL of DTBP

WS—50 µL Purified white blood cells , 50 µL Serum, avidin beads, 50 µL 1xPBS, 50 µL 1x biotinylated NMB α, 20 µL of DTBP

The reactions were then incubated at RT for 1 hour, afterwards pH8 Tris was added to a final concentration of 50 mM to stop the crosslinking reaction. The reaction was left to quench at RT for 30 minutes, before being centrifuged at 20000 *x g* to separate the supernatant from the beads. The supernatant was then removed and the beads were washed in 0.5 mL of 1xPBS. The beads and the supernatant were then analysed by SDS PAGE as outlined in section 2.1.9 using a 7.5% acrylamide gel.

2.5.5 Alpha-IαI pulldowns

For the pulldown reaction 0.2mg of Biotinylated alpha (section 2.5.1) was added to 100 µL of streptavidin beads (ThermoScientific Pierce) along with 0.1 mg of DTBP that was previously resuspended at a concentration of 5 mg/mL in 1xPBS. The reaction was incubated at RT for 1hr before being stopped with pH8 Tris at a final concentration of 50 mM. Once stopped 20 µL of avidin agarose beads (ThermoScientific Pierce) were added and the mixture was incubated for a further 1 hour at RT and then centrifuged at 20000 *x g* using a benchtop centrifuge in order to separate the beads from the supernatant. The beads were then washed with either 0.1 M NaCl $NaHCO_3$ buffer or 0.5 M NaCl $NaHCO_3$ buffer and centrifuged again at 20000 *x g*. 15µL of either beads or supernatant were mixed with 10 µL of loading dye before being boiled at 95°C for 3 minutes and loaded onto 7% (w/v) SDS gel.

2.5.5.1 Conditions used for the alpha and IαI pulldown assay

A pulldown assay was carried out as detailed in section 2.5.5. In order to characterise any possible interactions between the two proteins the following experiments were set up;

	Volume of α covered bead slurry	Volume of purified IαI in 1xPBS	Volume of 1xPBS	Volume of 0.1 mg/mL BSA
Tube 1	100 µl	200 µl	112.5 µl	0
Tube 2	200 µl	200 µl	12.5 µl	0
Tube 3	100 µl	200 µl	100 µl	12.5 µl
Tube 4	200 µl	200 µl	0	12.5 µl

2.6 PCR amplification and fusion of neisserial Gly1-ORF1 and Alpha protein

2.6.1 Buffers

A Tris base, acetic acid and EDTA buffer (TAE) was used as the running buffer for agarose gels; the TAE contained 40 mM Tris Base, 19 mM acetic acid and 1 mM EDTA. For eluting

DNA from columns during miniprep and also for resuspending and diluting primers DNA elution buffer was used and contained 20 mM Tris pH8 with 1 mM EDTA.

2.6.2 Primers

All primers were supplied by Eurofins and resuspended in DNA elution buffer and kept as a 100 pmol/µL stock which was diluted to 10 pmol/µL working stock. Once diluted in DNA elution buffer the primers were stored at -20°C. For primer list see Appendix I.

2.6.3 Miniprep of plasmid DNA

Plasmid purification was carried out using the QIAprep spin miniprep kit (Qiagen) following the centrifuge protocol as supplied with the kit. The protocol was carried out on 5ml of overnight cell culture made in LB containing 100 µg/ml of ampicillin as in section 2.1.4. The resulting purified plasmid was eluted from the miniprep column using DNA elution buffer and then stored at -20°C.

2.6.4 Sequencing

Sequencing was carried out by the Core Genomics facility located in the University of Sheffield Medical School (http://genetics.group.shef.ac.uk). A 20 µl sample of plasmid purified by miniprep (section 2.6.3) was provided to the facility and the sequencing M13 primers were used to sequence the plasmid insert. Data was retrieved and analysed by a combination of FinchTV (Geospiza) and Multalin software.

2.6.5 PCR amplification and mutagenesis

PCR amplification and mutation was carried out on both the NMB α protein, located in the pJONEX4_NMB_alpha plasmid, the *neisserial* IgA1P gene, located on the pJONEX4_IgAP_WT plasmid, and the *N. meningitidis* Gly1-ORF1 gene, located in the pJONEX4_neisserial_gly1_ORF1-his. Using a Phusion high-fidelity PCR kit (New England Biolabs) separate 50 µl reactions without DMSO were set up according to the manufacturer's instructions supplied with the kit. For these reactions 15-50 pg of the appropriate purified plasmid was used as a template, the template plasmids were isolated prior by miniprep carried out as described in section 2.5.3. In this study two different methods of PCR mutagenesis were used, 1 method used 4 primers (two internal and two external to achieve the mutation whilst the other used one pair which amplified the whole plasmid[124]. The former method was used initially however later in the study the latter method proved to be more reliable.

The PCR cycles used were generally: 95°C- 5 min, (95°C - 30s, 55°C - 1 min, 72°C - 1 min 30s) 20 cycles, 72°C - 5 min. Although the 55°C annealing step was often tweaked in order to achieve a purer end product. Products were analysed using a 2% agarose gel as described in section 2.5.6 and the bands of the correct size were extracted from the gel using a QIAEX II gel extraction kit (Qiagen) as per the manufacturer's instructions in order to remove contaminating templates, products and primers. The resulting pure plasmid was then transformed into chemically competent *E. coli* M72 cells as described in section 2.5.8.

2.6.6 Agarose gel

Agarose gels for DNA analysis were made using 2% (w/v) electrophoresis grade agarose (Sigma Aldrich) dissolved in TAE buffer heated in a microwave. Once cool 1 µl of 10 mg/ml ethidium bromide was added per 30 ml of gel solution and thoroughly mixed. This was then poured into a gel rig with a suitable comb in place to form 15 µl wells.

2.6.7 PCR fusion

Using a Phusion high-fidelity PCR kit (New England Biolabs) a 50 µl reaction without dimethyl sulfoxide (DMSO) was set up according to the manufacturer's instructions supplied with the kit in order to fuse the purified PCR products. For this 15-50 pg of purified α PCR amplification product and 15-50 pg of amplified Gly1-ORF1 PCR amplification product were used as a template in conjunction with the M13 uni and M13 rev primers. PCR cycle: 95°C- 5 min, (95°C - 30s, 50°C - 1 min, 72°C - 1 min 30s) 20 cycles, 72°C - 5 min. Products were analysed using a 2% agarose gel as described in section 2.5.6 and the bands of the correct size were extracted from the gel using a QIAEX II gel extraction kit (Qiagen) as per the manufacturer's instructions in order to remove contaminating templates, products and primers. The resulting pure plasmid was then transformed into chemically competent *E. coli* M72 cells as described in section 2.5.8.

2.6.8 Transformations

A 100 µL aliquot of chemically competent cells were mixed with 10-50 ng of DNA and incubated on ice for an hour, heat shocked at 42°C for 1 min and then rested on ice for 10 min. Cells were then plated on to LB amp plates.

2.7 Analysis of the alpha proteins potential as a carrier protein

2.7.1 Mouse antibody titre model

Six week old female BALB/C mice were immunised with a 10 µg dose of alpha, gamma, c-ɣ-α or 50:50 mixture of alpha and gamma. All solutions were at 50 µg/mL diluted in PBS. A 0.2

mL dose of PBS was also given to the negative control mice to rule out any immune response to the procedure. 4 mice were used in all of the experimental groups and 2 mice were used as a negative control. The mice were immunised by IP injection at days 0 and 13 and tail vein bleeds were carried out on days 14 and 28. Whole blood was centrifuged at 1000 *x g* for 10 minutes in order to obtain plasma, this was then frozen at -20^{o}C. ELISAs were later carried out on this plasma in order to quantify the antibody titres as explained in section 2.7.2

2.7.2 Antibody titre ELISA

Plasma was serially diluted to dilutions of 1:500, 1000, 2000, 5000 and 10000 in PBS. 96 well ELISA plates were coated with 100 µl of 10 µg/mL of alpha, gamma or PBS and incubated O/N at 4^{o}C to coat plates. Plates were then aspirated and washed with PBS tween 0.05% (w/v), then blocked with 100 µL of PBS with 5% (w/v) milk powder at RT for 1 hour. Plates were aspirated and washed with PBS-T, serial dilutions were then added to individual wells and the plates covered with parafilm (Bemis) and incubated with shaking at RT for 2 hours. Plates were then aspirated and washed with PBS-T. Next 100 µL of anti-mouse IgG linked to HRP (Abcam) at a 1:2000 dilution in PBS was added to wells and incubated for 1 hour at RT with shaking. Plates were then aspirated and washed with PBS-T before 100 µL of 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Aldrich), which quantifies HRP activity, was added to each well. This reaction was stopped with 1% (w/v) SDS solution after 2-5 min. The absorbance of each well was read at 405 nm using a plate reader.

2.7.3 Crosslinking alpha with Gly1 homologues and tbFEND183K

Alpha was crosslinked with a variety of Gly1 homologues, as well as the *Trypanosoma brucei* FEN D183K mutant. These crosslinked proteins would then be injected into female New Zealand white rabbits in order to quantify the potential of alpha as a carrier protein as explained in section 2.7.6. Both proteins to be cross-linked were dissolved in 1xPBS. Alpha was labelled using sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) (Thermo Scientific) at a 5 times molar excess. The sulfo-SMCC was dissolved in water and then added to Alpha in 1xPBS and incubated at RT for 30 min, afterwards the excess sulfo-SMCC was removed by buffer exchange using a Vivaspin column (Sartorius) with a MWCO of 5000. Gly1 MH0579/1205/para, neisserial gly1 and tbFEND183K were all labelled with N-succinimidyl-S-acetylthiopropionate (SATP) at a 5 times molar excess. SATP was first dissolved in 50-100 µL of DMSO and then added to the protein in 1xPBS and incubated at RT 30 mins, afterwards the excess SATP was removed by buffer exchange using a Vivaspin column (Sartorius) with a MWCO of 5000. The SATP was then deacetylated ready for

crosslinking by incubating it with 100-200 µL of deacetylation solution (0.5 M hydroxylamine, 25 mM EDTA in 1xPBS at pH 7.2-7.5) at RT for 2 hours. The deacetlyated SATP labelled protein was then mixed with the sulfo-SMCC labelled alpha protein and incubated at RT for 30 min or 4^{o}C O/N. Protein conjugates were purified using a size exclusion chromatography column attached to an ACTA rig as covered in section 2.7.4.

2.7.4 Size exclusion chromatography

A column packed with Sephacryl S-200 HR (GE Healthcare) was used in order to separate proteins based on molecular weight. Prior to loading on the column the protein sample was filtered through a 0.2 µm filter and the column was equilibrated with 10 column volumes of 1xPBS. The sample, with a maximum volume of 10% of the columns volume, was then loaded. Sterile 1xPBS was then used to wash the column and up to 60 fractions were collected. These were then analysed by SDS PAGE as described in section 2.1.9.

2.7.5 LPS assay

Prior to being injected the crosslinked proteins were checked for LPS contamination, this ruled out elevated immune responses as a result of contamination. LPS content in samples was measured using Limulus Amebocyte Lysate (LAL) QCL-1000 kit (Lonza). Samples were then analysed using the supplied test tube protocol and glass test tubes which were washed with distilled water and sterilised in an autoclave for 20 min at 120^{o}C.

Standards were made at 1, 0.5, 0.25, 0.1 and 0 EU/mL (endotoxin diluted in LAL reagent water). 50 µL of sample or standard was placed in a glass test tube at 37^{o}C then 50 µL of LAL was added and the reaction incubated at 37^{o}C for 10 min. 100 µL of chromogenic substrate was then added and incubated at 37^{o}C for 6 min. Finally 100 µL of 10% (w/v) SDS was added to stop the reaction. The absorbance of the samples and standards were measured at 405 nm using a NanoDrop ND-1000 spectrophotometer (Thermoscientific).

2.7.6 Generon New Zealand white rabbit Antibody model

Four female New Zealand white rabbits were immunised with the following mixtures of proteins;

Protein A- α crosslinked to the *Mannheimia haemolytica* Gly1 paralogue plus free *Mannheimia haemolytica* Gly1 homologue MH0579.

Protein B- α crosslinked to the *Mannheimia haemolytica* Gly1 homologue MH0579 plus free *Mannheimia haemolytica* Gly1 paralogue.

Protein C- α crosslinked to *Trypanosoma brucei* FEN D183K mutant plus free *Mannheimia haemolytica* Gly1 homologue MH1205.

Protein D- α crosslinked to the *Mannheimia haemolytica* Gly1 homologue MH1205 plus free *Trypanosoma brucei* FEN D183K mutant.

These immunisations would give data as to whether proteins crosslinked to the α protein elicited a larger immune response to those that were injected without conjugation to α. Rabbits were immunised on day 1 and 14 with 200 µg of the respective protein mixture plus complete Freuds adjuvant. On day 28 the rabbits were then immunised with 100 µg of the respective protein mixture in the presence of incomplete Freuds adjuvant. Rabbits then had small test bleeds carried out on days 38 and 41 in order to check antibody titres prior to injection with 100 µg of the respective protein mixture in the presence of incomplete Freuds adjuvant on day 42. Rabbits then had small test bleeds carried out on days 52 and 55 in order to check antibody titres prior to a repeat injection of 100 µg of the respective protein mixture in the presence of incomplete Freuds adjuvant on day 56. Rabbits then had a final test bleed carried out on day 66 prior to a terminal bleed of around 46ml on day 67. Serum was then shipped to the University of Sheffield for analysis.

2.7.7 Antibody purification

In order to purify antibodies around 10 mg of the protein to which the antibody bound was immobilised on a 2 mL AminoLink Plus column (ThermoFisher) at pH7.2 in 1xPBS as directed by the supplied protocol. This involved first equilibrating the column at pH 7.2 using 1xPBS then adding the protein of interest along with 40µL of cyanoborohydride, resulting in a 50mM final concentration of cyanoborohydride. The column was then incubated at RT for 6 hours whilst being rolled. Post incubation the column was washed in 1xPBS prior to use. The rabbit serum containing the antibody was diluted two fold in 1xPBS and loaded on to the column, after the column had first been equilibrated with 6 mL of 1xPBS. The column was then incubated with the serum for 1 hour at RT whilst rolling before being centrifuged at 800 *x g* for 1 minute at 10^{o}C, this flow through was then set aside. The pure antibody was the eluted from the column using 6 mL of elution buffer (0.1 M glycine.HCl at pH2.5), to the pure antibody 300 µL of neutralisation buffer was then added (1 M Tris HCl at pH 8.5). Purified antibody was then stored at -20^{o}C.

Chapter 3- Investigation of alpha-host interactions

3. Investigation of alpha-host interactions

3.1 Introduction

As was discussed in chapter 1 the α protein has been the subject of some structural and functional characterisation over the last decade [25,95,102,111,112,116] . However, many questions still remain to be answered. Chief among these questions is why the α peptide so highly conserved in neisserial species and does it play a functional role in any step of the invasion and virulence of the pathogen.

The function of the α protein has not been well characterised *in vivo* with previous studies hinting that α, through its possession of multiple nuclear localisation signals, could be able to target IgA1P into host cells in order for IgA1P to have maximum effect [116] . It has also been speculated that α could be involved in binding eDNA, a signal that could trigger neisserial biofilm formation in both blood vessels and at the mucosal layer [112] . This would mean that α could be involved in neisserial colonisation and survival within the host. Previous studies in the Sayers lab (Helena Parsons, MSc thesis, University of Sheffield, UK) have also shown that α may be involved in binding bikunin or the bikunin domain containing proteins such as Inter-α-inhibitor, a serine protease inhibitor known to inhibit the function of trypsin and chymotrypsin [117] . This could be relevant in preventing these proteins from inhibiting the cleavage of IgA1P therefore allowing it to carry out its function *in vivo*. This could also explain the co-expression of alpha and IgA1P in as much as when IgA1P production is upregulated it is obviously required by the pathogen and as such inhibition of the protease must be prevented, this could therefore be carried out by a co-secreted factor such as α.

The hypothesis of this work was that the alpha protein would interact with one or more serum based factors that could indicate a role in virulence determination, due to its co-expression with another virulence factor IgA1P. This may then lead to further study of the proteins role *in vivo* and a better understanding of why the protein is highly conserved.

In order to test this hypothesis the α protein was first expressed and purified and then a number of experiments were carried out using blood cells and serum derived proteins, from both human and animal samples as described below.

3.2 Results and discussion

3.2.1 Alpha protein expression

Cells were cultured in 6 ml of LB media as described in section 2.1.5 using *E. coli* M72 cells containing either the pJONEX4_NMB_cys_γ_α or the pJONEX4_NMB_α plasmid. A protein expression check was then carried out as described in section 2.1.6. This highlighted the optimal time for bacterial harvest in order to gain the maximum yield of the protein of interest.

In all cases multiple expression checks were carried out in order to confirm that the bacteria grew consistently and as such that the yield of protein and cells would be reproducible.

It was found from multiple trials that a plateau in yield was reached after between 12 and 24 hours of induction at 42°C, this is most likely due to the cells dying after this point. As a result, an overnight induction of the *E. coli* cells was routinely used (Fig 25).

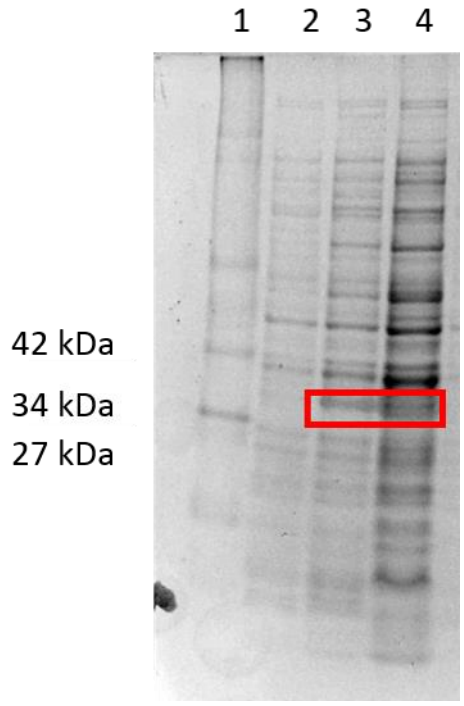

Figure 25. 10% (w/v) acrylamide SDS PAGE of a cys-ɣ-α expression time course. Lane 1 protein weight marker (New England Biolabs), lane 2 uninduced cys-ɣ-α cell pellet, lane 3 cys-ɣ-α cell pellet after a 1hr induction, lane 4 cys-ɣ-α cell pellet after a overnight induction. The expression of a protein at 35 kDa can clearly be seen post induction, this band corresponds to the 22 kDa cys-ɣ-α protein (Helena Parsons, MSc thesis, University of Sheffield, UK). The O/N induction was routinely used due to a plateau in expression being reached after 12 hours.

3.2.2 Alpha protein purification

Cells were cultured in a 5 L fermenter containing 5YT as described in section 2.1.7 using *E. coli* M72 cells containing either the pJONEX4_NMB_cys_γ_α or the pJONEX4_NMB_α plasmid. This allowed a large volume of cells to be obtained from which the protein could be purified. Bacterial cell pellets containing α or cys-ɣ-α were then treated as in section 2.2.2 in order to purify the protein of interest. Both α and cys-ɣ-α could be purified using the same protocol as the 26 peptide addition in the cys-ɣ-α did not significantly alter the attributes of the protein from those of the normal α protein. The initial steps of this protocol were carried out in order to obtain a pure cell lysate containing no whole bacterial cells as well as removing any contaminating DNA which may interfere with ion exchange chromatography. The α and cys-ɣ-α proteins were both calculated to have a pI of around 9.3 and as such a pH5.5 buffer was used during ion exchange chromatography utilising a heparin matrix column. This is due to the fact that heparin has a negatively charged matrix which is often used for purifying DNA binding proteins and so the protein needed to be positively charged in order to adhere to the column. When proteins were applied to the Heparin HP column they bound to the matrix, although some may have been found in the flowthrough due to column overloading. The proteins eluted in fractions 6-12 of the salt gradient, as seen in **Figure 26**, and corresponds to a salt concentration of between 145-415 mM.

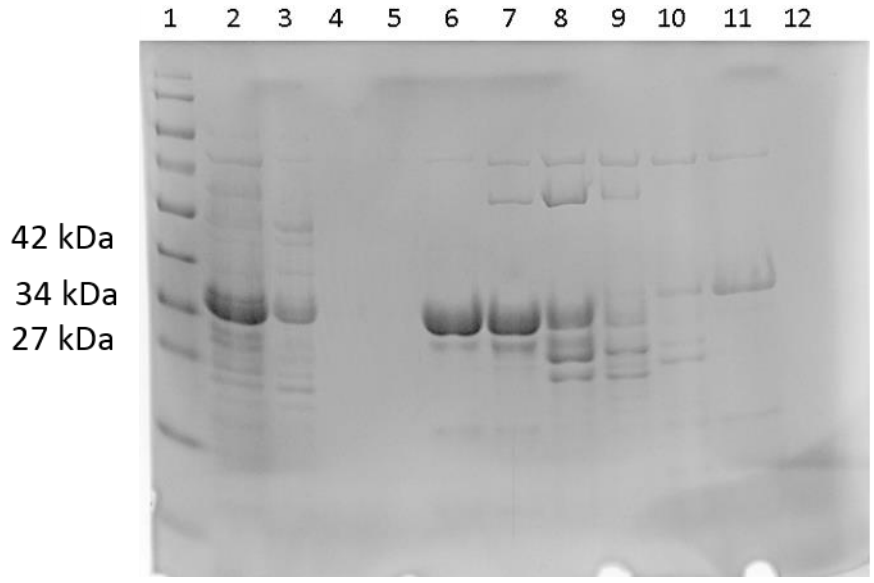

Figure 26. A 10% (w/v) acrylamide SDS PAGE gel showing purification of c-ɣ-α using a Heparin HP column at pH 5.5. In each case 10 µL of each sample was loaded onto the gel. Lane 1 protein weight marker (New England Biolabs), lane 2 crude protein extract, lane 3 tubing dead volume, lane 4 column flowthrough, lane 5 column wash, lane 6-12 fractions from a sodium chloride gradient ranging from 145 mM NaCl to 415 mM NaCl. Lanes 6-12 each correspond to a 1 column volume fraction.

After elution the fractions containing the protein of interest, in this case lanes 6-12, were pooled together. This pooled material contained the majority of the proteins of interest and so was dialysed and into Q column buffer and loaded on to an anion exchange column (Q-HP sepharose column GEhealthcare) at pH 8, in order to further purify the protein. As the Q column is contains anionic exchange resin the protein eluted from the column in the flowthrough fraction as seen in **Figure 27** whilst other cellular proteins bound to the resin, therefore purifying c-γ-α or α further.

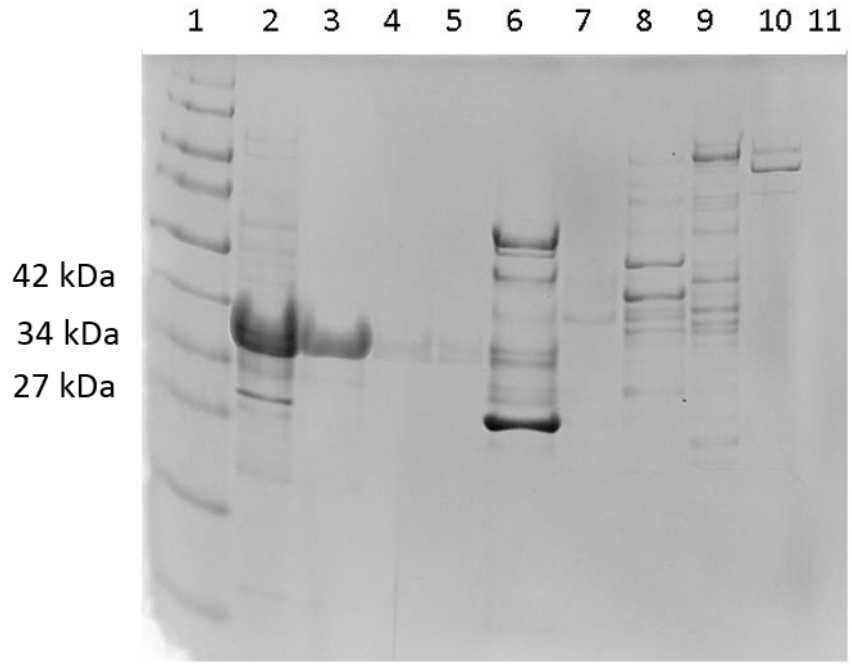

Figure 27. A 10% acrylamide SDS PAGE gel showing further purification of c-γ-α using an anion exchange column at pH 8. Lane 1 protein weight marker (New England Biolabs), lane 2 pooled protein fractions from first heparin column, lane 3 column flowthrough, lane 4 column wash, lane 5-12 fractions from a sodium chloride gradient ranging from 100 mM NaCl to 415 mM NaCl. Lanes 5-12 correspond to a 1 column volume fraction.

The flowthrough from the Q column was then dialysed into pH 5.5 potassium sulphate buffer and loaded onto another Heparin HP column .This repeat heparin column separated the pure c-γ-α or α from any remaining contaminants as seen in **Figure 28**.

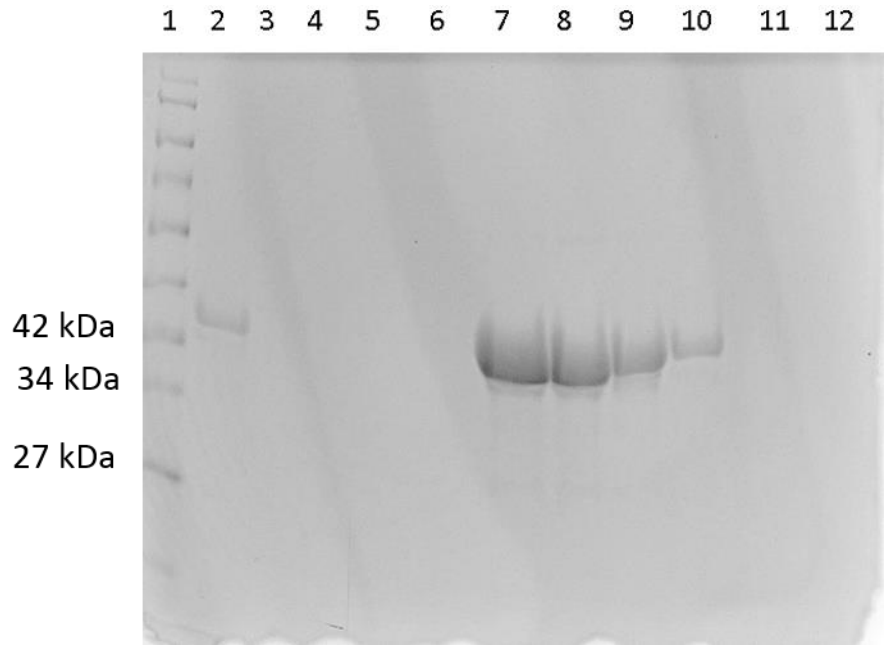

Figure 28. A 10% acrylamide SDS PAGE gel showing the final purification of c-γ-α using a Heparin HP column at pH 5.5. Lane 1 protein weight marker (New England Biolabs), lane 2 tubing dead volume, lane 3 column flowthrough, lane 4 column wash, lane 5-12 fractions from a sodium chloride gradient ranging from 145 mM NaCl to 460 mM NaCl. Lanes 5-12 correspond to a 1 column volume fraction.

The resulting purified protein was treated with 4 M ammonium sulphate in order to precipitate the protein and then centrifuged at 48000 x *g* for 20 min in order to pellet the protein. This pellet was then resuspended in 1-2 ml of pH 7 KPB in order to concentrate it. It was then quantified as described using either method from section 2.2.4 or 2.2.5. The quantified proteins were then tested for purity by western blot using a multiclonal anti-α rabbit antibody raised against purified α protein. This α protein used for antibody generation had previously been verified via mass spectrometry and the anti-α antibodies checked for specificity.

3.2.3 Measurement of Interactions between alpha and human blood cells using flow cytometry

FACS analysis of blood-protein interactions were carried out as described in section 2.4, the purpose of these experiments was to identify any potential cellular binding partners in the bloodstream for α or c-γ-α. These experiments both used FITC labelled proteins which could easily be detected using flow cytometry as well as a FITC labelled negative control. This allowed the identification and quantification of labelled proteins binding to either red blood cells or white blood cell populations.

3.2.3.1 Interaction between alpha and whole human blood

In this experiment FITC labelled α, c-γ-α and BSA, all at 0.4 mg/ml, were incubated with whole blood at RT for 20 min before being centrifuged and re-suspended in FACS buffer as outlined in section 2.4.3.1. This process of centrifugation and resuspension effectively washed the cells therefore ensuring a sample free of debris. The resulting mix was then analysed using a FACScalibur machine (BD Biosciences) and Cellquest software (BD Biosciences). For this experiment interactions with erythrocytes were measured and therefore all other cells were gated out using SSC and FSC readings, the SSC and FSC readings measure the size and granularity of the cells in the sample. Erythrocytes are easily gated due to their small size and low granularity. Interactions between erythrocytes and fluorescent proteins were then quantified using SSC and fluorescence values in order to work out percentage of cells labelled fluorescently, when compared with a blank, and also the mean fluorescence intensity of the sample.

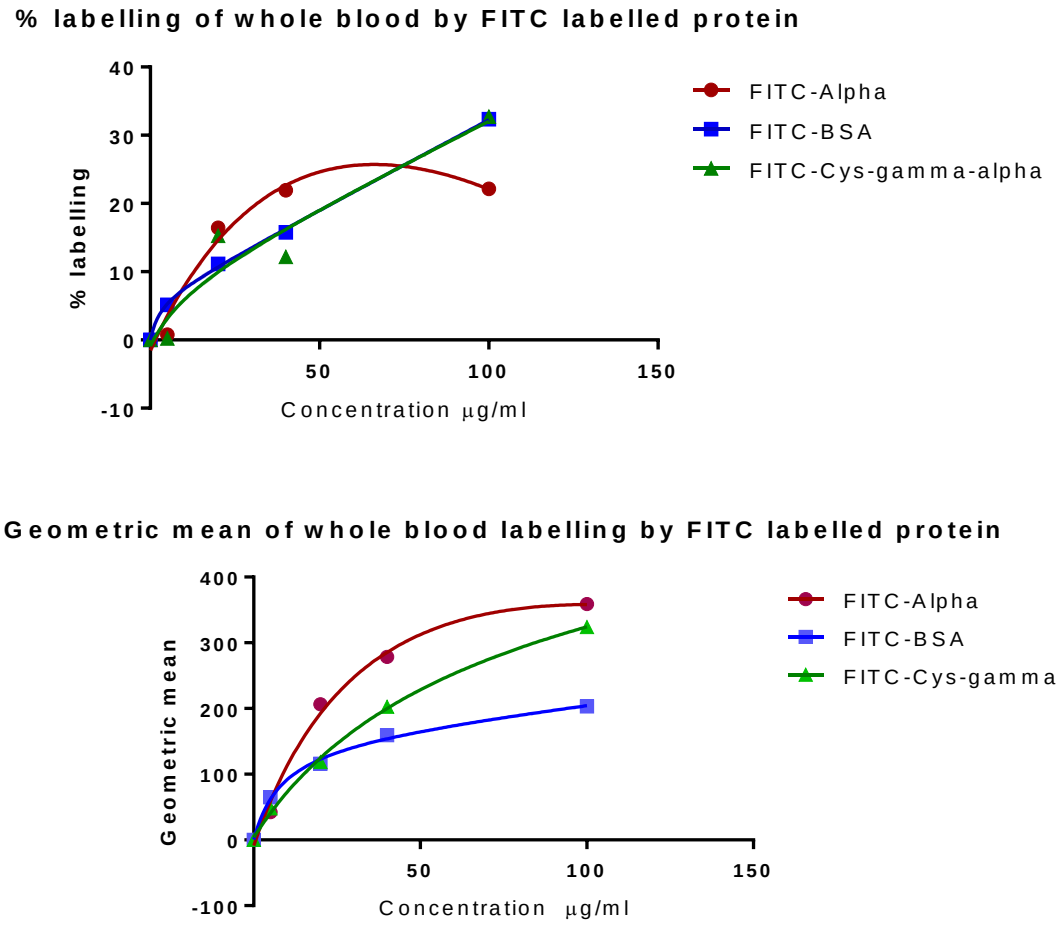

Figure 29. Analysis of the FACs data in Cellquest (BD Biosciences). The top graph shows that FITC labelled α and c-γ-α did not give a percentage labelling of erythrocytes higher than the negative control. However, the bottom graph shows that the average labelling intensity, as measured by geometric mean was higher for FITC labelled α and c-γ-α than for the negative control BSA.

The result of this experiment, **Figure 29** ,showed that the negative control, FITC labelled BSA protein, bound to erythrocytes at a much higher rate than expected which may hint at a non-specific interaction between FITC and erythrocytes. Despite this, labelled proteins of interest were shown to bind to erythocytes with a similar percentage of labelling but a much higher geomtric mean than the negative control. This indicates that, potentially, the labelling is more specific. This is due to the fact that the geometric mean effectively quantifies the number of labels per erythrocyte and so although the same quantity of erythrocytes are labelled they are labelled at a much higher intensity. This hints that the α and c-γ-α proteins have a higher affinity for whole blood than BSA as they more efficiently bind to whole blood than the FITC-BSA, despite labelling the same percentage of cells in the sample.

3.2.3.2 Interactions between alpha and human white blood cells

For this part of the experiment FACS interactions between FITC labelled α, BSA and c-γ-α, all at 0.4 mg/ml and white blood cells was measured. This was carried out as previous experiments in the Sayers group (University of Sheffield, UK) indicated that α protein may preferentially bind to neutrophils in the presence of serum based factors. To determine this the FITC labelled proteins were incubated at RT for 20 min with whole blood. At the end of this incubation erythrocytes were lysed using erythrocyte lysis buffer as described in section 2.4.3.2 and the mixture centrifuged and resuspended in FACS buffer in order to wash the cells and ensure a sample free of debris. This was then analysed using a FACScalibur machine (BD Biosciences) and Cellquest (BD Biosciences) software. As only protein interactions with white blood cells were being investigated cellular debris and any remaining erythrocytes were gated out using FSC and SSC readings, effectively gating the sample by size and granularity. The interactions were then quantified using SSC and fluorescence in order to work out the percentage labelling, above the blank, of the white blood cells by the protein as well as the mean fluorescence intensity.

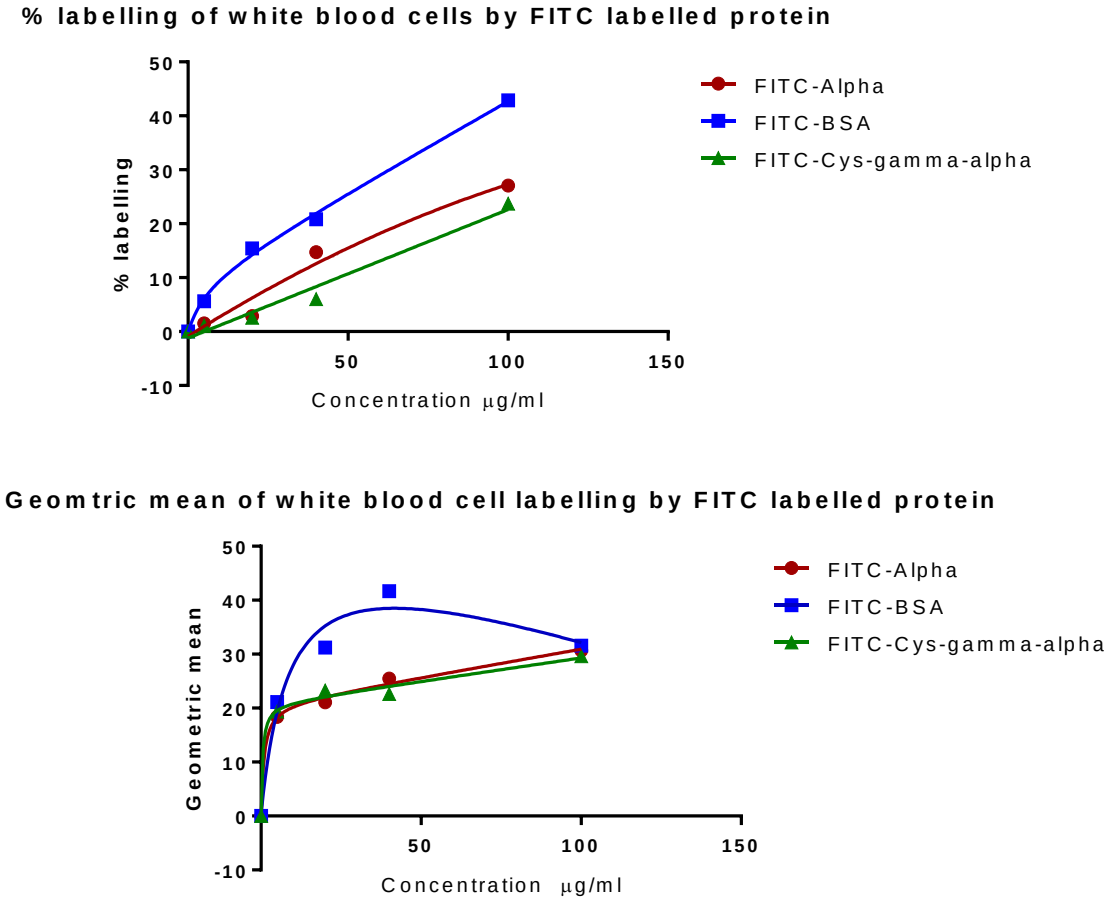

Figure 30. Analysis of the FACs data in Cellquest (BD Biosciences). The top graph shows that FITC labelled α and c-γ-α did not give a percentage labelling of leukocytes higher than the negative control. The bottom graph shows that the average labelling intensity, as measured by geometric mean was also lower for FITC labelled α and c-γ-α than for the negative control BSA.

The results shown in **Figure 30** showed that the negative control BSA had a higher percentage labelling of white blood cells than the proteins of interest. Also unlike in section 3.4.1 the geometric mean, which equates to labelling intensity, was lower in the reactions involving the test proteins compared to those reactions involving BSA. This indicates that the α and c-γ-α proteins exhibit no specific interactions with any white blood cell populations.

3.2.4 Measurement of interactions between alpha and human blood components using the electrophoretic mobility shift assay

The mobility shift assay described in this section was carried out in order to identify the size of any serum based binding partner for α or c-γ-α, which would not only validate previous experiments within the Sayers group that suggested a serum based binding partner but also help to identify such a protein. The mobility shift assay effectively measures the change in

molecular weight of a protein of interest in order to identify what size protein it has bound to. This gives an idea of what the potential binding partner could be and allows for further pulldown experiments to be done to isolate such a partner.

3.2.4.1 Interactions between alpha and serum or whole blood detected using an electrophoretic shift assay

An electrophoretic shift assay was carried out as described in sections 2.4.4, 2.4.4.1 and 2.4.4.2 then analysed using a western blot with rabbit anti-α antibody as the primary antibody and anti-rabbit HRP conjugated antibody as the secondary antibody. The primary focus of this experiment was to validate previous findings by the Sayers group that the α protein appears to have a serum based binding partner. For this experiment α and c-γ-α were mixed with a variety of different serums including human, equine and sheep. This is due to the fact that although human serum was likely to provide the most reliable binding data it was significantly more difficult to obtain than either of the non-human blood samples. It was hoped that α and c-γ-α would exhibit similar binding patterns in all of the samples allowing the use of animal serum in further experiments. The resulting image, **Figure 31**, showed that α was binding to a serum based factor with a molecular weight of at least 20-30 kDa. This was determined as α was detected at a higher molecular weight, between 50-60 kDa when cross-linked with human serum, compared with the molecular weight of 30-35 kDa at which it is normally seen to run on an SDS PAGE gel. However as there is no crosslinked control on this gel it cannot be ruled out that this is instead some multimerisation of alpha, although this is highly unlikely given the detection pattern on the western blot. However due to the poor running of the ladder it was not possible to determine the weight of the complex with any precision but there appears to be an increase in size of at least 30 kDa. Unfortunately, the lanes containing whole equine or sheep blood cross-linked to α showed poor detection by the anti-α antibody, possibly due to the inclusion of an anti-biotin antibody in order to detect the western blot ladder. The results from the human serum lanes further validate the previous findings of the Sayers group that both α and c-γ-α bind to a serum based factor which may in turn determine its cellular or systemic effects.

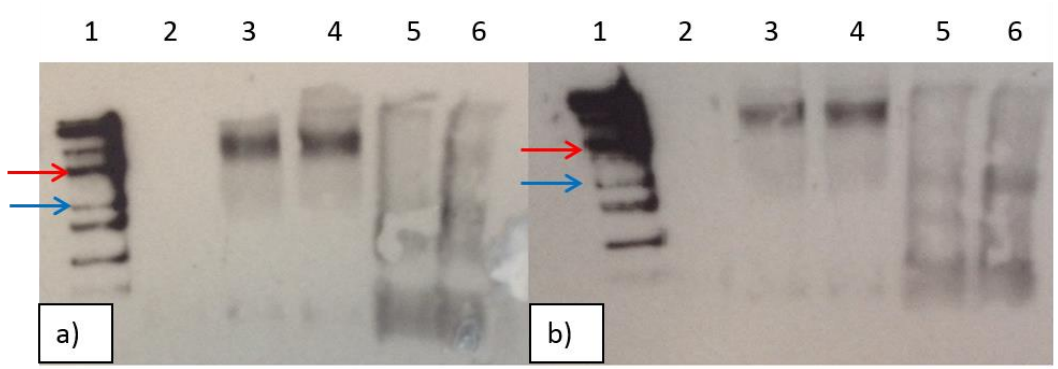

Figure 31. Western blot of an electrophoretic mobility shift assay between α or c-γ-α and whole blood. The red arrow indicates a 60 kDa protein marker, whilst the blue arrow indicates a 50 kDa marker. Gel a) shows the image of α cross-linking data, Gel b) shows c-γ-α cross-linking data. In both gels lane 1 shows a biotinylated protein weight ladder, lane 2 blank, lane 3 protein cross-linked to human serum sample 1, lane 4 protein cross-linked to human serum sample 2, lane 5 protein cross-inked to equine blood, lane 6 protein cross-linked to sheep blood.

To further evaluate potential serum based binding partners for the α protein a pulldown experiment was carried out as described in section 2.4.4 using citrated human whole blood obtained from the clinical research facility in the Royal Hallamshire hospital, Sheffield (ethics for this collection was granted by the local ethics committee under UoS No: SMBRER39 “How do microbes interact with human blood”). Some of the citrated blood was used to isolate serum whilst some was used to isolate white blood cells, these samples were isolated and used as described in section 2.5.4.

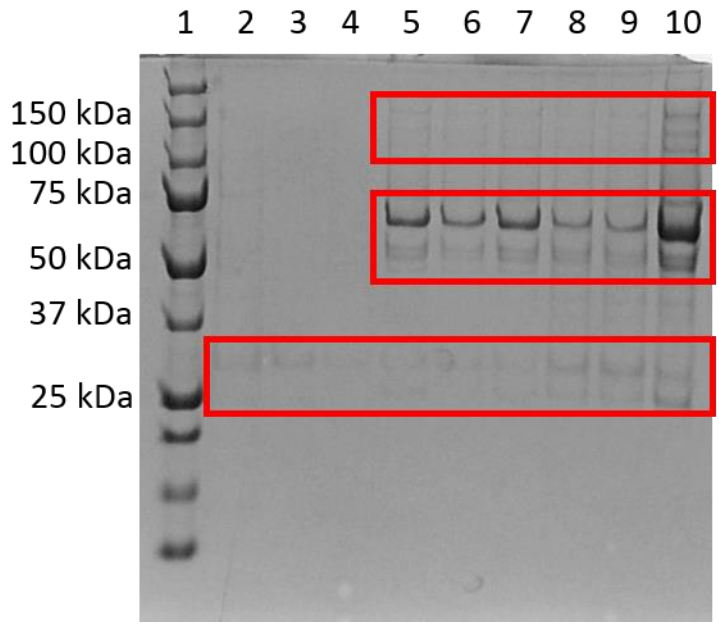

Figure 32. A 10% acrylamide SDS PAGE gel showing the interaction between the α protein and white blood cells and serum. Lane 1 precision plus protein all blue prestained ladder (Biorad), lanes 2-4 the α pulldown of white blood cells, lanes 5-7 the α pulldown of human serum and lanes 8-10 the α pulldown of white blood cells in the presence of human serum. The bands at around 30 kDa correspond to the α protein, whilst the bands at 50-75 kDa and 100-150 kDa correspond to serum based potential binding partners.

As can be seen in **Figure 32** the alpha protein is unable to interact and pulldown whole white blood cell, although this could be an artefact of the analysis by SDS PAGE, but is able to interact with a number of serum based proteins in the presence of a crosslinking agent. This crosslinking agent will stabilise transient interactions and so as a result some of these interactions may be false positives caused by stabilisation of non-specific interactions. What is clear however is that the α protein does seem to interact readily with a protein of around 60 kDa, most likely human serum albumin and a large protein at around 100-150 kDa. This large protein is particularly interesting as previous studies in the Sayer lab (Helena Parsons, MSc thesis, University of Sheffield, UK) have suggested a possible interaction between α and a large protein, around 120 kDa, known as inter-α-inhibitor (IαI). This result possibly backs up these findings and lead to the experiments carried out in section 3.6.

3.2.5 Measurement of interactions between the alpha protein and IαI from pig serum

As previously mentioned previous work in the Sayers lab has suggested that the binding partner for α may be inter-α-inhibitor (IαI) or a related molecule called bikunin, suggesting that α binds to the chondroitin-4-sulphated light chain (Helena Parsons, MSc thesis,

University of Sheffield, UK). These findings also appeared to be replicated in section 3.3.4.1. In order to confirm these observations a set of experiments were carried out with the hope of characterising the binding partner more fully. In all of these experiments human serum was substituted for pig (*Sus scrofa*) serum as it was much more readily available, this was deemed to be an acceptable substitution as the *Sus scrofa* IαI and bikunin proteins have a high similarity to those found in humans.

3.2.5.1 Confirming the interactions between alpha and IαI purified from pig serum using a pulldown assay

In order to help characterise this potential interaction IαI was purified from pig serum, section 2.5.2, and incubated with biotinylated α protein as described in section 2.5.5. This experiment would help to determine whether or not alpha interacts with IαI *in vitro* and as such whether this interaction was likely to occur *in vivo*.

In order to fully test the interaction between α and IαI multiple experimental conditions were used, these experimental conditions would include non-specific interaction controls and a variety of concentrations of the α protein, described in section 2.5.5.1.

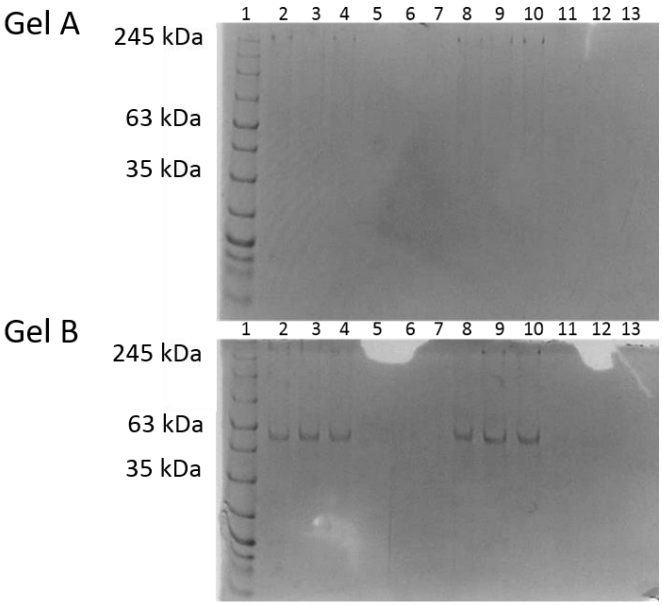

Figure 33. A 13% acrylamide SDS PAGE gel of an α-IαI pull down experiment. Biotinylated α was first bound to streptavidin beads and then used to pulldown purified IαI from pig serum. Gel A Lane 1 BLUeye prestained protein ladder (Gene flow), lanes 2-4 tube 1 supernatant, 5-7 tube 1 pulldown, 8-10 tube 2 supernatant, 11-13 tube 2 pulldown. Gel B Lane 1 BLUeye prestained protein ladder (Gene flow), lanes 2-4 tube 3 supernatant, 5-7 tube 3 pulldown, 8-10 tube 4 supernatant, 11-13 tube 4 pulldown. Tube 1 contained α and IαI mixed in a 1:2 ratio, tube 2 contained α and IαI mixed in a 1:1 ratio, tube 3 contained α and IαI mixed in a 1:2 ratio with the addition of 1.25 µg of BSA, finally tube 4 contained α and IαI mixed in a 1:1 ratio with the addition of 1.25 µg of BSA (Tube contents are detailed in section 3.2.1).

As shown in the gel above the α coated beads failed to bind to any IαI as it was all found to be in the supernatant fractions rather than the pulldown fractions, which were boiled off the beads post washing. The data from this experiment contradicts the data from the previous experiment, which showed that in free solution α and IαI interact with one another. The lack of interaction in this experiment may be a result of the way the α protein was annealed to the beads used to pulldown other proteins. It is possible that the α protein being bound to the beads created a steric hindrance effect which inhibited the interactions, it could also be that the interaction between the two proteins is transient and was stabilised in the previous experiment using a crosslinking agent.

3.2.5.2 Biolayer interferometry analysis of alpha and IαI interactions

In order to fully characterise the interaction between the α protein and the IαI protein biloayer interferometry was used (BLItz machine, Fortebio). This machine has interchangeable fibre optic probes with different binding surfaces at the tip, this allows a variety of proteins to be bound to the tip of the probe. The BlItz system works by sending white light down the probe, some of this light is then reflected by the protein molecules that form the binding surface on the tip of the probe and some is reflected by an internal control reflective surface. These reflections create an interference pattern which sets a baseline for the probe, when another protein or molecule interacts and binds to the tip this changes the interference pattern. The software then calculates the thickness of the layer of bound protein based upon the change in interference pattern. This allows for multiple step kinetic experiments to be carried out, in this case biotinylated α was bound to a streptavidin probe, this was then placed into a solution of free IαI, the software then calculated if there was any interaction between the two proteins by measuring the thickness of the protein layer bound to the probe.

The BLItz experiments were all carried out as described in section 2.5.3 using conditions described in section 2.5.3.1. Reactions were initially carried out using unpurified pig serum in the presence of biotinylated α (section 2.5.1) and in all cases a streptavidin probe was used.

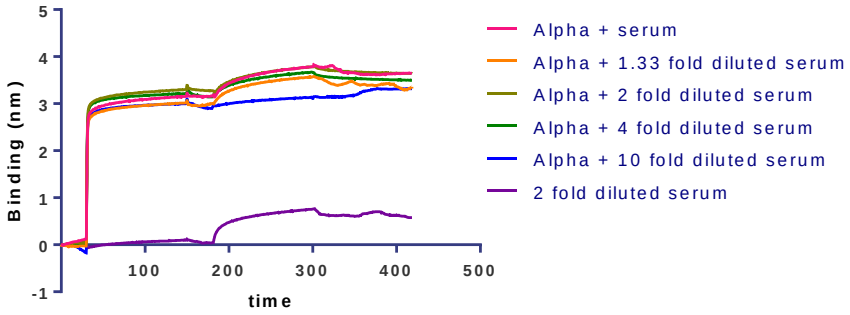

Figure 34. Graph of the interaction between α and IαI as determined by the BLItz system. Each run detailed above corresponds to a colour coded line, and corresponds to a single experiment. In all runs, except run 6 (purple), the α protein was incubated with the probe for 120 sec, then following a wash step IαI was incubated with the probe for 120 sec. In the case of the experiment using 2 fold diluted serum alone, the α binding step was replaced with a 120 sec 1xPBS wash step.

This graph at first glance appears to show an interaction between α and IαI, which appears to be concentration dependent, however inspection of run 6, the negative control run, shows that the serum itself is interacting directly with the streptavidin probe. This invalidated any interaction seen between α and IαI in this experiment.

It was believed that a variety of serum based factors could interact directly with the probe and so as a result IαI was purified directly from the pig serum, as described in section 2.5.2. This purified protein was then used to repeat the above experiment, it was hoped that this would remove the background non-specific interactions allowing for a more accurate study of the interaction between the two proteins.

In this experiment purified IαI was incubated with the biotinylated α for 1 hour at room temperature before being applied to the streptavidin probe. It was hoped that this may negate any steric hindrance effect caused when biotinylated α was bound to the streptavidin probe, this would allow the data to more accurately reflect the interaction between the two proteins. The experimental conditions for this are described in section 2.5.3.2 and in all cases a streptavidin probe was used.

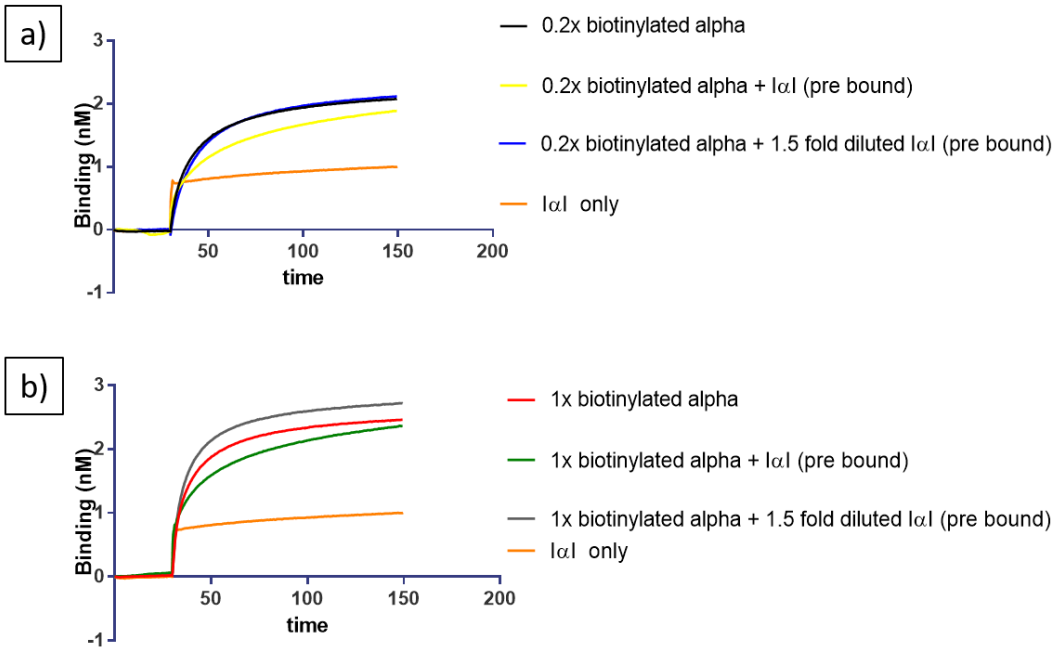

Figure 35. Interaction between the α protein and IαI as measured by the BLItz system. Each run detailed above corresponds to a colour coded line, and corresponds to a single experiment. a) Shows all the experiments 0.2x biotinylated α protein was used whilst b) shows all the experiments in which 1x biotinylated α protein was used.

As can be seen in **Figure 35**, above, no combination of alpha and IαI resulted in a significantly thicker layer being deposited on the BLItz probe than when alpha alone was loaded. This leads to the conclusion that alpha and IαI were not interacting on the surface of the probe.

3.2.6 Site directed mutagenesis of alpha

In order to facilitate some of the characterisation of the α protein a number of mutations were made within the α gene, either in terms of single point mutations or large additions. These mutations were carried out as described in either section 2.6.5 or 2.6.7 depending on what the desired outcome was.

The aim of these PCR mutagenesis experiments were threefold; 1. To create a protein which could be easily purified, 2. To create a protein which would stabilise multimer formation and 3. To create a fusion protein which would test the potential of α as a carrier protein.

The first of these goals was achieved by introducing a six residue histidine tag on the N terminus of the α protein. This histidine tag would therefore allow easier purification of the protein using a chromatography column packed with nickel resin, but would also allow for immobilisation of the protein. The immobilisation of the protein would therefore allow protein-protein interaction studies to be carried out using either a BLItz machine, as

previously described, or using nickel chelate resin. The insertion was carried out using the method outlined in section 2.6.5.

The second of these goals was to create series of mutants that would stabilise multimer formation, this wold then aid structural studies whilst also creating a molecule that was larger in solution. This increased size would possibly increase renal clearance times for any molecule attached to it which would have implications in pharmaceuticals. Due to the unique crystal structure of the α protein (**Figure 22**) formed from interlocked trimers of the α protein it was hypothesised that point mutations of residues 55 and 91 to cysteine residues would help to stabilise the multimer structure via disulphide bridging. Cysteine residues are not normally found within the α protein and so it was also hoped that these would provide specific points which could be labelled or used to link α to other proteins.

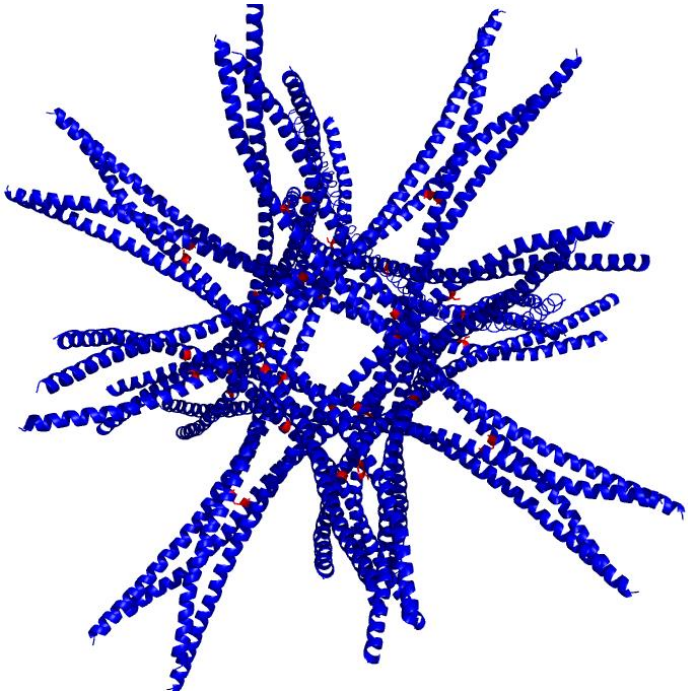

Figure 36. The unpublished crystal structure of α with residues 55 and 91 highlighted in red. As can be seen these residues, once mutated to cysteine residues, would be close enough to form disulphide bridges in a large multimer structure. This should help to stabilise the structure, making it more likely to be found under physiological conditions.

In order to create this construct pJONEX4 NMB α was mutated as described in section 2.6.5 in order to create two separate constructs, one containing a single cysteine point mutation at residue 55 and one containing a single cysteine point mutation at residue 91.

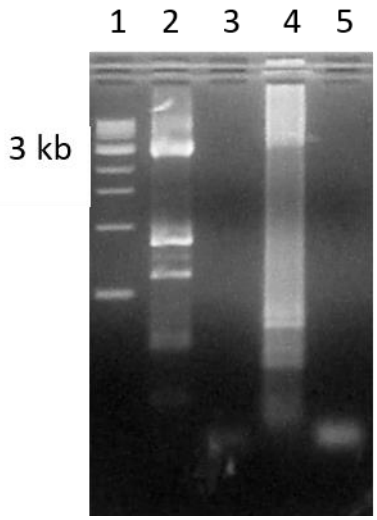

Figure 37. A 2% agarose gel on the products from the PCR mutagenesis reaction aiming to create a single cysteine point mutation within the α protein. Lane 1, New England Biolabs 1 kb ladder, lane 2 55C α positive PCR reaction, lane 3 55C α negative PCR reaction, lane 4 91C α positive PCR reaction, lane 5 91C α negative PCR reaction. The negative control reactions were set up as the positive reactions just without the addition of the template plasmid. The gel shows clear amplification at around 3.2 kb, this was the size of the product that was expected from this mutagenesis.

As can be seen in **Figure 37** the PCR reaction appeared to be successful and so the bands at around 3.2 kb were gel extracted, as described in section 2.6.5, and transformed into chemically competent M72 *E. coli* cells, as described in section 2.6.8. Following successful transformation bacteria were cultured in small scale and checked for expression, using the method outlined in section 2.1.6. A small culture of each mutant were also used to isolate the plasmid from the mutants, section 2.6.3, this was then sent for sequencing at the Core Genomics facility located in the University of Sheffield Medical School (http://genetics.group.shef.ac.uk)

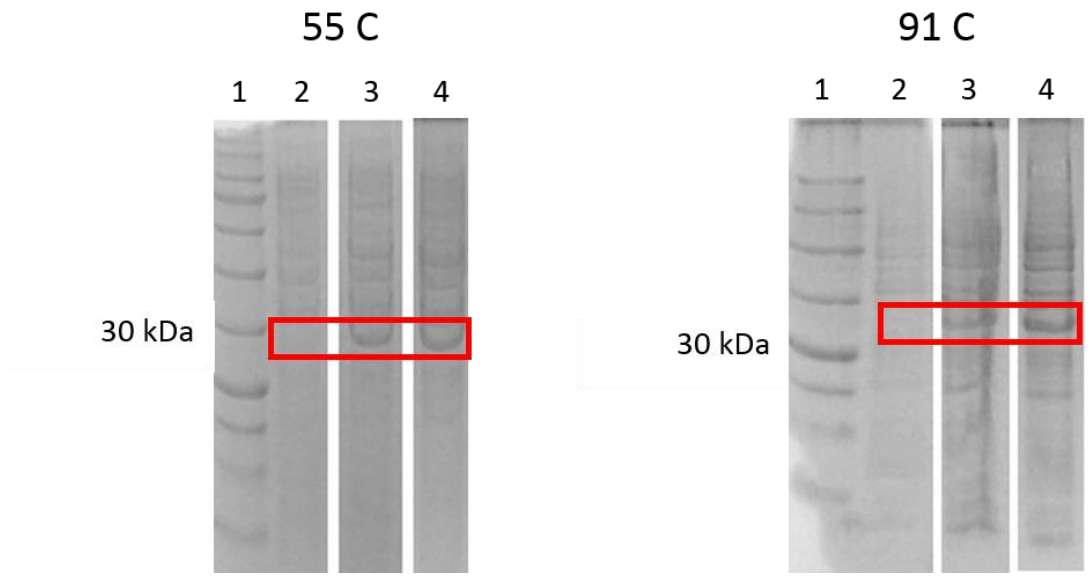

Figure 38. A 10% acrylamide SDS PAGE gel showing expression of the residue 55 (55 C) and the residue 91 (91 C) single cysteine α mutants. In both cases lane 1 is the BLUeye prestained protein ladder (Generon), lane 2 is the cell pellet of the uninduced bacteria, lane 3 is the cell pellet from bacteria that have been induced for 3 hours and lane 4 is the cell pellet from bacteria induced for 5 hours. What can clearly be seen is that both strains are strongly expressing a protein at around 30-35 kDa, the expected weight of α on an SDS PAGE gel.

As can be seen in **Figure 38** both strains appeared to be strongly expressing a protein of the correct weight. However, sequencing later revealed that both the mutation at residue 55 and 91 were unsuccessful (Appendix III).

3.2.7 Creation of an alpha-Gly1 fusion protein

In order to test the potential of α as a carrier protein a fusion of the α protein and the neisserial GLy1ORF1 protein were to be created. This fusion would fuse the N terminus of the Gly1 protein to the N terminus of the NMB α protein via a short 3 glycine and 1 serine linker and then the C terminus of Gly1 would then be fused to the C terminus of the NMB α protein via another short linker sequence. The aim was to create a large protein formed from two potential *neisserial* virulence factors which could be tested for immunogenicity, it was also hoped that this new fusion protein would be capable of multimer formation, similar to the α protein.

Figure 39. A monomer of the predicted structure of the α-Gly1 fusion protein (image created in PyMOL using structures provided by Prof JR Sayers and Prof PJ Artymiuk of the University of Sheffield, UK). The orange and red domains each correspond to half of a Gly-1 monomer attached to the ends of the α protein, shown in light blue.

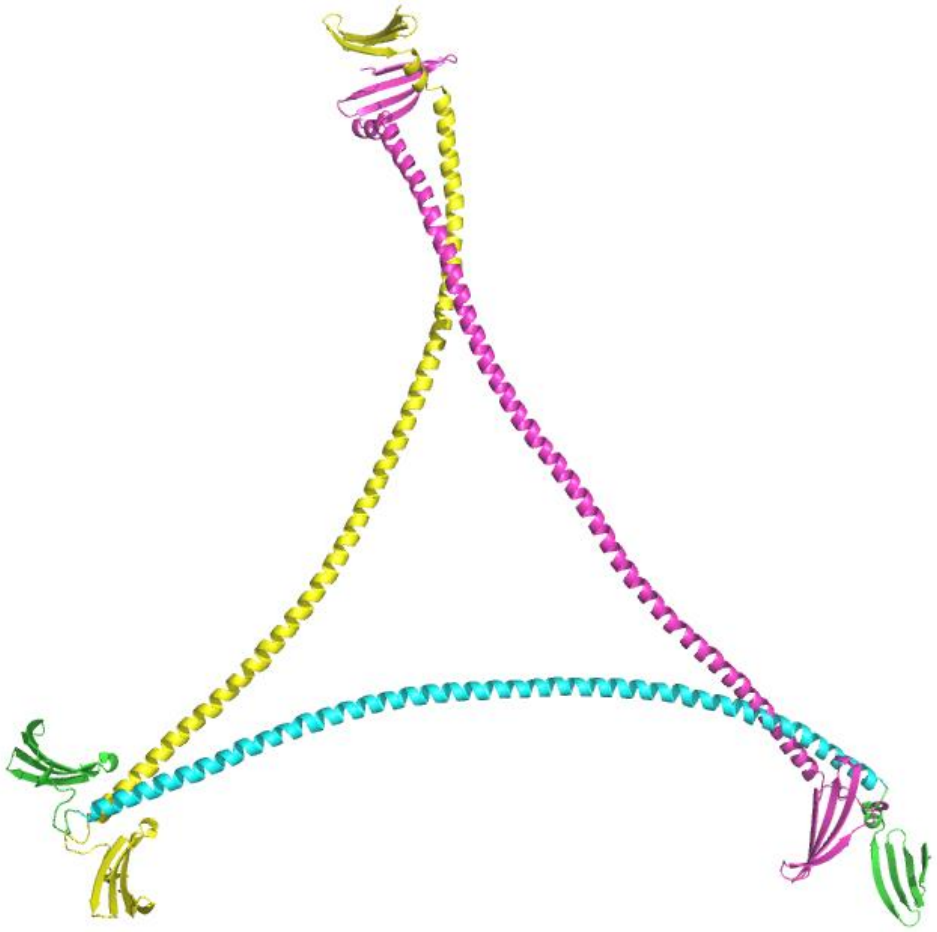

Figure 40. A prediction of the possible multimer formation by the α-Gly1 fusion protein. As in figure 39 the long alpha helices each have one half of the Gly-1 monomer attached at either end. This large structure should be readily formed due to αs propensity to form trimers, this would hopefully increase renal clearing time of the protein and its immunogenicity (image created in PyMOL using structures provided by Prof JR Sayers and Prof PJ Artymiuk of the University of Sheffield, UK). This formation of multimers should also bring the two halves of the Gly1 monomer, fused to the ends of the α protein, close together. It was hoped that the Gly1 domains could then interact and form a full Gly1 protein which would be more useful for antibody generation.

The fusion of these two proteins was to be achieved by introducing a XhoI restriction site in the centre of the Gly1ORF1 gene and also at the C terminus of the α gene, this would be complimented by a BtgI restriction site at the N terminus of the α gene and in the centre of the Gly1ORF1 gene. This would enable the Gly1 gene to be cut in two and then annealed to the end of the α protein, therefore creating the desired fusion protein.

Multiple rounds of PCR mutagenesis were carried out, as described in section 2.6.5, in the end the insertion of both restriction sites in the α gene were successful. This required creation of a mutant with the BtgI site inserted, this was then used as a template in the reaction to insert the XhoI site.

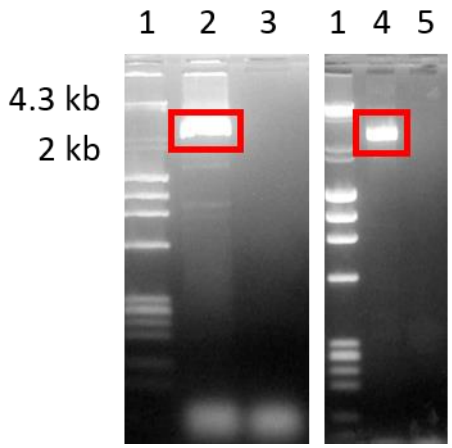

Figure 41. 2% agarose gels on the α BtgI and XhoI PCR products. Lane 1 Quick load broad range ladder (New England Biolabs), lane 2 α BtgI positive reaction, lane 3 α BtgI negative reaction, lane 4 α XhoI positive reaction and lane 5 α XhoI negative reaction. The negative reactions were set up as the positive reactions except the template plasmid was omitted.

The bands highlighted in **Figure 41** were gel extracted as described in section 2.6.5 and sent for sequencing at the Core Genomics facility located in the University of Sheffield Medical School (http://genetics.group.shef.ac.uk). This sequencing revealed that the α protein had the correct restriction sites inserted in the desired places. Unfortunately, multiple rounds of PCR were unable to insert either of the restriction sites into the Gly1ORF1 gene. This could possibly be rectified by redesigning the primers, however it was decided that chemical fusion of the α protein to a complete neisserial Gly1 would achieve the same result in a more time efficient manner.

3.3 Conclusions

The α protein has been widely acknowledged as a co-expressed protein conserved across a number of neisserial and other meningococcal bacteria with a role in targeting the IgA1P protein into host cells [111,116] . What has remained unclear until now is whether the alpha protein has any other functions within the pathogen and how it elicits any such responses. The work shown in this chapter has helped to expand the current state of the knowledge base regarding the α protein and its function whilst also raising more question requiring answers and further investigation.

The vector and *E. coli* strain chosen for this project were shown to express strongly, leading to an easy to purify product. The pJONEX4 vector utilises a heat shock promoter which is activated above 42°C, this plasmid was shown to give significant protein yields from a 5 L fermenter culture allowing rapid purification and experimentation to be carried out.

Pulldown interaction shown in this chapter demonstrates that the α protein is capable of interacting with a number of human serum based factors between 50-75 kDa and 100-150 kDa. The most likely candidates for these interactions are human serum albumin at around 65 kDa, which has been shown to bind a wide variety of proteins and IαI at around 125 kDa, despite the fact IαI is often found at around 220 kDa the active trypsin inhibiting form has shown to be around 125 kDa [118]. These proteins would explain the result shown in the latter half of section 3.2.4.1 whilst in the first half of section 3.2.4.1 alpha interacted with a protein of around 30kDa, this is most likely to correspond to bikunin based upon previous work carried out on α. Bikunin proteins have been found to contain 2 Kunitz type inhibitor domains [119], one of which has been shown to be an inhibitor of other serine proteases such as trypsin and chymotrypsin. Free bikunin tends to be only found at low circulating levels under normal conditions due to its short half-life [120], instead pre-alpha inhibitor is the dominant protein found in the bloodstream containing a bikunin domain. This pre-alpha inhibitor is a 220kDa protein which is cleaved into a functional 125 kDa protein known as IαI, which has been shown to be a serine protease inhibitor but which may also have other roles in the host [119]. It is therefore possible that the interaction between α and bikunin and IαI plays a role in preventing the proteins from inhibiting IgA1P and allowing IgA1P to cleave targets within the host.

Flow cytometry analysis of alphas interaction with blood cells revealed an unexpected interaction between red blood cells and α. Although the cells were not labelled to a greater degree than when incubated with the negative control protein, they were labelled with greater intensity when incubated with the labelled α. This hints that the interaction between α and red blood cells could be more specific than the interaction between red blood cells and BSA, what remains unclear is whether this is just an artefact of the high concentration of αused. The interaction between α and the red blood cells is particularly unexpected as the α protein contains multiple NLS which target α into cells nuclei [116], which are of course lacking in red blood cells and platelets. Previous work in the Sayers lab (Helena Parsons, MSc thesis, University of Sheffield, UK) showed a possible interaction between α and a group of cells thought to be neutrophils. This interaction was confirmed at concentrations of 20 μg/ml and above as was expected from the previous work. The potential interaction with neutrophils is particularly interesting as it has been shown that bikunin readily locates to the membrane of neutrophils [121], this would provide the alpha protein with a point to attach to on the neutrophillic membranes, thereby explaining the interaction. This is because previous work carried out by Helena Parsons indicated that the α protein was capable of interacting with

bikunin and inter-alpha-inhibitor (IαI), via the incorporated bikunin domain most likely. This bikunin interaction may also allow the targeting of IgA1P to neutrophils whereby it can cleave target proteins such as TNFRII [100]. This may lead to an increase in cytokine production leading to the increase in pro-inflammatory cytokines such as IL-6, IL-8 and TNFα that is classically associated with meningococcal disease [84]. The IgA1P targeted to neutrophils would also have access to LAMP proteins found in neutrophils and cleavage of these proteins may help to protect the bacteria from phagocytic killing in both the bloodstream and at the mucosal layer [97,98]. As a result, the role of the α protein may be that of a targeting protein, directing IgA1P with an α protein still attached to host cells where it can have an effect.

In order to fully characterise and confirm the interaction between α and IαI/bikunin a number of methods were used, including pulldown and BLItz assays. Under both of these conditions α failed to interact with purified pig IαI. The reasons for this could be three fold, firstly the purified IαI was around 220 kDa, in humans the form with this size is the inactive pre-alpha inhibitor form and not the 125 kDa active form. This might explain why α was unable to target it, as *in vivo* targeting the inactive form of the inhibitor would serve no function whilst being a burden on the pathogen. Secondly, despite the pig IαI protein being suspected to be highly similar to the equivalent protein in humans it is possible that α is unable to target it. *Neisseria meningitidis* is an obligate human pathogen and as a result its inability to target homologous proteins from other species should not be discounted. This issue has been raised in the past and it has been shown multiple times that a neisserial animal model is very difficult to achieve due to its specificity for a human host. Finally, in both the BLItz and the pulldown assay the α protein was biotinylated and immobilised to a streptavidin matrix. This biotinylation and immobilisation could cause problems in a number of ways. Chiefly amongst those problems could be the biotin groups masking interaction sites between the two proteins, this cannot be ruled out and in the future another form of tagging and immobilisation could be used, possibly N terminal histidine tagging which would allow immobilisation in a very specific place. One of the major issues that arose with the Biotinylation of the α protein is the lack of fine control over how many biotin groups have labelled each protein. This may result in a protein with multiple labels which effectively binds flush to the surface of the probe, therefore preventing interaction between the two test proteins. Once more this could be rectified using specific N terminal or C terminal labelling or a labelling method which only reacts with a single point on the protein, such as a cysteine residue. A further problem with the immobilisation to the matrix is that being immobilised to a matrix may cause steric hindrance, this would prevent the two proteins from interacting

fully resulting in a failure to show an affinity between the two proteins. Most of these problems could be solved in the future with the use of the human IαI protein and a different method of immobilisation or capture of the proteins.

The final part of this study was to mutate the α protein in two specific ways, firstly the introduction of a cysteine group and secondly the fusion of α to Gly1ORF1. The first of these two mutations would help to stabilise multimer formation, which could have large implications for the α proteins potential as a carrier whilst also facilitating structural study. The second of these mutations was purely to be used to test the potential of α as a carrier protein for neisserial vaccine development. Unfortunately, in both cases it appears that the PCR mutagenesis failed, this is most likely down to issues with primer design which could be rectified in future attempts or indeed fusion of the proteins could be achieved chemically rather than through PCR.

Overall the work shown in this chapter has helped to build the knowledge base relating to the function of the α protein *in vivo*. Multiple possible roles have been suggested based upon this work and the work of others including; targeting IgA1P to host cells via the NLS [111,116], inhibition of serum based serine protease inhibitors and a possible interaction with red blood cells. During this study a reliable expression and purification method has been demonstrated allowing further work to be carried out which can address some of the issue discussed above and identify the role of α in host invasion and survival.

Chapter 4- Characterisation of the structure of the neisserial alpha protein and its potential as a carrier protein

4. Characterisation of the structure of the neisserial alpha protein and its potential as a carrier protein

4.1 Introduction

As was explained in section 1.7 the α protein has so far been studied very little and as a result there is little evidence for its function *in vivo* or its structure. The name alpha and secondary and tertiary structure prediction have shown that the α protein is comprised of 1 α helix of around 200-400 amino acids long. However, very little experimental characterisation has been carried out. At the University of Sheffield Prof Artymiuk's group crystallised the α protein (unpublished) at around 100 mg/mL, this crystal structure (**Figure 22**) does indeed show that α is thin alpha helical and forms multimers of up to 24 monomers. However this crystal structure was formed at very high concentration under non-physiological conditions, as a result this chapter aims to investigate the structure of α at lower concentrations, as well as in solution. This should help to define the structure of the α protein under more physiological conditions.

This chapter also set out to investigate the potential of α as a carrier protein for vaccine formulation. The little characterisation of α that has been carried out has shown that the α protein contains a number of T cell helper epitopes [25,102] . These regions are short 7-10 amino acids sequences which are capable of CD4+ helper T cells which causes maturation of B cells into memory B cells [25] . This activation results in a long lasting immune system memory of the protein, it is thought therefore this characteristic of alpha could be exploited in vaccine development in a similar way to other carrier proteins. Carrier proteins are normally highly immunogenic proteins such as tetanus toxoid which boost the immune systems response to proteins that they are conjugated to [2,23] . This has the effect of increasing the stimulation of the immune system, leading to longer lasting antibody production[23]. It is thought that α could function in a similar way when conjugated to another protein by boosting the immunogenicity of that protein and causing production of memory B cells, leading to a very long lasting immunity.

In order to answer a number of these questions this chapter set out with a number of aims;

1. To characterise the structure of α at lower concentrations and in solution using a variety of techniques.
2. To assess the potential of α as a carrier protein for vaccine development using a number of *in vitro* models.

4.2 Results and discussion

4.2.1 Characterisation of the neisserial Alpha proteins structure

The structure of the α protein is not well characterised, with only 1 unpublished crystal structure being available (supplied by Prof PJ Artymiuk of the University of Sheffield UK). This structure shows a multimer formed from 24 α monomers which form a large 3 dimensional star made of interlocking trimers. In order to further study this structure a number of experiments were carried out. These were designed with the aim of testing the capability of α to form multimers in solution as well as confirming the structure of α both in solution and *in crystallo*.

4.2.1.1 Alpha multimer formation

A multimer formation was checked using both FITC labelled alpha protein, section 2.4.2, which was then run on an SDS PAGE gel, 2.1.9 and also a western blot, section 2.4.4.1, with a rabbit anti-α primary antibody and an anti-rabbit secondary antibody.

The first method involved running FITC labelled proteins on an SDS PAGE gel and then imaging it using both standard white light and a secondary imaging technique which imaged the fluorescence, by imaging the SDS PAGE gel with a long exposure time in the dark.

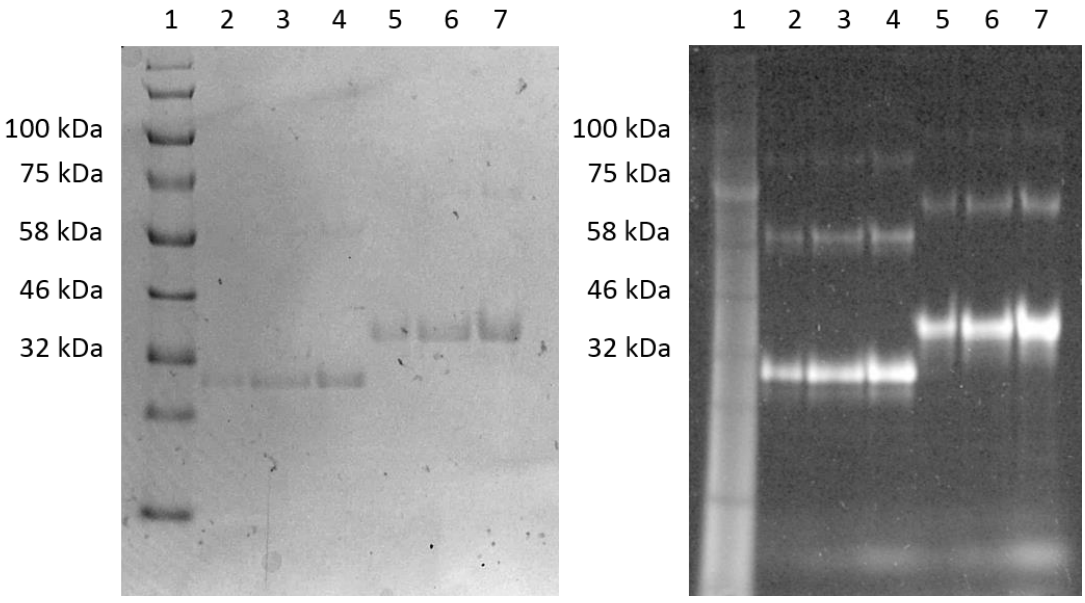

Figure 42. Crosslinking of labelled α and cys-ɣ-α in solution. Analysis of proteins on 10% SDS PAGE gels: Left panel Coomassie stained, right panel fluorescence visualisation. Lane 1 Blue prestained broad range ladder (New England Biolabs), lanes 2-4 FITC labelled α in increasing concentrations, lanes 5-7 FITC labelled cys-ɣ-α in increasing concentrations.

The right panel of **Figure 42** shows that α and cys-ɣ-α are capable of forming multimers in solution. This is due to the fact that α and cys-ɣ-α proteins usually appear at between 30-35

kDa on a SDS-PAGE gel, therefore it can be assumed that the bands at 58-~65 kDa equate to α/cys-ɣ-α dimers whilst the bands at between 80-100 kDa equates to trimers of the proteins.

The second trial utilised western blotting in order to confirm the multimer formation as this would rule out non-specific interactions between the FITC labels as the cause of multimer formation. This trial also served as a test in order to identify which in house rabbit anti-α antibodies, which had been raised previously in the Sayers lab against α that had been verified by mass spectrometry, had the highest affinity for α and least non-specific labelling. For this experiment 10 µL of 10 mg/mL α protein was loaded onto a 10% SDS-PAGE gel and then treated as described in section 2.4.4.1.

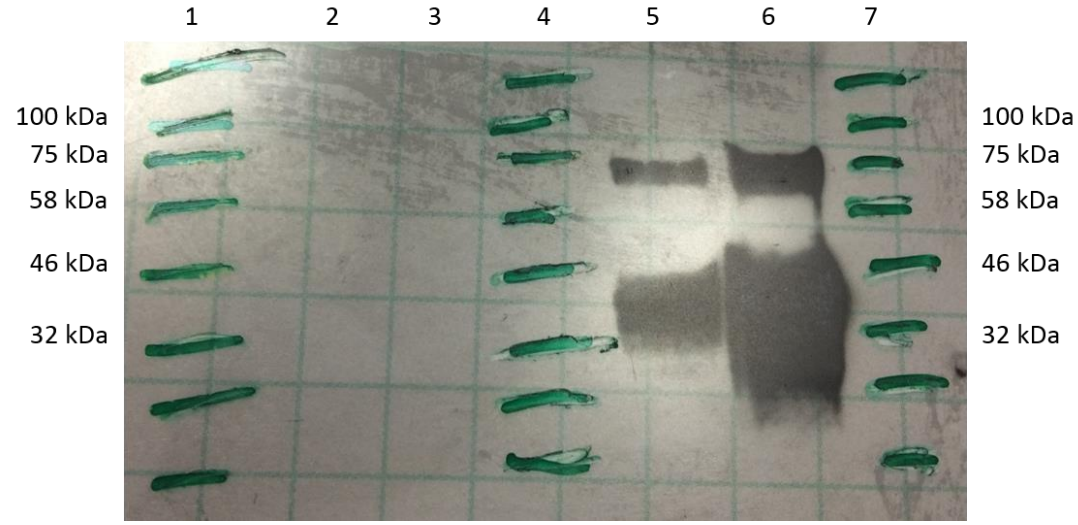

Figure 43. Western blot showing α multimer formation. Lanes 1,4 and 7 Blue prestained broad range ladder (New England Biolabs), lanes 2, 3, 5 and 6 all contained the α protein but were probed with different primary antibodies.

The two in house rabbit anti-α antibodies were able to detect α monomer efficiently and also provided further evidence of dimer formation in solution. This confirms the previous observation that alpha is capable of forming multimers in solution, at concentrations of around 10 mg/ml. What is absent in this second assay, compared to **Figure 43**, is the presence of trimers. This could indicate that they do not form readily or, more likely, could indicate that the larger trimers have not efficiently transferred to the membrane.

4.2.1.2 Circular dichroism analysis of the alpha secondary structure

In order to confirm the crystal structure observation that the α protein was purely alpha helical, circular dichroism was performed as described in section 2.3.4. Alongside the circular dichroism of α, a sample of cys-ɣ-α was also analysed. This would not only give further insight

into the structure of α but may also determine what structure, if any the short ɣ peptide forms in solution.

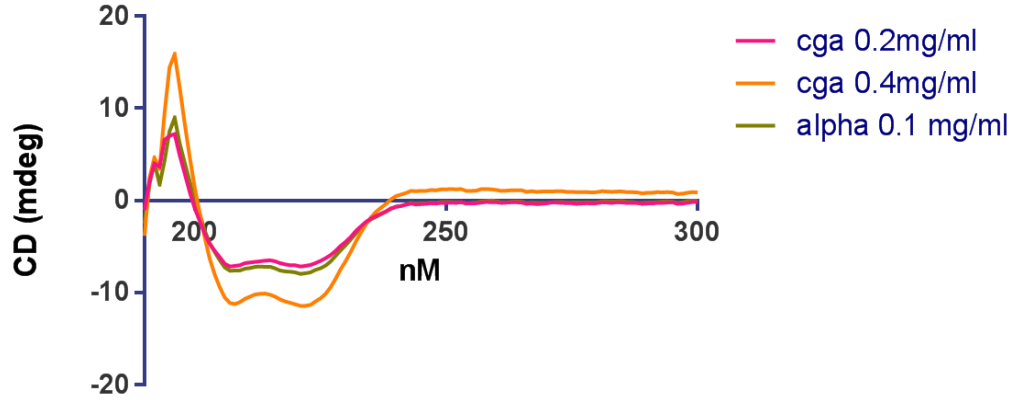

Figure 44. CD spectra of the α and cys-ɣ-α proteins at a variety of different concentrations. Both α and cys-ɣ-α at all concentrations produce a spectra which very closely resembles the reference spectrum given by purely alpha helical proteins.

The figure above shows the spectrum produced by performing circular dichroism on both α and cys-ɣ-α, the spectra clearly shows a form very similar to that produced by a pure alpha reference protein[123]. Due to time constraints the precise percentage of the structure that was α helical was not calculated, although its similarity to the alpha reference spectrum hinted that it would be around 100%. What should be noted however is it requires a much higher concentration of cys-ɣ-α in order to produce a spectrum with similar intensity to that of 0.1 mg/ml α. Also of note is the fact that the α and cys-ɣ-α do not overlap perfectly, this indicates that there may be some subtle structural difference caused by the addition of the ɣ peptide.

4.2.1.3 Crystallography trials on the alpha protein

Crystallisation studies were carried out on both c-ɣ-α and α in order to determine the structures of the proteins which could then be used to validate any binding partners or mechanisms determined at a later date. In order to do this the crystallography screens described in section 2.3.2 were set up using an automated system which placed 0.2 µl of either 5.48 mg/ml α or 9.24 mg/ml c-ɣ-α in to the wells. The crystallography screens were then left at 17°C for two weeks allowing crystal formation to occur. However, for both proteins crystals only appeared in wells containing calcium or potassium salts. This gave an indication that the crystals were most likely composed of calcium phosphate or potassium phosphate, this was later confirmed as the case using methylene blue dye.

4.2.2 Investigation into the potential of the neisserial alpha protein as a carrier protein

Previous studies have shown that the α proteins contain a number of T cell helper epitopes which may allow it to acts as a carrier protein in vaccine development. The function of these carrier proteins is to boost the immunogenicity of vaccine antigens in the formulation whilst also giving rise to long lasting protective immunity. The α protein seems uniquely suited to this task, section 1.7, and so as a result a number of experiments were designed in order to test the carrier protein potential of α *in vivo*.

4.2.2.1 Alpha carrier potential analysis in a female BALB/C mouse model

In order to determine the potential of α as a carrier protein it was injected into female 6 week old BALB/C mice as described in section 2.7.1 and then antibody tires were analysed by ELISA as described in section 2.7.2. The ELISA analysis used for this experiment quantified the specific anti-α or anti-ɣ IgG found in each serum sample collected from the mice. This highlighted whether the α protein could boost the immunogenicity of ɣ, a very weakly immunogenic peptide, when injected as either a fusion of ɣ and α or a mixture of ɣ and α. In order to decide if α was boosting the immunogenicity of ɣ the anti-ɣ antibody titres of mice injected with a mixture or fusion of α and ɣ were compared to the antibody tires of those injected with α or ɣ alone or mice injected with 1xPBS only. This showed any difference in immune response achieved by injecting α alongside ɣ and determined the potential of α as a carrier protein. The fusion of α and ɣ was particularly important as it mirrors how carrier proteins are used in vaccine formulation, as often poorly immunogenic antigens are directly conjugated to a carrier protein, allowing them to boost the immune response to the target antigen.

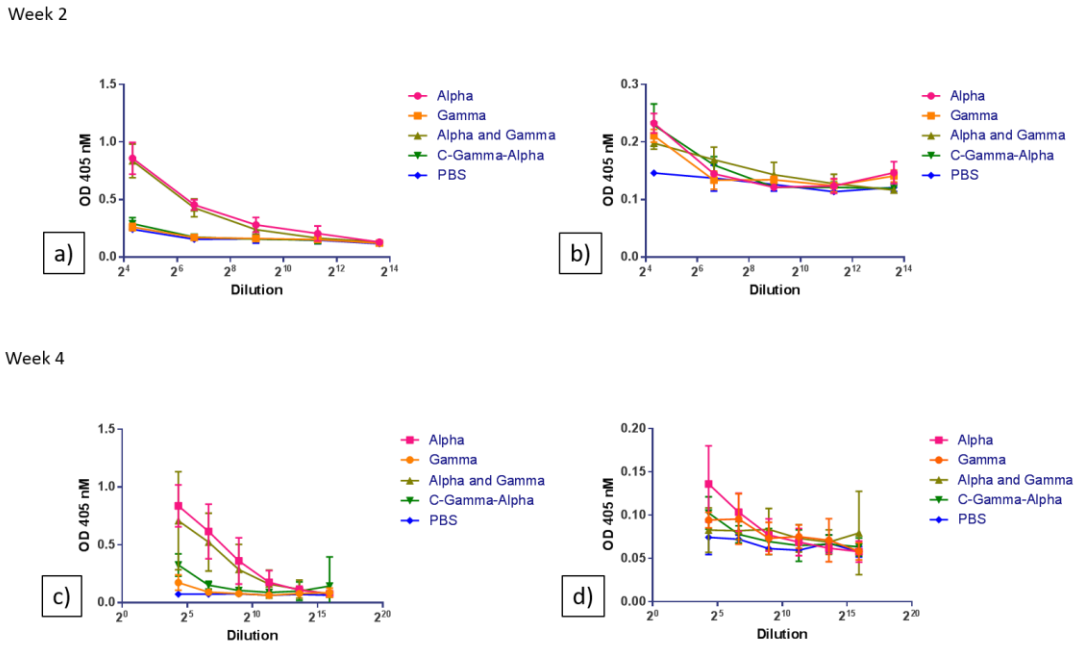

Figure 45. Antibody titres derived from ELISA data on mouse serum 2 and 4 weeks post injection. Graphs a) and b) are antibody titres against α (a) and ɣ (b) two weeks post inoculation with the proteins. Graphs c) and d) are antibody titres against α (c) and ɣ (d) four weeks post inoculation with the proteins.

Figure 45 Shows an interesting trend in both antibody tire in the mice against the α protein as well as against the ɣ protein. At both two and four weeks post injection it can clearly be seen that both α and a mixture of α and ɣ produce a very high antibody titre against alpha. This increased antibody titre is detectable above the baseline control injection of PBS up to a 2500 fold dilution of serum. This proves that the α protein is highly immunogenic *in vivo*, as predicted by its multiple T cell helper epitopes. With regards to the ɣ protein it appears that at week 2 a mixture of α and ɣ are able to provide a noticeable increase in antibody titre above the baseline up to a 500 fold dilution of serum. Unfortunately, by week 4 post immunisation this trend appears to be less clear cut, the mixture of α and ɣ do give an antibody titre that on average is higher than the baseline up to a 500 fold dilution of serum.

Post analysis of this data an LPS assay was carried out upon the samples used in order to rule out any immunogenic effects of LPS contamination, as described in section 2.7.5, this quantified any LPS if any was present in the samples. It was highly important to have as little LPS contamination as possible as LPS is known to be highly immunogenic and therefore could skew any data obtained from the study. The results of the LPS assay confirmed that there was no endotoxin present in the samples used, therefore ruling out the possibility that the immune response was as a result of endotoxin contamination.

4.2.2.2 Crosslinking of Alpha to the Gly1 homologues or FEN D183K

In order to more fully test the potential of α as a carrier protein a new animal model was designed. This model would use female New Zealand white rabbits which would be injected with a conjugate protein made up of the α protein covalently attached to another potential virulence factor from *Mannheimia haemolytica* or *Trypanosoma brucei*, section 2.7.3-6.

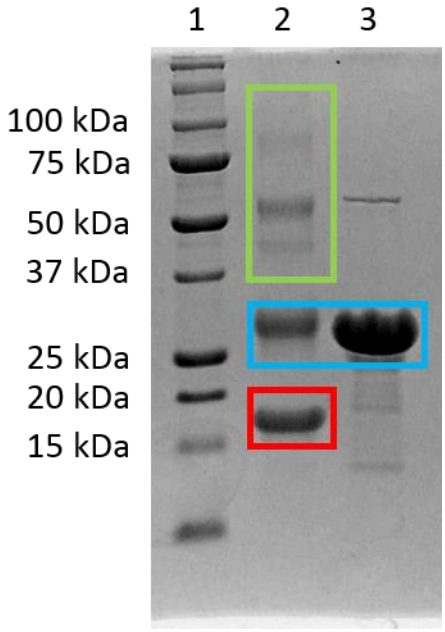

Figure 46. A 10% acrylamide SDS PAGE of the result of crosslinking α to MH0579 form *Mannheimia haemolytica.* Lane 1 Precision plus prestained all blue protein ladder (Biorad), lane 2 α-MH0579 crosslink, lane 3 SMCC labelled α. The red box indicates MH0579 that has not crosslinked to α and the blue box corresponds to free α, the green box highlights fusion products created in the reaction.

It can be seen in **Figure 46** that the crosslinking reaction, carried out as described in section 2.7.3, was partially successful. Although not all of the SMCC labelled α reacted with the SATP labelled MH0579 the reaction yielded sufficient product to purify and isolate for use in rabbit inoculation, as described in section 2.7.6. This reaction was replicated for each protein conjugate desired and was successful in all cases. Success was determined by measuring the shift in size of the conjugated protein compared to the original size of the two constituent parts. In order to purify the conjugated proteins size exclusion chromatography was used, section 2.7.4. This separates proteins based upon molecular weight, with larger proteins being eluted in earlier fractions. Due to the large size difference between the conjugated proteins and the constituent proteins this was the most efficient way to purify the desired conjugate.

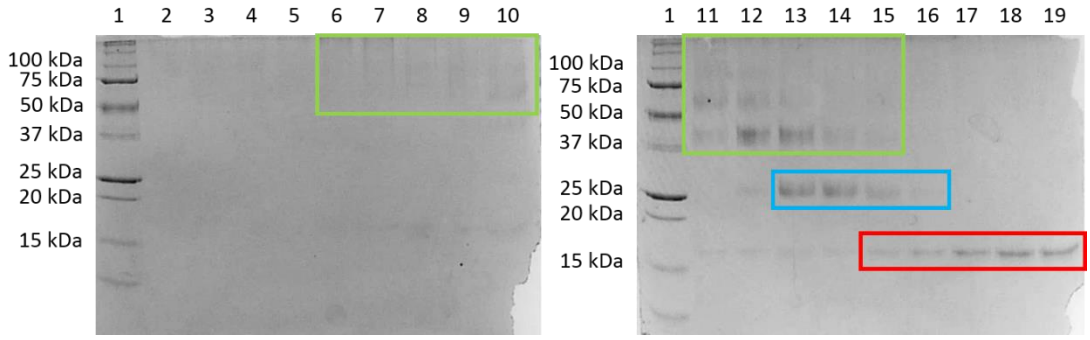

Figure 47. A Coomassie stained 13% acrylamide SDS PAGE showing the elutions from a size exclusion chromatography column packed with Sephacryl S-200 HR. Lane1 Precision plus prestained all blue protein ladder (Biorad), Lanes 2-19 elutions from size exclusion column. The green box highlights the α-MH1205 conjugates, the blue box highlights the unconjugated α and the red box indicates unconjugated Gly1 MH1205. Each elution corresponds to a 0.5 mL fraction eluted at 0.5 mL/min, as the fraction number increases the mass of the protein collected should decrease with each fraction containing proteins within a 40-50 kDa range. This range could be further decreased by eluting fractions from the column at a slower rate, however this broader range gave sufficient separation of high weight conjugates from lower molecular weight contaminants.

Figure 47 shows that using size exclusion chromatography alone pure conjugate protein was obtained that could be used for inoculation. These proteins were then concentrated and mixed into the designated inoculation mixes as described in 2.7.6 and sent to Generon, a contract research organisation, for injection into female New Zealand white rabbits as detailed in section 2.7.6.

4.2.2.3 Purification of antibodies from the rabbit serum

In order to isolate each antibody produced from the conjugate proteins an antibody purification step was carried out, section 2.7.7. This needed to be done as each rabbit was inoculated with a mixture of 1 alpha conjugate protein and 1 free protein, as listed in section 2.7.6. These free proteins (MH0579, MHPara, MH1205 and tbFEND183K) were various proteins used by the lab group, in the case of the MH protein they were all Homologues of the Gly1 protein found in *Mannheimia haemolytica*, whilst tbFEND183K was a mutated flap endonuclease from *Trypanosoma brucei*. The purification process separated the various antibodies form the individual sera received, therefore allowing their affinity to be tested.

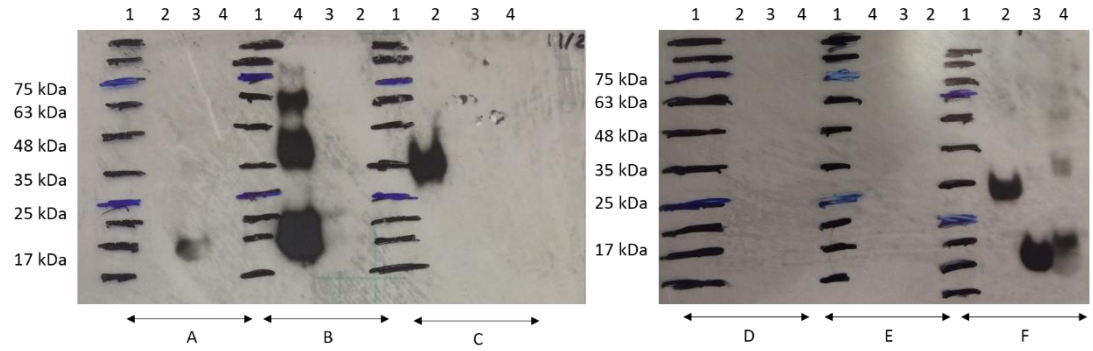

Figure 48. Western blot using a variety of purified antibodies. Lane 1 BLUeye prestained protein ladder (Geneflow), lane 2 neisserial α protein, lane 3 Gly1 paralogue MHPara from *Mannheimia haemolytica,* lane 3 Gly1 homologue MH0579 from *Mannheimia haemolytica*. Each of the sections utilised a different primary antibody; section A probed with anti-MHPara, B probed with anti-MH0579, C probed with anti-α, D probed with anti-α-MHPara, E probed with anti-α-MH0579 and F was probed with unpurified serum.

As can be seen in **Figure 48** the western blot showed that the whole serum contained antibodies capable of labelling all of the expected proteins and that pure anti-MHPara anti-MH0579 and anti-α were obtained. However, probes D and E revealed that there were no proteins present capable of labelling both the α and the Gly1 homologue or paralogue. This was an expected outcome as any antibody able to do that would have to react with both proteins and the short chemical linker, this control was carried out to prove that no such antibody would be formed.

4.3 Conclusions

The research contained within this chapter has helped to expand the knowledge of both the structural and functional role of the neisserial α protein, whilst also assessing its potential as a vaccine tool. This study takes what was previously known about α and expands it whilst providing proof that the α protein may have some use as a carrier protein in neisserial and possibly other vaccines.

As has been discussed very little published work has studied the structure of α, other than to state that it is α helical, and the unpublished work used concentrations and conditions that in no way mirrored those found physiologically. As a result this work aimed to define the structure of α under more physiological conditions and in solution. The results of studying α in solution showed that as seen in the crystal structure (**Figure 22**) α is capable of forming multimers, most often dimers or trimers. This mirrors what can be seen in the crystal structure, which is made up of 8 interlocking trimers. This confirms the ability of α to multimerise, which may indicate that its role *in vivo* relies someway on multimerisation. It

also hints that an α-protein conjugate may be able to multimerise, this would make it a highly useful carrier protein as it would greatly increase the immunogenicity of the attached protein whilst simultaneously increasing renal clearing time.

These two factors combined will be worth investigating further in order to assess its potential as a carrier protein. Further structural work using circular dichroism also confirmed previously published data that α was a purely α helical structure. However, this research built upon this knowledge by also carrying out circular dichroism on a α-ɣ conjugate. This work has helped to elucidate the structure, if any, of the short ɣ peptide that is also co-secreted with IgA1P and α. It appears that the ɣ peptide has a slight structure as it caused a shift in the spectrum away from a α helical baseline. However, it is most likely that this is a random coil or globular domain. Unfortunately, crystallisation of α at a lower concentration was unsuccessful due to salts being present in the buffer, in the future this could be repeated with α in a more crystallography friendly buffer. The aim of this would be to discern whether α would still form 24-mers or whether at a lower concentration its crystal structure might more closely mimic that seen in solution and on western blots.

Previous studies carried out on α have highlighted a number of T cell helper epitopes within the proteins, these function by activating CD4+ T helper cells causing maturation of B cells into memory B cells [25,102] . In this research it was hypothesised that these epitopes could be utilised by using α as a carrier protein, similar to tetanus toxoid[2,23]. These carrier proteins increase the immunogenicity of a protein they are conjugated to and help to improve the immune response against proteins contained within the vaccine. It was also hypothesised that the ability of α to form multimers, which was confirmed earlier, would increase the immunogenicity of target proteins further by creating large complexes with long renal clearing times.

The BALB/C mouse model used in this study has provided interesting data that appears to confirm this hypothesis, certainly at 2 weeks post inoculation but also possibly at 4 weeks post inoculation. What can be seen in **Figure 45** is that at week 2 a mixture of the α and ɣ proteins leads to an increased antibody titre against ɣ than when either are injected into the mouse alone. This seems to suggest that the presence of α is indeed boosting the immune response against ɣ leading to a higher detectable antibody titre. What should be noted however is that the conjugate of α and ɣ, cys-ɣ-α, did not have this effect, or at least the effect was less noticeable than with the mixture. This was an unexpected result as most carrier proteins must be directly conjugated to the protein in order to have an effect, what

should also be noted is that the conjugated protein failed to elicit a response to the α protein either, which is normally highly immunogenic. This would suggest that the conjugated protein was possibly not purified or injected properly, in order to confirm this theory the study would have to be repeated with more stringent purification and injection conditions. At 4 weeks post inoculation these trends were much less defined, still the conjugate failed to elicit a response to α, whilst both the mixture of α and ɣ and α alone did. What is unusual is that the anti-ɣ antibody titres seem to be highly variable and do not mirror that seen at two weeks post injection. Still there is some indication that the mixture of αand ɣ causes an increased immune response to α, but the variability in the data and the fact that the α alone causes antibody generation against ɣ means that this speculative trend should probably be disregarded. The reasons for the large variation in the samples at week 4 are still unclear and could be multi-factorial, for instance the mice might have stopped responding to the proteins or the injections may have been carried out improperly or samples could have been mixed up during blood sampling. As a result of this the data from week 4 should likely be discarded and the experiment repeated, however the data collected from week 2 seems to support the hypothesis that α could be used as a carrier protein in vaccine development. The antibodies gained from the New Zealand white rabbit model, may confirm these findings, however due to a lack of time the antibody titres were not calculated. This could be rectified quickly by carrying out ELISAs on the serum samples obtained and may help to support the case for α as a carrier protein.

Overall the research contained in this chapter has gone some way to building upon the previous knowledge of the structure of α by confirming that α is capable of forming multimers in solution and that it is a highly α helical protein with very high immunogenicity that could be exploited in the future. This chapter also provides the first glimpse at a possible structure for the ɣ peptide, hinting that it is most likely globular or made up of a short random coil.

Chapter 5- neisserial IgA1P mutation and structural characterisation

5. Neisserial IgA1P mutation and structural characterisation

5.1 Introduction

The IgA1 protease is a protease found in a number of meningococci which has been shown to be capable of cleaving a number of host proteins involved in the immune response against invasive pathogens [85,98,100]. Previous study of IgA1P has found that it is upregulated heavily in invasive isolates when compared to commensal strains and so it is thought to be a potential virulence factor [34,35]. Despite being a founding member of the type Va autotransporter protein family very little structural characterisation has been carried out on the neisserial IgA1P, with all of the structural data available to date having been derived from experimentation on the *Haemophilus influenzae* IgA1P [75]. As a result, the core aim of this section of research was to create a number of neisserial IgA1P mutants which were easy to purify and express and which stayed stable over a long period of time post purification.

Throughout the course of this research a number of mutant IgA1P proteins were produced with a number of aims in mind. Firstly, less active or inactive mutants were created as under normal conditions the IgA1P protein has been found to readily cleave itself, making structural work difficult to carry out. Therefore, in order to gain useful structural data on the neisserial IgA1P it was necessary to slow or stop the activity of the protease, this was achieved by mutating the active site serine at residue 288 to either a cysteine or an alanine. The effect of the cysteine mutation was to slow cleavage down by around 100-1000 fold, whilst the alanine mutation would effectively create a dead mutant incapable of cleavage. Secondly more easily purifiable IgA1P was desired, this was achieved via two separate mutations. In some cases a 6 histidine tag was added to the end of the protease domain, before the ɣ peptide, this would allow easy purification using nickel coated affinity resin. In conjunction with this some mutants had the β core domain removed, the effect of this was to prevent the passage of the protease through the outer membrane allowing it to be purified from the periplasm. These periplasm preparations contain much fewer proteins than full cell lysis preparations, therefore making protein purification simpler.

These mutant strains would then enable a variety of structural studies to be carried out on the protein in order to get an accurate neisserial IgA1P structure, rather than the predicted structures in use currently.

The aims of this section were 3 fold;

1. To create a number of IgA1P mutants with increased stability which would be easier to purify.
2. To express and purify these mutant proteases
3. To begin structural characterisation of the mutant IgA1 proteases.

The hypothesis of this chapter was that the cysteine and alanine mutant IgA1 protease would show decreased cleavage and increased stability, whilst the histidine tagged or Δβ core mutants would be easier to purify. Also that the neisserial IgA1P would have a high structural similarity to the IgA1P from *Haemophilus influenza* but would lack a unique loop D insertion.

5.2 Results and discussion

5.2.1 Expression of the neisserial IgA1P protein

The wildtype neisserial IgA1P was previously cloned into a pJONEX4 plasmid with a heatshock promoter, this was then transformed into M72 *E. coli* cells, as described in section 2.6.8. Cells were cultured as in 2.1.6 and checked for protein expression.

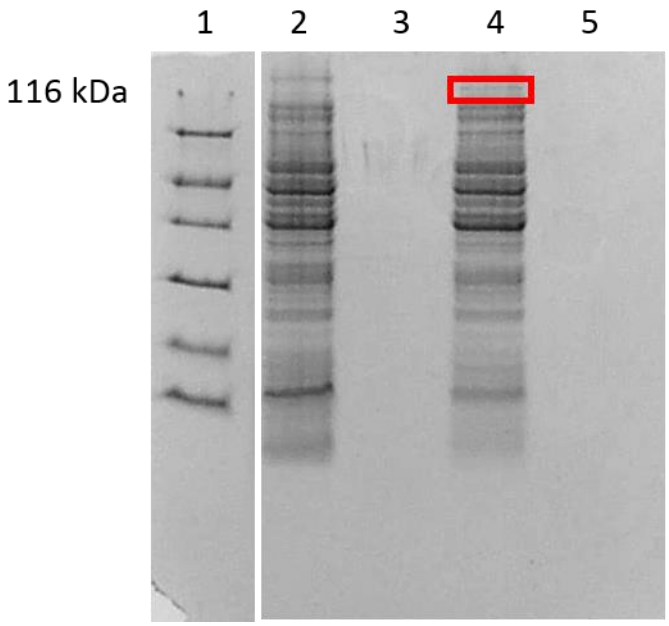

Figure 49. A 12% acrylamide SDS PAGE showing the expression of the wildtype neisserial IgA1P. Lane 1 Pierce unstained protein MW marker (ThermoFisher), lane 2 uninduced IgA1P WT pellet, lane 3 uninduced IgA1P WT supernatant, lane 4 induced IgA1P WT pellet, lane 5 induced IgA1P WT supernatant. A band can be seen to appear in the post induction cell pellet at around 110 kDa, this is around the expected size of IgA1P and so the protein was determined to be expressing well. Both lane 3 and 5 are blank as the supernatant collected from the cell culture was very dilute and few proteins are expressed extracellularly in this strain of *E. coli* as a result any proteins in the supernatant were likely below the detection threshold for coomassie stain on an SDS-PAGE gel.

As can be seen in **Figure 49** the wildtype neisserial IgA1P was found to express when induced for 16 hours at 42°C. Although the expression was not particularly strong it was deemed sufficient for purification and mutation. However due to time constraints the band was never formally identified using mass spectrometry or western blotting. The protein was however verified to a reasonable degree using its molecular weight along with the fact that this band appears only post-induction in these M72 cells containing a heat inducible IgA1P containing plasmid..

5.2.2 Purification of the neisserial IgA1P protein

The neisserial IgA1P was either purified using size exclusion chromatography or if the protein contained a histidine tag nickel coated resin was used, sections 2.2.3 and 2.7.4. This resulted in pure protein being obtained, however the yields were generally very low due to the low expression of IgA1P when compared to the α protein.

5.2.3 PCR site directed mutagenesis of the neisserial IgA1P gene in the pJONEX4 plasmid

PCR mutagenesis of IgA1P was carried out using the method outlined in section 2.6.5. This resulted in the production of a number of IgA1P mutants;

Wildtype- full length and Δβ core

Alanine mutant- full length, Δβ core and Δβ core with 6 histidine tag

Cysteine mutant- Δβ core and with 6 histidine tag

Both the alanine and the cysteine point mutations cause the active site serine to lose an oxygen molecule which is a key nucleophile in the catalysis of the cleavage reaction. In the alanine mutant this is replaced with an unreactive carbon, whilst in the cysteine mutant the oxygen is replaced with a thiol group which is a much weaker nucleophile. This gives a fully inactive mutant and a weakly active mutant, both of which could then be used for structural characterisation studies.

The production of all of these mutants was confirmed by sequencing at the Core Genomics facility located in the University of Sheffield Medical School (http://genetics.group.shef.ac.uk). These were then all transformed into M72 *E. coli* cells ready for protein expression testing, as described in section 2.6.8

5.2.4 Expression of the IgA1P mutant strains

Expression of the mutant strains was characterised using the method outlined in section 2.1.6. In this case the IgA1P proteins were expressed overnight for around 16 hours at 42°C before being analysed using SDS PAGE.

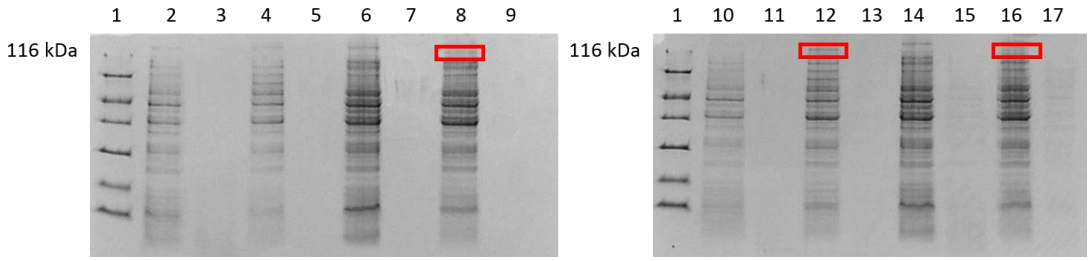

Figure 50. A 12% acrylamide SDS PAGE showing the expression of various IgA1P constructs. The lane on the left of the paired lanes indicates the pellet obtained after centrifugation whilst the right hand lane corresponds to the supernatant. Lane 1 Pierce unstained protein MW marker (ThermoFisher), lanes 2-3 Uninduced full length IgA1P alanine mutant, lanes 4-5 induced full length IgA1P alanine mutant, lanes 6-7 uninduced wildtype IgA1P with a β core deletion, lanes 8-9 induced wildtype IgA1P with a β core deletion, lanes 10-11 uninduced IgA1P cysteine mutant with a β core deletion, lanes 12-13 induced IgA1P cysteine mutant with a β core deletion, lanes 14-15 uninduced IgA1P alanine mutant with a β core deletion and a 6 histidine tag, lanes 16-17 induced IgA1P alanine mutant with a β core deletion and a 6 histidine tag.

As can be seen above multiple different strains were tested for expression, after many trial runs a set of conditions were found under which most mutant proteases would express. What should be noted is that once more the expression level is not very high, but it was sufficient for structural analysis by circular dichroism to be carried out, as described in section 5.2.5. The wildtype IgA1P and the created IgA1P mutants were purified from induced lysed cell pellets. Cell pellets were lysed and treated as in section 2.2.3 and purified using a nickel affinity resin (section 2.2.3) or size exclusion chromatography as detailed in section 2.7.4. Unfortunately, due to the low level of expression exhibited by both the wildtype and mutant IgA1P proteins any images of SDS PAGE gels containing the pure protein were very faint and thus unusable in this document. IgA1P was not verified by mass spectrometry, however the size of the protein along coupled with the fact that the band only appeared in induced bacteria containing an IgA1P plasmid indicated that the protein was most likely IgA1P.

5.2.5 Analysis of the neisserial IgA1P protein using circular dichroism

Purified IgA1P cysteine mutant at 0.18 mg/ml was used in a circular dichroism experiment as described in section 2.3.4.

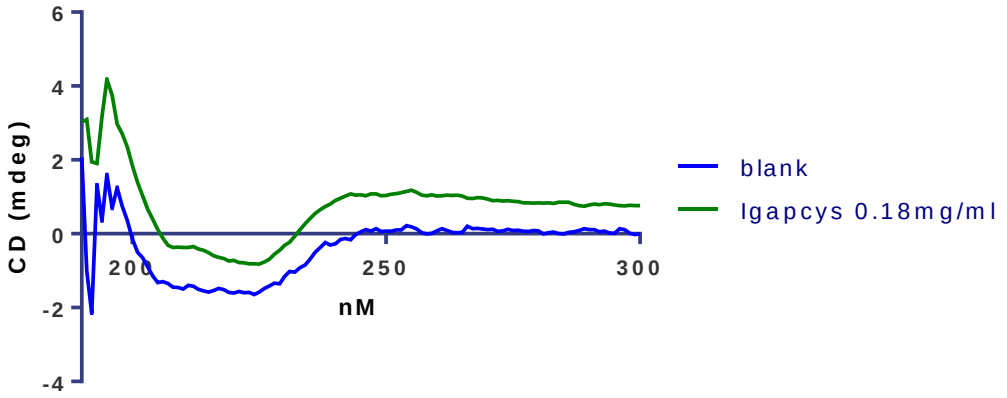

Figure 51. The circular dichroism spectrum for IgA1P cysteine mutant. The spectrum unfortunately shows very little data as the blank (1xPBS) actually absorbed more greatly than the protein.

Figure 51 shows that despite the relatively high concentration of IgA1P the circular dichroism spectrum shows very little absorbance, but the little spectra that can be seen gives a mostly α helical spectra. This contradicts what is predicted for the structure of IgA1P, but the very low intensity of the absorbance means that the result should likely be discarded.

5.3 Conclusions

Neisserial IgA1P is a widely studied protein in terms of function but as of yet there is very little known about its structure [34,35,85,96]. The information that is predicted for the neisserial structure comes from extrapolation of the *Haemophilus influenzae* IgA1P structure [75] and so is at best a guess. The work contained within this chapter goes someway to setting up a wide variety of structural analyses in the future, by creating more easily purifiable proteases as well as more stable protease which can be used for crystallography and spectroscopy trials. These mutants will allow a much greater understanding of the IgA1P structure, rather than the threading or prediction based models that are currently in use [81].

These mutants are predicted to be much more stable than the wildtype IgA1P and as a result are also more useful in vaccine and drug design. IgA1P is a predicted meningococcal virulence factor predicted to be involved in the switching of the pathogen from commensal to invasive [34,35], whilst simultaneously protecting the bacteria from the effects of the immune system through a number of cleavage interactions [35,84,100]. As a result, the IgA1P is thought to be a potential vaccine candidate for meningococcal disease and in particular disease caused by *Neisseria meningitidis*. The increased stability of these new mutant IgA1P proteins would allow them to be included in vaccine trials more easily as they will no longer be capable of degrading themselves, in theory this should increase the effectiveness of the vaccine. What

has also been shown about IgA1P is it is capable of creating a very large immunogenic response when expressed during meningococcal disease [25,101] , probably due to its large size, this again raises the question about whether it would be a good addition to a neisserial or meningococcal vaccine.

Unfortunately attempts to study the IgA1P cysteine mutants using circular dichroism failed to provide and useful data. Normally proteins for analysis using circular dichroism are used at a concentration of 0.1 mg/ml, however in this study the IgA1P mutant was at nearly double that concentration but still failed to absorb properly. This could be due to the large size of IgA1P, this large size increases its molecular weight and decreases the number of protein molecules found at such a low concentration. For example, in 100 µl of α at 0.1 mg/ml there would be around $3.2x10^{17}$ molecules present whereas for IgA1P at the same concentration the would be around $5.0x10^{16}$ molecules, a 10 fold reduction in the number of molecules present, therefore in order to get a similar intensity spectrum the IgA1P would need to be at 0.9-1 mg/ml. In the future this could be easily be achieved using one of the new IgA1P mutant strains that is much easier to purify.

This chapter whilst not answering many of the research questions it set out to has instead laid the groundwork for future study of neisserial IgA1P by creating a number of IgA1P mutant strains that have a predicted decrease in activity and are easier to express an purify. In the future this will allow much greater study of the proteases structure and allow for more thorough investigations into its potential as a vaccine candidate.

Chapter 6- Conclusions and future work

6. Conclusions and future work

As has been discussed in previous chapters the experimental results in this chapter have helped to add to the existing published work on neisserial α and IgA1P whilst building a platform for ongoing experimental work.

The α protein currently has very little published structural and functional data. Previous studies have outlined its general secondary structure and that it may be involved in targeting IgA1P into host cells using NLS and that it may have a role in binding eDNA [111,112]. In this thesis this role has been expanded to also include host protein interactions, via bikunin or IαI and a high immunogenicity and ability to invoke a long lasting immune system memory [25]. These role shave helped to expand the understanding of the neisserial α protein and also hinted that it could be used in future vaccine development as a carrier protein or as a potential tool for biotechnology as a host cell targeting protein. These roles were previously thought of, but this study has helped to provide the data necessary to confirm these hypotheses.

With regards to the structural characterisation of α previous studies have shown that it is a long α helical monomer containing multiple NLS and T cell helper epitopes [25,111], whilst as yet unpublished data from the University of Sheffield has also shown that the protein is capable of forming large multimers *in crystallo*. The research carried out in this thesis has helped to confirm the observation that α can multimerise by showing multimerisation in solution using multiple different methods. The multimerisation shown in solution however is markedly different from the multimerisation *in crystallo*, in that *in crystallo* multimers comprised of up to 24 monomers are formed whilst in solution this drops to 2-3. In order to confirm this crystal structure the crystallography trials should be repeated as before but at a much lower concentration. The crystal structure shown in this thesis (**Figure 22**) was obtained at around 100 mg/ml, in order to more closely mirror physiological conditions whilst still retaining the ability to form crystals these trials should be repeated a lower concentration, possibly around 10-15 mg/ml. It is hypothesised that under these conditions the crystals will more likely show trimers of α that are similar to those found in solution.

The development of multiple cysteine labelled α proteins has helped to lay the groundwork for further study both structurally and into this ability to act as a carrier protein. The cysteine residues will both provide single points for thiol modification allowing 1:1 cross linking of the α protein to target proteins, whilst also helping to stabilise multimer formation in solution. This multimer formation is thought to be key to its ability to induce a large immunogenic response and increased renal clearing times, as a result these cysteine modified proteins

should provide a great foundation for studying the *in vivo* effects of α as a carrier protein. This multimer stabilisation should aid crystallisation as well as multimer formation, this will allow research to be carried out to determine whether multimer formation is crucial to the function of the α protein. This could be studied by measuring the affinity of alpha binding to a substrate, such as bikunin, in both monomer and multimer form using technology such as the BLItz system. If any change in affinity for the substrate is detected in either form, this may indicate that α protein has a functional form *in vivo.*

Further to these mutant strains the research contained in this thesis has shown that the protein itself coupled with its T cell helper epitopes seem to be able to elicit a large immune response in a mouse model. Until now the ability to use α as a tool for biotechnology or a carrier protein had been speculated, but this work provides the first proof that α could certainly be used as a carrier protein. In order to further prove this the as yet untested serum provided from the New Zealand white rabbit immunisations may provide further evidence for this carrier protein role, these will have to be tested via ELISA to produce antibody titre data. This antibody titre data can then help to confirm the observations made in the BALB/C mouse model by indicating that in the presence of α the immunogenicity of other proteins is increased. If the rabbit sera confirms the observations made in the mouse model the next step would be to compare the efficacy of α to commonly used carrier proteins such as *tetanus* toxoid or *diphtheria* toxoid, both in terms of ability to induce a large immune response upon inoculation and the ability to produce a long lasting immune system memory. This data would be invaluable in evaluating the potential of the neisserial α protein as a carrier protein in vaccine development.

The relatively little functional data published on neisserial α has shown its ability to bind eDNA and its ability to target IgA1P to host cells [111,112] . These most likely cover the major roles of α in vivo but until now very little work has been done on elucidating a protein binding partner in the host. In this study the observation that α most likely interacts with IαI through a bikunin domain has been further investigated. The data contained in this thesis shows that whilst α in unable to bind IαI from pig serum it does interact with a number of proteins from human serum. In order to further characterise this interaction, proteins that interact with α should be isolated using a pull down experiment and subjected to mass spectroscopy. This would give a much clearer understanding of how the α protein elicits a response *in vivo* and what its major role is in infection and colonisation.

IgA1P is the founding member of the type Va autotransporter family, which since its discovery in 1973 has been well researched both functionally and structurally[72]. Research into the export pathway has shown that IgA1P, despite its family name, requires multiple other factors in order to export properly and carry out its function *in vivo* [103,104]. This function has been well characterised thus far and seems to involve triggering invasive disease by protecting the bacteria from neutralisation by the host immune system whilst also causing the inflammatory response associated with invasive meningitis[34,35,84,100]. The only area with lacking research regarding neisserial IgA1P is structural studies, there is currently only one published structure for IgA1P and that is from *Haemophilus influenzae*[75]. Whilst these proteins share homology to one another in order to carry out proper drug design and characterisation study an accurate structure is required. The research contained within this thesis on neisserial IgA1P, whilst not adding greatly to the current published knowledge has created a number of mutant proteins and a reliable way of expressing and purifying protein which should enable rapid further study of IgA1P, both structurally and functionally.

Currently the only available structures of neisserial IgA1P are derived from prediction based models utilising the published *Haemophilus influenzae* IgA1P structure [75] . While this are a useful starting point for protein characterisation they are often flawed and so reliable and accurate structural data is required if further research is to be carried out on the protein. The plasmid construct and purification methods contained in chapter 5 have shown that a pure IgA1P protein can be obtained relatively easily, although the yield of this purification is very low. In order to begin structural characterisation circular dichroism and crystallography trials should be carried out using the inactive or very weakly active mutant IgA1P proteins created in this research. These proteins would enable the protease to be more easily purified and crystallised, as the WT IgA1P has a tendency to undergo auto proteolytic cleavage and quickly degrade itself in solution. This means that under normal conditions the WT IgA1P cannot be used to produce accurate structural data, due to its incomplete nature. These structural trials should provide an accurate IgA1P structure that can then be used in further research. In conjunction with this dynamic light scattering trials should also be carried out in the presence of IgA. Dynamic light scattering is a fast but low resolution method of determining the orientation in which a substrate is bound to a protein. It uses the backwards scattering of incident laser light caused when the light strikes the particle to determine the dimension known as hydrodynamic diameter, this equates to the size of the molecule [122] . Due to the large size of the substrate the orientation of substrate binding will alter the reported

hydrophobic diameter, it is therefore possible to calculate the orientation of binding as well as any dimerisation of substrate or protein.

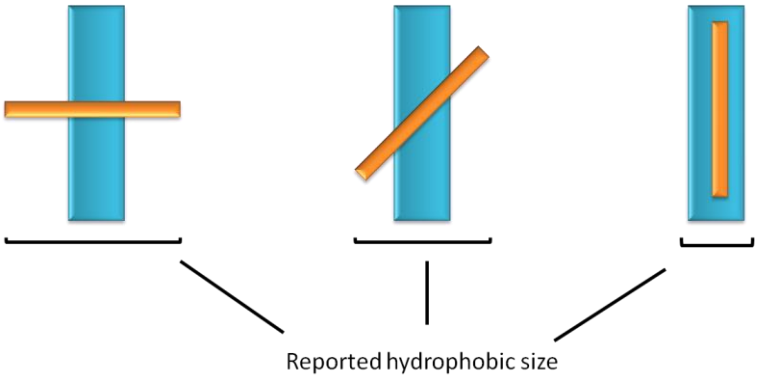

Figure 52. A pictorial representation of the principle of dynamic light scattering. Depending upon the orientation of binding the reported hydrophobic size alters, therefore using the reported hydrophobic size along with the dimensions of the proteins in question it is possible to elucidate the orientation of binding and whether any dimerisation has occurred.

This would give an indication as to where the substrate binds the protease and whether the smaller domains in the protease are involved in orientating the substrate, at the time of writing there are many theories on substrate binding orientation but very little evidence for any of them. This experiment would provide the much needed evidence that would answer how the IgA1P protein binds its substrates.

Further to this structural study more functional trials could be carried out on neisserial IgA1P with regards to its substrate specificity. It has been previously published that neisserial IgA1P exhibits a broad range of substrates in vitro, including IgA1, LAMP and TNFRII. However, the affinity of IgA1P for these substrates has never been demonstrated, as such the affinity for IgA1P for each substrate could be measured *in vitro*. This would provide a crucial indication as to whether or not these interactions are likely to occur at physiological concentrations or whether they are an artefact of *in vitro* methodology.

Alongside this structural and functional work, the potential of IgA1P as a vaccine candidate could be evaluated using a serum bactericidal assay. In this assay anti- neisserial IgA1P antibodies could be incubated with a variety of commensal and invasive isolates grown in conditions similar to that found in the nasopharynx or bloodstream to test whether the inhibition of IgA1P is sufficient to slow bacterial growth or promote bacterial death.

Overall this work has helped to expand the current published knowledge on both the *Neisseria meningitidis* α and IgA1P proteins whilst creating several mutant proteins which will enable further rapid study of both proteins.

References

1. Stephens, D., Greenwood, B. & Brandtzaeg, P. Epidemic meningitis, meningococcaemia, and Neisseria meningitidis. *Lancet* **369,** 2196–2210 (2007).
2. LaForce, F., Konde, K., Viviani, S. & Preziosi, M. The meningitis vaccine project. *Vaccine* **25,** A97–A100 (2007)
3. WorldHealthOrganisation, W. Meningococcal fact sheet. (2011).
4. Ginsberg, L. Difficult and recurrent meningitis. *Journal of Neurology Neurosurgery and Psychiatry* **75,** I16–I21(2004)
5. HSCIC. UK Hospital episode statistics.
6. Fraser, P. K., Bailey, G. K., Abbott, J. D., Gill, J. B. & Walker, D. J. The meningococcal carrier-rate. *Lancet* **1,** 1235–7 (1973).
7. Cartwright, Stuart, Jones & Noah. The Stonehouse survey: nasopharyngeal carriage of meningococci and Neisseria lactamica. *Epidemiol Infect* **99,** 591 (1987).
8. Jeppesen, C. A. *et al.* Meningococcal carriage in adolescents in the United Kingdom to inform timing of an adolescent vaccination strategy. *J. Infect.* **71,** 43–52 (2015).
9. Jones *et al.* Dynamics of carriage of Neisseria meningitidis in a group of military recruits: subtype stability and specificity of the immune response following colonization. *J Infect Dis* **178,** 451–9 (1998).
10. Gasparini, R. *et al.* Molecular and serological diversity of Neisseria meningitidis carrier strains isolated from Italian students aged 14 to 22 years. *J. Clin. Microbiol.* **52,** 1901–10 (2014).
11. Broderick, M., Faix, D., Hansen, C. & Blair, P. Trends in Meningococcal Disease in the United States Military, 1971–2010. *Emerg Infect Dis* **18,** 1430–7 (2012).
12. The Diversity of Meningococcal Carriage Across the African Meningitis Belt and the Impact of Vaccination With a Group A Meningococcal Conjugate Vaccine. *J. Infect. Dis.* **212,** 1298–307 (2015).
13. Jafri, R. *et al.* Global epidemiology of invasive meningococcal disease. *Popul Heal Metrics* **11,** 1–9 (2013).
14. Hill, D., Griffiths, N., Borodina, E. & Virji, M. Cellular and molecular biology of Neisseria meningitidis colonization and invasive disease. *Clin Sci Lond Engl 1979* **118,** 547–564 (2010).
15. Catenazzi, M. *et al.* A large genomic island allows Neisseria meningitidis to utilize propionic acid, with implications for colonization of the human nasopharynx. *Mol Microbiol* **93,** 346–355 (2014).
16. Johswich, K. O. *et al.* In vivo adaptation and persistence of Neisseria meningitidis within the nasopharyngeal mucosa. *PLoS Pathog.* **9,** e1003509 (2013).
17. Laver, J. R., Hughes, S. E. & Read, R. C. Neisserial Molecular Adaptations to the Nasopharyngeal Niche. *Adv. Microb. Physiol.* **66,** 323–55 (2015).
18. Swartley, J. S. *et al.* Capsule switching of Neisseria meningitidis. *Proc Natl Acad Sci* **94,** 271–276 (1997).
19. Nolan, T., O'Ryan, M., Wassil, J., Abitbol, V. & Dull, P. Vaccination with a multicomponent meningococcal B vaccine in prevention of disease in adolescents and young adults. *Vaccine* **33(36),** 4437-4445 (2015).
20. Perrett, K. P. *et al.* Immune responses to a recombinant, four-component, meningococcal serogroup B vaccine (4CMenB) in adolescents: a phase III, randomized, multicentre, lot-to-lot consistency study. *Vaccine* **33,** 5217–24 (2015).
21. Delbos, V. *et al.* Impact of MenBvac, an outer membrane vesicle (OMV) vaccine, on the meningococcal carriage. *Vaccine* **31,** 4416–4420 (2013).

22. McIntyre, P., O'Brien, K., Greenwood, B. & van de Beek, D. Effect of vaccines on bacterial meningitis worldwide. *Lancet* **380,** 1703–1711 (2012).
23. Berti, F. & Adamo, R. Recent Mechanistic Insights on Glycoconjugate Vaccines and Future Perspectives. *Acs Chem Biol* **8,** 1653–1663 (2013).
24. Gupta, S. *et al.* In silico CD4+T-cell epitope prediction and HLA distribution analysis for the potential proteins of Neisseria meningitidis Serogroup B-A clue for vaccine development. *Vaccine* **28,** 7092–7097(2010)
25. Jose, Wölk, Lorenzen, Wenschuh & Meyer. Human T-Cell Response to Meningococcal Immunoglobulin A1 Protease Associated α-Proteins. *Scand J Immunol* **51,** 176–185 (2000).
26. Read, R. C. *et al.* Effect of a quadrivalent meningococcal ACWY glycoconjugate or a serogroup B meningococcal vaccine on meningococcal carriage: an observer-blind, phase 3 randomised clinical trial. *Lancet* **384,** 2123–31 (2014).
27. World Health Organisation. *Menigococcal fact sheet*. (2011).
28. Maiden, M. C. *et al.* Impact of meningococcal serogroup C conjugate vaccines on carriage and herd immunity. *J. Infect. Dis.* **197,** 737–43 (2008).
29. Vesikari, T. *et al.* Immunogenicity and safety of an investigational multicomponent, recombinant, meningococcal serogroup B vaccine (4CMenB) administered concomitantly with routine infant and child vaccinations: results of two randomised trials. *Lancet* **381,** 825–835 (2013).
30. McQuaid, F. *et al.* Persistence of specific bactericidal antibodies at 5 years of age after vaccination against serogroup B meningococcus in infancy and at 40 months. *CMAJ* **187(7),** E215-223(2015).
31. Frosi, G. *et al.* Bactericidal antibody against a representative epidemiological meningococcal serogroup B panel confirms that MATS underestimates 4CMenB vaccine strain coverage. *Vaccine* **31,** 4968–74 (2013).
32. Vogel, U. *et al.* Predicted strain coverage of a meningococcal multicomponent vaccine (4CMenB) in Europe: a qualitative and quantitative assessment. *Lancet Infect Dis* **13,** 416–25 (2013).
33. Arnold, R., Galloway, Y., McNicholas, A. & O'Hallahan, J. Effectiveness of a vaccination programme for an epidemic of meningococcal B in New Zealand. *Vaccine* **29,** 7100–7106 (2011).
34. Vitovski, S., Read, R. C. & Sayers, J. R. Invasive isolates of Neisseria meningitidis possess enhanced immunoglobulin A1 protease activity compared to colonizing strains. *FASEB J.* **13,** 331–7 (1999).
35. Vitovski, S., Dunkin, K., Howard, A. & Sayers, J. Nontypeable Haemophilus influenzae in carriage and disease - A difference in IgA1 protease activity levels. *Jama-Journal of the American Medical Association* **287,** 1699–1705 (2002)
36. Proft, T. & Baker, E. Pili in Gram-negative and Gram-positive bacteria - structure, assembly and their role in disease. *Cellular and Molecular Life Sciences* **66,** 613–635 (2009)
37. Tzeng, Y.-L. L., Thomas, J. & Stephens, D. S. Regulation of capsule in Neisseria meningitidis. *Crit. Rev. Microbiol.* 1–14 (2015).
38. Swain, C. L. & Martin, D. R. Survival of meningococci outside of the host: implications for acquisition. *Epidemiol. Infect.* **135,** 315–20 (2007).
39. Leimkugel, J. *et al.* Clonal waves of Neisseria colonisation and disease in the African meningitis belt: eight- year longitudinal study in northern Ghana. *PLoS Med.* **4,** e101 (2007).

40. Virji, M., Makepeace, K., Ferguson, D. J., Achtman, M. & Moxon, E. R. Meningococcal Opa and Opc proteins: their role in colonization and invasion of human epithelial and endothelial cells. *Mol. Microbiol.* **10,** 499–510 (1993).
41. Malorny, B., Morelli, G., Kusecek, B., Kolberg, J. & Achtman, M. Sequence diversity, predicted two-dimensional protein structure, and epitope mapping of neisserial Opa proteins. *Journal of Bacteriology* **180,** 1323–1330 (1998)
42. Prince, S. M., Achtman, M. & Derrick, J. P. Crystal structure of the OpcA integral membrane adhesin from Neisseria meningitidis. *Proc. Natl. Acad. Sci. U.S.A.* **99,** 3417–21 (2002).
43. De Vries, F. P., Cole, R., Dankert, J., Frosch, M. & van Putten, J. P. Neisseria meningitidis producing the Opc adhesin binds epithelial cell proteoglycan receptors. *Mol. Microbiol.* **27,** 1203–12 (1998).
44. Sadarangani, M., Pollard, A. & Gray-Owen, S. Opa proteins and CEACAMs: pathways of immune engagement for pathogenic Neisseria. *Fems Microbiology Reviews* **35,** 498–514 (2011)
45. Callaghan, M. J., Jolley, K. A. & Maiden, M. C. Opacity-associated adhesin repertoire in hyperinvasive Neisseria meningitidis. *Infect. Immun.* **74,** 5085–94 (2006).
46. Virji, M., Makepeace, K. & Moxon, E. R. Distinct mechanisms of interactions of Opc-expressing meningococci at apical and basolateral surfaces of human endothelial cells; the role of integrins in apical interactions. *Mol. Microbiol.* **14,** 173–84 (1994).
47. Virji, M. *et al.* Opc- and pilus-dependent interactions of meningococci with human endothelial cells: molecular mechanisms and modulation by surface polysaccharides. *Mol. Microbiol.* **18,** 741–54 (1995).
48. Griffiths, N., Bradley, C., Heyderman, R. & Virji, M. IFN-gamma amplifies NF kappa B-dependent Neisseria meningitidis invasion of epithelial cells via specific upregulation of CEA-related cell adhesion molecule 1. *Cellular Microbiology* **9,** 2968–2983 (2007)
49. Lee, H. S. *et al.* Neisserial outer membrane vesicles bind the coinhibitory receptor carcinoembryonic antigen-related cellular adhesion molecule 1 and suppress CD4+ T lymphocyte function. *Infect. Immun.* **75,** 4449–55 (2007).
50. Sarantis, H. & Gray-Owen, S. D. The specific innate immune receptor CEACAM3 triggers neutrophil bactericidal activities via a Syk kinase-dependent pathway. *Cell. Microbiol.* **9,** 2167–80 (2007).
51. Chen, T. & Gotschlich, E. C. CGM1a antigen of neutrophils, a receptor of gonococcal opacity proteins. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 14851–6 (1996).
52. Pantelic, M. *et al.* Neisseria gonorrhoeae kills carcinoembryonic antigen-related cellular adhesion molecule 1 (CD66a)-expressing human B cells and inhibits antibody production. *Infect. Immun.* **73,** 4171–9 (2005).
53. Helaine, S., Dyer, D., Nassif, X., Pelicic, V. & Forest, K. 3D structure/function analysis of PilX reveals how minor pilins can modulate the virulence properties of type IV pili. *Proceedings of the National Academy of Sciences of the United States of America* **104,** 15888–15893 (2007)
54. Merz, A. J., Enns, C. A. & So, M. Type IV pili of pathogenic Neisseriae elicit cortical plaque formation in epithelial cells. *Mol. Microbiol.* **32,** 1316–32 (1999).
55. Chamot-Rooke, J. *et al.* Posttranslational modification of pili upon cell contact triggers N. meningitidis dissemination. *Science* **331,** 778–82 (2011).

56. Mikaty, G. *et al.* Extracellular bacterial pathogen induces host cell surface reorganization to resist shear stress. *PLoS Pathog.* **5,** e1000314 (2009).
57. Merz, A. J., So, M. & Sheetz, M. P. Pilus retraction powers bacterial twitching motility. *Nature* **407,** 98–102 (2000).
58. Bambini, S. *et al.* Neisseria adhesin A variation and revised nomenclature scheme. *Clin. Vaccine Immunol.* **21,** 966–71 (2014).
59. Comanducci, M. *et al.* NadA, a novel vaccine candidate of Neisseria meningitidis. *J. Exp. Med.* **195,** 1445–54 (2002).
60. Comanducci, M. *et al.* NadA diversity and carriage in Neisseria meningitidis. *Infect. Immun.* **72,** 4217–23 (2004).
61. Capecchi, B. *et al.* Neisseria meningitidis NadA is a new invasin which promotes bacterial adhesion to and penetration into human epithelial cells. *Mol. Microbiol.* **55,** 687–98 (2005).
62. Bullen, J. THE SIGNIFICANCE OF IRON IN INFECTION. *Reviews of Infectious Diseases* **3,** 1127–1138 (1981).
63. Anzaldi, L. & Skaar, E. Overcoming the Heme Paradox: Heme Toxicity and Tolerance in Bacterial Pathogens. *Infection and Immunity* **78,** 4977–4989 (2010)
64. Wilks, A. & Burkhard, K. Heme and virulence: How bacterial pathogens regulate, transport and utilize heme. *Natural Product Reports* **24,** 511–522 (2007)
65. Tong, Y. & Guo, M. Bacterial heme-transport proteins and their heme-coordination modes. *Archives of Biochemistry and Biophysics* **481,** 1–15 (2009)
66. Rohde, K. H., Gillaspy, A. F., Hatfield, M. D., Lewis, L. A. & Dyer, D. W. Interactions of haemoglobin with the Neisseria meningitidis receptor HpuAB: the role of TonB and an intact proton motive force. *Mol. Microbiol.* **43,** 335–54 (2002).
67. Bidmos, F. A. *et al.* Investigation into the Antigenic Properties and Contributions to Growth in Blood of the Meningococcal Haemoglobin Receptors, HpuAB and HmbR. *PLoS ONE* **10,** e0133855 (2015).
68. Moynie, L. *et al.* The AEROPATH project targeting Pseudomonas aeruginosa: crystallographic studies for assessment of potential targets in early-stage drug discovery. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **69,** 25–34 (2013).
69. Serruto, D. *et al.* Neisseria meningitidis GNA2132, a heparin-binding protein that induces protective immunity in humans. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 3770–5 (2010).
70. Menozzi, F. D. *et al.* Enhanced bacterial virulence through exploitation of host glycosaminoglycans. *Mol. Microbiol.* **43,** 1379–86 (2002).
71. Duensing, T. D., Wing, J. S. & van Putten, J. P. Sulfated polysaccharide-directed recruitment of mammalian host proteins: a novel strategy in microbial pathogenesis. *Infect. Immun.* **67,** 4463–8 (1999).
72. Mehta, S., Plaut, A., Calvanic.Nj & Tomasi, T. HUMAN IMMUNOGLOBULIN-A - PRODUCTION OF AN FC-FRAGMENT BY AN ENTERIC MICROBIAL PROTEOLYTIC-ENZYME. *Journal of Immunology* **111,** 1274–1276 (1973).
73. Mistry, D. & Stockley, R. The cleavage specificity of an IgA1 protease fromHaemophilus influenzae. *Virulence* **2,** 103–110 (2011).
74. Mistry, D. & Stockley, R. A. IgA1 protease. *Int. J. Biochem. Cell Biol.* **38,** 1244–8 (2006).
75. Johnson, T. A., Qiu, J., Plaut, A. G. & Holyoak, T. Active-site gating regulates substrate selectivity in a chymotrypsin-like serine protease the structure of

haemophilus influenzae immunoglobulin A1 protease. *J. Mol. Biol.* **389,** 559–74 (2009).
76. Jose, J., Jahnig, F. & Meyer, T. COMMON STRUCTURAL FEATURES OF IGA1 PROTEASE-LIKE OUTER-MEMBRANE PROTEIN AUTOTRANSPORTERS. *Molecular Microbiology* **18,** 378–380 (1995)
77. Pohlner, J., Halter, R., Beyreuther, K. & Meyer, T. F. Gene structure and extracellular secretion of Neisseria gonorrhoeae IgA protease. *Nature* **325,** 458–62 (1987).
78. Benz, I. & Schmidt, A. Structures and functions of autotransporter proteins in microbial pathogens. *Int J Med Microbiol* **301,** 461–468 (2011).
79. Henderson, I., Navarro-Garcia, F., Desvaux, M., Fernandez, R. & Ala'Aldeen, D. Type V Protein Secretion Pathway: the Autotransporter Story. *Microbiol Mol Biol R* **68,** 692–744 (2004).
80. Kilian, M., Mestecky, J., Kulhavy, R., Tomana, M. & Butler, W. T. IgA1 proteases from Haemophilus influenzae, Streptococcus pneumoniae, Neisseria meningitidis, and Streptococcus sanguis: comparative immunochemical studies. *J. Immunol.* **124,** 2596–600 (1980).
81. Kelley, L. & Sternberg, M. Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc* **4,** 363–371 (2009).
82. Bachovchin, W. W., Plaut, A. G., Flentke, G. R., Lynch, M. & Kettner, C. A. Inhibition of IgA1 proteinases from Neisseria gonorrhoeae and Hemophilus influenzae by peptide prolyl boronic acids. *J. Biol. Chem.* **265,** 3738–43 (1990).
83. Plaut, A. G., Genco, R. J. & Tomasi, T. B. Isolation of an enzyme from Streptococcus sanguis which specifically cleaves IgA. *J. Immunol.* **113,** 589–91 (1974).
84. Lorenzen, D. *et al.* Immunoglobulin A1 protease, an exoenzyme of pathogenic Neisseriae, is a potent inducer of proinflammatory cytokines. *Journal of Experimental Medicine* **190,** 1049–1058 (1999)
85. Vitovski, S. & Sayers, J. R. Relaxed cleavage specificity of an immunoglobulin A1 protease from Neisseria meningitidis. *Infect. Immun.* **75,** 2875–85 (2007).
86. Smith, J., Smith, N., Orourke, M. & Spratt, B. HOW CLONAL ARE BACTERIA. *Proceedings of the National Academy of Sciences of the United States of America* **90,** 4384–4388 (1993)
87. Woof, J. M. & Kerr, M. A. The function of immunoglobulin A in immunity. *J. Pathol.* **208,** 270–82 (2006).
88. Chintalacharuvu, K. *et al.* Cleavage of the human immunoglobulin A1 (IgA1) hinge region by IgA1 proteases requires structures in the Fc region of IgA. *Infection and Immunity* **71,** 2563–2570 (2003)
89. Childers, N., Bruce, M. & McGhee, J. MOLECULAR MECHANISMS OF IMMUNOGLOBULIN A DEFENSE. *Annual Review of Microbiology* **43,** 503–536 (1989).
90. Almogren, A. & Kerr, M. Irreversible aggregation of the Fc fragment derived from polymeric but not monomeric serum IgA1—Implications in IgA-mediated disease. *Mol Immunol* **45,** 87–94 (2008).
91. Kilian, M., Reinholdt, J., Lomholt, H., Poulsen, K. & Frandsen, E. Biological significance of IgA1 proteases in bacterial colonization and pathogenesis: Critical evaluation of experimental evidence. *Apmis* **104,** 321–338 (1996)

92. Murphy, T. *et al.* A Clonal Group of Nontypeable Haemophilus influenzae with Two IgA Proteases Is Adapted to Infection in Chronic Obstructive Pulmonary Disease. *Plos One* **6(10),** e25923 (2011)
93. Fernaays, M., Lesse, A., Cai, X. & Murphy, T. Characterization of igaB, a second immunoglobulin A1 protease gene in nontypeable Haemophilus influenzae. *Infection and Immunity* **74,** 5860–5870 (2006)
94. Grundy, F. J., Plaut, A. G. & Wright, A. Localization of the cleavage site specificity determinant of Haemophilus influenzae immunoglobulin A1 protease genes. *Infect. Immun.* **58,** 320–31 (1990).
95. Mansa, B. & Kilian, M. Retained antigen-binding activity of Fab alpha fragments of human monoclonal immunoglobulin A1 (IgA1) cleaved by IgA1 protease. *Infection & Immunity* **52,** 171–4 (1986).
96. Parsons, H. K., Vitovski, S. & Sayers, J. R. Immunoglobulin A1 proteases: a structure-function update. *Biochem. Soc. Trans.* **32,** 1130–2 (2004).
97. Lin, L. *et al.* The Neisseria type 2 IgA1 protease cleaves LAMP1 and promotes survival of bacteria within epithelial cells. *Molecular Microbiology* **24,** 1083–1094 (1997)
98. Hauck, C. R. & Meyer, T. F. The lysosomal/phagosomal membrane protein h-lamp-1 is a target of the IgA1 protease of Neisseria gonorrhoeae. *FEBS Lett.* **405,** 86–90 (1997).
99. Clementi, C. F., Håkansson, A. P. & Murphy, T. F. Internalization and trafficking of nontypeable Haemophilus influenzae in human respiratory epithelial cells and roles of IgA1 proteases for optimal invasion and persistence. *Infect. Immun.* **82,** 433–44 (2014).
100. Beck, S. & Meyer, T. IgA1 protease from Neisseria gonorrhoeae inhibits TNF alpha-mediated apoptosis of human monocytic cells. *Febs Letters* **472,** 287–292 (2000)
101. Thiesen, B., Greenwood, B., Brieske, N. & Achtman, M. Persistence of antibodies to meningococcal IgA1 protease versus decay of antibodies to group A polysaccharide and Opc protein. *Vaccine* **15,** 209–219 (1997)
102. Tsirpouchtsidis, A., Hurwitz, R., Brinkmann, V., Meyer, T. & Haas, G. Neisserial immunoglobulin A1 protease induces specific T-cell responses in humans. *Infection and Immunity* **70,** 335–344 (2002)
103. Voulhoux, R., Bos, M., Geurtsen, J., Mols, M. & Tommassen, J. Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science* **299,** 262–265 (2003)
104. De Cock, H., Brandenburg, K., Wiese, A., Holst, O. & Seydel, U. Non-lamellar structure and negative charges of lipopolysaccharides required for efficient folding of outer membrane protein PhoE of Escherichia coli. *J. Biol. Chem.* **274,** 5114–9 (1999).
105. Long, S., Phan, E. & Vellard, M. C. The expression of soluble and active recombinant Haemophilus influenzae IgA1 protease in E. coli. *J. Biomed. Biotechnol.* **2010,** 253983 (2010).
106. Estrada Mallarino, L. *et al.* TtOmp85, a β-Barrel Assembly Protein, Functions by Barrel Augmentation. *Biochemistry* **54,** 844–52 (2015).
107. Oomen, C. J. *et al.* Structure of the translocator domain of a bacterial autotransporter. *EMBO J.* **23,** 1257–66 (2004).
108. Veiga, E., Sugawara, E., Nikaido, H., Lorenzo, V. & Fernandez, L. Export of autotransported proteins proceeds through an oligomeric ring shaped by C-terminal domains. *Embo Journal* **21,** 2122–2131 (2002)

109. Barnard, T. J., Dautin, N., Lukacik, P., Bernstein, H. D. & Buchanan, S. K. Autotransporter structure reveals intra-barrel cleavage followed by conformational changes. *Nat. Struct. Mol. Biol.* **14,** 1214–20 (2007).
110. Oliver, D., Huang, G., Nodel, E., Pleasance, S. & Fernandez, R. A conserved region within the Bordetella pertussis autotransporter BrkA is necessary for folding of its passenger domain. *Mol. Microbiol.* **47,** 1367–1383 (2003).
111. Pohlner, J., Langenberg, U., Wolk, U., Beck, S. & Meyer, T. UPTAKE AND NUCLEAR TRANSPORT OF NEISSERIA IGA1 PROTEASE-ASSOCIATED ALPHA-PROTEINS IN HUMAN-CELLS. *Molecular Microbiology* **17,** 1073–1083 (1995)
112. Arenas, J., Nijland, R., Rodriguez, F. J., Bosma, T. N. & Tommassen, J. Involvement of three meningococcal surface-exposed proteins, the heparin-binding protein NhbA, the α-peptide of IgA protease and the autotransporter protease NalP, in initiation of biofilm formation. *Mol. Microbiol.* **87,** 254–68 (2013).
113. Oconnor, C. & Timmis, K. HIGHLY REPRESSIBLE EXPRESSION SYSTEM FOR CLONING GENES THAT SPECIFY POTENTIALLY TOXIC PROTEINS. *Journal of Bacteriology* **169,** 4457–4462 (1987)
114. Sayers, J. & Eckstein, F. A SINGLE-STRAND SPECIFIC ENDONUCLEASE ACTIVITY COPURIFIES WITH OVEREXPRESSED T5 D15 EXONUCLEASE. *Nucleic Acids Research* **19,** 4127–4132 (1991)
115. Dong, X., Stothard, P., Forsythe, I. & Wishart, D. PlasMapper: a web server for drawing and auto-annotating plasmid maps. *Nucleic Acids Research* **32,** W660–W664 (2004)
116. Besbes, A. *et al.* Hyperinvasive Meningococci Induce Intra-nuclear Cleavage of the NF-κB Protein p65/RelA by Meningococcal IgA Protease. *PLoS Pathog.* **11,** e1005078 (2015).
117. Salier, J., Rouet, P., Raguenez, G. & Daveau, M. The inter-alpha-inhibitor family: From structure to regulation. *Biochemical Journal* **315,** 1–9 (1996)
118. Daveau, M. *et al.* Human inter-alpha-inhibitor family in inflammation: simultaneous synthesis of positive and negative acute-phase proteins. *Biochem. J.* **292 (Pt 2),** 485–92 (1993).
119. Xu, Y., Carr, P. D., Guss, J. M. & Ollis, D. L. The crystal structure of bikunin from the inter-alpha-inhibitor complex: a serine protease inhibitor with two Kunitz domains. *J. Mol. Biol.* **276,** 955–66 (1998).
120. Lindström, K. E., Blom, A., Johnsson, E., Haraldsson, B. & Fries, E. High glomerular permeability of bikunin despite similarity in charge and hydrodynamic size to serum albumin. *Kidney Int.* **51,** 1053–8 (1997).
121. Kobayashi, H., Gotoh, J., Fujie, M. & Terao, T. Characterization of the cellular binding site for the urinary trypsin inhibitor. *J. Biol. Chem.* **269,** 20642–7 (1994).
122. Zölls, S. *et al.* Particles in therapeutic protein formulations, Part 1: overview of analytical methods. *J Pharm Sci* **101,** 914–35 (2012).
123. Whitmore, L. & Wallace, B.A. Protein Secondary Structure Analyses from Circular Dichroism Spectroscopy: Methods and Reference Databases. *Biopolymers* **89 (5)**, 392-400 (2007)
124. Xu, J.Z. & Zhang, W.G. Strategies used for genetically modifying bacterial genome: Site-directed mutagenesis, gene inactivation, and gene over-expression. *J. Zheijang.Univ.Sci.B.* **17(2)**, 83-99 (2016)

125. Holst, J. et al. Vaccines against meningococcal serogroup B disease containing outer membrane vesicles (OMV): lessons from past programs and implications for the future. *Hum. Vaccin. Immunother*. **9(6)**, 1241-1253 (2013)
126. Mora, M. et al. Reverse Vaccinology. *Drug Discovery Today*. **8(10)**, 459-464 (2003)
127. Rappuoli, R. Reverse Vaccinology, *Curr. Opin. Microbiol*., **3(5)**, 445-450 (2000)
128. Hood, D.W. et al. DNA repeats identify novel virulence genes in Haemophilus influenzae. *Proc. Natl. Acad. U.S.A*, **93(20)**, 11121-11125 (1996)
129. Serruto, D. et al. Genome-based approaches to develop vaccines against bacterial pathogens. *Vaccine*. **27(25-26)**, 3245-3250 (2009)
130. Lee, E.Y. et al. Global proteomic profiling of native outer mebrane vesicles derived from Escherichia coli. *Proteomics*. **7(17)**, 3143-3153 (2007)

Appendix I- PCR primers

Alpha N His insertion

AlphaNHis_ndeR2 5’ AGCAGCCAACTCTGCAGCTATTTAAGCTGTATCACGGTGGTAT 3’

AlphaNHis_ndeF 5’AAATACATATGCATCATCACCACCATCATAGCCCGCAGGCAAATCAAGCCGAA 3’

Short_alphaNH 5’ ATAAACATATGCATCATCACCACCATCACAGCCCGCAGGCAAATC 3’

Alpha_revstop 5’ GATGAAGCTTTATTACGGTGGTATAGCCCTGCG 3’

Alpha_RBH2 5’ gatgaagcggatccccggtggtatagccctgcg 3’

alphaNhis_F 5’ caccaccacAGCCCGCAGGCAAATCAA 3’

alphaNhis_R 5’ atgatgatgCATAATTAAATCCTCGATGAATTCTGATGTG 3’

Alpha-gly fusion

Alpha_fus_rev2 5’ ACGACGTTTGGGTTGGCCTCCCCACGGACCGCCAGATTGTGCTGCTTG

M13 uni (-21) 5’ TGTAAAACGACGGCCAGT

Gly1_Fus_F2 5’ CAAACCGTGTGGGCGGGCGGTCCGTGGGGAGGCGATACGGTGTTTTCCT

M13 rev (-49) 5’ GAGCGGATAACAATTTCACACAGG

Gly1_fus_F1 5’ TGGAGGAGGTCGAACTGATACGGTGTTTTCCTGTAAAACGG 3’

Alpha_fus_Rev1 5’ ACTTCCACCACCTCCACATTGTGCTGCTTGGC 3’

NeisGly_Btg1F 5’ TTCCGACAGGccgcggGGTATGGGCAGCGG 3’

NeisGly_Btg1R 5’ CGCCCCAACAGGTCAGCT 3’

NeisGly_Xho1F 5’ AGGTATGGGCctcgagCGTTGGGCAACGATG 3’

NeisGly_Xho1R 5’ TGCCACCTGTCGGAACGC 3’

Alpha499Btg1F 5’ ggcggatcaAGCCCGCAGGCAAATCAA3’

Alpha499Btg1R 5’ tccccgcggAATTAAATCCTCGATGAATTCTGATGTG 3’

Alpha1024Xho1F 5’ tcactcgagCCAAAGCTTGGCGTAATC 3’

Alpha1024Xho1R 5’ tccgcctccCCTGCGGTTACGACGTTT 3’

Cysteine mutations within alpha

55C_alpha_F 5’ AAAAGCAGAATGTGAACGCAGCAGCGCCGAACTTGC 3’

55C_alpha_R 5’ TGGCGGGGTCGCCAGCTCT 3’

91C_alpha_F 5’ TCAAGCATTGTGTGTACGCCGAAAAGCAGAAGCC 3’

91C_alpha_R 5’ GCCCTCACGTTCGGCTTCG 3’

IgAP beta spine truncation

IgAP trunc 753 F 5’ caccactagtagGTTAATCAATCCGCATCATTCTC 3’

IgAP trunc 753 R 5’ gtgatgatgatgCGCAATTTCTGCGGCTTTG 3’

IgAP trunc 818 F 5’ caccactagtagGCGACACAGATTAACGGC 3’

IgAP trunc 818 R 5’ gtgatgatgatgTCCAAAGCTGTTTAAAGCC 3’

IgAP SA mutation

Ex_forIgAP1 5’ CAACCAGTGCAGGCGGCGGCGTTGAAACCTATAAAGATAAAAACCGT 3’

SArev_1 5’ CAAATAATGGTGAACCGGCATCGCCTAACACAG 3’

SAfor_1 5’ TGCTGTGTTAGGCGATGCCGGTTCACCATTATTTG 3’

Ex_revIgAP1 5’ GCATCCAAACGACCGCCACGGAAACCGAAATATAGG 3’

Adding 6 his tag to IgA1P

IgAPprism6H 5’ ATAAACATATGCATCATCACCACCATCACGGAGCGACACAGATTA 3’

IgAPγHis_F 5’ tcatcaccaccacCCTGCCACAAACACGGCT 3’

IgAPγHis_R 5’ tgatgggatccgccAGACGGGACCGGTTTGAC 3’

Appendix II- Plasmid sequences and inserts

pJONEX4

TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACA
GCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGG
CGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATA
TGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCA
TTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCT
GGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCAC
GACGTTGTAAAACGACGGCCAGTGAATTAGATAACCATCTGCGGTGATAAATTATCTCTGGC
GGTGTTGACATAAATACCACTGGCGGTGATACTGAGCACATCAGAATTCGAGCTCGGTACCC
GGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCGTAATCATGGTCATAGCTGTT
TCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGT
GTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCC
GCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAG
AGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCG
TTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCA
GGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAA
GGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGAC
GCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGA
AGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCT
CCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGG
TCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTA
TCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGC
CACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGT
GGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTT
ACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGG
TTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGA
TCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATG
AGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAAT
CTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTA
TCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACT
ACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTC
ACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTC
CTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGT
TCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTC
GTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCC
CCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTG
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pJONEX4 with NMB α Insert

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pJONEX4 with Cys-γ-α insert

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pJONEX with wildtype neisserial IgA1P insert (based on IgAp from accession number AF235032.1)

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pJONEX with the gene for Gly1-ORF1 from *N. meningitidis inserted*

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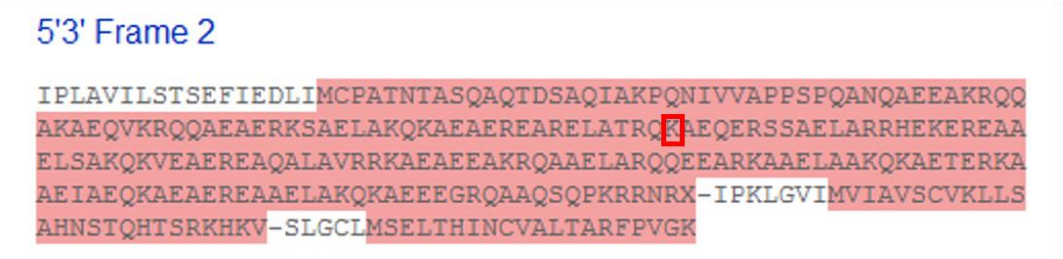
ACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTC

Appendix III- Sequencing result for the single cysteine α mutants

As can be seen from the figure below, the point mutations at both residue 55 and 91 were unsuccessful. The figure below shows the sequencing results translated back into single letter amino acid code.

55C



91C