Engineering self-assembling proteins to produce a safe and effective vaccine for Porcine Reproductive and Respiratory Syndrome

Ondre H. Harper, The University of Western Ontario

Supervisor: Menassa, Rima, Agriculture and Agri-Food Canada
Co-Supervisor: Hill, Kathleen, The University of Western Ontario
A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology
© Ondre H. Harper 2019

Follow this and additional works at: https://ir.lib.uwo.ca/etd
Part of the Biochemistry Commons, and the Biology Commons

Recommended Citation
https://ir.lib.uwo.ca/etd/6670

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlsadmin@uwo.ca.
Abstract

Commercially available vaccines for porcine reproductive and respiratory syndrome virus (PRRSV) provide some control over the virus but none are ideal since they either are not completely safe for use, lack efficacy in promoting long-lasting immunity or provide no protection from heterologous PRRSV strains. Innovative approaches to designing vaccines are being pursued to overcome these drawbacks. One example is the use of nanoparticles to present a dense array of antigenic epitopes to the immune system which can effectively stimulate antibody producing cells (B cells) and T cells, resulting in long-lasting immunity. Here, I genetically fused antigenic epitopes from PRRSV to multiple self-assembling protein nanoparticles and assessed their ability to be produced recombinantly in *E. coli*. The most successful candidate was purified to homogeneity and demonstrated via multiple biochemical and biophysical techniques to assemble like the native nanoparticle. Immunological testing will reveal the candidate’s efficacy as a vaccine against PRRSV.

Keywords

porcine reproductive and respiratory syndrome, subunit vaccine, protein nanoparticles, *Brucella* lumazine synthase
Summary for Lay Audience

The goal of my project is to produce a safe and effective vaccine for porcine reproductive and respiratory syndrome virus (PRRSV), a devastating disease in pigs that accounts for over $600 M in losses per year in the US. Commercially available vaccines for PRRSV provide some control over the virus but none are ideal since some contain damaged but living viruses that in time may cause disease in the pigs, some contain killed viruses or pieces of the virus that lack efficacy in promoting protection against the native virus both short-term and long-term, and some provide no protection against multiple strains of the virus. Vaccines containing whole viruses may be less safe but are generally more effective than vaccines containing free pieces of the virus. My approach to designing a new PRRSV vaccine that is safe and effective involves presenting specific protein sequences from the PRRS virus to the pig’s immune system in a virus-like fashion, without the virus. I will be using stable protein nanostructures as carriers for the chosen peptides from PRRSV; these nanostructures can effectively mimic the architecture of the virus while having no potential for replication. In my work, I genetically fused peptides from PRRSV that are known to stimulate the pig immune system to multiple protein nanostructures and determined their ability to be produced in and purified from a bacterial expression host. The most promising nanostructure was purified and studied using techniques that allow us to determine whether the attachment of the chosen PRRSV peptide to the nanostructure affected its assembly and stability. I demonstrated that I can successfully attach an immunoreactive peptide from PRRSV on to a very stable nanostructure and this product has the potential of being both safe as it cannot cause disease, and effective as it may stimulate a strong protective immune response against PRRSV.
Acknowledgments

I would like to thank my amazing supervisors, Dr. Christopher Garnham and Dr. Rima Menassa, for giving me the opportunity to learn from them over these years and for giving me constant guidance and encouragement. They have improved my abilities as a researcher by challenging me to think more critically, write concisely and convey my thoughts effectively; and for that I am truly grateful.

Many thanks to my co-supervisor, Dr. Kathleen Hill, and my committee member, Dr. Robert Cumming, for their valued comments, suggestions and perspective regarding my work. For their assistance in the lab, I would like to thank Dr. Patrick Telmer and Shane Butler.

I would like to thank Dr. Richard Gardiner for training on the electron microscope and Lee-Ann Briere for training on the circular dichroism spectropolarimeter and assisting in the collection and interpretation of my ultracentrifugation data.

Finally, to my family and friends, thank you for the immense love and support throughout this milestone in my life.
# Table of Contents

Abstract........................................................................................................................................... ii

Summary for Lay Audience.................................................................................................................. iii

Acknowledgments............................................................................................................................... iv

Table of Contents .............................................................................................................................. v

List of Tables ....................................................................................................................................... viii

List of Figures ...................................................................................................................................... ix

List of Abbreviations ........................................................................................................................ xii

Chapter 1 ........................................................................................................................................... 1

1 Introduction ...................................................................................................................................... 1

1.1 Porcine reproductive and respiratory syndrome virus (PRRSV) ................................................. 2

1.1.1 The two major envelope proteins of PRRSV .......................................................................... 4

1.2 Vaccines and vaccination approaches ......................................................................................... 7

1.2.1 PRRSV vaccines ...................................................................................................................... 8

1.3 Virus-like particles ......................................................................................................................... 8

1.4 Platforms of interest .................................................................................................................... 15

1.4.1 Brucella lumazine synthase ..................................................................................................... 15

1.4.2 Aquifex aeolicus lumazine synthase ......................................................................................... 15

1.4.3 Ferritin ...................................................................................................................................... 16

1.4.4 Small heat shock protein .......................................................................................................... 17

1.5 Rationale and goal ....................................................................................................................... 17

1.6 Objectives ................................................................................................................................... 17

Chapter 2 ........................................................................................................................................... 19

2 Experimental Procedures ............................................................................................................. 19

2.1 Construction of chimeras ............................................................................................................. 19
2.2 Expression and purification .................................................................................. 22
  2.2.1 Expression and lysis ..................................................................................... 22
  2.2.2 Immobilized metal affinity chromatography ............................................. 22
  2.2.3 Anion-exchange chromatography ............................................................. 23
  2.2.4 Size-exclusion chromatography ............................................................... 23
2.3 Characterization .................................................................................................. 24
  2.3.1 Transmission electron microscopy ............................................................ 24
  2.3.2 Circular dichroism spectroscopy ............................................................... 24
  2.3.3 Sedimentation velocity ............................................................................. 25

Chapter 3 .................................................................................................................... 26
3 Results ....................................................................................................................... 26
  3.1 The GP5-antigen chosen is conserved and immunogenic ............................ 26
  3.2 Nanoparticles fused with the GP5-antigen are produced but are insoluble .... 29
  3.3 Nanoparticles are enriched and partially purified by immobilized metal-affinity chromatography ................................................................. 32
  3.4 The new M-GP5-BLS construct is an improvement on GP5-BLS ................. 35
  3.5 Size-exclusion chromatography indicates multimeric assembly of BLS and M-GP5-BLS ................................................................................................................. 40
  3.6 Sedimentation velocity analysis indicates that M-GP5-BLS is elongated while BLS is globular ............................................................. 42
  3.7 Secondary structure and thermal stability are conserved in the M-GP5-BLS chimera ................................................................................................. 45
  3.8 Transmission electron microscopy shows pentameric assembly of both BLS and M-GP5-BLS ........................................................................................................... 49

Chapter 4 ....................................................................................................................... 51
4 Discussion ................................................................................................................ 51
  4.1 Selection of candidate PRRSV vaccine ........................................................ 51
  4.2 Structural characterization of BLS and M-GP5-BLS ................................... 55
4.3 Conclusion and future directions ................................................................. 59

References .................................................................................................................. 61

Appendices .................................................................................................................. 73

Curriculum Vitae ......................................................................................................... 75
List of Tables

Table 1. Output summary from CDPro showing percentages of secondary structure assigned to each protein by three different programs (CONTINLL, CDSSTR and SELCON3) using the CDPro protein reference set SMP56. .................................................. 48
List of Figures

Figure 1. Schematic showing the structure of the PRRS virion. ................................................. 3

Figure 2. Schematic diagram showing the predicted topology of the proteins M and GP5 of PRRSV VR-2332 within the virus envelope. ................................................................. 6

Figure 3. Summary of the different types of vaccines highlighting key drawbacks of each. 12

Figure 4. Surface representation of the candidate antigen carriers displayed in PyMOL ........ 14

Figure 5. Surface images of the Brucella lumazine synthase (BLS) fused to the extended representation of the GP5 antigen, displayed in PyMOL ......................................................... 18

Figure 6. Schematic of the designed GP5-fusions ....................................................................... 21

Figure 7. Multiple sequence alignment of the GP5 ectodomain of the PRRS virus from the North American genotype and the European genotype ............................................... 28

Figure 8. Expression and solubility of the wild-type or GP5 engineered protein nanoparticles of interest ..................................................................................................................... 30

Figure 9. Expression and solubilization of engineered nanoparticles with urea............. 31

Figure 10. Immobilized metal affinity chromatography .......................................................... 33

Figure 11. Refolding of engineered nanoparticles ..................................................................... 34

Figure 12. Introduction to M-GP5-BLS construct ................................................................. 37

Figure 13. SDS-PAGE gel analysis of the expression, solubility, nickel purification and refolding of M-GP5-BLS and GP5-M-BLS. ................................................................. 39

Figure 14. Size-exclusion chromatography ............................................................................. 41

Figure 15 Sedimentation coefficient distribution of BLS and M-GP5-BLS ....................... 44

Figure 16. Circular dichroism (CD) spectroscopy of BLS and M-GP5-BLS ....................... 47
Figure 17. Negative stain electron microscopy analysis of BLS and M-GP5-BLS.............. 50
List of Appendices

Appendix 1. Amino acid sequences of all proteins produced recombinantly.............................. 73
List of Abbreviations

AaLS: *Aquifex aeolicus* lumazine synthase

AUC: analytical ultracentrifugation

BLS: *Brucella* lumazine synthase

CD: circular dichroism

EAV: equine arteritis virus

EU: European

GGS: glycine-glycine-serine

GP5: glycoprotein 5

HBcAg: hepatitis B core antigen

His: histidine

IBs: inclusion bodies

IMAC: immobilized metal affinity chromatography

IPTG: isopropyl β-D-1-thiogalactopyranoside

LDV: lactate-dehydrogenase elevating virus

MW: molecular weight

NA: North American

Ni: nickel

Ni-NTA: nickel-charged nitrilotriacetic acid

ORF: open reading frame

PRRSV: porcine reproductive and respiratory syndrome virus

RNA: ribonucleic acid

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

sHSP: small heat shock protein

SV: sedimentation velocity

TEM: transmission electron microscopy
$T_m$: melting temperature

Tris-HCl: tris (hydroxymethyl) aminomethane hydrochloride

UV: ultraviolet

VLP: virus-like particle
Chapter 1

1 Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a viral disease first observed in the US in the 1980s where it was referred to as the mystery swine disease (Benfield et al., 1992; Wensvoort et al., 1991) and it quickly spread throughout North America, being reported in Canada in 1992 and in several European countries thereafter. Currently PRRS is endemic in swine herds worldwide and spread between and within farms occurs most commonly via the introduction of infected pigs to the farm, and direct contact with nasal secretions, mammary secretions, semen, urine and feces of an infected pig. Although less common today, due to the current biosecurity measures, transmission via artificial insemination of sows with imported semen from an infected boar was a major cause of introduction of the virus to herds in other countries (Nathues et al., 2016).

PRRS generally presents itself in two ways, as a reproductive disease in sows and a respiratory disease in growing pigs. Sows infected with the virus have high rates of abortions, stillbirths and even the delivery of mummified piglets. As for the piglets that make it to term, they are generally weak and have low life expectancies (Christianson, 1992). In young pigs infected with the virus, fever, sneezing, pneumonia and stunting of growth are common symptoms (Botner et al., 1997). Currently, PRRS is the most economically significant swine disease affecting the US food industry. In 2012, annual losses due to PRRS were estimated in the US to be $664 million, an increase from the 2005 estimate of $560 million per year (Holtkamp et al., 2013; Neumann et al., 2005). In Canada PRRS was estimated to cost the swine industry $130 million per year (Johnson, 2012).
1.1 Porcine reproductive and respiratory syndrome virus (PRRSV)

PRRSV is a small enveloped RNA virus (50-65 nm) in the genus Arterivirus. Closely-related arterivirus species include equine arteritis virus (EAV) and lactate-dehydrogenase elevating virus (LDV) whose infection of horses and mice respectively, also leads to respiratory failure and abortion (Snijder et al., 2013). Two genotypes of PRRSV exist, the European (genotype 1; EU) and the North American (genotype 2; NA) and they are genetically and antigenically distinct. The prototypic European Lelystad virus and North American VR-2332 isolates, possess only about 60% nucleotide identity and this divergence is thought to be a result of their evolution on separate continents (Allende et al., 1999; Nelsen et al., 1999). In addition, there is a great deal of genomic variation within PRRS viruses isolated from within a continent and even within a given country or state. PRRS viruses of the NA genotype isolated in Central China have been shown to share as low as 88% nucleotide identity genome-wide and as low as 72% identity when comparing individual genes (CAN et al., 2016). This heterogeneity is due to the error-prone nature of the PRRSV RNA polymerase, recombination events and selective pressure in the field (Murtaugh et al., 2010). For this reason, there continues to be genetic and subsequently antigenic difference between PRRS virions, and this poses the greatest challenge to vaccine developers today, and to the general control of the disease.

Both North American and European isolates have a nearly identical genome organization. It consists of a linear positive sense, single stranded RNA genome 15 kb in length with eight open reading frames (ORFs). ORF1a and b encode non-structural proteins involved in replication, while ORF2-7 code for structural proteins. ORFs 2 to 4 encode the minor structural glycoproteins GP2, GP3 and GP4, respectively while ORFs 5 to 7 encode the major structural proteins glycoprotein 5 (GP5), membrane protein (M) and nucleocapsid (N), respectively (Nelsen et al., 1999). Both GP5 and M dominate the virion’s surface and form a heterodimeric structure that is important for virus assembly (Mardassi et al., 1996; Verheije et al., 2002) (Figure 1).
Figure 1. Schematic showing the structure of the PRRS virion. M and GP5 dominate the surface of the virion as a heterodimer while minor structural proteins GP2, GP3, and GP4 exist as a complex within the lipid envelope.
1.1.1 The two major envelope proteins of PRRSV

GP5 is the major glycosylated envelope protein of PRRSV and originates from one of the most variable regions in the viral genome, the ORF5 gene. The ORF5 gene of one PRRSV isolate can share as little as 84% nucleotide identity with that of another isolate from the same genotype (Chen et al., 2006). ORF5 encodes the transmembrane protein GP5 which is typically 200 amino acids long. The first 28-30 amino acids make up the putative signal peptide, which is assumed to be cleaved to give rise to the mature protein (Mardassi et al., 1996). Following this is the domain of GP5 that exists outside the virus, the ectodomain, which is approximately 31 residues in length with two potential N-glycosylation sites. After the ectodomain, there is a long hydrophobic region of about 70 residues that spans the membrane three times, and finally a long hydrophilic domain existing within the virus, the endodomain, of approximately 68 residues (Figure 2). PRRSV GP5 is a target of most of the neutralizing antibodies in an infected animal (Dea et al., 2000), and this is not surprising because it lies exposed at the surface of the virion and is highly abundant. These immunoreactive epitopes involved in virus neutralization are ideal candidates for display on nanoparticles. With production of neutralizing antibodies against PRRSV, uninfected pigs can be protected from viral challenge and infection, while the viral load of infected pigs can be reduced. Using the sera of PRRSV infected pigs and a series of overlapping peptides derived from the GP5 ectodomain of PRRSV VR-2332 strain, Plagemann and colleagues (2002) identified an antibody binding site between amino acids 36 and 52. In agreement with this data, amino acids 37 to 45 were identified as a conserved region that is reactive with pig sera containing high levels of neutralizing antibodies, and is recognized by a known PRRSV-neutralizing antibody (Ostrowski et al., 2002). Neutralizing antibodies have also been shown to target residues 29-35 of the GP5 protein (Wissink et al., 2003) further signifying the potential of the GP5 ectodomain as a target in my vaccine design.

The M protein is the other major structural protein found in the envelope of PRRSV and it originates from the ORF6 gene (Mardassi et al., 1995). M is typically 174 amino acids long and has a similar membrane topology to GP5; it begins with an ectodomain of about 18 residues, traverses the PRRSV membrane three times, and ends
with a C-terminal endodomain of about 72 amino acids (Figure 2). Unlike GP5, M is not glycosylated and considered as the most conserved structural protein of PRRSV (Dea et al., 2000). However, despite being abundant at the surface of the PRRS virion, there are no identified neutralizing epitopes in the ectodomain of M. The large endodomain of M is thought to be the most immunoreactive region of the protein, having B cell epitopes within residues 150-174, however, none of these epitopes are involved in the neutralization of the virus (de Lima et al., 2006). Jiang and colleagues showed that the presence of M, although not inducing neutralizing antibodies against itself, can amplify the immune response and production of neutralizing antibodies against GP5 when co-expressed as a fusion protein (M-GP5) in mice (Jiang et al., 2006).

For PRRSV and two closely related arteriviruses, lactate-dehydrogenase-elevating virus and equine arteritis virus, researchers demonstrated that GP5 and M homologs from all three viruses respectively; coprecipitate when targeted by anti-GP5 and anti-M monoclonal antibodies and comigrate on agarose gels in non-reducing conditions. Upon exposure to reducing conditions, GP5 and M proteins are resolved during electrophoresis indicating their interaction via a disulfide linkage (de Vries et al., 1995; Faaberg et al., 1995; Mardassi et al., 1996). GP5-M heterodimers, formed by the disulfide bridge between their ectodomains (Figure 2), are thought to be essential for virion assembly (Snijder et al., 2003; Verheije et al., 2002).
Figure 2. Schematic diagram showing the predicted topology of the proteins M and GP5 of PRRSV VR-2332 within the virus envelope. Their dimerization via a disulfide bond is shown.
1.2 Vaccines and vaccination approaches

Vaccines are biological agents that allow for the presentation of one or more recognizable features of a disease-causing micro-organism (pathogen) to the immune system. Since the late 18th century with the introduction of the first vaccine by Edward Jenner (Riedel, 2005), vaccines have played a pivotal role in the prevention of infectious diseases and death. The ideal vaccine stimulates a strong immune response and leads to the development of immunological memory cells that essentially remain in the recipients, readily equipping them with the means to prevent disease if exposed to the recognizable pathogen in the future.

Traditionally, vaccination approaches began with the development and administration of chemically inactivated forms of a virus (termed killed vaccines), or by reducing the virulence of a virus by passage in tissue culture or animal hosts (termed ‘live-attenuated’ vaccines). While these approaches have resulted in the development of many effective vaccines, they have their drawbacks (Plotkin, 2014). Killed viruses do not tend to stimulate a sufficiently strong immune response thus it is usually required that recipients receive multiple doses and/or adjuvants to acquire desired immunity. Conversely, live-attenuated viruses are typically very efficient immunological stimulants, but they replicate in the host and can acquire a collection of mutations that has the potential to cause reversion to a disease-causing virus. Another disadvantage associated with some live-attenuated vaccines is that, despite their reduced virulence, these viruses continue to produce proteins that modulate host cell responses to invading viruses, thus preventing an adequate immune response (Renukaradhya et al., 2012).

With an obvious desire for improved vaccines, and an increased understanding of immunobiology, in the early 20th century came the development of subunit vaccines which contain purified or recombinantly-produced proteins from a pathogen. The proteins from a pathogen that induce an immune response are commonly referred to as antigens. While being safer than both killed and live-attenuated vaccines, subunit vaccines lack the ability to effectively stimulate the immune system and possible reasons for this include improper folding of the antigen, inadequate presentation to the immune system and the general instability of the soluble antigens (Chua et al., 2011; Liu et al.,
Like killed vaccines, more doses at higher concentrations of antigens and adjuvants are necessary for protective immunity and thus subunit and killed vaccines are considerably more expensive than live-attenuated vaccines (Liu et al., 2018; Noad and Roy, 2003).

### 1.2.1 PRRSV vaccines

Currently there are two types of PRRSV vaccines commercially available, a live-attenuated and a killed version. As mentioned previously, although successful as vaccines, live-attenuated PRRSV vaccines carry risks associated with their use since there is a potential for reversion to virulence (Botner et al., 1997; Plummer and Manchester, 2011). As for the killed PRRSV vaccines, they are generally associated with insufficient B cell activation, unsatisfactory viral clearance and in controlled experiments they have been shown to provide no protection from disease upon viral challenge (Piras et al., 2005; Renukaradhy et al., 2015). For this reason, killed PRRSV vaccines are no longer used in the United States. Also, since both PRRSV genotypes are distributed worldwide and their divergence can significantly affect vaccine efficacy, many vaccines fail to provide cross-protective immunity; i.e. immunity against heterologous strains of the PRRS virus (Lager et al., 1999; Martelli et al., 2009; Mengeling et al., 2003). With these drawbacks in mind, there is a need for new and innovative vaccines for PRRS.

### 1.3 Virus-like particles

A major advance for subunit vaccines came in the late 20th century when Hepatitis B core antigen (HBcAg), which forms the nucleocapsid of the hepatitis B virus, was expressed in *E. coli*, purified and visualized by electron microscopy (Richmond and Cohen, 1982). HBcAg is formed from 240 copies of a single capsid protein and Cohen and Richmond (1982) were able to show that HBcAg self-assembled to form virus-like particles (VLPs) that were indistinguishable from native Hepatitis B viral core and had comparable antigenic properties. By definition, VLPs are composed of viral structural proteins that
assemble into nanostructures that ultimately resemble a virus. VLPs vary in their level of complexity and HBcAg is an example of a simple VLP (Richmond and Cohen, 1982). More complex VLPs involve the assembly of multiple copies of two or more different structural proteins, for example, the VLP derived from the *Salmonella typhimurium* bacteriophage P22 (VLP P22). VLP P22 is formed from 430 copies of a coat protein along with 100-300 copies of a scaffolding protein (Yoshimura et al., 2016). Beyond VLPs consisting of viral structural proteins alone, there are VLPs composed of viral structural proteins within a lipid envelope (Figure 3). These envelope VLPs occur in cases where viral proteins that are involved in budding of the native virus from infected cells are recombinantly expressed. An example of such a protein is the Gag polyprotein of the human immunodeficiency virus type 1 (Cervera et al., 2013).

Soon after the discovery of HBcAg, researchers took a similar approach to construct and characterize various self-assembling viral proteins and found that several had very attractive features for vaccine development (Christianson, 1992; McAleer et al., 1984; Miyanohara et al., 1986; Thuenemann et al., 2013). They mimicked the structure of the native infectious viruses; they lacked a viral genome (no capacity of self-replication) and they elicited high antibody titers in animals (good immunostimulants). Immediately, VLPs became very appealing candidates as vaccines for the virus from which they originated especially since they also contained no viral proteins that could downregulate the immune system. This meant that lower doses were expected to be necessary to mount a desirable protective immune response. The potency of VLPs in stimulating and sustaining the immune system to the point of long-lasting protection is largely due to the dense repetitive structure of the exposed surface and their particulate nature (Chaplin, 2010).

The immune cells of the adaptive immune response, the B and T cells, are responsible for continued protective immunity and they are both well stimulated upon exposure to VLPs. The polymeric, repetitive surface of VLPs ensures multivalent presentation of epitopes that induce B cells, and the particulate nature of VLPs encourages internalization by antigen presenting cells that later activate T cells (Chaplin, 2010). In comparison, vaccines based on free soluble antigens generally do not stimulate immune cells.
adequately because they have weak, short-lasting interactions with immune cell receptors. A VLP on the other hand, is an assembly of multiple copies of an antigen or antigens that together provide multiple interactions at the surface of immune cells cumulatively strengthening the interaction (López-Sagasta et al., 2016). This long-lasting interaction promotes effective intracellular signaling which leads to a strong immune response and long-lasting immunity. To date, there are successful US FDA approved VLP vaccines for human papillomavirus including Gardasil® (Merck and Co. Inc.) and Cervarix® (GlaxoSmithKline) (Rodríguez-Limas et al., 2013) and VLP vaccines in clinical trials for influenza virus (Roldão et al., 2010), norwalk virus (Roldão et al., 2010) and chikungunya virus (Sun et al., 2010). Figure 3 summarizes the key drawbacks of the different vaccines being used today.

While VLPs have a strong potential for presenting antigens to the immune system in a structured, repetitive and safe format, producing VLPs in general still poses an issue. Unlike HBcAg, where self-assembly is induced simply by increasing salt concentration (Bundy et al., 2008), there are many cases where VLPs require additional biomolecules such as RNA or even additional proteins to induce their assembly. One such example is the VLP made from the coat protein of the bacteriophage MS2. These bacteriophage VLPs consist of 180 copies of a single coat protein that, in native conditions, hold the RNA genome. MS2 bacteriophage VLPs have been successfully used as platforms for a multitude of molecules including antibody fragments, glycoproteins and nucleic acids, however, their assembly relies on a specific stem-loop structure found in the RNA genome (Patel and Swartz, 2011). Production of MS2 VLPs is typically a strenuous procedure involving culturing the virus in E. coli, purification of the virus itself, disassembly of the virus using denaturant, careful precipitation and removal of genomic RNA, removal of the denaturant by dialysis, then reassembly with the addition of the stem-loop RNA (Ashley et al., 2011). Another issue that arises with the use of VLPs is that some VLPs have restrictions on where they can be produced; not all VLPs can be produced in E. coli and yeast, the most common protein expression systems. Cow pea mosaic virus VLPs for example must be produced in plant or insect cells where its initial coat protein polypeptide can be properly processed by a proteinase into its component L and S coat proteins (Saunders et al., 2009).
The difficulty in assembling most VLPs, compounded with the fact that there are limitations on what molecules can be accommodated on any one specific VLP, encouraged the study of nanostructures based on non-viral proteins. Exploring non-viral protein nanoparticles as platforms opened up the possibility of finding more stable and versatile platforms that can carry antigenic peptides that could not be produced as VLPs previously (Frietze et al., 2016). Today, researchers use many different protein nanoparticles as platforms or cages to display chosen antigens in a polymeric and repetitive fashion similar to VLPs, or entrap antigens for delivery (Dalmau et al., 2008; Domingo et al., 2001; Flenniken et al., 2003).
Figure 3. Summary of the different types of vaccines highlighting key drawbacks of each.
Figure 4. Surface representation of the candidate antigen carriers. A. Brucella lumazine synthase (PDB code: 1t13) highlighting from left to right its monomers within the pentamer, the dimer of pentamers and the N-termini (green) of all 10 monomers. B. *Aquifex aeolicus* lumazine synthase (PDB code: 1nqx_1) highlighting from left to right its five monomers within the spherical structure, the C-termini (red) of the five monomers and the C-termini of all 60 monomers. C. *Helicobacter pylori* ferritin (PDB code: 3bvf) highlighting from left to right, two monomers within the spherical structure, the N-termini (green) of the two monomers and the N-termini of all 24 monomers. D. *Methanococcus jannaschii* small heat shock protein (PDB code: 4i88) highlighting from left to right four monomers within the spherical structure, the C-termini (red) of the four monomers and the C-termini of all 24 monomers. Scale bar: 5 nm. All imaged produced in PyMOL.
1.4 Platforms of interest

My goal is to present antigens from porcine reproductive and respiratory syndrome virus (PRRSV; described below) in a multimeric virus-like fashion using self-assembling protein nanoparticles. The nanoparticles to be tested include lumazine synthase from *Brucella*, lumazine synthase from *Aquifex aeolicus*, ferritin from *Helicobacter pylori* and the small heat shock protein from *Methanococcus jannaschii* (Figure 4).

1.4.1 *Brucella* lumazine synthase

Lumazine synthase (LS) is a polymeric bacterial enzyme involved in riboflavin synthesis and exists in various quaternary forms depending on the organism from which it originates. LS isolated from *Brucella* spp. (BLS) can be found in its more commonly known pentameric form (Braden et al., 2000) or as a stable dimer of pentamers joined head to head (Zylberman et al., 2004) (Figure 4A). BLS is well studied and has been shown to be an effective immunostimulant and good candidate for use in vaccination against brucellosis since it can generate both a strong humoral and cell-mediated immune response even in the absence of adjuvants which generally accompany subunit vaccines to establish efficacy (Rossi et al., 2015; Velikovsky et al., 2003, 2002). Structural analysis and previous work on BLS have shown that it is highly resistant to chemical and thermal denaturation and it contains a disordered N-terminus of around 10 amino acids that can be replaced with foreign peptides with no apparent effect on the folding capabilities or stability of the decamer (Bellido et al., 2009; Cassataro et al., 2007; Zylberman et al., 2004) (Figure 4A). As such, it has become a prominent protein carrier of foreign peptides in vaccine development and other biomedical applications (Laplagne et al., 2004; Rosas et al., 2006).

1.4.2 *Aquifex aeolicus* lumazine synthase

LS isolated from the hyperthermophile *Aquifex aeolicus* (AaLS) exists as a hollow icosahedral structure with a diameter of about 15.4 nm made up from 12 LS pentamers (Zhang et al., 2001) (Figure 4B). AaLS has predominantly been used as a cargo system whereby negatively or positively charged amino acid residues are introduced to the interior of the cage to accommodate oppositely charged cargo via electrostatic attraction.
A few examples of such cargo include proteins such as HIV protease (Worsdorfer et al., 2011) and “supercharged” GFP (Worsdorfer et al., 2012), nucleic acids (Lilavivat et al., 2012) and anticancer drugs (Wang et al., 2018). To target drug delivery to certain cells, Min and colleagues successfully added cell targeting peptides to both the C-terminus of AaLS, and within a loop structure of the AaLS monomer that is surface exposed in the final icosahedral structure (Min et al., 2014). By doing this, they showed that AaLS can accommodate foreign peptides in these exterior positions without altering its overall architecture.

1.4.3 Ferritin

Ferritins are a family of globular iron storage proteins that maintain iron homeostasis in organisms from all kingdoms. Free iron is toxic to cells since it participates in the production of harmful reactive oxygen species. Ferritin prevents this by sequestering and converting the harmful ferrous (Fe$^{2+}$) form of iron to its ferric (Fe$^{3+}$) form which is safely compartmentalized as iron (III) oxide (Jameson et al., 2002). Structurally, ferritin is a 24-subunit hollow nanoparticle with a diameter of about 12 nm; dimers form initially and later self-assemble into a dodecameric cage (Cho et al., 2009) (Figure 4C). This cage is thermostable up to 85°C, structurally sound in a wide pH range (3.4 to 10) and resistant to denaturation in relatively high levels of urea and guanidinium at neutral pH (Kim et al., 2011; Linder et al., 1989; Otsuka et al., 1980). Over the years the surface of ferritin has been extensively used as a platform for several molecules including fluorescent dyes, targeting peptides and antibodies (Truffi et al., 2016). This functionalization of ferritin is performed either by chemical conjugation or genetic fusion. Ferritin has been shown to accommodate genetic fusions of foreign peptides at both its termini and in the middle of a flexible loop exposed on its surface (Kanekiyo et al., 2013; H. J. Kang et al., 2012; Y. J. Kang et al., 2012). An N-terminal genetic fusion protein of ferritin has previously been produced whereby the influenza virus hemagglutinin was fused to the surface of the \textit{Helicobacter pylori} ferritin resulting in a more potent influenza vaccine than one that was commercially available (Kanekiyo et al., 2013).
1.4.4 Small heat shock protein

The small heat shock protein (sHSP) is a stress response protein that assists the folding of proteins by stabilizing their folding intermediates. In the thermophilic archaeon, *Methanococcus jannaschii*, sHSP exists as a 24-subunit hollow sphere with a diameter of about 12.4 nm (Quinlan et al., 2013) (Figure 4D). This cage is stable up to 70°C, within a pH range of 5 to 11 and amenable to both genetic and chemical modification (Bova et al., 2002; Flenniken et al., 2006, 2005). Flenniken and colleagues (2006) engineered a cell-targeting protein nanoparticle using *M. jannaschii* sHSP (MjHSP16.5) as a platform by genetically fusing an integrin ligand to the C-terminus of the MjHSP16.5 monomer. The presence of the ligand on each monomer did not affect assembly of the MjHSP16.5 cage; the ligands were displayed on the cage’s exterior and were functional (Flenniken et al., 2006). They also demonstrated that MjHSP16.5 is such a robust structure that it tolerates the chemical conjugation of a functional, cell-targeting monoclonal antibody onto its surface.

1.5 Rationale and goal

Previous work on these nanoparticles of interest demonstrates they are amenable to genetic fusion enabling the creation of stable multivalent structures with a uniform distribution of foreign peptides on their surfaces. The goal of my thesis is to develop a novel PRRSV vaccine prototype via the genetic fusion of antigenic PRRSV peptides to each of the aforementioned proteins.

1.6 Objectives

My objectives are to select a candidate peptide from the PRRS virion to display on the protein nanoparticles of interest, design the fusions between it and the protein monomers, express these recombinant fusion proteins in *E. coli*, identify ones that are well expressed and can be purified, and finally characterize these fusions along with the unfused nanoparticles. Figure 5 shows one example of the fusion proteins I have produced, a surface representation of the extended form of the GP5 peptide displayed on the surface of BLS.
**Figure 5.** Surface images of the Brucella lumazine synthase (BLS) fused to the extended representation of the GP5 antigen. From left to right; side view of BLS, side view of GP5-BLS fusion and top view of GP5-BLS fusion. Black, flexible linker (x4GGS); green, GP5-antigen; red, purification tag (x6His-tag). Scale bar: 5 nm. All images produced in PyMOL.
Chapter 2

2 Experimental Procedures

Genetic constructs were designed and sent to Gene Universal Inc. for synthesis within expression plasmids to overexpress the self-assembling proteins being tested, either fused or unfused to an antigen from PRRSV. These expression plasmids were transformed into Escherichia coli and the various proteins were overexpressed, purified and characterized.

2.1 Construction of chimeras

Amino acids 1 to 60 from the GP5 protein of 110 North American PRRSV strains were aligned to reveal a well conserved region in the ectodomain of GP5. The selected GP5 amino acid sequence was NASNDSSSHQLIYNLCELNGTD, corresponding to the well conserved region (amino acids 30-54) of the GP5 protein (accession number: AAO13196.1) from PRRSV strain VR-2332. The selected M amino acid sequence was MGSSLDFFCHDSTAPQKV, corresponding to the entire first ectodomain (amino acids 1-18) of the M protein (accession number: AAO13196.1) from PRRSV strain VR-2332. The flexible linker chosen was a tetrapeptide repeat of GGS and amino acids 8-158 of BLS (PDB code: 1T13) was used as the BLS monomer. Amino acid sequences were converted to DNA sequences and optimized by Gene Universal Inc. for protein expression in E. coli.

Fused chimeras were synthesized as either N-terminal fusions (GP5-linker-BLS, GP5-linker-Ferritin, M-linker-GP5-linker-BLS) or C-terminal fusions (HSP-linker-GP5, AaLS-linker-GP5); refer to Figure 6. N-terminally fused constructs were cloned into the expression plasmid pET-28a(+) while C-terminally fused constructs were cloned into pET-24a(+) by Gene Universal Inc. Six histidine residues were fused to the N-terminal or C-terminal end of each of the proteins during cloning into pET-28a(+) or pET-24a(+), respectively. BLS was kindly donated by Fernando Bravo-Almonacid (Alfano et al., 2015), amplified using oligonucleotide primers CPG010F (5’-TACTTCCAATCCAAATCGGACACATCCTT-TAAAATCGC-3’) and CPG010R (5’F-TTATCCACTT-CCAATGTTATTAGACAAG-CGCGATGCGGCTGCG-3’R),
isolated and cloned into pET His6 TEV LIC cloning vector (1G); a gift from Scott Gradia (Addgene plasmid # 29655). Site-directed mutagenesis using primers OHH017F (5’-GCAGCAGCCGATCG-CGGCCCTTTGCTT -AATAACATTGG-3’) and OHH017R (5’-CCAATGTTATTAGACAAAGGGCCCGGA -TGCGGCTGC-3’) was performed to replace a missing alanine at amino acid position 156 that is native to BLS.
Figure 6. Schematic of the designed GP5-fusions. From left to right on the N-terminal fusion, the 6x-histidine tag, GP5 amino acids 30-54, 4x GGS linker, monomer of nanoparticle. BLS and ferritin were designed as N-terminal fusions while HSP and AaLS were designed as C-terminal fusions.
2.2 Expression and purification

2.2.1 Expression and lysis

All expression plasmids were transformed into BL21 (DE3) E. coli cells (NEB) and one colony was grown overnight at 37°C in 5 ml of Luria-Bertani broth containing 50 μg/mL kanamycin (LBkan). The overnight culture was used to inoculate a 500 ml culture grown the following day. After an OD of 0.8-1.0 was reached, protein expression was induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 hours at 37°C, with shaking at 250 rpm. The induced cells were harvested by centrifugation using the Sorvall RC5B Plus at 5,465 x g for 10 minutes at a temperature of 4°C.

Cells expressing wild-type recombinant proteins were resuspended in buffer A (50 mM Tris-HCl (pH 8.5), 500 mM NaCl) while those expressing chimeras were resuspended in buffer B (50 mM Tris-HCl (pH 8.5), 500 mM NaCl, 8 M urea, 14.3 mM 2-mercaptoethanol). In the following steps, 14.3 mM 2-mercaptoethanol was present in buffers used to process all engineered recombinants if not stated otherwise. Lysis was performed by sonication (QSonica) at 30W for 5 minutes, using 30/30 seconds on/off rounds. After centrifugation at 20,000 x g for 30 minutes at 4°C, the cleared cell lysate was collected, and imidazole was added to a final concentration of 5 mM.

2.2.2 Immobilized metal affinity chromatography

Immobilized metal affinity chromatography (IMAC) was performed to enrich for His6-tagged proteins since histidine residues have a high affinity for the Ni²⁺-charged resin in the chromatography column. IMAC was performed by passing the cleared cell lysate through 5 mL Ni Sepharose 6 Fast Flow (GE Healthcare) resin in the column then washing with buffer A or buffer B containing first 5 mM imidazole, then 25 mM imidazole. The proteins were eluted in buffer A or B containing 100mM NaCl and 500 mM imidazole. Proteins were analyzed by SDS-PAGE using a 4-20% gradient polyacrylamide gel (Bio-Rad), a Tris-Tricine running buffer (Bio-Rad) and visualized by staining the gel with Coomassie Blue. Eluates containing the proteins of interest were
pooled and dialyzed thoroughly against 50 mM Tris-HCl (pH 8.5), 100 mM NaCl. However, due to the amount of precipitate formed in the dialysis buffer used for the other chimeras (50 mM Tris-HCl (pH 8.5), 100 mM NaCl, 14.3 mM 2-mercaptoethanol), the eluate of the chimera GP5-HSP was dialyzed repeatedly in 50 mM Tris-HCl (pH 8.5), 100 mM NaCl, 2 mM dithiothreitol (DTT); 2 mM DTT was kept in the buffers in future steps.

2.2.3 Anion-exchange chromatography

The dialyzed samples were further enriched by anion-exchange chromatography using an NGC™ Quest plus Fast-Performance Liquid Chromatography (FPLC) system (Bio-Rad) and either an Enrich mono Q 5 x 50 column (Bio-Rad) or HiTrap Q HP 5 x 1 ml column (GE Healthcare). Columns were equilibrated with 50 mM Tris-HCl (pH 8.5), 100 mM NaCl while the elution buffer was 50 mM Tris-HCl (pH 8.5), 1 M NaCl. Typically, runs were carried out at 1 ml/min with a gradient of 0-100% elution buffer over 25 ml and 0.45 ml fractions were collected. Eluted fractions were analyzed by SDS-PAGE and fractions containing the desired protein were pooled and further purified by size-exclusion chromatography.

2.2.4 Size-exclusion chromatography

Size-exclusion chromatography (SEC) was performed to further purify the proteins and acquire an average molecular weight for the self-assembled protein structures. An ENrich SEC 650 10 x 300 mm column (Bio-Rad) equilibrated in 50 mM Tris-HCl (pH 8.5), 100 mM NaCl was used.
2.3 Characterization

2.3.1 Transmission electron microscopy

Pure protein (5 ul) following size-exclusion chromatography was dropped onto 400 mesh copper grids coated with formvar carbon film (Electron Microscopy Sciences), washed with 50 mM Tris-HCl (pH 8.5), 100 mM NaCl then negatively stained for one minute with 2% uranyl acetate. The structure of the purified nanoparticles was imaged using the Jeol JEM-1200EXII transmission electron microscope with an accelerating voltage of 60 kV.

2.3.2 Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy was carried out at the Biomolecular Interactions and Conformations Facility at Western University. Pure BLS at a concentration of 0.79 mg/ml and pure M-GP5-BLS concentrated using Vivaspin 500 (GE Healthcare) to a concentration of 0.77 mg/ml were used for CD analysis. CD spectra were obtained on a Jasco J-810 spectropolarimeter using a 0.1 mm path length quartz cell. Scans were recorded from 260 to 190 nm with a step size of 0.5 nm, a scanning speed of 100 nm/min, a response time of 0.5 s, a data pitch of 0.5 nm, and a band width of 1 nm at 20 °C. To reduce background noise, fifteen spectra were recorded for each sample and the average spectrum obtained as raw data. Buffer scans were subtracted from the raw data and corrected values were converted to molar ellipticity using the concentration values determined from their absorbance at 280 nm measured on a NanoDrop One spectrometer (Thermo Scientific). The melting temperature (T_m) of individual constructs was monitored at 222 nm using a temperature gradient from 50 °C to 105 °C using a step size of 1 °C at a temperature change rate of 1 °C/min. To determine the T_m, the data were fitted by non-linear regression to the equation:

\[
y_{obs} = \frac{(y_n + m_n T) + (y_d + m_d T) \left( e^{\frac{\Delta H_m}{R} \left( \frac{1}{T_m} - \frac{1}{T} \right)} \right)}{1 + e^{\frac{\Delta H_m}{R} \left( \frac{1}{T_m} - \frac{1}{T} \right)}}
\]
In Equation 1, \( y_{\text{obs}} \) is the absorbance observed during the experiment, \( y_n \) and \( y_d \) are the y-intercepts of the native and denatured baselines respectively, \( m_n \) and \( m_d \) are the slopes of the native and denatured baselines respectively, \( T \) is the temperature in degrees Kelvin, \( T_m \) is the melting temperature, \( R \) is the gas constant and \( \Delta H_m \) is the enthalpy of unfolding.

### 2.3.3 Sedimentation velocity

Sedimentation velocity studies were performed to provide information about the molecular weight and shape of the purified nanoparticles. These studies were carried out at the Biomolecular Interactions and Conformations Facility at Western University, using a Beckman Optima XL-A Analytical Ultracentrifuge. Samples were loaded into a double-sector cells with Epon charcoal centerpieces and centrifugation was carried out at 20 °C using an An60Ti rotor. Absorbance was monitored at 280 nm and scans were taken every 10 minutes for a total of 45 scans, in 0.002 cm radial steps. BLS was sedimented at a speed of 30,000 rpm and M-GP5-BLS at 25,000 rpm. Three samples of BLS and M-GP5-BLS at various concentrations were analyzed by sedimentation velocity in buffer containing 50 mM Tris-HCl and 250 mM NaCl. Prior to sedimentation, for both BLS and M-GP5-BLS, concentrations were adjusted to 0.3, 0.6 and 0.9 mg/ml assuming an \( \text{OD}_{280} \) of 1 is equal to 1 mg/ml; measured on the NanoDrop One (Thermo Scientific). Partial specific volumes (vbar) of BLS (vbar = 0.740) and M-GP5-BLS (vbar = 0.727) were calculated from their amino acid compositions using the program SEDNTERP. Buffer density (1.0099 g/mL) and viscosity (0.0104 Poise) were also calculated with SEDNTERP. Sedimentation data were analyzed using the c(s) distribution model in Sedfit and frictional ratios for BLS \((f/f_0=1.3)\) and M-GP5-BLS \((f/f_0=1.5)\) were estimated by non-linear regression in order to get the best-fit c(s) distribution. Data were normalized in GUSSI and exported to GraphPad Prism for figure making.
Chapter 3

3 Results

3.1 The GP5-antigen chosen is conserved and immunogenic

When selecting a PRRSV peptide sequence to genetically fuse to the nanoparticles of interest, there are several characteristics I sought to increase the likelihood of obtaining an effective vaccine. Ideally, the peptide should be immunogenic, abundantly displayed on the surface of the virus, and conserved across strains. An immunogenic peptide will effectively induce the immune response and increase the likelihood of long-lasting protection against PRRSV. A peptide that is abundantly displayed on the surface of the virion will provide multiple binding points for antibodies and increase the chances of PRRSV neutralization upon infection. Finally, a peptide sequence that is conserved, common to many strains of a virus, is likely to induce the production of antibodies that will target this sequence in all the different strains of the virus in which this target sequence lies. Therefore, a conserved, immunogenic and surface exposed peptide is ideal in my vaccine design as it will have the greatest potential to provide protection against multiple strains, and potentially genotypes (North American and European) of the virus.

I selected a peptide from the ectodomain of the GP5 protein because it dominates the surface of the virion, and within its ectodomain lies an immunogenic and conserved region (Mardassi et al., 1996; Ostrowski et al., 2002; Plagemann et al., 2002). Figure 7 illustrates the conservation present within the PRRSV GP5 ectodomain from both genotypes. One-hundred and ten isolates from each genotype (NA and EU) were aligned and results showed that within each genotype, the ectodomain of GP5 is well conserved. Amino acids 40-56 of the NA genotype and 42-58 of the EU genotype have the consensus sequence Q(L/Y)IYNLT(I/L)CELNGTDWL, whereby only two amino acids in their sequences usually differ. This strongly indicates conservation of the GP5 ectodomain between genotypes. Figure 7 also shows that the putative transmembrane signal peptide, the first 28-30 amino acids of GP5 that are cleaved from the mature GP5 protein (Mardassi et al., 1996), contains many hydrophobic amino acids.
I chose amino acids 30-54 of the GP5 membrane protein from the NA genotype which excludes the hydrophobic signal peptide and includes the well conserved region present within the NA genotype and the conserved region between both genotypes.
North American

European

**Figure 7.** WebLogo (Crooks et al., 2004; Schneider et al., 1990) representation of the multiple sequence alignment of the GP5 ectodomain of the PRRS virus from the North American genotype and the European genotype. 110 isolates were aligned for both genotypes and the height of the letters indicates the sequence conservation at that position. Hydrophobic amino acids are colored black, charged blue and neutral green. The chosen GP5-antigen is boxed in red.
3.2 Nanoparticles fused with the GP5-antigen are produced but are insoluble

Initial screening of the non-engineered nanoparticles showed robust over-expression following induction with IPTG and most of these nanoparticles were soluble post-lysis (Figure 8; BLS, sHSP 16.5, Ferritin). Lumazine synthase from *Aquifex aeolicus* (gel not shown) overexpressed well, however, it was almost completely insoluble. Conversely, the engineered nanoparticles, bearing GP5 genetically fused at specific termini express, however, all were insoluble post lysis as indicated by the presence of the protein exclusively in the pelleted fraction (Figure 8; GP5-BLS, sHSP 16.5-GP5, GP5-Ferritin).

To resolubilize these engineered constructs, I added 8M urea to the lysis buffer. With this addition, a significant portion of the nanoparticle chimeras were resolubilized (Figure 9).
Figure 8. Expression and solubility of the wild-type or GP5 engineered protein nanoparticles of interest. Coomassie blue-stained SDS-PAGE gel analysis of each nanoparticle overexpressed in *E. coli* BL-21 (DE3) cells with 0.5 mM IPTG for 3 hours at 37°C and lysed in TBS (50 mM Tris-HCL (pH 8.5), 500 mM NaCl). Pr, pre-induction; Po, post-induction; Sup, supernatant; Pell, pellet; BLS, *Brucella* lumazine synthase; sHSP 16.5, small heat shock protein. Red arrows highlight the monomer that ran on the gel at its expected size (BLS, 18.8 kDa; GP5-BLS, 22.4 kDa; sHSP 16.5, 17.5 kDa; sHSP 16.5-GP5, 21 kDa; Ferritin, 21.6 kDa; GP5-Ferritin, 25.1 kDa).
Figure 9. Expression and solubilization of engineered nanoparticles with urea.

Coomassie blue-stained SDS-PAGE gel analysis of each chimera overexpressed in *E. coli* BL-21 (DE3) cells with 0.5 mM IPTG for 3 hours at 37°C and lysed in TBS-urea (50 mM Tris-HCL (pH 8.5), 500 mM NaCl, 8 M urea, 14.3 mM 2-mercaptoethanol). Pr, pre-induction; Po, post-induction; Sup, supernatant; Pell, pellet; BLS, *Brucella* lumazine synthase; sHSP 16.5, small heat shock protein. Red arrows highlight the monomer that ran on the gel at its expected size (GP5-BLS, 22.4 kDa; sHSP 16.5-GP5, 21 kDa; GP5-Ferritin, 25.1 kDa).
3.3 Nanoparticles are enriched and partially purified by immobilized metal-affinity chromatography

All soluble recombinant proteins were initially purified by immobilized metal affinity chromatography (IMAC). IMAC allows for the enrichment of histidine-tagged recombinant proteins since the polyhistidine tag (usually a hexahistidine tag) has a high affinity for the resin when charged with nickel ions. The interaction between the polyhistidine tag and these immobilized metal ions is independent of the protein’s fold and the fold of the tag itself. Since there is no specific conformation needed, this allows for purification of the engineered constructs to occur under denaturing conditions such as 8 M urea. The nickel-charged nitrilotriacetic acid (Ni-NTA) resin binds His-tagged proteins with high affinity but interacts poorly with other non-tagged proteins.

The IMAC step shows that wild-type nanoparticles can be enriched, with BLS and sHSP 16.5 showing the greatest enrichment, followed by ferritin where a good portion was found in the flow-through and thus did not bind (Figure 10A). As for the chimeras, there is a clear enrichment of sHSP-16.5-GP5 and GP5-Ferritin even though some of each is lost during the process to the flow-through and the washes (Figure 10B); perhaps as a consequence of the high concentration of urea or the protein was not properly resolubilized to expose the His-tag. GP5-BLS in general did not express well (Figure 8) and was difficult to enrich via IMAC (Figure 10B).

To refold the engineered constructs following IMAC, each one was dialyzed exhaustively in buffer containing no urea. Figure 11 shows that a significant proportion of each construct was resolubilized following dialysis to remove urea. However, a large fraction of each construct could not be resolubilized. In an effort to increase the yield of my potential vaccine candidates, constructs were redesigned taking into account evidence that shows that the ectodomain of the PRRSV GP5 protein interacts with that of the M protein ectodomain via a disulfide linkage (de Vries et al., 1995; Faaberg et al., 1995; Mardassi et al., 1996).
Figure 10. Immobilized metal affinity chromatography. Coomassie blue-stained SDS-PAGE gel analysis of fractions collected during the purification of the soluble fraction of each cell lysate. A. Purifications in the absence of urea. B. Purifications in the presence of 8M urea. S, sample loaded; FT, flow-through; 5, 25 and 500 refer to the mM concentration of imidazole present in the buffers used for washing and eluting the His-tagged protein from the Ni$^{2+}$ resin. Each numbered lane corresponds to a 5 ml wash or elution fraction. BLS, *Brucella* lumazine synthase; sHSP 16.5, small heat shock protein. Red arrows direct you to the monomer which ran on the gel at its expected size (BLS, 18.8 kDa; GP5-BLS, 22.4 kDa; sHSP 16.5, 17.5 kDa; sHSP 16.5-GP5, 21 kDa; Ferritin, 21.6 kDa; GP5-Ferritin, 25.1 kDa).
**Figure 11.** Refolding of engineered nanoparticles. Coomassie blue-stained SDS-PAGE gel analysis of the supernatant and pelleted fractions post-centrifugation of each dialyzed sample. Elutions from the nickel purifications of each protein were pooled and dialyzed against 50 mM Tris-HCl, 100 mM NaCl with 14.3 mM 2-mercaptoethanol (GP5-BLS and GP5-Ferritin) or 2 mM dithiothreitol (HSP-16.5-GP5). BLS, *Brucella* lumazine synthase; sHSP 16.5, small heat shock protein; sup, supernatant; pell, pellet.
3.4 The new M-GP5-BLS construct is an improvement on GP5-BLS

Previous research indicates that the structural proteins M and GP5 form a complex in the outer-envelope of the virus, and this complex is assisted via a disulfide bond between their solvent-exposed ectodomains (Mardassi et al., 1996) (Figure 2). It is distinctly possible that this interaction creates unique antigenic epitopes not present in their individual linear sequences. Unfortunately, a disulfide bond interaction in *E. coli* is unlikely to be replicated since the cytoplasm of the bacterium is generally maintained as a reducing environment where disulfide bonds are uncommon. However, if this interaction is favorable and beneficial to the overall structure and solubility of the ectodomains of these proteins, the presence of the M ectodomain paired with my chosen GP5 antigen may improve refolding of the engineered particles in a non-reducing environment.

Therefore, in an attempt to maximize the amount of properly folded engineered nanoparticles acquired that display antigenic epitopes in a more natural manner, I designed two constructs using BLS as the carrier; M-GP5-BLS and GP5-M-BLS. BLS was chosen as the carrier for the new antigenic epitopes because compared to GP5-Ferritin and HSP-GP5, GP5-BLS displayed greater homogeneity and stability during size-exclusion chromatography (SEC). SEC results of GP5-Ferritin and HSP-GP5 showed multiple elution peaks containing those proteins indicating greater dissociation of their monomers compared to GP5-BLS, which generally eluted in one homogeneous peak (data not shown).

In the case of M-GP5-BLS, starting from the N-terminus of the fusion protein, the first ectodomain of the M protein, linked to the chosen antigenic GP5 sequence by a flexible linker, and finally, GP5 is linked to the N-terminus of BLS via the same flexible linker (Figure 12). With the GP5-M-BLS construct, the amino acid sequence of GP5 and M is switched to test if the orientation of their amino acid sequences may affect their interaction via the disulfide bond. The first ectodomain of M was chosen from the M protein of the same PRRSV strain we selected the GP5-antigen (PRRSV strain VR-2332).
With these new constructs synthesized, transformed into *E. coli* BL-21 cells and expression induced, M-GP5-BLS expressed very well while GP5-M-BLS did not (Figure 13A). Upon lysis of these cells in non-denaturing conditions, it was evident that similarly to all the other engineered nanoparticles, they were insoluble (Figure 13B) and thus misfolded to form inclusion bodies within the *E. coli* cells. Therefore, to retrieve these proteins, urea was included in the lysis buffer prior to IMAC. Analysis of the eluted fractions from the nickel column shows that M-GP5-BLS eluted with high purity while GP5-M-BLS was noticeably less pure in comparison. While there was one prominent protein in the elution of M-GP5-BLS that ran on the SDS-PAGE gel at the expected MW (25.2 kDa), there were three prominent protein species in that of GP5-M-BLS (Figure 13C). The highest prominent band on the GP5-M-BLS SDS-PAGE gel ran at the expected size of GP5-M-BLS (25.3 kDa) while the second highest around 23 kDa and the smallest, around 18 kDa (Figure 13C). These lower molecular weight proteins are suspected to be degradation products of GP5-M-BLS and therefore M-GP5-BLS appears to be the more favorable and stable orientation in the context of BLS as the carrier.

M-GP5-BLS was then dialyzed exhaustively against buffer without urea or reducing agent (50 mM Tris-HCl (pH 8.5), 100 mM NaCl). Analysis of the supernatant and pellet after removal of urea shows that a large fraction of M-GP5-BLS refolded and regained solubility (Figure 13D). When dialyzed in a reducing environment a larger fraction of M-GP5-BLS recovered its solubility upon removal of urea (Figure 13D). Therefore, there was no clear indication that a disulfide bridge between the GP5 antigen and first ectodomain of the M protein aided in the refolding of this new construct. However, there was a clear improvement in expression, purification and dialysis of M-GP5-BLS compared to the GP5-BLS construct. There was greater expression of M-GP5-BLS, its purity following IMAC was markedly improved and upon removal of urea, a majority of M-GP5-BLS remained soluble unlike GP5-BLS. M-GP5-BLS was chosen as my vaccine candidate for future purification and characterization.
**Figure 12.** Schematic of the designed M-GP5-BLS construct, from left to right the 6x-hisitidine tag, M protein amino acids 1-18, 4x GGS linker, GP5 protein amino acids 30-54, 4x GGS linker, BLS amino acids 8-158.
A.  

<table>
<thead>
<tr>
<th></th>
<th>GP5-M</th>
<th>M-GP5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pr Pr</td>
<td>Po Po</td>
<td>Po Po</td>
</tr>
</tbody>
</table>

B.  

<table>
<thead>
<tr>
<th></th>
<th>M-GP5</th>
<th>GP5-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sup Pell</td>
<td>Sup Pell</td>
<td>Sup Pell</td>
</tr>
</tbody>
</table>

C.  

<table>
<thead>
<tr>
<th></th>
<th>GP5-M-BLS</th>
<th>M-GP5-BLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>S 5 25 500 500</td>
<td>S 5 25 500 500</td>
<td>S 5 25 500 500</td>
</tr>
</tbody>
</table>

D.  

<table>
<thead>
<tr>
<th></th>
<th>M-GP5-</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLS</td>
<td>BLS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Sup Pell</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-reducing</td>
<td>reducing</td>
</tr>
</tbody>
</table>
**Figure 13.** Coomassie blue-stained SDS-PAGE gel analysis of the expression, solubility, nickel purification and refolding of M-GP5-BLS and GP5-M-BLS. A. Each chimera was overexpressed in *E. coli* BL-21 (DE3) cells with 0.5 mM IPTG for 3 hours at 37°C; Pr, pre-induction; Po, post-induction. B. Supernatant versus pelleted fraction of cell lysates lysed in in TBS (50 mM Tris-HCL (pH 8.5), 500 mM NaCl); Sup, supernatant; Pell, pellet. C. IMAC of the soluble fraction of each cell lysate obtained under denaturing conditions; S, sample loaded; FT, flow-through; 5, 25 and 500 refer to the mM concentration of imidazole present in the buffers used for washing and eluting the His-tagged protein from the Ni\(^{2+}\) resin. D. Supernatant and pelleted fractions of each sample dialyzed against either 50 mM Tris-HCL (pH 8.5), 100 mM NaCl, 14.3 mM 2-mercaptoethanol (reducing) or 50 mM Tris-HCL (pH 8.5), 100 mM NaCl (non-reducing). Red arrows points to the monomer which ran on the gel at its expected MW (M-GP5-BLS, 25.2 kDa; GP5-M-BLS, 25.3 kDa).
3.5 Size-exclusion chromatography indicates multimeric assembly of BLS and M-GP5-BLS

Following IMAC and dialysis of BLS and M-GP5-BLS into low salt buffers, they were further purified by anion-exchange chromatography using a salt gradient from 100 mM to 1 M NaCl. Fractions bearing wild-type and engineered constructs were pooled and concentrated for analysis by size-exclusion chromatography. The expected decameric molecular weights of BLS and M-GP5-BLS are 188 kDa and 252 kDa respectively.

The elution profile of BLS shows a single, monodisperse peak with an elution volume of 13 ml corresponding to an apparent MW of approximately 172 kDa. BLS was the only protein in the eluted fraction as indicated by SDS-PAGE (Figure 14). The elution profile of M-GP5-BLS contains a prominent peak of pure M-GP5-BLS at 11.6 ml, corresponding to an apparent MW of 505 kDa. Additional peaks and valleys were also observed (Figure 14), however, SDS-PAGE analysis revealed no additional proteins were present. The over-estimated MW of the prominent protein species in the M-GP5-BLS sample may be due to the shape of the M-GP5-BLS decamer (See section 4.2).
Figure 14. Size-exclusion chromatography. Elution profiles of BLS (black line) and M-GP5-BLS (blue line) monitored at a wavelength of 280 nm. Insets of SDS-PAGE gels indicate purity of each peak with a monomer migrating at the appropriate size; 25.2 kDa for M-GP5-BLS and 18.8 kDa for BLS. S represents each sample prior to size-exclusion chromatography. Elution volumes of the protein standards used to calibrate the size-exclusion column are indicated above the chromatogram (670 kDa, thyroglobulin; 158 kDa, bovine γ globulin).
3.6 Sedimentation velocity analysis indicates that M-GP5-BLS is elongated while BLS is globular

Sedimentation velocity (SV) is an analytical ultracentrifugation method whereby molecules in solution are subjected to a high centrifugal force. The rate at which they move in response to this force is measured and provides information about the molecular weight (MW) and shape of those molecules. Therefore, SV experiments were performed to characterize and compare the shape and MW of BLS and M-GP5-BLS. The sedimentation coefficient $c(s)$ distribution of BLS standardized to conditions corresponding to pure water at 20°C ($s_{20,w}$) shows a singular, sharp peak at an $s_{20,w}$ value of 8.1 S (Figure 15A). This monodispersed distribution, with no other apparent peaks, indicates that BLS forms a single structure that is stable and well-behaved in 50 mM Tris-HCl (pH 8.5), 250 mM NaCl at 20°C, with no formation of aggregates. With this type of $c(s)$ distribution, a proper molecular mass distribution can be determined and with that, the average molecular mass of BLS was calculated to be 178 kDa. This calculation is a close approximation of the MW of decameric BLS whose expected MW is 188 kDa. The frictional ratio of BLS was estimated to be 1.3 and this value informs on the globular and symmetrical shape of the structure. In general, a perfectly spherical, compact and smooth protein would have a minimum value of 1.0 and as the protein structure shifts from globular to elongated, there is an increase in frictional ratio. Globular proteins typically have frictional ratios ranging from 1.05 to 1.30 (Unzai, 2018).

The $c(s)$ distribution of M-GP5-BLS in Figure 15B shows three peaks with the majority of the engineered protein existing as a structure with the $s_{20,w}$ value of 8.8 S. This tells us that the majority of M-GP5-BLS assembles into a structure larger than BLS, as indicated by the larger $s_{20,w}$ value. The presence of multiple peaks indicates the presence of aggregates in the cell. Although the $c(s)$ distribution can be converted to molecular mass distribution, with the existence of multiple peaks, calculation of the molecular mass of each species at each peak is not expected to be accurate due to the assignment of one frictional ratio to all species in one given run. However, the average molecular mass of M-GP5-BLS calculated for the $s_{20,w}$ peak at 8.8 was 220 kDa. This calculation is agreeable with the MW of decameric M-GP5-BLS whose expected MW is 252 kDa. As
for the shape of M-GP5-BLS, its frictional ratio of 1.5 indicates that it is not globular in nature but rather an elongated protein structure.
Figure 15 Sedimentation coefficient distribution of BLS (A.) and M-GP5-BLS (B.). Normalized sedimentation coefficient distribution, c(s), is plotted against the sedimentation coefficient, $s_{20,w}$. Sedimentation velocity experiments were conducted at an initial protein concentration of 0.78 mg/ml (BLS) and 0.74 mg/ml (M-GP5-BLS) in 50 mM Tris-HCl (pH 8.5), 250 mM NaCl at 20°C. Data were collected at rotor speeds of 30,000 rpm and 25,000 rpm for BLS and M-GP5-BLS respectively. The calculated values of the weight-average $s_{20,w}$ and frictional ratio of BLS are 8.1 and 1.3, respectively. The calculated values of the weight-average $s_{20,w}$ and frictional ratio of M-GP5-BLS are 8.8 and 1.5.
Secondary structure and thermal stability are conserved in the M-GP5-BLS chimera

The far ultraviolet (UV)-circular dichroism spectra of BLS was compared with that of both the BLS and chimeric M-GP5-BLS constructs which were extracted and processed by IMAC in denaturing buffer (contained 8M urea) before being refolded by dialysis. All steps following, anion-exchange chromatography and size-exclusion chromatography were performed similarly to BLS not exposed to urea. The spectra of wild-type BLS purified in the presence or absence of urea were virtually identical (Figure 16A.). Both BLS spectra had two minima, one located at 220.5 nm and the other at 211 nm. As for the chimera M-GP5-BLS, the overall shape of the spectra resembled that of BLS, with its minima falling at 220 nm and 210 nm.

CDPro, a software package containing three different programs often used for secondary structure assignment of proteins, facilitated the analysis of the CD spectra obtained for each protein (Table 1). All analytical programs assigned a majority (>65%) of α-helical content to BLS and BLS refolded with very little (<4.5%) β-sheet prediction, along with about 15-20% unordered structure. These values are not what was expected since based on crystal structures of BLS, it is 50-53% α-helical and 18-19% β-sheet (Kabsch et al., 1983; Klinke et al., 2005; Zylberman et al., 2004). Since the α-helical content of BLS was overestimated and β-sheet content underestimated, this indicates that the software prediction used herein could not accurately discern the percentages of secondary structure using the CD spectra obtained. However, assuming that α-helical content will be overestimated and β-sheet content underestimated between different samples, changes in secondary structure content between samples will still provide valid information.

The majority of M-GP5-BLS, like BLS, is also predicted to be α-helical however there is a general decrease in the α-helical nature assigned to M-GP5-BLS compared to that of BLS alone; with a 55% α-helical prediction being the highest assigned (Table 1 CDSSTR). M-GP5 consists of 87 amino acids including a His-tag, the first ectodomain of PRRSV M protein, a 4x GGS linker, the chosen GP5 antigen followed by another 4x GGS linker (Figure 12). Phyre2 secondary structure prediction software predicted the secondary structure of M-GP5 to be 16% α-helical, 25% β-sheet and 60% disordered
(Kelley et al., 2015). This prediction agrees with the analytical programs that predicted an increase in percentages of β-sheet, turn and disordered structure upon addition of M-GP5 to BLS. The changes are likely due to the addition of the antigen affecting the cumulative absorbance of the BLS core structure.

To compare the thermal stability of BLS with that of the engineered counterpart, M-GP5-BLS, their CD signals were monitored at 222 nm as a function of temperature. Figure 16 B and C show the thermal denaturation curves of BLS and M-GP5-BLS respectively. The estimated melting temperature for BLS is 92.1 °C while that of M-GP5-BLS is 91.5 °C which indicates that the addition of the antigen did not affect the thermal stability of the carrier, BLS.
Figure 16. Circular dichroism (CD) spectroscopy of BLS and M-GP5-BLS. A. Far UV CD spectra of BLS (solid line), BLS refolded (dashed line) and M-GP5-BLS (dotted line). Results are representative of three experiments each with absorbances averaged before conversion to mean residue ellipticity (MRE). B. CD signal of BLS measured at 222 nm as a function of increasing temperature; melting point estimated by non-linear regression fit at 92.1 °C. C. CD signal of M-GP5-BLS measured at 222 nm as a function of increasing temperature; melting point estimated by non-linear regression fit at 91.5 °C.
**Table 1.** Output summary from CDPro showing percentages of secondary structure assigned to each protein by three different programs (CONTINLL, CDSSTR and SELCON3) using the CDPro protein reference set SMP56.

<table>
<thead>
<tr>
<th>CONTINLL</th>
<th>Secondary structure assignment</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α- helix (%)</td>
<td>β-sheet (%)</td>
<td>Turn (%)</td>
<td>Unordered (%)</td>
</tr>
<tr>
<td>BLS</td>
<td>66.1</td>
<td>4.6</td>
<td>9.2</td>
<td>20.1</td>
</tr>
<tr>
<td>BLS refolded</td>
<td>68.7</td>
<td>3.4</td>
<td>8.5</td>
<td>19.4</td>
</tr>
<tr>
<td>M-GP5-BLS</td>
<td>48</td>
<td>10.2</td>
<td>15.5</td>
<td>26.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CDSSTR</th>
<th>Secondary structure assignment</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α- helix (%)</td>
<td>β-sheet (%)</td>
<td>Turn (%)</td>
<td>Unordered (%)</td>
</tr>
<tr>
<td>BLS</td>
<td>67.8</td>
<td>4.5</td>
<td>9</td>
<td>19.1</td>
</tr>
<tr>
<td>BLS refolded</td>
<td>72.2</td>
<td>2</td>
<td>5.9</td>
<td>15</td>
</tr>
<tr>
<td>M-GP5-BLS</td>
<td>54.9</td>
<td>9.7</td>
<td>12.2</td>
<td>23</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SELCON3</th>
<th>Secondary structure assignment</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α- helix (%)</td>
<td>β-sheet (%)</td>
<td>Turn (%)</td>
<td>Unordered (%)</td>
</tr>
<tr>
<td>BLS</td>
<td>67.7</td>
<td>4.1</td>
<td>9.1</td>
<td>19.4</td>
</tr>
<tr>
<td>BLS refolded</td>
<td>71.2</td>
<td>3.3</td>
<td>7.7</td>
<td>19.1</td>
</tr>
<tr>
<td>M-GP5-BLS</td>
<td>53</td>
<td>7.9</td>
<td>15.1</td>
<td>25.2</td>
</tr>
</tbody>
</table>
3.8 Transmission electron microscopy shows pentameric assembly of both BLS and M-GP5-BLS

Nanoparticle assembly was visualized by transmission electron microscopy (TEM) for both BLS and M-GP5-BLS. The two proteins, purified by size-exclusion chromatography, were negatively stained with uranyl acetate and examined. Both BLS and M-GP5-BLS appear to form pentameric structures with an approximate diameter of 5-7 nm (Figure 17). TEM images showed no noticeable difference in sizes between BLS and M-GP5-BLS decamers. However, images obtained for BLS generally contained less clusters than those obtained for M-GP5-BLS indicating that BLS is less likely to form unwanted aggregates.
Figure 17. Negative stain electron microscopy analysis of BLS (A.) and M-GP5-BLS (B.) purified by size-exclusion chromatography. Pentameric structures with diameters between 5-7 nm were observed for both BLS and M-GP5-BLS. Scale bar: 50 nm.
Chapter 4

4 Discussion

To create a vaccine candidate for porcine reproductive and respiratory syndrome (PRRS), four protein nanoparticles were screened as potential carriers for antigens derived from the PRRS virus (PRRSV). These engineered nanoparticles were screened to discover ones that were well overexpressed, soluble, stable and easily purified. Here, I report the generation, purification and characterization of a PRRS vaccine candidate comprising a fusion between *Brucella* lumazine synthase and select peptides from both major structural proteins of PRRSV, the M protein (M) and glycoprotein 5 (GP5).

4.1 Selection of candidate PRRSV vaccine

Initially, all potential carriers (BLS, AaLS, Ferritin, sHSP-16.5) were genetically fused with a GP5 peptide derived from the N-terminal ectodomain of the PRRSV GP5 membrane protein. In theory, the ectodomain of GP5 is an ideal peptide to display on the nanoparticles of interest as it is conserved and immunoreactive (Mardassi et al., 1996; Ostrowski et al., 2002; Plagemann et al., 2002; Wissink et al., 2003). I chose to display residues 30 to 54 of GP5, encompassing the neutralizing epitopes identified previously (Ostrowski et al., 2002; Plagemann et al., 2002; Wissink et al., 2003).

These fusion proteins expressed well in *E. coli* BL-21 cells when induced, however, they aggregated and formed inclusion bodies which hindered further purification by IMAC. Inclusion bodies (IBs) are dense, insoluble protein aggregates that are commonly observed in *E. coli* cells expressing recombinant proteins (Baneyx and Mujacic, 2004). IBs are generally considered to be a negative aspect of recombinant protein production because proteins concentrated within IBs require additional processing such as solubilization using a protein denaturant, and subsequent protein refolding steps. A protein’s solubility has long been considered a major indicator of it having reached its proper conformation. Thus, in this case where the fused nanoparticles are all almost completely insoluble (Figure 8), it appears that the foreign peptide is preventing them from folding properly to reach their usually soluble conformation.
The GP5 peptide I selected is composed of residues 30-54 of the GP5 membrane protein from PRRSV and this peptide was selected for display on the nanoparticles of interest because the sequence is exposed on the surface of the virion, well conserved and immunogenic (Ostrowski et al., 2002; Plagemann et al., 2002). However, this sequence includes four potential N-glycosylation sites at positions 30, 33, 44 and 51. In general, N-glycosylation is important for proper folding and solubility of proteins and N-glycans also play a role in the protein’s biological activity by serving as a recognition tag that allows these proteins to interact with receptors involved in transmembrane signaling (Helenius and Aebi, 2004). With respect to GP5, previous work showed that the potential N-glycosylation sites N33, N44 and N51 are all occupied by glycan moieties in fully matured PRRSV GP5 (Ansari et al., 2006). The glycosylation site N44 has been repeatedly shown to be essential for viral particle formation; mutations at this site drastically reduce formation, release and infectivity of PRRSV (Meulenberg et al., 1995; Wissink et al., 2005). Since I used E. coli as an expression system, those N-glycans are absent from the fusion proteins produced and it is likely that their absence is negatively affecting the solubility of the PRRSV GP5 sequence chosen for display. Also in respect to the absence of N-glycans present on the candidate vaccines, their efficacy is a concern since in the case where neutralizing antibodies are produced against the chosen ectodomain sequence, there is still the possibility that N-glycans present on the native GP5 membrane protein will prevent binding of these antibodies to that ectodomain sequence on the surface of the PRRS virion (Vu et al., 2011).

To overcome the insolubility of the engineered proteins and acquire soluble protein for purification and future characterization, urea, a strong denaturant, was included in the lysis buffer preparation at a concentration of 8M (Figure 9). The use of urea is acceptable in laboratory-scale production for research purposes, however, from the standpoint of industrial production, use of urea in the production of a vaccine would be greatly discouraged. Urea essentially denatures proteins that form IBs and upon purification of the desired recombinants proteins, refolding steps must be performed to recover structured, functional recombinants. A significant portion of the GP5 engineered proteins are lost during the refolding steps (Figure 11) and therefore proteins produced in IBs are not marketable products; recovery is low, and the functionality of the product is
questioned. It has been common practice to discard recombinant proteins produced in IBs (Baneyx and Mujacic, 2004). However, there are some recovery and refolding strategies for proteins solubilized by urea but they are often only successful on a case by case basis so there is still no standard for refolding and recovery of potential therapeutic proteins from IBs (Ferrer-Miralles et al., 2009). To recover the recombinant proteins from their denatured state in urea, I first performed stepwise dialysis in 2M increments from 8M urea to no urea, but acquired similar recovery levels as with direct dialysis into 50 mM Tris-HCl (pH 8.5), 100 mM NaCl, 14.3 mM 2-mercaptoethanol, so the latter was kept as routine.

I was able to enrich for the engineered proteins by Ni-NTA chromatography under denaturing conditions (Figure 10B) and later dialyze eluates to recover soluble engineered proteins (Figure 11). Although my objective is to have one of these nanoparticles serve as a platform for the PRRSV antigen, it would be preferable that the antigen be accepted without disrupting the native folding of the core structure of the nanoparticles. Initially, I sought to achieve this by genetically fusing the chosen antigen to the solvent exposed N-termini (BLS, Ferritin) or C-termini (AaLS, HSP) of our nanoparticles of interest. However, it was evident by the amount of aggregation that folding was disrupted and therefore, at this point, I investigated options to minimize aggregation. General approaches to minimize recombinant protein aggregation in E. coli include chaperone protein co-expression, temperature and transcription control, and protein engineering (Garcia-Fruitos et al., 2012). As a first approach, expression of all engineered proteins was induced at 16°C overnight instead of the usual 37°C for 3 hours, however, there was no apparent increase in the yield of soluble protein. Next, two genetic constructs were designed using BLS as the platform; M-GP5-BLS and GP5-M-BLS. Briefly, these constructs contain the first ectodomain of the PRRSV M protein, linked to my chosen GP5 antigen by a flexible linker, and finally this M-GP5 or GP5-M sequence, is linked genetically to the N-terminus of BLS via a flexible linker (Figure 12).

The decision to produce an antigen with both M and GP5 ectodomain sequences was made based on past research indicating that the GP5-M heterodimers that dominate the envelope membrane of PRRSV are linked via a disulfide bond between their ectodomains
Apart from increasing the yield of the soluble proteins, it is possible that with this disulfide bond within my vaccine candidates, I may present epitopes that are specific to the GP5-M heterodimer that could not be targeted with the display of the GP5 ectodomain alone. Both participating cysteines are included in the constructs. However, since I used E. coli as the expression system, this interaction will not occur upon production of the fusion proteins since, the reducing environment maintained in E. coli cells will prevent disulfide bond formation. Therefore, I hypothesized that the pairing of M and GP5 on the nanoparticles may help minimize aggregation at the refolding stage in the presence of non-reducing environment where disulfide bond formation can readily occur and potentially assist in folding of the fusion proteins.

Expression plasmids containing the new constructs, either M-GP5-BLS and GP5-M-BLS, were transformed into E. coli BL-21 cells, and expressed and purified by IMAC. M-GP5-BLS appeared to overexpress very well in E. coli while GP5-M-BLS did not (Figure 13A). As with the previous fusion proteins, which contained the GP5 antigen alone, I found that both M-GP5-BLS and GP5-M-BLS fusion proteins formed inclusion bodies in E. coli (Figure 13B) and thus, urea was needed for their extraction. Upon nickel purification of M-GP5-BLS, it was acquired with a high level of purity with one prominent protein species at its expected size on the SDS-PAGE gel. However, upon purification of GP5-M-BLS, there were three prominent protein species with the highest at the expected size of GP5-M-BLS and two lower molecular weight species (Figure 13C). These lower molecular weight species are thought to be degradation products of GP5-M-BLS since they do not appear on the SDS-PAGE gel of M-GP5-BLS nickel purification (Figure 13C). Both GP5-M-BLS and M-GP5-BLS were cloned into identical expression plasmids (pET-28a+) and transformed into identical E. coli strains. If these lower molecular weight protein species were not associated with expression of the GP5-M-BLS fusion protein, they would be purified in a similar fashion from the E. coli cells transformed with the plasmid containing M-GP5-BLS. Therefore, I concluded that the fusion protein GP5-M-BLS was prone to degradation and thus, the order in which the selected M and GP5 sequences were fused to BLS affected its stability. Although
insoluble, the fusion protein M-GP5-BLS expressed well in *E. coli*, was stable and could be significantly enriched via Ni-NTA chromatography.

To investigate whether the presence of the ectodomain of the PRRSV M protein helped with the refolding of the new fusion protein M-GP5-BLS, the nickel purified sample was dialyzed exhaustively against Tris buffer containing no denaturant with either reducing agent or no reducing agent (Figure 13D). Supernatant and pelleted fractions of both dialyses did not indicate any advantage of dialyzing without reducing agent so there was no indication that interaction between M and GP5 via a disulfide bridge helped in the refolding of this fusion protein. Comparing the fractions of each dialysis separately, I saw that without reducing agent, a large percentage of M-GP5-BLS aggregated while with reducing agent, a significantly smaller percentage of M-GP5-BLS aggregated (Figure 13D). Therefore, it appeared that the presence of reducing agent helped M-GP5-BLS retain solubility upon removal of urea, as it did with the fused nanoparticles previously tested and other recombinantly expressed fusion proteins of BLS (Bellido et al., 2009). My best candidate thus far is M-GP5-BLS; it overexpressed well in *E. coli*, was stable, and most of it remained soluble upon removal of urea following Ni-NTA enrichment.

### 4.2 Structural characterization of BLS and M-GP5-BLS

Both BLS and M-GP5-BLS were purified further and analyzed by size-exclusion chromatography (SEC). The elution profile of the recombinantly-produced BLS showed a single, narrow, monodispersed peak eluting at an apparent molecular weight slightly higher than 158 kDa (Figure 14C). Since this peak when analyzed by SDS-PAGE contained the pure monomer of BLS which has a molecular weight of 18.8 kDa, it is evident that monomers of BLS assembled into the expected decameric form with a molecular weight of 188 kDa. The molecular weight of BLS was estimated to be 172 kDa based on the linear calibration curve created by plotting the elution volumes of the protein standards used to calibrate the column versus the \( \log_{10} \) of their molecular weights. Based on the elution profile of M-GP5-BLS, it is evident that this fusion protein assembles into a multimeric complex. However, its elution profile shows a peak at a
much earlier volume (11.6 ml) than both the peak of 158 kDa protein marker (13.11 ml) and that of BLS (13 ml). Based on the SEC calibration curve, M-GP5-BLS is estimated at a size of 505 kDa. Estimating the molecular weight of a protein using SEC is recommended only when the protein takes a shape similar to the proteins used to calibrate the column; in this case globular proteins were used for calibration. This is recommended because SEC elution profiles are affected by both the size and shape of molecule. Two molecules with identical or very similar molecular weights will not have the same elution volume if one has a rod-like shape and the other is a compact sphere (Sorensen et al., 2001). The rod-shaped molecule will pass through the SEC column at a faster rate because it has less access to the small pores within the resin of the column than a molecule that is compact and globular. BLS is cylindrical in shape, referred to as spool-like (Zylberman et al., 2004) but it is still a small compact molecule and this is likely why its size estimation was close to its calculated molecular weight; BLS does not appear to depart much from a globular shape. Since M-GP5 is predicted as mainly disordered (Kelley et al., 2015) it is likely that it extends randomly from the BLS core and causes a great departure from the compact spool-like shape of the BLS molecule. This change in shape to a more elongated molecule would explain the surprisingly large shift in elution volume between BLS and M-GP5-BLS, and the potentially over-estimated molecular weight of 505 kDa.

To acquire more precise estimates of the shape and molecular weights (MWs) for BLS and M-GP5-BLS sedimentation velocity (SV) AUC was performed. Along with MW estimation, SV experiments also estimate the frictional ratio of a sample and this value gives a clear indication of the shape of a molecule in solution. Results from my SV experiment for BLS (Figure 15A) agreed with the SEC data, wherein BLS has a singular, monodispersed size distribution with an estimated molecular weight of 178 kDa. As for the frictional ratio of BLS, it was estimated to be 1.3. By definition, the frictional ratio is a measure of the resistance experienced by a molecule during sedimentation in relation to the resistance experienced by an ideal sphere of the same molecular weight (Smith, 1988). Therefore, an ideal sphere of any molecular weight will theoretically have a frictional ratio of 1.0 and if this sphere shifts towards a more elongated form, it will experience more resistance and an increase in frictional ratio. BLS therefore departs from
the ideal sphere and is more elongated but it is still considered to be globular since globular proteins typically have frictional ratios ranging from 1.05 to 1.30 (Unzai, 2018). The globular nature of BLS explains the relatively accurate estimation of its MW by SEC. Based on the SV experiments of M-GP5-BLS, the engineered construct sediments more quickly than BLS, having a sedimentation coefficient of 8.8 compared to the 8.1 calculated for BLS. Multiple peaks in the sedimentation plot of M-GP5-BLS (Figure 15B) indicated that the engineered construct formed aggregates. Estimation of molecular mass by SV experiments is accurate and considered most appropriate when analyzing heterogenous samples with molecules having similar frictional ratios or when a sample has a single major peak in its c(s) distribution plot (Dam and Schuck, 2004) as seen with BLS (Figure 15A). In the case of M-GP5-BLS, since there is aggregation and the appearance of multiple species, a weight-average frictional ratio is calculated to represent the frictional ratio of all species therein. It was calculated to be 1.58, indicative of an elongated molecule no longer considered as globular. Although the weighted-average frictional ratio represents the frictional ratio of the most abundant species in a sample well, the molecular mass estimate is not expected to be as accurate. The estimated molecular mass of M-GP5-BLS by SV is 220 kDa, which is 32 kDa less than the expected MW of 252 kDa, however, within the experimental uncertainty, this is consistent with a decamer of M-GP5-BLS.

In order to evaluate and compare the secondary structure of BLS and M-GP5-BLS, their circular dichroism (CD) signal in the far UV (190–260 nm wavelength range) was measured. The far UV-circular dichroism spectra of BLS was practically superimposable with that of the BLS which was extracted with urea and later refolded (Figure 16A). This shows us that refolding by dialysis allowed for the proper folding of BLS with no disruption of its native secondary structure and therefore it is inferred that refolding of M-GP5-BLS follows this trend and so its BLS core structure folds properly. Although the spectra of BLS and M-GP5-BLS do not overlay (Figure 16A), they do have an identical overall shape and a shared minimum of 220 nm, indicating that the overall secondary structure of BLS is not changed by the addition of M-GP5. Both spectra correspond to a protein with majority alpha helical structure and some beta secondary structure, which agrees with the crystallographic structure of BLS (Zylberman et al., 2004).
Analysis of the thermal stability of BLS and M-GP5-BLS by CD spectroscopy and the transmission electron microscopy (TEM) images taken of these two recombinant proteins indicate that the presence of the PRRSV antigenic sequence M-GP5 does not disrupt the overall structure of BLS. Both BLS and M-GP5-BLS share similar melting temperatures ($T_m$s), 92.1°C and 91.5°C respectively (Figures 15B and 15C), and their high $T_m$s are typical of a protein from a thermophilic organism. The thermal stability of these two recombinant proteins also speaks to their oligomeric state. Based on the reported $T_m$s of lumazine synthases with pentameric or icosahedral (60 subunits) assemblies, my decameric BLS is expected to have an intermediate $T_m$. Zhang and colleagues (2001) reported the melting temperature of the pentameric lumazine synthase isolated from *Saccharomyces cerevisiae* as 74.1°C and the melting temperature of the icosahedral lumazine synthase isolated from *Aquifex aeolicus* as 119.9°C. The $T_m$ of BLS I have estimated based on my CD experiments is 92.1°C; it lies between 74.1°C and 119.9°C and is very similar to the BLS $T_m$ of 88 ± 2°C estimated by the CD experiments of Zylberman and colleagues (2004). Therefore, it is clear that the M-GP5-BLS chimera produced in this work maintains the thermal stability of BLS and likely assembles into a decamer similar to BLS. Negative stain TEM images show that both BLS and M-GP5-BLS appear to form pentameric structures with an approximate diameter of 5-7 nm (Figure 17). However, my results from SEC, SV and the thermal denaturation experiments strongly indicate the formation of a higher order, more stable decameric species, not the pentamer visualized by TEM. Looking from above the decameric barrel of BLS, it has a diameter of 7 nm and from the side, a height of 8 nm (Zylberman et al., 2004). Because of the similarity in size of these two major orientations, it is likely that in the TEM images BLS and M-GP5-BLS are in several orientations, but the top view is easiest to interpret. Also, it is possible that the low pH of the uranyl acetate (pH 4-4.5) used to stain the samples caused the dissociation of decameric BLS and M-GP5-BLS into their pentameric state. Zylberman and his colleague (2004) report dissociation of decameric BLS into pentamers between the pH range 4.0-5.0 at room temperature. Visualizing the engineered parts of M-GP5-BLS and thus distinguishing BLS from M-GP5-BLS by TEM was not possible; likely due to the disordered nature of the fused M-GP5 sequence.
4.3 Conclusion and future directions

To conclude, I show that I have successfully produced a Brucella lumazine synthase nanoparticle that carries antigenic peptide sequences from the ectodomains of both major structural proteins of the PRRS virus; M protein and glycoprotein 5. The antigenic sequences (M-GP5) were genetically fused as one foreign peptide to the N-terminus of BLS and my results demonstrated that the secondary structure of BLS was conserved in the chimeric protein M-GP5-BLS, the thermal stability of BLS was unaltered and that M-GP5-BLS assembles into a decamer similar to the wild-type BLS.

The next step for this project will be to determine whether M-GP5-BLS induces an immune response against PRRSV. This can be studied in mice by subcutaneous injections of the recombinant protein followed by monitoring the production and level of PRRSV-specific antibodies and neutralizing antibodies (Yu et al., 2016). Typically, a laboratory-bred strain of mice called BALB/c mice would be the test subjects; this strain of house mice is routinely used in animal experimentation and will lack exposure to PRRSV. The test group would be immunized with my test vaccine, blood sera would be collected, and antibody activity determined using indirect enzyme-linked immunosorbent assay (ELISA) kits coated with PRRSV antigens. Sera containing PRRSV-specific antibodies can then be tested to determine whether they can prevent the virus from infecting susceptible cells. M-GP5-BLS is expected to stimulate neutralizing antibodies against the immunoreactive domain of GP5 and since this is region is conserved across PRRSV strains of the NA genotype (Figure 7), it is likely that these antibodies will bind the GP5 ectodomain of various strains of the NA genotype. With respect to the divergent EU genotype, protection may be elicited if antibodies target specifically the region that is conserved between both genotypes (Figure 7). To improve on my potential vaccine, a counterpart M-GP5-BLS could be made that is based on the M and GP5 membrane proteins of PRRSV strains from the EU genotype. A mixture of these two M-GP5-BLS constructs is likely to increase the potential of protection against a vast range of PRRSV isolates.
Another direction that may be explored is the production of my engineered proteins in plants. Transgenic *Arabidopsis* and tobacco plants are common expression systems in which glycosylation of my constructs can be controlled via subcellular targeting (An et al., 2018; Pereira et al., 2014) and with these glycosylated constructs purified, their antigenicity can be tested and compared with that of the non-glycosylated constructs produced in *E. coli*. Glycosylation may affect the antigenicity of our constructs since in some cases, glycans are involved in the interaction between antibody and epitope and in other cases, glycans shield epitopes from antibodies and protect viruses from the immune system (Desrosiers et al., 2004; Lisowska, 2002; Vu et al., 2011). In the case where the non-glycosylated constructs induce antibodies against an epitope shielded by a glycan on the surface of native PRRSV, immunological testing will reveal the efficacy of those constructs as vaccines. It’s possible that the shielded epitope is exposed along the course of the virus’ infectious cycle and upon exposure of the epitope, antibodies may bind and provide control of the virus.

In summary, I used BLS as a platform for PRRSV antigens to create a PRRS vaccine candidate. Its efficacy must be investigated, and improvements will likely be necessary to promote cross-protective immunity. Alternative methods of protein production will also be explored to investigate the importance of glycosylation of the PRRSV antigens on the antigenicity and efficacy of the vaccine candidate.
References


Hsia, Y., Bale, J.B., Gonen, S., Shi, D., Sheffler, W., Fong, K.K., Nattermann, U., Xu, C., Huang, P.-S.,


https://doi.org/10.1017/S1466252312000023


## Appendices

**Appendix 1.** Amino acid sequences of all proteins produced recombinantly.

### Brucella lumazine synthase (BLS):

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGSSHHHHHHENLYFQSNAKTSFKIAFIQARWHADIVDEARKSFVAELAAKTGGSVEVEIFDVPGAYEIPLHAKTLARTGRYAAIVGAADFVIDGGIYRHDFVATAVINGMMQVQLETEVPVLSVVLTPHHFHESKEHDFHFAHFKVKGVEAAHAALQIVSERSRIAALV</td>
<td>BLS</td>
</tr>
</tbody>
</table>

### GP5-BLS:

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGSSHHHHHHENLYFQSNANASNDSSSHLQLIYNLTLCELNGTDGGSGGSGGSGKSFKIAFIQARWHADIVDEARKSFVAELAAKTGGSVEVEIFDVPGAYEIPLHAKTLARTGRYAAIVGAADFVIDGGIYRHDFVATAVINGMMQVQLETEVPVLSVVLTPHHFHESKEHDFHFAHFKVKGVEAAHAALQIVSERSRIAALV</td>
<td>GP5-BLS</td>
</tr>
</tbody>
</table>

### GP5-M-BLS:

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGSSHHHHHHENLYFQSNANASNDSSSHLQLIYNLTLCELNGTDGGSGGSGGSGGSGKSFKIAFIQARWHADIVDEARKSFVAELAAKTGGSVEVEIFDVPGAYEIPLHAKTLARTGRYAAIVGAADFVIDGGIYRHDFVATAVINGMMQVQLETEVPVLSVVLTPHHFHESKEHDFHFAHFKVKGVEAAHAALQIVSERSRIAALV</td>
<td>GP5-M-BLS</td>
</tr>
</tbody>
</table>

### M-GP5-BLS:

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGSSHHHHHHENLYFQSNAMGSSLDFFCHDSTAPQKVGSGGSGGSGGGSNASNDSSSHLQLIYNLTLCELNGTDGGSGGSGGSGKSFKIAFIQARWHADIVDEARKSFVAELAAKTGGSVEVEIFDVPGAYEIPLHAKTLARTGRYAAIVGAADFVIDGGIYRHDFVATAVINGMMQVQLETEVPVLSVVLTPHHFHESKEHDFHFAHFKVKGVEAAHAALQIVSERSRIAALV</td>
<td>M-GP5-BLS</td>
</tr>
</tbody>
</table>

### Aquifex aeolicus lumazine synthase (AaLS):

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGSSHHHHHSSGLVPGSHPGHMQIYEKGKTAELRGFIVASRFNHALVDRLVEGAIDCIVRHGGREEDITLVRPGSWEIPAELRADIAIAVGVRIGATPHFDAESEVSKGLANLSLELRKPIITFGVITADLQIAIERAGTKHGNKGVEAAALSIEHMANLFKSLRLE</td>
<td>AaLS</td>
</tr>
<tr>
<td>Protein Name</td>
<td>Sequence</td>
</tr>
<tr>
<td>------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>AaLS-GP5:</strong></td>
<td>MQIYEGKLTAEGLRFGIVASRFNHALVDRLVEGAIDCIVRHGGREEDITLVRVPGSWEIPVAA</td>
</tr>
<tr>
<td></td>
<td>GELARKEDIDAVIAIGVILRGATPHFHDYIASEVSKGLANLSLELRKPTFGVITADTEQAIERA</td>
</tr>
<tr>
<td></td>
<td>GTKHGNGKWEAAALSAIEMANLFKSLRGGSGGSGGSGSNASNDSSSHLQLIYNLTLCELNGTDLEHHHHHH</td>
</tr>
<tr>
<td><strong>Methanococcus jannaschii</strong></td>
<td>small heat shock protein (sHSP-16.5):</td>
</tr>
<tr>
<td></td>
<td>MFGRDPFDLSLFERMFKEFFATPMGTITMIQSSTGIQISGKFMPISIIEGDQHIKVIAWLPGVN</td>
</tr>
<tr>
<td></td>
<td>KEDILNAVGDITLEIRAKRSPLMITESERIIYSEIEPEEEIYRTIKLPATVKEENASAKFENGVLS</td>
</tr>
<tr>
<td></td>
<td>VILPKAESSIKKGINIELEHHHHHH</td>
</tr>
<tr>
<td><strong>sHSP-16.5-GP5:</strong></td>
<td>MFGRDPFDLSLFERMFKEFFATPMGTITMIQSSTGIQISGKFMPISIIEGDQHIKVIAWLPGVN</td>
</tr>
<tr>
<td></td>
<td>KEDILNAVGDITLEIRAKRSPLMITESERIIYSEIEPEEEIYRTIKLPATVKEENASAKFENGVLS</td>
</tr>
<tr>
<td></td>
<td>VILPKAESSIKKGINIEGSGGGSGGSGGSNASNDSSSHLQLIYNLTLCELNGTDLEHHHHH</td>
</tr>
<tr>
<td><strong>Helicobacter pylori</strong></td>
<td>ferritin (ferritin):</td>
</tr>
<tr>
<td></td>
<td>MGSSHHHHHHHSSGLVPRGSHMLSDKDIKLLEQVNKEMNSSNLYMSMSSWCYTHSLLGDAGL</td>
</tr>
<tr>
<td></td>
<td>FLFDHAEEYEHAKKLIVFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYGHEQHISESINNIV</td>
</tr>
<tr>
<td></td>
<td>DHAIKSDKDHATFNFLQWYVAEQHEEEVLKFDILDKIELIGNENHGLYLADQYVKGIAKSRKS</td>
</tr>
<tr>
<td><strong>GP5-ferritin:</strong></td>
<td>MGSSHHHHHHHSSGLVPRGSHNASNDSSSHLQLIYNLTLCELNGTDGSGSGSGGSMLSK</td>
</tr>
<tr>
<td></td>
<td>DIIKLLNEQVNKEMNSSNLYMSMSSWCYTHSLLGDAGLFLFDHAEEYEHAKKLIVFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYGHEQHISESINNIVDHAIKSDKDHATFNFLQWYVAEQHEEEVLKFDILDKIELIGNENHGLYLADQYVKGIAKSRKS</td>
</tr>
</tbody>
</table>
Curriculum Vitae

Name: Ondre Harper

Post-secondary Education and Degrees:
Master of Science in Biology 2017- present
Western University, London, Ontario

Thesis title: “Engineering self-assembling proteins to produce a safe and effective vaccine for Porcine Reproductive and Respiratory Syndrome”

Bachelor of Medical Sciences Honors (4 Year) 2017
Western University, London, Ontario
Honors Specialization in Biochemistry
Major in Microbiology and Immunology
Honors thesis title: “Investigation of the dormant IS200 transposon conserved in Salmonella”

Honors and Awards:
Western University Continuing Admission Scholarship 2013-2016

Related Work Experience:
Teaching Assistant, Methods in Biology (Bio 2290), Western University 2017-2019

Poster Presentations: