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INDUSTRIAL OPTIMIZATION OF THE PRODUCTION OF CARGO LOADED PIV5 VIRUS-LIKE PARTICLES

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ABSTRACT

A novel protein delivery system involving cargo loaded paramyxovirus-like particles is currently being studied. Despite showing promise as a biotech platform, production of these cargo loaded virus-like particles (VLPs) is costly. Currently, production relies on expensive transfection reagents and adherent mammalian cell lines, neither of which are practical in large scale production. In order for this promising technology to take the next step towards industrial or clinical use, new methods of production must be developed. In this study, evidence from pilot-scale experiments suggests the viability of large-scale production of cargo loaded PIV5 VLPs via the use of polyethylenimine (PEI) transfection reagents in the producer suspension cell line, Expi 293F. The VLPs produced with these new methods were quality tested in their ability to deliver proteins, and these delivery experiments confirmed VLP functionality.

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Chapter 1

Introduction

1.1 The Paramyxoviridae Family

Paramyxoviruses, or members of the *Paramyxoviridae* family, are large, enveloped, negative sense RNA, viruses whose linear single-stranded genomes typically encode 6-10 proteins. Within the family, *Paramyxoviridae*, there are five genera: *Morbillivirus, Avulavirus, Respirovirus, Rubulavirus,* and the genus commonly associated with newly emerging zoonoses, *Henipavirus* (Amarasinghe et al., 2017) (Figure 1).

Common members of *Paramyxoviridae* family include, parainfluenza virus 5 and mumps viruses of the *Rubulavirus* genus, the agriculturally relevant Newcastle disease virus of the *Avulavirus* genus, the measles virus of the *Morbilivirus* genus, and the Hendra and Nipah viruses of the *Henipavirus* genus, which are highly lethal pathogens requiring BSL-4 clearance. These viruses, among others within the family, are relevant in terms of human health, agricultural productivity, and social issues surrounding vaccinations.

The major structural proteins of paramyxoviruses include matrix protein (M), some variant of a nucleocapsid protein (N/NP), hemagglutinin-neuraminidase glycoprotein (HN), and the fusion glycoprotein (F) (Figure 2). M serves as an adaptor protein bridging the tails of the glycoproteins to NP, NP complexes with the viral genome, and the glycoproteins have various functions on the exterior of the virion.

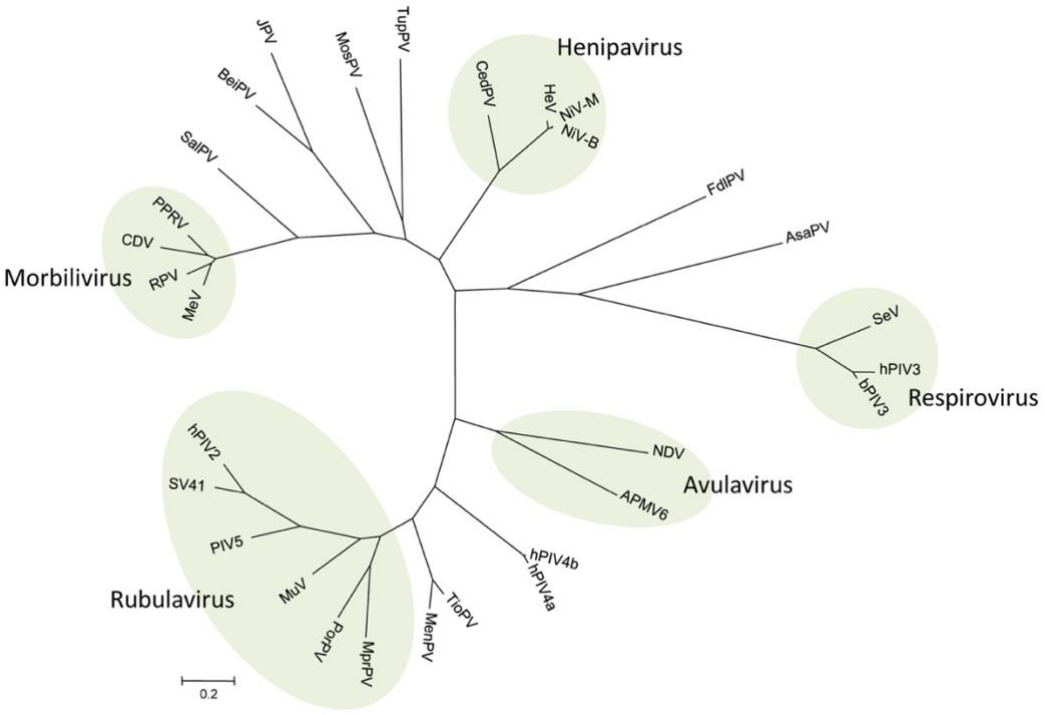

Figure 1: *Paramyxoviridae* Phylogenetic Tree. This phylogenetic tree represents the genetic divergence of genus and species within the *Paramyxoviridae* family. The analysis and grouping was done based on similarity of nucleocapsid protein (NP) sequences. The genus *Morbilivirus* includes PPRV (peste-de-pestis ruminants), CDV (canine distemper virus), RPV (rinderpest virus, which has since been eradicated), and MeV (measles virus). The genus *Henipavirus* includes NIV-B (Nipah virus-Bangladesh strain), NIV-M (Nipah virus-Malaysia strain), HeV (Hendra virus), and CedPV (Cedar virus). The genus *Respirovirus* includes bPIV3 (bovine parainfluenza virus 3), hPIV3 (human parainfluenza virus 3), and SeV (Sendai virus). The genus *Avulavirus* includes APMV6 (avian paramyxovirus 6), and NDV (Newcastle disease virus). The genus *Rubulavirus* includes hPIV2 (human parainfluenza virus 2), SV41 (simian parainfluenza virus 41), PIV5 (parainfluenza virus 5), MuV (mumps virus), PorPV (porcine rubulavirus), and MprPV (Mapuera virus). Several paramyxoviruses did not meet the classification of these categorized genera, and they are as follows: MenPV (Menangle virus), TioPV (Tioman virus), hPIV4a (human parainfluenza virus 4a), hPIV4b (human parainfluenza virus 4b), AsaPV (Atlantic salmon paramyxovirus), FdlPV (Fer-de-lance virus), TupPV (Tupaia paramyxovirus), MosPV (Mossman virus), JPV (J virus), BeiPV (Beilong virus), and SalPV (Salem virus). It is important to note that PIV5 is the most relevant virus to this study (Marsh et al., 2012).

Paramyxoviruses typically follow a relatively simple life cycle initiated by attachment and fusion to their target cell via viral glycoprotein binding to their cellular receptors. Following

fusion to the plasm membrane, viral ribonucleoprotein (VRNP) is delivered directly to the cytosol where the RNP undergoes uncoating. The negative sense RNA genome is capable of undergoing transcription and translation immediately in addition to viral genome replication, which all takes place in the cytosol. Following production of additional genome copies and viral proteins, viral assembly is guided to the plasma membrane where M, NP/N, and glycoprotein interactions direct assembly of the whole, infectious, virion. Following assembly, the virion buds outwards and is released from the cell (Figure 3).

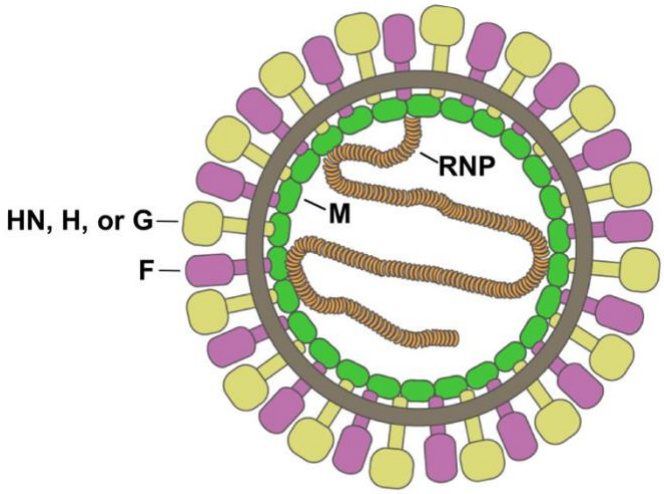

Figure 2: Schematic depicting the structural components of a paramyxovirus particle. This schematic illustrates the major structural components of a paramyxovirus particle. M (matrix) protein pictured in green is thought to be the viral component that drives assembly. M protein self assembles at the membrane with binding sites for other M protein, for the cytoplasmic tails of HN, H, or G, as well as for F, and has other binding sites for NP/RNP. M protein's structural role can be thought of as an adaptor linking the outer and inner components of the cell that provides structural integrity. RNP (ribonucleoprotein) is a complex including the viral RNA genome and viral NP (nucleocapsid) protein. Where M serves as an adaptor between inner and outer proteins, NP serves as an adaptor between protein and nucleic acid within the interior of the virion that ensures proper packaging. The glycoproteins labelled HN, H, or G are a variety of attachment proteins with varying targets and are pictured in gold. Another glycoprotein pictured in purple, F (fusion) protein, is responsible for fusion of the viral membrane with host cell plasma membrane and entry into the cell. For PIV5 virions, either HN of F must be coexpressed with M an NP for the particle to assemble (Harrison et al., 2010).

One member of the *Paramyxoviridae* family, parainfluenza virus 5 (PIV5), is extremely useful for the study of both life cycle processes and general structure. PIV5 is primarily a canine pathogen associated with kennel cough, and is not capable of causing symptomatic human infection (Dubovi et al. 2011). In addition to the safety of studying this virus, the virus shares relevant structural similarities to other paramyxoviruses and replicates to high titers in cell

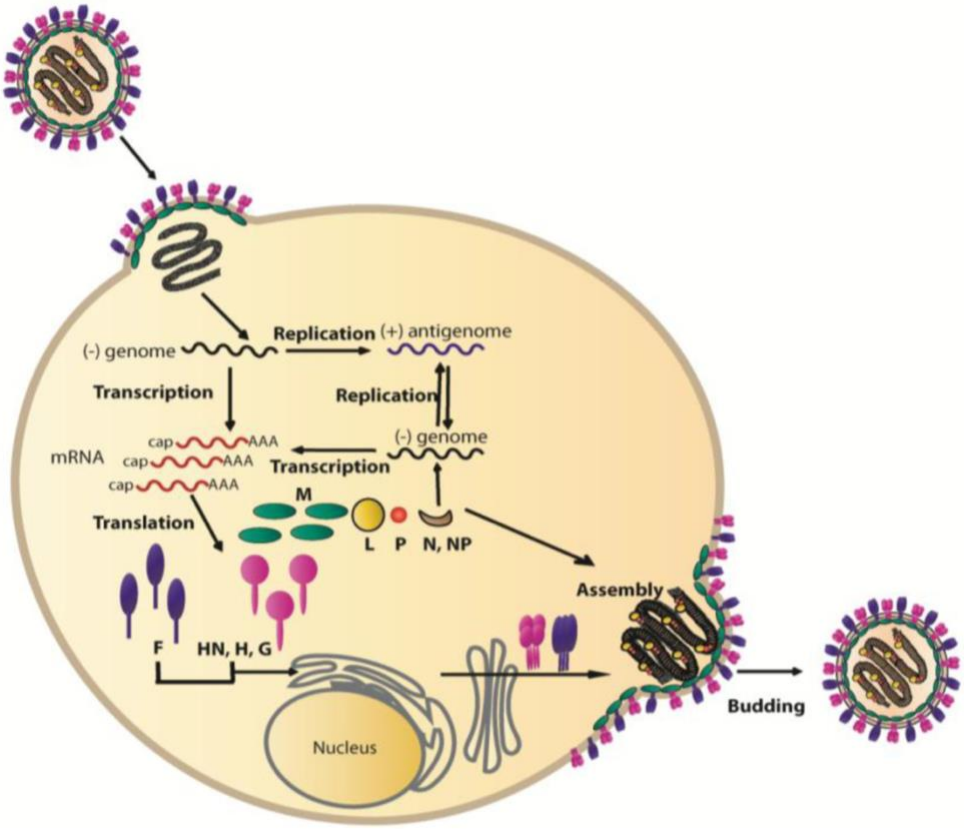

Figure 3: Paramyxovirus Life Cycle. This schematic depicts the major steps of the protypical paramyxovirus life cycle: attachment and fusion, uncoating, genome replication, transcription, assembly, and budding. After binding to the virus's receptor via H, HN, or G attachment protein, a structural change is induced in the F protein. The F protein then fuses the virion's outer membrane with the host membrane and releases the contents of the virion directly into the host cytoplasm. The virus's (-) sense genome can then either be directly transcribed or undergo genome replication. Once the necessary viral components have been produced, the components self assemble at the host cell membrane and bud outwards to form a mature virion. Note that this cycle begins and ends with an extracellular, infectious virion (Najjar et al., 2014).

culture. Furthermore, PIV5 displays a broad tropism for various mammalian cell types due to the binding of the relatively ubiquitous sialic acid as a target for attachment.

1.2 Paramyxovirus Assembly and Budding

One step of the paramyxovirus life cycle that has garnered recent attention is assembly. The matrix (M) protein is prototypically the central organizer of the assembly process. Once M is directed to the plasma membrane, it serves as an adaptor to link vRNP, which consists of Nucleocapsid (NP) protein and viral genome, on the inner surface and glycoproteins on the outer surface of a budding virion (Harrison et al. 2010) (Figure 4).

After the structural proteins of the paramyxovirus have gathered at the host cell plasma membrane and self-assembled, the virion must bud in order to complete its infectious life cycle. The virion buds away from the cytoplasm and becomes enveloped by the plasma membrane. The final step of the budding process is accomplished when the membrane pinches off and separates

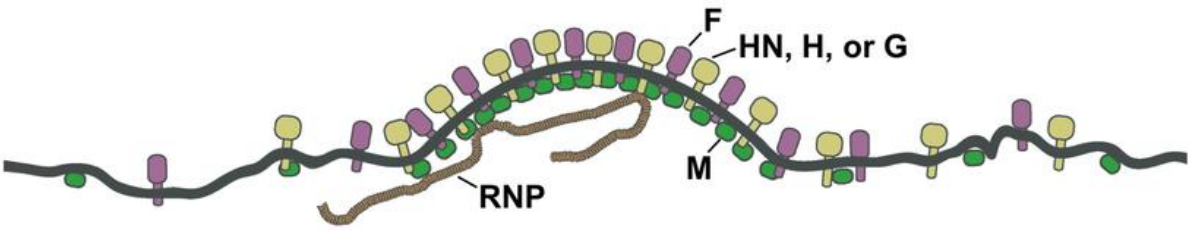

Figure 4: Schematic depicting viral assembly at the host plasma membrane. During assembly, structural viral proteins are directed to multiple points on the host cell plasma membrane. M serves as the central adaptor and drives assembly through M-NP interactions as well as M-glycoprotein interactions. NP can be though of as the adaptor bridging genomic RNA to the M protein through M-NP interaction. These self-assembing viral proteins eventually complete assembly and bud outwards from the host cell plasma membrane, pinching off the membrane anchoring the virion to the cell in order to become infectious virions (Harrison et al., 2010).

the enveloped virion from the host membrane. The product is an infectious, mature virion ready to repeat its life cycle (Harrison et al. 2010) (Figure 5).

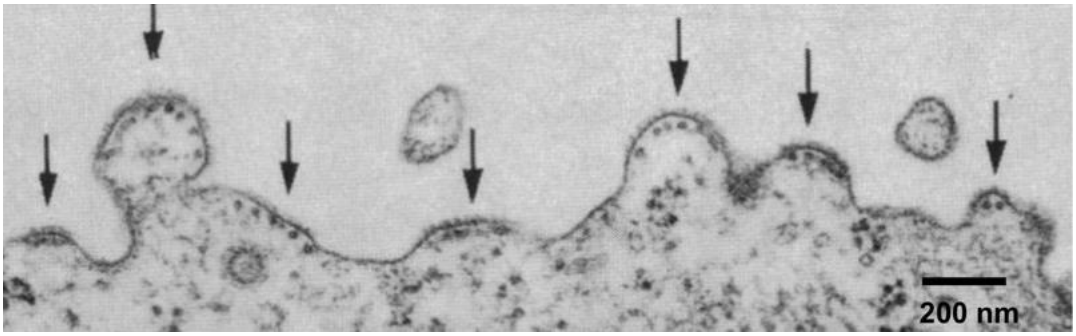

Figure 5: Paramyxovirus budding. This figure is a transmission electron micrograph that has captured virions budding from the apical surface of an MDCK cell. The arrows indicate sites of viral budding (Boulan et al. 1978).

1.3 Virus-Like Particles

Virus-like particles (VLPs) are noninfectious nanoparticles derived from viral proteins. These particles are produced via expression of one or more viral components that have the capacity to self-assemble within a cell (Fuenmeyer et al. 2017). Once assembled, VLPs resemble infectious virions and can even retain certain functions associated with authentic virus (Cai et al. 2016). VLP generation has opened doors for new technologies in vaccine development, immune modulation, gene therapy, and protein delivery. With the potential for advancement in so many different areas of study, understanding the structure and function of viral components has become even more important.

Like other families of virus, many VLPs have been derived from various species of paramyxovirus. For some paramyxoviruses, such as NDV, Nipah, and Hendra, expression of M

alone is sufficient for the generation of VLPs (Pantua et al. 2007). For other paramyxoviruses, such as PIV5 and Mumps, VLP generation is more complex. For PIV5, M, NP, and one of its two glycoproteins must be expressed in order for VLP assembly to efficiently take place (Schmitt et al. 2002).

1.4 Paramyxovirus-Like Particles as a Viable Protein Delivery Platform

The concept of packaging exogenous cargo into a budding VLP is not new, but most of the research into this topic is in terms of gene therapy vectors. Lentiviral and adenoviral gene vectors are being studied clinically as vehicles for the delivery of therapeutic genes (Parker et al. 2020). Useful applications for these gene therapies would include the treatment of genetic diseases caused by a systemic deficiency in a certain gene or genes. The risks associated with viral gene therapy include insertion of exogenous DNA near a protooncogene, insertional mutagenesis, dissemination into nontarget tissues, etc. (Kay et al. 2001).

Delivery of proteins via a VLP vehicle is an area that remains less studied. Components of Lentiviruses, such as the structural protein, gag, have been used as protein delivery vehicles with varying success. In concept, these cargo loaded VLPs are generated by coexpression of plasmids encoding viral components together with cargo inside of a cell. Once assembled, the particles undergo budding and can be isolated. The isolated, cargo loaded VLPs are used to attach and enter target cells where they ideally begin uncoating and deliver their protein cargo.

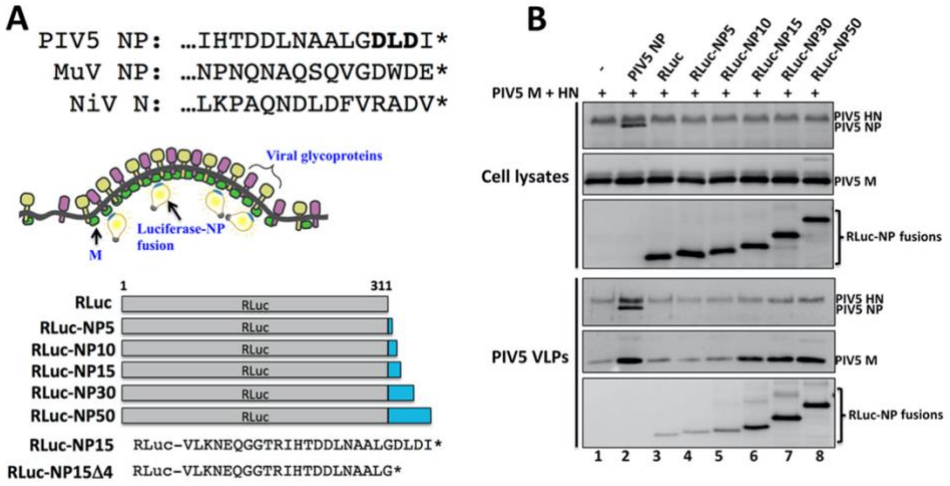

Figure 6: Incorporation of cargo proteins into VLPs. (A) This schematic represents the process by which foreign cargo proteins are incorporated into budding VLPs. The cargo protein, *Renilla* Luciferase (RLuc) in this case, has been modified by the addition of a c-terminal portion of NP protein. The minimal modification required of the cargo protein does not interfere with the cargo molecule's function. The NP sequence retains its prototypical interaction with M protein, helping to guide assembly. As assembly continues, the cargo protein would be incorporated into a budding VLP on the inner surface as if it were vRNP. (B) A western blot was performed and shows that the 15 amino acid modification was sufficient to package cargo within the VLPs. While RLuc is used here, there is also unpublished data suggesting a wide range of potential cargos are viable (Ray et al., 2016).

Even though the natural lifecycle of Lentiviruses involves direct fusion and entry into the cytoplasm, there is some evidence that proteins delivered by lentivirus carrier molecules can become entrapped in the endosome (Richard et al. 2002). Another limitation is that the cargo protein must be completely contained within the Gag polyprotein, which could potentially interfere with function once inside the cell. Furthermore, the gag component of these cargo loaded VLPs can be toxic at high doses, which is another concern to be accounted for when adapting the technology to clinical trials (Chai et al. 2017). While this technology has continuously been improved over the years, the barriers associated with gag-based protein delivery persist today.

Previous work from the Schmitt lab has uncovered the ability to package exogenous cargo proteins into budding paramyxovirus-like particles. After studying M and NP interactions, it was discovered that a segment of the C-terminal end of PIV5 NP protein was necessary to coordinate M-NP interaction in viral assembly (Schmitt et al 2010.). With this information in mind, it was hypothesized that truncation of this critical region of NP to an exogenous cargo could direct a cargo molecule into budding VLPs. After investigation, it was determined that fusion of 15 or more amino acid residues from the C-terminal end of NP to cargo protein (Renilla Luciferase) significantly augmented VLP production together with incorporation of cargo into budding particles (Fig. 6). In effect, these findings suggest that many a variety of cargo molecules could potentially be tagged with NP-derived packaging sequences for VLP budding induction and incorporation of cargo into particles. The cargo loaded VLPs were then shown have the ability to attach, fuse with, and deliver their minimally modified cargo to target cells in vitro (Panthi et al. Manuscript in preparation). The possibility of functional cargo loaded paramyxovirus VLPs is exciting for several reasons. Foremost, knowing that the paramyxovirus life cycle includes direct fusion with the target cell membrane and cytosolic uncoating, the cargo within the VLP would be delivered directly into the cytoplasm and avoid endosomal entrapment (Figure 3). Additionally, these methods would avoid the possibility of host genomic manipulation, a problem faced by several other viral platforms. The direct delivery, low risk, and the minimal modifications of cargo required all warrant further study of the paramyxovirus-like particle protein delivery system in hopes of finding both clinical and industrial applications for this platform.

When considering the viability of cargo loaded paramyxovirus VLPs as an industrially relevant technology, the production process must be considered. VLPs are most often generated

by one of four ways: 1. Viral proteins are expressed in bacterial or yeast cells through the use of plasmids 2. VLPs are generated in transgenic plants and isolated from live tissue 3. Genetic information encoding viral proteins is stored in a baculovirus, which injects its genetic information into an insect cell line to then generate VLPs or 4. Transient expression of viral components in mammalian cell lines via a transfection process (Fuenmeyer et al. 2017). VLP generation in bacterial or yeast cells is the lowest cost method of VLP generation, but the process is limited by the producer cell lines' lack of machinery for appropriate post-translational modification. Additionally, components of bacterial LPS can be incorporated into budding, enveloped VLPs, which would make clinical application potentially toxic. This method is used primarily for more simple, non-enveloped VLPs (Fuenmeyer et al. 2017). Production of VLPs via a baculovirus system is more expensive, but it comes with the added benefit of more accurate post-translational modification. The drawbacks of the baculovirus system are that downstream VLP purification is difficult due to baculovirus fragments in the media and that post translational modification is still not identical to that in mammalian cell lines (Gutiérrez et al. 2018). Transfection of mammalian cell lines is the most expensive way to produce VLPs as it requires expensive transfection reagents and laborious cell maintenance, but post translational modification takes place in a more relevant mammalian cell and the result should be as similar as possible to how a virion is produced in vivo in a mammalian organism (Gutiérrez et al. 2018).

The central premise of transfection is that genetic material must be delivered to a cell in a way that causes temporary production of the protein encoded for. Delivery of the genetic material to mammalian cell lines in transfection is most commonly achieved via a viral vehicle, physical process, or through various chemical methods (Kim et al. 2010). Viral delivery is more common in permanent delivery of genes in a clinical setting, but still remains a viable

transfection vehicle if the need is present. Physical methods of transfection include direct injection, laser irradiation, magnetic nanoparticles, etc.. In general, these methods require specialized equipment, risk damaging the nucleic acids, and rely on a large amount of technical skill needed of the researcher (Kim et al. 2010). Chemical transfection is most commonly used in industrial practices. A wide variety of chemical transfection reagents exist premade, and there are economically feasible options with high transduction efficiency (Gutiérrez et al. 2018).

Within the category of chemical transfection methods, there are many chemical reagents available. Lipid-based and polymer-based reagents are amongst the most common. While lipid-based reagents boast high transfection efficiency and ease of use, the cheaper, polymer-based reagents are used almost exclusively in industry (Gutiérrez et al. 2018). In addition to the large-scale economic feasibility of polymer-based transfection reagents, their inferior transfection efficiency has been challenged by recent data (Rahimi et al. 2018). In many cell lines, including common industrially relevant suspension cells, polymer-based transfection reagents can equal or even exceed the transfection efficiency of lipid-based reagents (Rahimi et al. 2018).

In the process of transfection, the cell line used to produce the desired protein is another important factor. Adherent mammalian cell culture is less labor intensive than suspension culture and is very consistent in terms of results. Adherent cells adhere to the dish used to incubate and expand two-dimensionally to fill only the bottom surface of their reaction vessel. Suspension cells, however, are suspended in their media and can expand three-dimensionally to occupy a much larger volume in their reactor. Transfection of suspension cell lines therefore produces much higher yields than the transfection of adherent cell lines per volume unit. For this reason, suspension cell lines are used exclusively in industry whereas adherent lines are mainly used for

proof of concept studies. Currently, two extremely common mammalian suspension cell types used for viral bioproduction in industry are 293 and CHO family lines (Gutiérrez et al. 2018). Presently, production of these cargo loaded VLPs relies on transfection of adherent cells with the costly, lipid-based Lipofectamine reagent. For this technology to become industrially and economically realistic, the production process of these VLPs must be converted to a suspension cell line transfected with a polymer-based transfection reagent. Bioproduction of viral components on an industrial scale is most commonly carried out using suspension variants of 293 and CHO cells where the cells are transfected with the polymer-based reagent, polyethylenimine (PEI) (Gutiérrez et al. 2018). Optimization of PEI transfection conditions and selection of a suitable producer suspension cell line would bring this promising technology even closer industrial use. Furthermore, quality assurance of VLPs produced via these means would be needed to prove the merit of the new process.

Chapter 2

Materials and Methods

2.1 Plasmids

The plasmids, pCAGGS-PIV5 M, pCAGGS-PIV5 NP, pCAGGS-PIV5 HN, and pCAGGS-SV5 F were generated by subcloning components of infectious PIV5 into eukaryotic expression vectors. These methods have been previously described (Schmitt, 2002). As has been previously described, the pCAGGS-RLuc-NP30 plasmid was generated via subcloning of cDNA corresponding to RLuc, a double glycine linker, and a 15 amino acid sequence from the C-terminal end of PIV5 NP into a eukaryotic expression vector (Greeshma et al. 2016).

2.2 Cell Culture

293T and CHOK1 cells used in production experiments were grown in 6-cm dishes using Dulbecco's modified Eagle's medium (DMEM), high glucose, supplemented with 10% Fetal Calf Serum (fcs) and 1% penicillin and streptomycin. CHO-K1S cells were grown in SFM4CHO for maintenance and transfected in Hycell TransFx-C medium. Expi 293F cells were grown in Expi293 Expression Medium; the same medium was also used for transfection. Suspension cells were maintained by splitting every two-three days to a density of $0.5x10^6$ cells/ml. Viable cell counts were determined by Trypan Blue dye exclusion assay. The suspension cell line, CHO-K1S, was adapted from CHO-K1. Expi 293F suspension cells were purchased from Life Technologies (PN A14527). A549 cells used for delivery experiments were grown in a 24 well plate using DMEM supplemented with 10% fcs and 1% penicillin and streptomycin.

2.3 Antibodies

Monoclonal antibodies, M-f and NP-125 were used as the primary antibodies in the detection of PIV5 M and NP proteins, respectively. The polyclonal antibodies SDS-HN and Fsol, were used as the primary antibodies to detect PIV5 HN and F, respectively. Detection of Renilla Luciferase was achieved using a polyclonal antibody purchased from MBL International. Alkaline-phosphatase conjugated goat anti-mouse antibodies were used as secondary antibodies to detect M-f and NP 125 antibodies and alkaline-phosphatase conjugated goat anti-rabbit antibodies were used as secondary antibodies to detect SDS-HN and Fsol antibodies for western blotting.

2.4 VLP Production

A day prior to transfection, cells were split to a density of $1.5x10^6$ cells/ml. On the day of transfection, the cells should have achieved a density of roughly $3x10^6$ cells/ml. The amount of DNA used for transfection was 1 μg/10^6 cells. The proportional ratio of PIV5 proteins used was M:F:HN:NP 4:8:8:1. Ratios of DNA:PEI Max ranged from 1:1 to 1:8 as described in each experiment. Ratios of 1:2 or 1:3 proved to be the most effective. Transfection complexes were prepared in OptiMEM medium. Briefly, DNA was added to OptiMEM medium and swirled to mix. PEI Max was then added and gently swirled to mix. The reaction was allowed to incubate at room temperature for 10 minutes. Afterwards, the mixture was added to the cells. Cells were placed in a 37^oC incubator with 8% CO_2 and were shaken at 110 RPM on an Infors HT Celltron. Unless specified otherwise, VLP harvest and isolation from media was conducted four days post-transfection.

For collection/isolation, the media and cells were first collected. The media was centrifuged at 9,000 RPM for 2 minutes to remove cellular debris. The supernatant containing VLPs was collected and layered onto 20% sucrose cushions. The tubes containing layered media and sucrose cushions were centrifuged at 140,000 x g for 1.5 hours at 4 degrees Celsius. The pellet was resuspended in 0.9 ml of 1x PBS and mixed with 2.4 ml 80% sucrose. Layers of 3.6 ml of 50% and 0.6 ml of 10% sucrose were applied to the tops of the gradients and centrifuged at 140,000 x g for 3 hours at 4 degrees Celsius. 4 ml volumes of top fractions were collected and VLPs contained in the fraction were pelleted again by centrifugation at 190,000 x g for 1.5 hours. The pellet is collected and resuspended in protein lysis buffer in the presence of DDT. The sample is then boiled for 5 minutes and subsequently stored at -20 degrees Celsius. To prepare cell lysates, 100 μl of cells from each sample were lysed with 0.1 ml of SDS-PAGE loading buffer. The lysates were centrifuged through QIAshredder homogenizers (Qiagen, Germantown, MD) to break apart cell debris. PLB-lysed cell lysates were boiled for 5 minutes and stored at -20$_{o}$C. For the delivery experiment, we generated and isolated two types of cargo-loaded VLPs as described above with the exception that the pellet obtained after centrifugation was suspended in filter-sterilized 1xPBS. One type had both attachment (HN) and fusion (F) glycoproteins and another type had only attachment (HN) glycoprotein. In theory, VLPs having both glycoproteins are capable of delivering cargo into target cells, whereas, VLPs lacking F proteins are entry-defective due to their inability to fuse with target cell membrane.

2.5 SDS-PAGE and Western Blot

Purified protein samples were fractionated by SDS-PAGE using 10% SDS-Polyacrylamide gels. Samples were loaded and run at 60V for 15 minutes, and then run at 120V until the dye reaches the bottom of the gel (approximately 1-1.5 hours) in 1X Tris-Glycine buffer. PVDF membranes were wetter in methanol, briefly rinsed in de-ionized water, and equilibrated in the buffer used for transfer. After running, the gel was also allowed to equilibrate in transfer buffer for minutes.

To transfer, the lattice was prepared and components were layered as follows: bottom lattice, sponge, Whatman's paper x2 saturated in transfer buffer, gel, PVDF membrane, Whatman's paper x2 saturated in transfer buffer, sponge, and top lattice. After each layer was added, a plastic roller was run over the surface of the stack in order to ensure that no air bubbles were present. The transfer apparatus was then secured in the transfer box in the appropriate orientation and run at 80V for 1 hour.

The membrane was placed in blocking solution for one hour on a rocker (1x PBS with 0.3% Tween and 5% dry milk). After blocking, the membrane was washed three times for 5 minutes, 10 minutes, and 10 minutes in 1x PBS with 0.3% Tween on a shaker. Following washing, the appropriate primary antibodies were added to the membrane and allowed to incubate on the rocker overnight. The membrane was then washed three times for 5 minutes, 10 minutes, and 10 minutes in 0.3% PBS-T and the secondary, alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit antibody was added and allowed to incubate for 1 hour. 100uL ECF substrate were added to the membrane and incubated for 2-3 minutes. A FUJI FLA700 phosphorimager was used to scan the blot.

2.6 Luciferase Activity Measurements

Cells were seeded one day before delivery. On the day of delivery, the culture medium was removed, and a fraction of RLuc-loaded VLPs diluted in DMEM/2% FCS were added and incubated for 24 h. After incubation cells were washed extensively with 1X PBS to remove residual unfused VLPs and lysed in 1X of passive lysis buffer (Promega, Madison, WI) and subjected to Renilla luciferase assay, per the instructions of the manufacturer. Renilla luciferase activity was measured using a Veritas microplate luminometer (Turner BioSystems, Sunnyvale, CA)

Chapter 3

Results

3.1 VLP Production Optimization

Expi 293F cells achieved high production of VLPs with very little optimization beyond manufacturer's instructions. It was tested to determine if varying the DNA:PEI ratio would affect VLP production. -NP groups, or groups where the NP protein was not expressed during

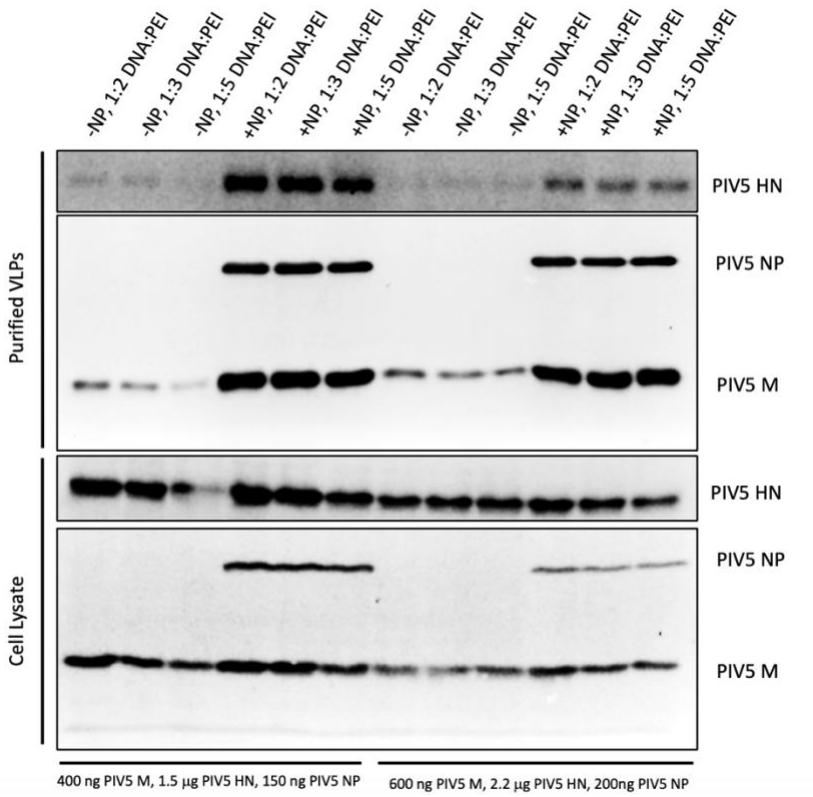

Figure 7: DNA:PEI ratio optimization in Expi 293F cell transfection. Expi 293F cells were transfected with either HN and M or HN, M, and NP. -NP samples serve as a negative control and should exhibit markedly decreased VLP production. Here, DNA:PEI ratios of 1:2, 1:3, and 1:5 were used knowing that these ratios should be viable for this type of VLP production. Purified, isolated VLPs were examined via immunoblotting as well as a cell lysate fraction. While the ratios appear similar, a slight in increased productivity was observed in the 1:2 group, and the 1:2 ratio was selected for future Expi 293F experiments. No statistical analysis could be performed with n=1 repetitions.

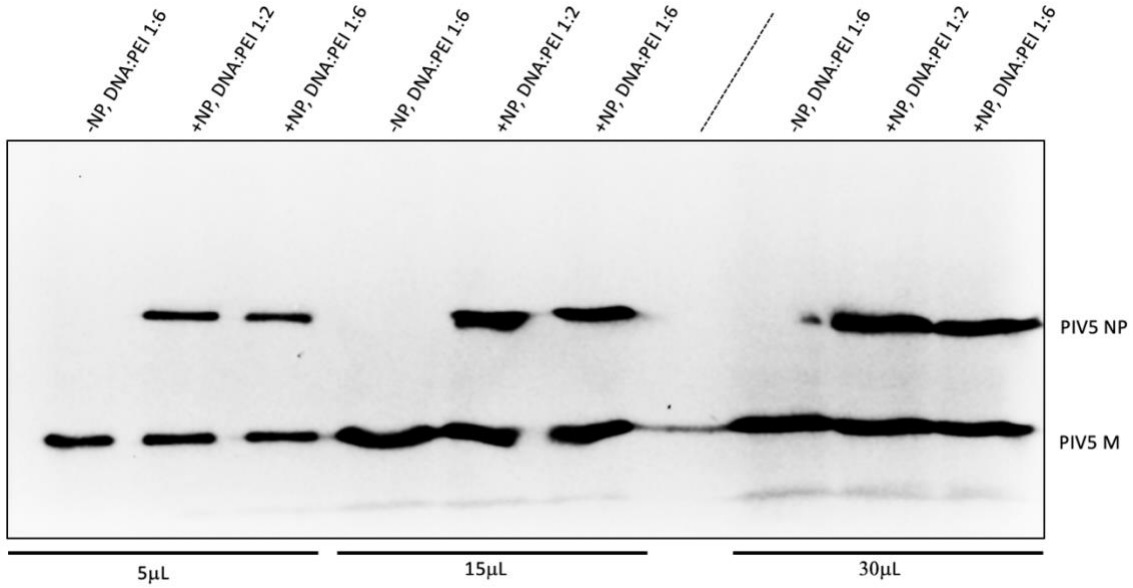

Figure 8: Optimization of DNA:PEI Ratios in CHO-K1S Transfection. Based on previous literature, 1:2 and 1:6 DNA:PEI ratios should be acceptable for CHO-K1S transfection. In order to determine if there was any difference in PIV5 VLP production for our purposes, we tested both ratios. We could not detect a difference in VLP production between the ratios and proceeded with a 1:3 ratio based on our observations and known guidelines for CHO transfection. Statistical analysis was not possible with n=1 repetitions.

transfection, were used as negative controls throughout these experiments because a lack of NP almost totally blocks VLP production. For these -NP samples, protein production can still be observed in the cell lysate, but none (or very little) of the protein should bud as VLPs and be observed in the purified VLP fraction. The trends for this experiment in the cell lysate sample were similar to the trends present in the purified VLP fraction. Very slight differences between ratios were observed suggesting that a 1:2 DNA:PEI ratio is optimal in the transfection of Expi 293 F cells (Figure 7). No further optimization was performed for Expi 293 F cells for the purposes of this study as strong signal detection was consistently observed. It should also be noted that the culture of these cells was user-friendly and easily reproduceable, especially when compared to CHO-K1S cells.

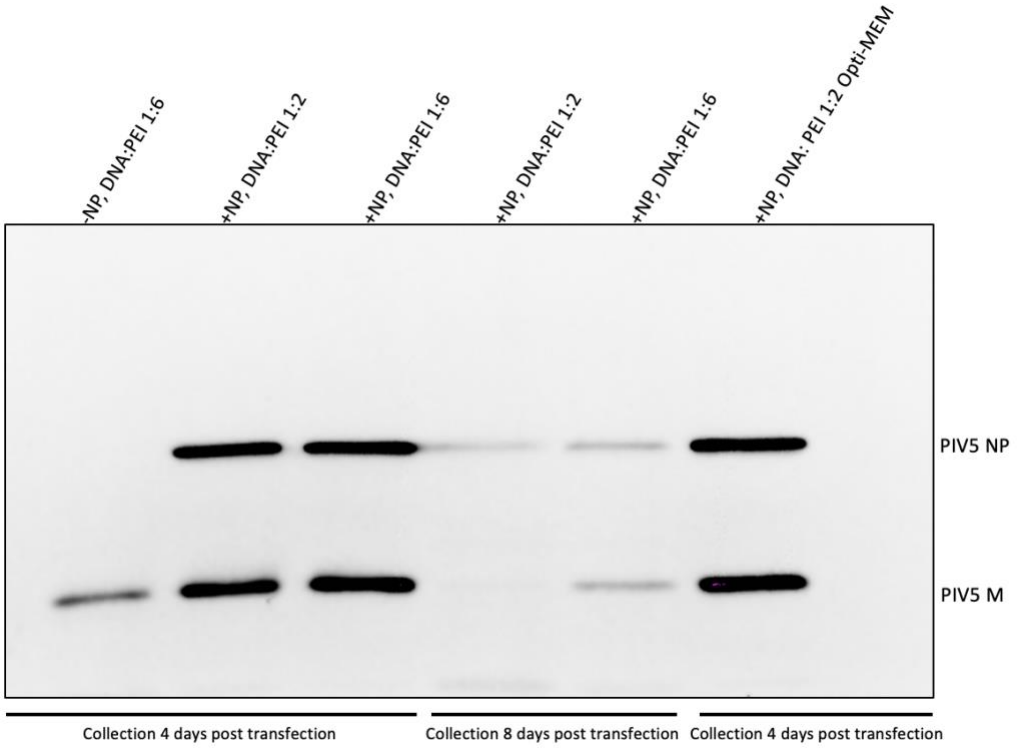

Figure 9: CHO-K1s Variation of Collection Time and Media. CHO-K1S cell transfection had previously not been detectable on the same blots as Expi 293F (not pictured), so they required separate optimization. Because it was more challenging to get CHO-K1S cells to express our desired proteins, additional conditions were optimized. CHO-K1S cells were transfected with either M, F, and HN only or M, F, HN, and NP. In an effort to find additional ways to boost CHO-K1S VLP production, DNA:PEI ratios were retested in addition to varying transfection to collection time and different media. Again, no difference was observed in production relative to DNA:PEI ratios. Collection 8 days post-transfection caused a marked decreased VLP collection. Varying media from to Hycell TransFxC to Opti-MEM appeared to have no effect on VLP production. The original transfection conditions were kept. Statistical analysis was not possible with n=1 repetitions.

CHO-K1S cells were weened off of serum and had initial difficulties with aggregation. After troubleshooting CHO-K1S cell culture, transfection was attempted and was barely detectable on the same blot as Expi 293F (not pictured). Because of the initial difficulties in CHO-K1S cells, additional avenues of optimization were explored. Based on previous literature, it is known that 1:2 and 1:6 DNA:PEI ratios should be at the upper and lower ends of acceptable ratios in CHO transfection. After testing, no discernable difference was detected between 1:2 and

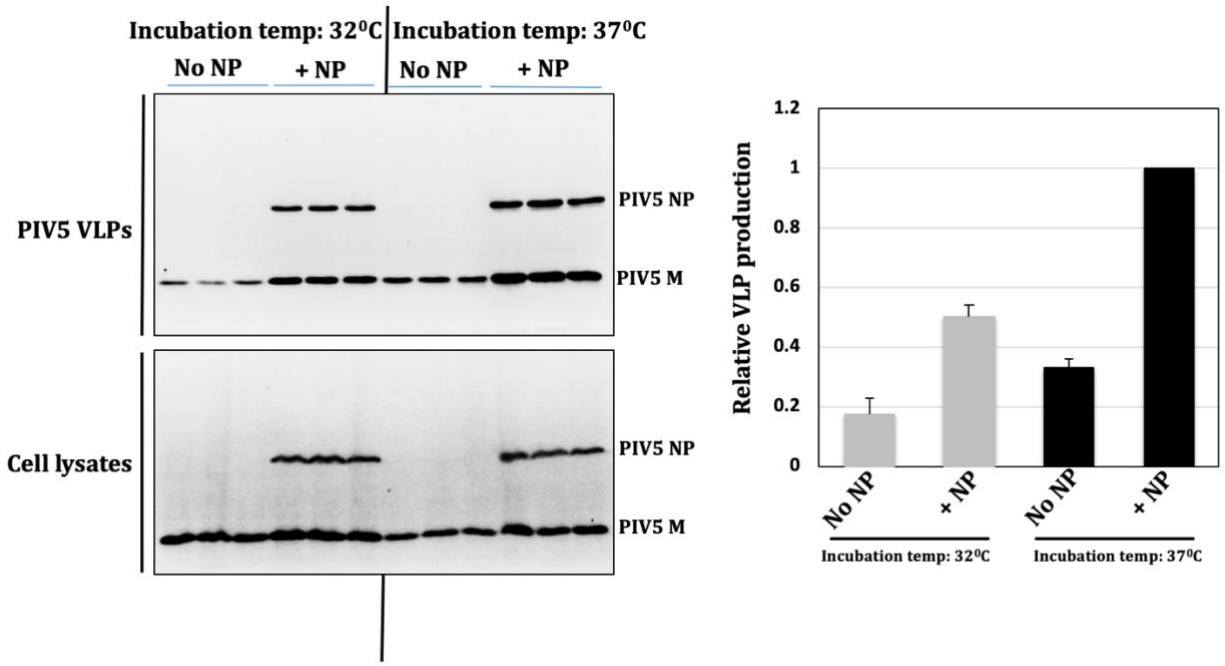

Figure 10: Varying Incubation Temperature and the Effect on CHO-K1S Transfection Production. Due to the previous poor transfectability of CHO-K1S cells observed, temperature was reduced from a standard 37 ₒC to 32ₒC to determine whether a lower temperature could lead to higher yields. Again, a -NP control was used. +NP at 32 ₒC and at 37 ₒC were significantly increased over the -NP respective controls ($P<0.005$) (n=3). The original incubation temperature, 37 ₒC, yielded significantly higher VLP production than the 32 ₒC group. 37 ₒC was selected as the incubation temperature for CHO cells moving forward.

1:6 ratios and either would be acceptable for further experimentation (Figure 8). Based on these results as well as on commonly practiced CHO transfection methods, a 1:3 ratio was selected for both CHO-K1 and CHO-K1S for the remainder of the experiments. While a 1:3 ratio was not specifically tested, it is within the range determined to be effective by these experiments. In an effort to maximize the yield of VLPs produced in CHO-K1S cells, it was attempted to collect the sample 8 days-post transfection instead of 4 days post-transfection. An 8 day-post transfection collection was performed alongside a 4 day post-transfection VLP collection and VLP yield was quantified via Western Blot. The 8 day post-transfection collection showed marked decrease in VLPs collected, so that question was not explored further (Figure 9). By 8 days, the VLPs had

begun to degrade and actually decreased the overall yield. Additionally, there was some question over the type of media being used. Transfection was attempted in Hycell TransFxC media as well as in Opti-MEM to test whether or not media was interfering with the transfection process . There was no detectable difference and transfection was continued the Hycell TransFxC media (Figure 9). One final optimization experiment involved lowering the temperature from 37$_o$C to 32$_o$C. In previous mammalian cell transfection studies, it was demonstrated that a 1.5-fold

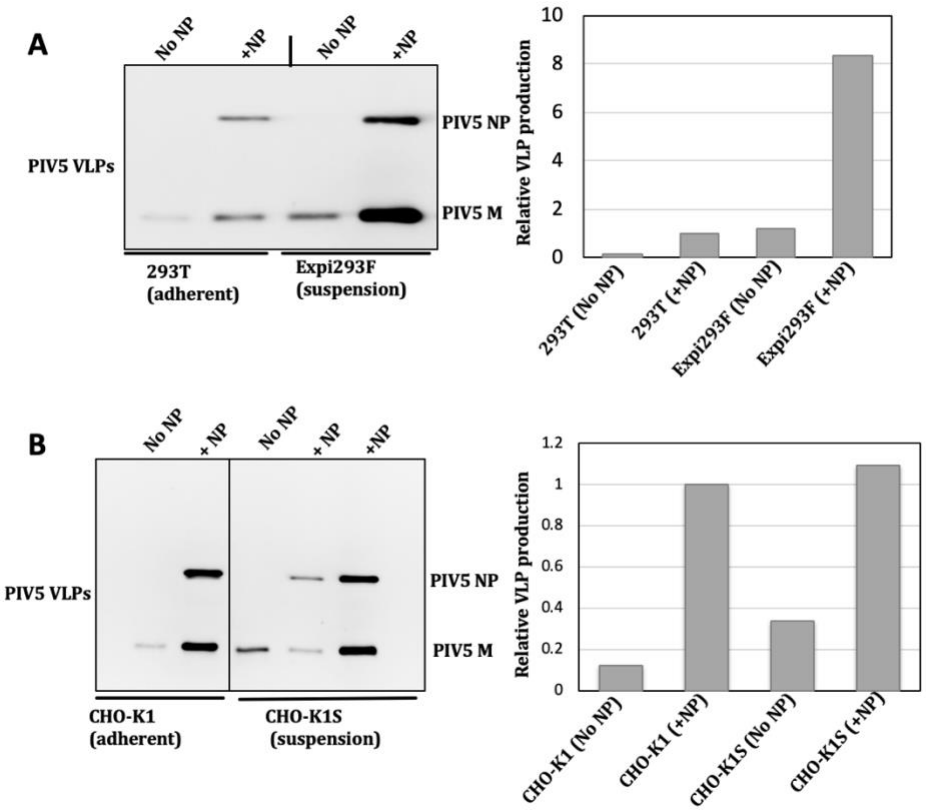

Figure 11: Comparing PIV5 VLP Production between Suspension Cell Line and Adherent Equivalent. Expi 293F is a suspension variant from 293T, the original producer for this PIV5 VLP platform. CHO-K1S suspension cells were adapted directly from adherent CHO-K1 cells. In order to test the effectiveness of each cell line in comparison with adherent counterparts, Western Blots normalized for incubator volume and sample loaded were performed and quantified. (A) Expi 293F cells outproduced adherent 293T cells yielding over an 8-fold increase in production. (B) CHO-K1S cells did not outperform adherent CHO-K1 cells in VLP production in an appreciable way in this experiment. Although the yield was higher, the difference was close enough that no statement could be made confidently about their performance without further experimentation and a statistical analysis. No statistical evidence could be gleaned from this experiment due to n=1 repetitions being performed.

increase in protein expression was attained by lowering incubation temperature to 32oC. We could not see any improvement in VLP yield with a lower temperature. Rather, yield was decreased by approximately 2-fold at 32oC when compared to the standard, 37oC incubation temperature (Figure 10).

3.2 Comparison of Candidate Cell Lines for VLP Production

When comparing suspension cell lines to their adherent counterparts, it is expected that equivalent samples should reveal more VLP production in the suspension cell line. Expi 293F

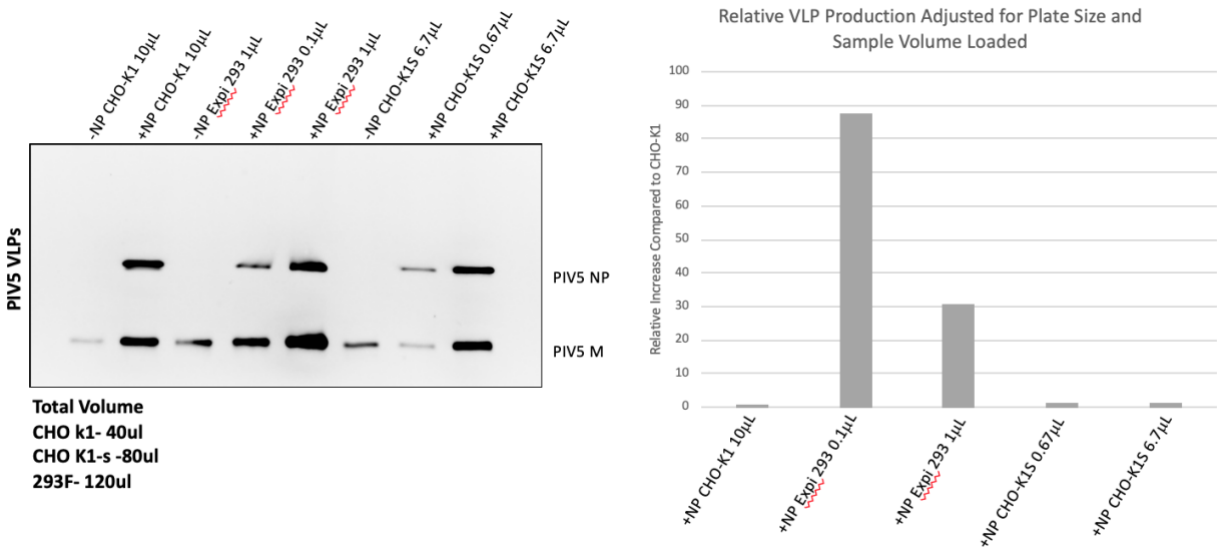

Figure 12: Comparison of VLP production between CHO-K1, Expi 293F, and CHO-K1S cell lines. Adherent CHO-K1, suspension Expi 293F, and suspension CHO-K1S cells were all transfected using their previously optimized transfection conditions. Various amounts of VLP samples were loaded due to expected differences in VLP production based on previous experiments. VLP production was normalized across samples based on volume of reactor and % fraction of total sample loaded. The.1uL sample loaded from Expi 293F cells showed a nearly 90-fold increase as compared to the adherent CHO-K1 control. It is important to note that the 1uL sample of Expi 293F resulted in a much lower production measurement (approximately a 30-fold increase compared to the control). This number is lower due to our scanner's inability to image the amount of signal output from this sample. The scanner was oversaturated due to the large amount of protein produced.

was compared to 293T, which is both Expi 293F's adherent counterpart and the original producer cell line used for this platform. In one trial, Expi 293F production yielded approximately an 8-fold increase in VLP production as compared with 293T production (Figure 11). These results suggest that Expi 293F produces a much higher VLP yield compared to 293T. In the future, more repetitions should be performed to determine, with statistical confidence, to what extent Expi 293F outperforms 293T cells in this regard. When a similar experiment was conducted comparing adherent CHO-K1 VLP production with suspension CHO-K1S VLP production, the results were less clear (Figure 11). In one repetition, CHO-K1S barely outperformmed its adherent CHO-K1 counterpart. It is not possible to conclude that CHO-K1S truly does outperform its control, and the slight increase in production could have been stochastic variation. In the future, more repetitions and a statistical analysis would be needed to reach a conclusion.

After some optimization, Expi 293F and CHO-K1S suspension cell lines were compared against each other using an adherent CHO-K1 cells as a control. The cell lines were compared via Western Blotting and imaged on one membrane. After normalization based on volume of reactor and the volume loaded, Expi 293F produced nearly 90 times more VLPs than an equivalent sample from CHO-K1 or CHO-K1S (Figure 12). CHO-K1S, however, produced similar amounts of VLPs to CHO-K1 and therefore did not achieve increased VLP production compared to the adherent control. It is important to note that the CHO-K1 control is not a control against the standard production methods for this technology, but was just used as a point of comparison. Expi 293F outperformed CHO-K1S by nearly a 90-fold increase in production, but it is likely that even the adherent 293T line is a better producer than CHO-K1S. The important conclusion gleaned from these data, therefore, is that Expi 293F is a vastly superior producer candidate when compared with CHO-K1S for the purpose of generating paramyxovirus VLPS.

As shown earlier, Expi 293F also appears to be a superior candidate when compared to the technology's original producer, 293T (Figure 11).

3.3 Quality Testing VLPs Via Renilla Luciferase Delivery

After selecting Expi 293F as the producer cell line due to its exceptional VLP yields, we proceeded to the delivery portion of this study. Until this point, all VLP production experiments have used full-length NP protein, which is optimal for production. For the following delivery experiments, VLPs were generated with RLuc-NP30 instead. A 30-amino acid segment of NP is responsible for directing M-NP interaction as well as packaging of the RLuc, which is illustrated in Figure 6. RLuc was selected because its bioactivity is easily measurable via a luminometer

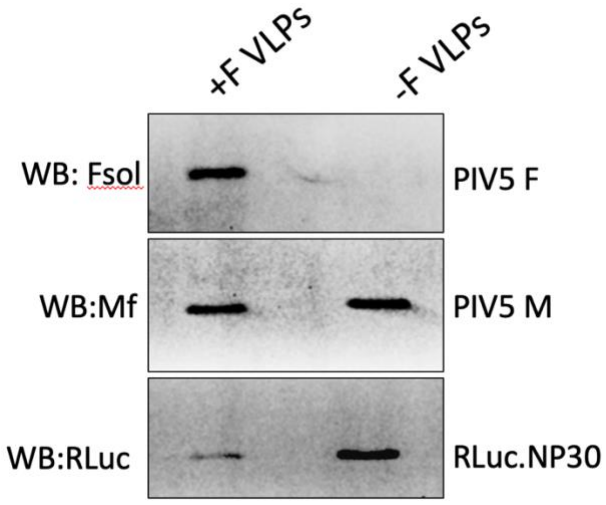

Figure 13: RLuc-loaded PIV5 VLP Production in Expi 293F Cells. Before proceeding to luminometer experiments to test delivery competency, a Western Blot was performed to ensure proper production of experimental and control groups. The VLPs were purified using a 3 spin purification. -F and +F VLPs were produced as appropriate groups for the luminometer experiments. RLuc.NP30 and PIV5M were detected in both VLP groups. F was detected in the +F group and no band was detected in the -F group, showing appropriate assembly each group of VLPs.

and its use as a cargo in this platform has been previously characterized (Greeshma et al. 2016). As a negative control for the luminometer delivery assay, -F VLPs were produced in addition to the experimental +F VLPs. -F VLPs have HN protein, so they retain the ability to attach to target cells, but they lack the F protein required for cellular entry. In theory, -F VLPs should be unable to deliver their cargo into the target cell and are an effective negative control to ensure that the luminometer does not read extracellular RLuc levels. Generation of both +F and -F VLPs containing RLuc-NP30 was carried out using the optimized conditions in Expi 293F suspension cells determined earlier in this study. The VLPs harvested were examined via Western Blot to ensure proper production. After scanning, M was detected in the purified VLPs to ensure the production of the particles and was substantial for both groups. RLuc-NP30 was also detected within the VLPs confirming proper cargo delivery. F was detected in the +F group but not in the -F group, ensuring that the control for the following experiment is valid (Figure 13).

After generation of +F and -F RLuc-NP30 VLPs was confirmed, the VLPs were used to deliver their cargo to A549 cells. After incubation with A549 cells and extensive washing, the samples were examined with a luminometer to determine intracellular RLuc activity. The luminometer readings are proportional to the amount of RLuc delivered to the cell. After measuring both samples, it was found that the +F VLPs delivered 40,000-times more RLuc to A549 cells than the -F VLPs. These results were as expected, where the fusion capable VLPs delivered their cargo and the fusion-incapable VLPs did not. The results of the delivery experiment confirmed that cargo loaded VLPs generated with our optimized methods are indeed capable of delivering cargo to A549 cells. These data indicate that the increased production of VLPs observed in Expi 293F cells was not at the cost of quality, as these particles are also delivery-competent.

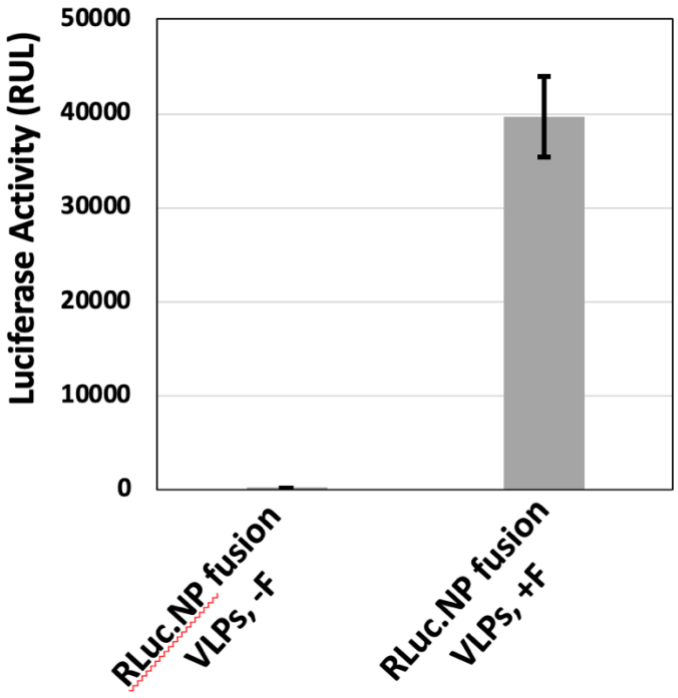

Figure 14: Luciferase Activity in A549 Cells Incubated with RLuc-Loaded PIV5 VLPs. The cargo loaded VLPs produced by Expi 293F suspension cells were tested in their ability to deliver quality via a luminometer assay. After incubation of VLPs with A549 cells and extensive washing, data from the luminometer was obtained. The negative control, the fusion-incompetent -F VLPs, did not elicit any substantial luminometer readings. The experimental group, the fusion-competent +F cargo loaded VLPs, elicited high luminometer readings. The 40,000-fold increase in luminometer readings from the +F group indicates that these VLPs are delivery-capable ($P<0.0001$) ($n=3$).

Chapter 4

Discussion

4.1 Suspension Culture VLP Production Evaluation

After only modest optimization of Expi 293F transfection, these cells outperformed CHO-K1 and CHO-K1S cells by nearly a 90-fold increase in VLP production. In addition to the major increase in production, Expi 293F cells were vastly more user friendly and more consistent. In spite of these advantages, the cell media required for Expi 293F culture/transfection is 4-times more expensive than the media required for CHO-K1S culture/transfection. If CHO-K1S cells could have produced even 25% of the VLP quantities of Expi 293F, they would have been considered as a producer cell line for our PIV5 VLPs. This reasoning is why additional optimization attempts were made for CHO-K1S that were not used for Expi 293F optimization. Because the gap in VLP production was so great and Expi 293F cells were generally less problematic, Expi 293F was determined to the be cell line of choice for producing high-volume PIV5 VLP production.

When compared against the original producers of this type of VLP, 293T cells, Expi 293 cells also outproduced the former by over 8-times in one trial. These results are promising and indicate that industrial plausibility may be attainable upon more rigorous optimization experiments.

4.2 Reliability of Data Obtained in Optimization/Production Experiments

This study is not intended to be a comprehensive optimization and analysis of suspension cell/PEI production of PIV5 VLPs. Instead, these experiments were intended to gather information and identify any potential issues with upcoming larger scale experiments. Most of these pilot experiments were not repeated to obtain statistically significant data and should not be considered definitive proof of the values we have obtained. Additionally, true optimization required more than two data points in order to find the peak of a curve. Rather, this data is sufficient for our in-house uses. For example, in conjunction with our subjective observations about scanner signal strength, pellet size, cell health throughout transfection, etc., the massive differences in VLP production justify our use of cell types. While these results may still be considered preliminary, they are useful for setting the stage for more expensive, publishable experiments.

4.3 Quality Testing VLPs Produced Via New Methods

While there was not expected to be any difference in the delivery capabilities of VLPs produced in adherent or suspension cells, luminometer delivery assay were performed regardless. Even if the new production methods produced unrealistically high yields of VLPs, if the VLPs are not functional, then the production increase is not useful. However, these luminometer experiments showed with statistical significance that our VLPs do deliver cargo to A549 cells. Based on the current data, we cannot determine with certainty that the 8-fold increase in VLP production translates directly into an 8-fold increase in delivery capability. However, the data compiled so far is consistent with the idea that suspension cell-produced VLPs have similar

delivery activity to adherent cell-produced VLPs. Further experimentation is required to prove the degree to which VLPs generated via these varying methods are similar or different, but the current results are promising.

4.4 Viability of Cargo Loaded PIV5 VLPs as a Protein Delivery Platform

The ability of PIV5 VLPs to self-assemble and incorporate minimally modified cargo protein into budding VLPs is promising as a protein delivery platform. One barrier faced by synthetic nanoparticle protein delivery is endosomal entrapment. Because PIV5 VLPs fuse directly with host cell plasma membranes and uncoat directly in the cytosol, their cargo would avoid endosomal entrapment. In addition to direct delivery to the cytosol, this VLP protein delivery system enjoys the benefits of avoiding genetic manipulation of target cells and the minimal modification (15-30 amino acids) of cargo proteins required to induce VLP packaging.

In order to industrialize this technology, the production methods for these VLPs needs an refinement. Making the switch from Lipofectamine and adherent producer cells to PEI and suspension producer cells is an essential step in defending the potential of this platform. Though there is not yet enough statistically significant evidence to prove the viability of these production methods, this pilot experiment has yielded promising results. In the future, larger scale experiments with statistical analyses would cement the viability of these production methods and bring this protein delivery platform even closer to industrial feasibility.

Appendix A

Abbreviations

CHO-K1 – Strain of adherent mammalian culture cells derived from Chinese hamster ovary cells

CHO-K1S – Strain of suspension mammalian culture cells derived from Chinese hamster ovary cells

DMEM – Dulbecco's modified Eagle's Medium

ECF – substrate used for Western Blotting

Expi 293F – Strain of suspension mammalian culture cells derived from canine kidney cells

F – Fusion glycoprotein

FBS – Fetal bovine serum

HN – Hemagglutinin-neuraminidase glycoprotein

LPS - Lipopolysaccharide

M – Matrix protein

MDCK – Madine-Darby canine kidney cells, adherent mammalian cell culture line

NDV – Newcastle Disease virus

NP – Nucleocapsid protein

Opti-MEM – minimal essential media lacking fbs

PBS – phosphate buffered saline

PEI – Polyethylenimine, a transfection reagent

PIV5 – Parainfluenza virus 5

PLB – Protein lysis buffer

PVDF – Polyvinylidene fluoride

RLuc – *Renilla* Luciferase

SDS-PAGE – Sodium dodecyl sulfate – Polyacrylimide gel electrophoresis

VLP – Virus-like particle

vRNP – Viral ribonucleoprotein

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- Honduras Medical Mission Trip – Aided in logistical preparation and tear down, acted as a pharmaceutical assistant, and performed light translation work as a part of a medical mission trip to Guadalupe Carne, Honduras (2016).

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