EFFECT OF SERUM AND CELLULAR PROTEINS ON HYBRID VECTOR TRANSDUCTION EFFICIENCY

By

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EFFECT OF SERUM AND CELLULAR PROTEINS

ON HYBRID VECTOR TRANSDUCTION EFFICIENCY

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TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

1.1 INTRODUCTION

The concept of gene therapy, which involves introduction of DNA (or RNA) in the cell to cure or prevent diseases associated with defective gene expression, has opened up endless possibilities in the fields of bioengineering and genetics. Altering the genes by activating, silencing, introducing or knocking out, has opened the door to finding a cure for many diseases[1]. When the genes are transported to the nucleus of the cells, there is a possibility of the genes undergoing degradation because of presence of various degrading acids and enzymes present in the cells[2]. It becomes essential to incorporate the gene to be delivered in a proper gene delivery vehicle which protects it from this environment and delivers it to the nucleus. Viral vectors and synthetic vectors are major gene delivery carriers used today. Despite having high efficiency, viral vectors have a risk of reversion during replication of live viruses or even mutation to a more pathogenic state[3]. Non-viral vectors, however, have a disadvantage of low efficiency. To overcome the drawbacks of viral and synthetic vectors, the concept of a hybrid vector has been proposed. The vector is composed of a synthetic material and a virus-like particle (VLP). The hybrid vector carries the gene to be delivered but lacks the envelope protein which is essential for recognizing and binding a specific cell receptor and mediating entry into the cell. The

synthetic part of the vector is composed of a polymer which fulfils the function of the envelope protein.

The hybrid vector, however, has low efficiency compared to the viral vectors used in gene delivery. We hypothesize that the presence of proteins during the formation of the hybrid vector complex has a negative effect on the transduction efficiency of the hybrid vector. To improve the efficiency of the hybrid vector, we intend to purify the VLPs from these proteins and form the hybrid vector using purified VLPs.

1.2 LITERATURE REVIEW

1.2.1 Viral Vectors

Most of the gene performed currently uses viral vectors. Retrovirus, adenovirus and adeno-associated virus are the major viral vectors used for gene delivery. One of the major advantages of using viral vectors is the extremely high efficiency of these viruses in delivery of genes to the target cells.

Using viral vectors has not been without consequences. In a major setback to the use of viral vectors, a patient died after being administered with adenoviral vector for the treatment of partial deficiency of ornithine transcarbamylase[4]. The death was attributed to the acute inflammatory response to the adenoviral vector.

Another major setback to the use of viral vectors occurred when children suffering from Severe Combined Immunodeficiency Disease (SCID) were treated with a gene-carrying retrovirus and developed leukemia. The retrovirus inserted the therapeutic gene and its

promoter near an oncogene of eleven patients. Three of the patients developed leukemia which led to a complete halt of viral gene therapy trials on human beings[5].

1.2.2 Synthetic Vectors

Synthetic vectors include carrier vehicles like polyplexes[6], micelles composed of block copolymers like poly(ethylene glycol)-*block*-polycations[7], lipopolyplexes and lipoplexes[8], matrix-degrading metalloproteinases[9] and lipids. The use of copolymers for non-viral delivery of DNA was studied by d'Ayala *et al*[10]. In their study, the researchers synthesized a copolymer of polyethylenimine (PEI) and poly (E-caprolactum). This copolymer was used to form a complex with DNA and the complex used to transfect on human cervix epithelial carcinoma (HeLa) cells. They observed that though PEI by itself is cytotoxic, use of a PEI-based copolymer was less cytotoxic and helped to improve gene delivery. They also proved that they could have better control on gene delivery by controlling particle size and surface charge by modifying experimental condition and conditions at which the polymers were made. Despite the advantage of being relatively less toxic and less immunogenic, synthetic vectors have a drawback of low efficiency compared to viral vectors[11].

1.2.3 Hybrid Vectors

Hybrid Vectors are composed of viral and non-viral (i.e. synthetic) components. The viral part performs the functions of intracellular trafficking, nuclear import and gene integration while the synthetic part does the function of viral envelope and facilitates endocytosis and endosomal escape.

Han *et al* tried to improve the transduction efficiency of recombinant adenovirus with a new synthetic polymer based on PEI[12]. They synthesized a copolymer of PEI with diethylene glycol (DEG). Adenoviruses need a coxsackie adenoviral receptor (CAR) receptor on the surface of the cell to undergo endocytosis. Complexes containing different ratios of polymer and adenovirus were formed and infected on Lewis Lung carcinoma (LLC), MDCK and A549 cell lines. The MDCK cell line is deficient. Enhancement in gene transfer efficiency was observed on native CAR positive target cells. In presence of copolymer, the complex also was able to infect the CAR negative cells. The study showed the ability of adenovirus to overcome intracellular barriers and the high efficiency of copolymer to endosomal escape were major factors which helped in improving the efficiency of the vector. Thus the combination of viral and non-viral vectors, which created a hybrid vector, was found to be effective in combining the advantages of both the vectors.

Another approach for producing a hybrid vector is the use of viral component called virus-like particles. VLPs consist of proteins that form the outer shell and the surface protein of the virus, without the RNA required for replication. These particles resemble the virus from which they are derived but lack the envelope protein. This makes the VLPs non-infectious. To make these non-infectious VLPs infectious, we add synthetic component, either lipid or polymer.

Friedmann *et al* showed that non-infectious VLPs produced from Moloney murine leukemia virus (MoMLV) packaging cells can be made infectious by addition of lipofectin reagents[13]. The VLPs produced from MoMLV lack the viral envelope protein necessary infection. The envelope protein performs two important functions, recognition

of receptor for specific binding of the virus to a cell and fusion, which permits the release of uncoated virus and the viral RNA from the endosome into the cytoplasm. To make the non-infectious VLPs infectious, the lipofectin was added to the VLP solution. These VLPs and lipofectin were incubated and then added to target cells which were transfected. As controls, virus pseudotyped with vesicular stomatitis virus G protein (VSV-G) and VLPs without addition of lipofectin, were used to infect target cells. Reporter gene expression from the infection of lipofectin VLPs was compared with that from VSV-G pseudotyped viruses. VLPs without lipofectin did not show any gene expression. While the VLPs with lipofectin showed high gene expression.

Ramsey *et al* formed hybrid vector complexes from retrovirus VLPs, derived from the Moloney murine leukemia virus (MoMLV-VLP), and either poly-L-lysine (PLL) or PEI and studied their transduction on HEK-293 cells[14]. MLV-VLPs were produced from the GP-293 Luc producer cell line which expresses the MLV *gag-pol* genes which assemble and form the MLV-VLPs. These VLPs bud from the producer cells. The supernatant containing VLPs was collected and PEI and PLL were added to the supernatant. Complexes were formed with different molecular weights of PEI and PLL and at different ratios of polymer/VLPs. Both PEI and PLL complexed VLPs showed good transduction. They studied the particle size with dynamic light scattering and particle morphology using transmission electron microscopy (TEM). The particle size was observed to be approximately 1,000 nm. Infection assay showed that the VLP/polymer complex was integrated in the cells. The large sized particles underwent successful transduction. This was due to the fact that large size would result in increased contact between complex and target cells. TEM micrographs showed particle morphology

showed a complex containing VLPs surrounding polymer aggregate. This morphology was hypothesized due to the presence of serum proteins in the VLP supernatant. The serum proteins were observed to take part in the complex formation and hence the size of complex was large.

CHAPTER II

PURIFICATION OF VIRUS-LIKE PARTICLES FROM SERUM AND CELLULAR PROTEINS

2.1 INTRODUCTION

The relatively low hybrid vector efficiency observed by Ramsey *et* al may be due to the presence of serum and cellular proteins during the formation of complex between VLPs and polymer[14]. The VLPs were collected in supernatant containing serum and cellular proteins and PEI was added to this supernatant to form complexes. Cationic PEI is expected to form electrostatic complex with positively charges VLPs and proteins. The presence of serum and cellular proteins was also supposed to influence the morphology of complex formation. The complex was observed to be large in size and also showed a mixture of VLPs surrounding a mass of proteins and PEI. We decided to purify the VLPs from the serum and cellular proteins and then form complexes using purified VLPs and PEI and study their transduction. We decided to adopt and modify a purification strategy that would enable us to separate the proteins from VLPs. In absence of proteins, we expect a small sized complex to be formed which we expect to improve the transduction efficiency.

2.2 PURIFICATION STRATEGY

2.2.1 Literature Survey

A study of purification methods has shown that different techniques like cesium chloride ultracentrifugation, heparin affinity chromatography, size exclusion chromatography and high performance liquid chromatography (HPLC) have been used for purifying viruses[15]. Transfiguracion *et al*, in their efforts to separate VSV-G virus from serum proteins, used the technique of HPLC.

Edelstein *et al* used sucrose density gradient centrifugation to separate Ross River Virus and Barmah Forest Virus from proteins[16]. Although the titers obtained for both type of viruses were quite high, this method has a drawback in that it cannot be used for large scale purification. Also, the infectivity of the recovered particles depends on the susceptibility of the particular pseudotyped retroviral vectors to hydrodynamic shear. Additionally, cellular debris, host nucleic acid and serum proteins that co-purify with the virus need to be removed in subsequent steps.

Segura *et al* exploited heparin affinity chromatography to purify retroviral vectors from proteins[17]. They subjected the supernatant containing viral particles and proteins to ultra-centrifugation, and the concentrated virus was added to a heparin affinity chromatography column. The viral particles were washed and then eluted using a NaCl gradient buffer. Despite obtaining high titers of viral vectors, the cost prohibits the use of this technique on large scale.

2.2.2 Purification Strategy

The purification system we chose to purify the VLPs was adopted from Transfiguracion *et al*, who used the approach to purify VSV-G virus[18]. The process involves concentrating virus, digesting cellular RNA and DNA and separating virus from the supernatant proteins using size exclusion chromatography.

VLPs are known to have an average size of 135-150 nm which was confirmed from DLS[19]. The proteins present in the medium have an average size of less than 10 nm. The proteins tend to agglomerate and form protein-protein complexes which had an average size of 40 nm (Figure 2.1). Thus the VLP supernatant was found to be comprised of particles with three different size categories: VLPs, 135 – 150 nm; proteins, 4 – 8 nm; protein agglomerates, 40 – 50 nm.

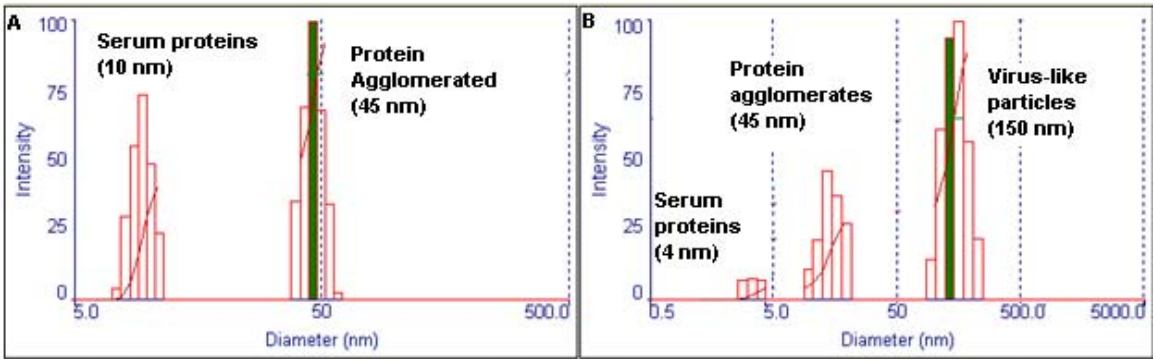

Figure 2.1: DLS data showing size distribution of FBS in DMEM (A) and FBS and VLPs in DMEM (B)

This size difference between VLPs and proteins was used to purify VLPs from proteins. Figure 2.2 gives an overview of our purification strategy. The purification process first involved concentrating the VLPs using an ultrafiltration cell with a polyvinylidene fluoride (PVDF) membrane having a MWCO of 300,000. This was followed by digestion of DNA and RNA using Benzonase nuclease. The last step used size exclusion chroma-

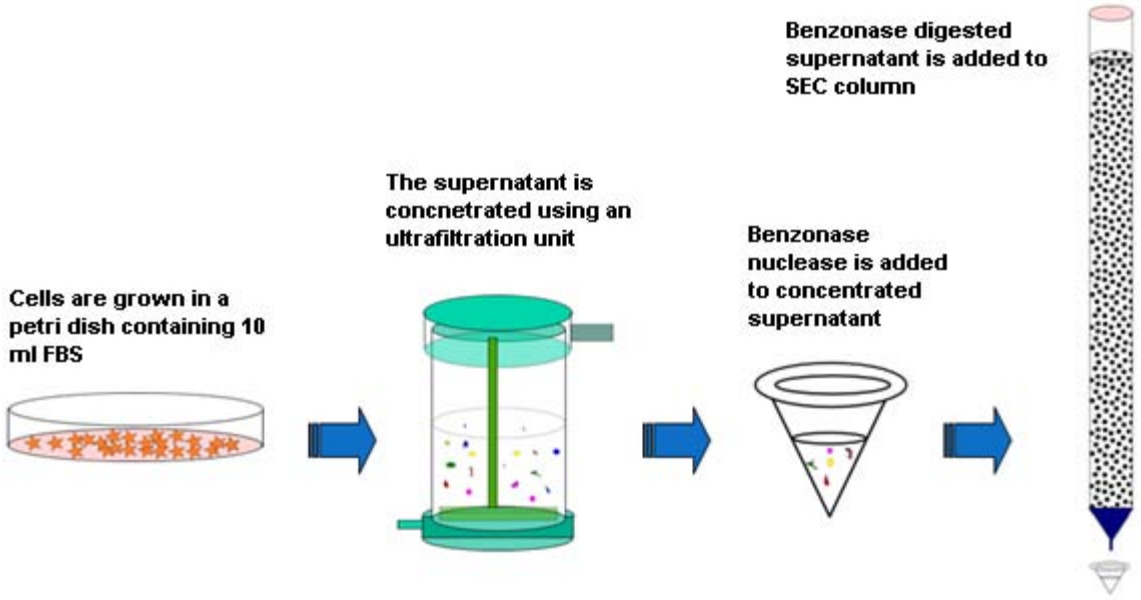

Figure 2.2: Brief Description of Purification Process

tography, which separates molecules based on size, large molecules are eluted first and small molecules are eluted last. Tris-EDTA-NaCl (TEN) buffer is used for elution from size exclusion chromatography column. These VLPs are subjected to different steps of purification as described. The volume of VLP supernatant and the amount of infective VLPs change with each step of purification. The process also changes the concentration of proteins relative to the VLPs. As a result there are a number of factors changing that must be accounted for at the each step of purification. As such, we standardized our purification system to account for these factors using VSV-G virus. The VSV-G virus is naturally infective and does not require PEI. As such, the presence of proteins does not affect the infectivity of VSV-G virus. The VSV-G virus was subjected to the same purification process and the percent recovery of infective VSV-G virus was calculated.

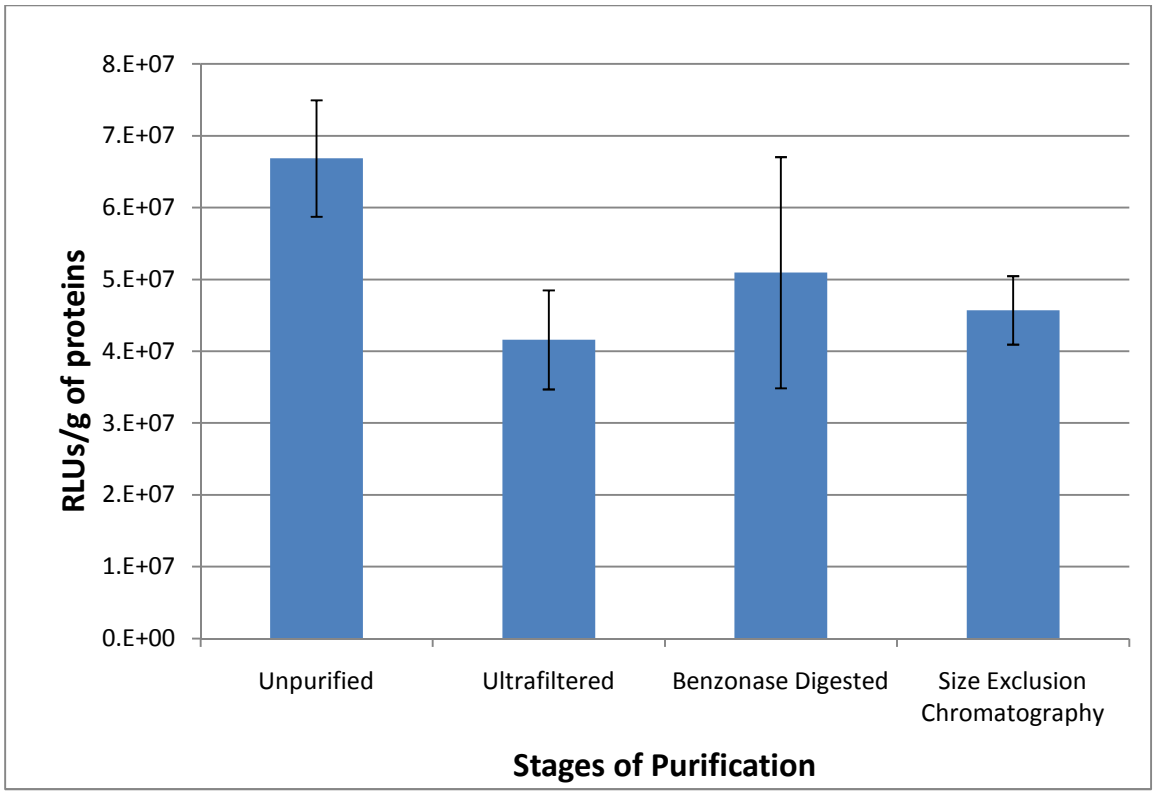

Figure 2.3: Purification of VSV-G virus (Avg. of three readings. P-values: 0.0077, 0.41, 0.808)

The percent recovery accounted for loss of virus and loss of activity (Figure 2.3).

2.3 RESULTS AND DISCUSSION

We initially identified SEC fractions containing VLPs and proteins. Both VLPs and proteins show high absorbance at 280 nm. We observed two sets of fractions showing high absorbance (Figure 2.4 A). To validate that the fractions eluted first contain VLPs, we performed an infection using all 30 fractions (Figure 2.4 B). The first few set of fractions showing high absorbance was found to give high values for infection. The second set of fractions showing high absorbance did not show any infection results which validated the fact that VLPs were eluted in the earlier fractions.

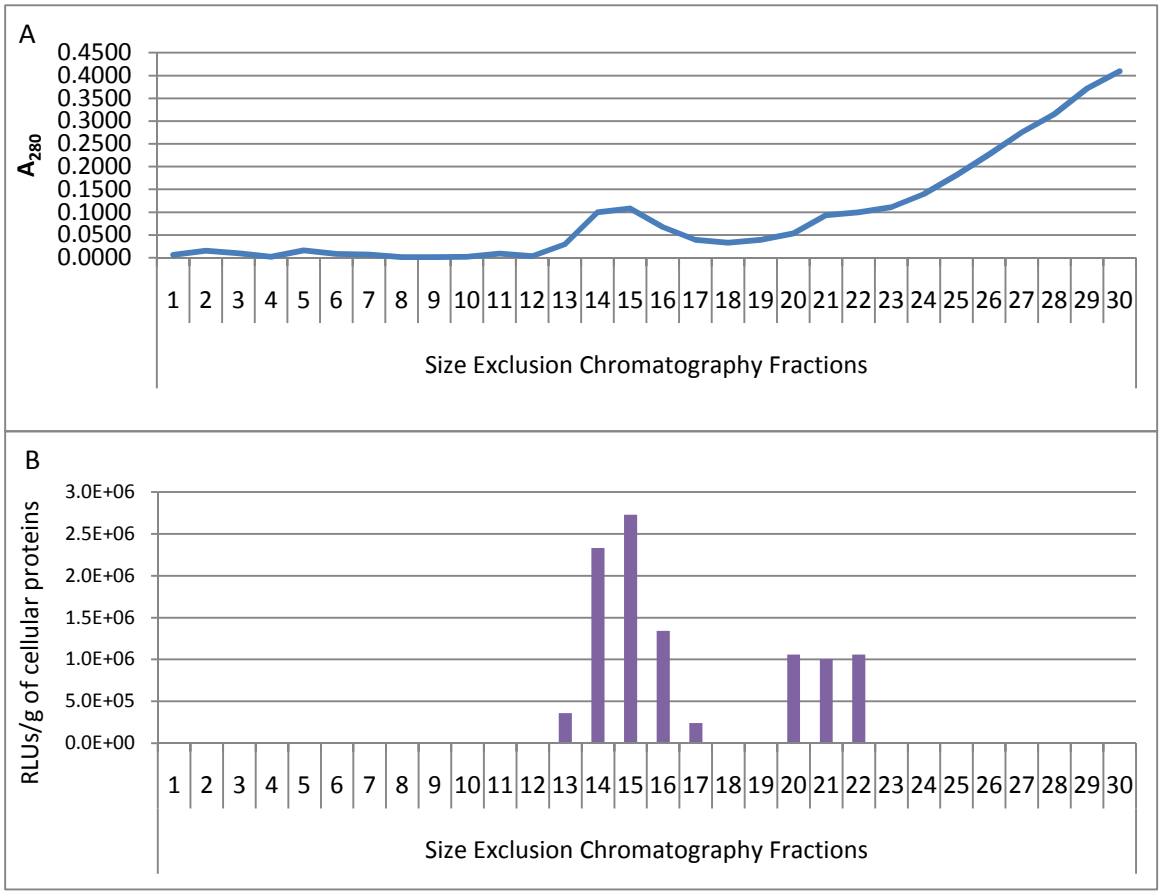

Figure 2.4: Absorbance (A) and infection (B) analysis of fractions obtained from size exclusion chromatography column (Avg. of 3 readings for absorbance, single reading for infection)

The protein concentration was quantified at each step of the purification process using a BCA assay. The supernatant was diluted to the concentration of unpurified VLPs. The protein content in unpurified VLP supernatant was 2.95 µg/µl. The protein concentration calculated in the final purified VLPs was below the lower limit of the BCA assay kit (< 0.02 µg/µl) (Table 2.1).

Table 2.1: Protein concentration after each step of purification

Type of VLPs	Supernatant Protein Concentration (µg/ µl)
Unpurified VLPs	2.95
Stirred Cell Purified VLPs	22.01
Benzonase Digested VLPs	14.56
Size Exclusion Chromatography VLPs	< 0.02

To further demonstrate that the purification strategy separated VLPs and proteins, we carried out dynamic light scattering analysis of the purified supernatant and unpurified VLP supernatant (Figure 2.5). Based on the results the unpurified VLP supernatant contain particles with average diameters of 4 nm, 45 nm and 150 nm. These peaks correspond to proteins, protein agglomerates and VLPs. The peaks at 4 nm and 45 nm were absent in the purified VLP supernatant.

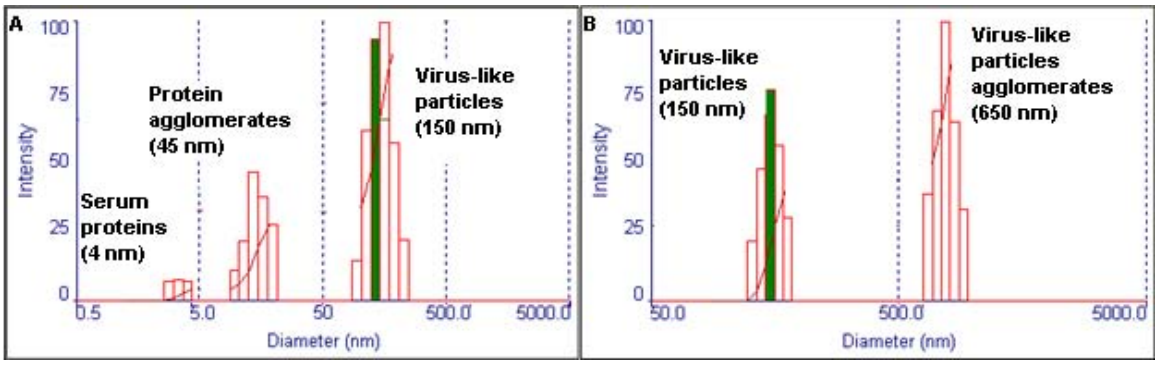

Figure 2.5: DLS data showing unpurified (A) and purified (B) VLP supernatant

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was carried out on samples collected at different stages of purification. The SDS-PAGE results indicate the presence of proteins and VLPs in the unpurified, ultrafiltered and benzonase digested samples. The concentration of VLPs in the final purified sample, however, was too low to be detected (Figure 2.6 A). In order to overcome this difficulty,

we used western blot analysis to detect purified VLPs. Rat monoclonal antibody for p10 capsid protein and horse-radish peroxidase conjugated anti-rat IgG1 secondary antibodies were used. Detection of the labeled bands was carried out using a chromogenic detector and 3,3',5,5' –tetramethylbenzidine (TMB) substrate (Figure 2.6 B).

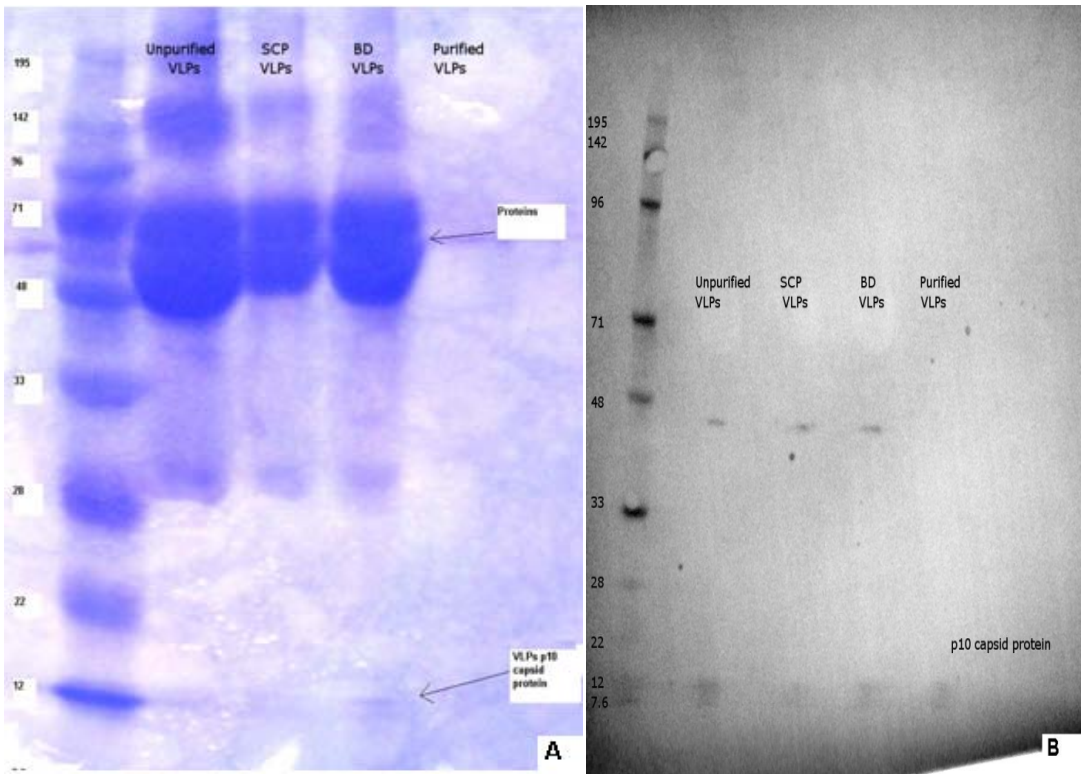

Figure 2.6: SDS-PAGE (A) and Western Blot (B) analysis of VLPs at different stages of purification

We used TEM to study the morphology of complexes formed using PEI and either unpurified or purified VLPs. Complexes were formed using both purified and unpurified VLPs and PEI and these complexes were fixed on a carbon-nickel grid. The grid was stained using phosphotungstic acid (PTA) and micrographs were obtained. The TEM micrographs for both purified and unpurified VLPs showed different morphology. For the complex formed from unpurified VLPs and PEI, a huge cluster of VLPs was observed. The cluster also contained some PEI and proteins. However, the cluster showed

morphology of PEI and proteins was surrounded by VLPs (Figure 2.7 A). In case of complex formed from purified VLPs and PEI, the morphology was different. It showed a single VLP particle surrounded by PEI (Figure 2.7 B). The absence of proteins prevented the formation of agglomerates and as such smaller complexes were formed.

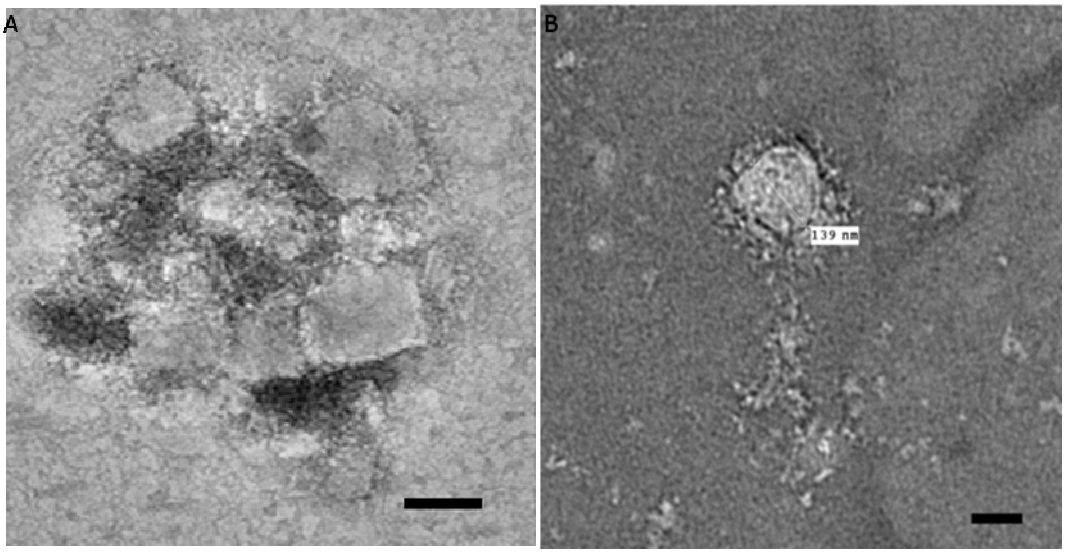

Figure 2.7: TEM micrographs showing complexes formed using unpurified VLPs and PEI (A) and purified VLPs and PEI (B) (size bar is 100 nm)

This finding is supported by the DLS data for complexes formed using PEI and either unpurified or purified VLPs (Figure 2.8). The unpurified VLP-PEI complex gives a complex size of 2300 nm. While the complex formed from purified VLP-PEI complex gives a complex size 650 nm. This small complex size is due to absence of proteins in the complex.

After each step of purification, the concentration of VLPs is changes. VLPs also undergo degeneration as they undergo the purification process. To compensate for above factors,

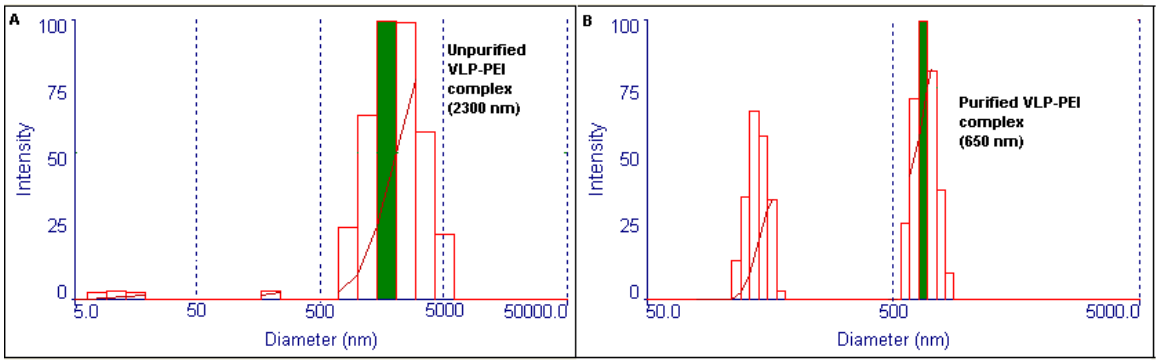

Figure 2.8: DLS data showing complex formation using unpurified (A) and purified (B) VLPs with PEI

we used the percent recovery of the VSV-G pseudotyped virus to adjust the volume of VLPs used for infection after each step of purification. Based on the VSV-G data, the amount of infective VLPs after each step of purification was normalized to that of unpurified VLPs. However, there was uncertainty in measuring the percent recovery of VSV-G virus after each step of purification. Hence, the overall recovery of VSV-G virus was calculated, which was 65 %. This percent recovery was used for normalizing concentration of VLPs after each step of purification.

After normalizing the concentration of VLPs obtained at each step of purification, we formed complexes using VLPs and PEI. These complexes were incubated for two hours and then transfected on HEK-293 cells seeded at 1×10^6 cells/well in a six-well plate. After 48 hours, the HEK-293 cells were lysed and luciferase assay was performed to determine the amount of infection (Figure 2.9). Graph shows that after each step of purification, the transduction efficiency of VLPs goes on reducing. The transduction efficiency of final purified VLPs is less as compared to the transduction efficiency of unpurified VLPs.

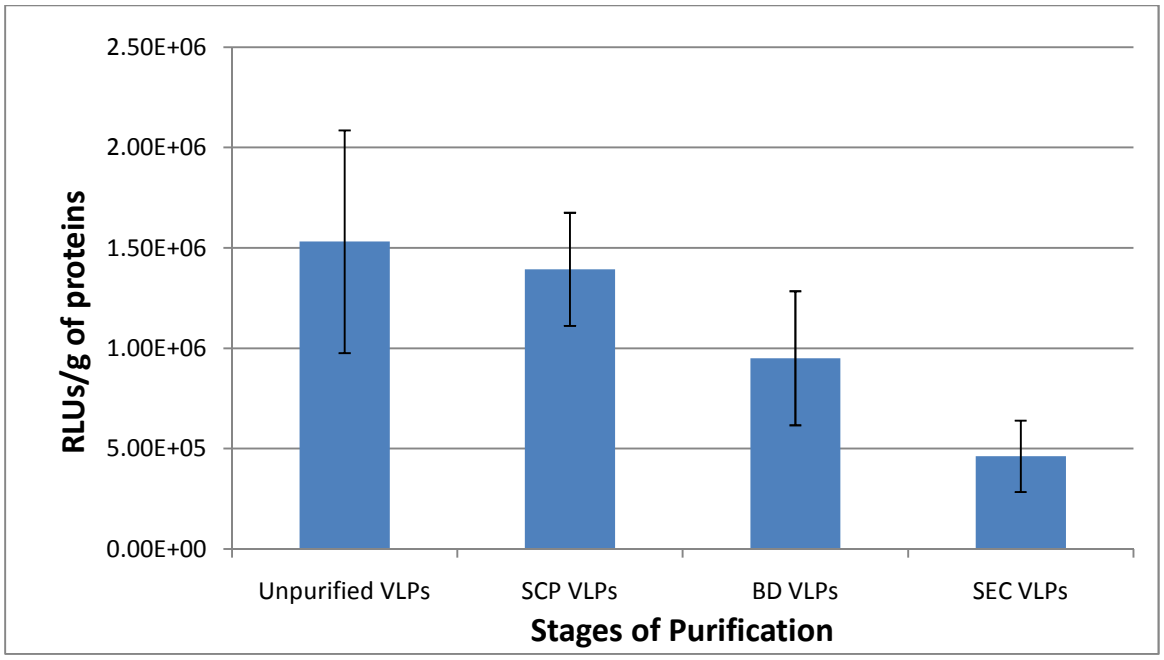

Figure 2.9: Infectivity of VLPs after each step of purification (Average of 3 readings, P-values: 0.66, 0.12, 0.069)

BCA assay showed us that the protein concentration is changing after each step of purification (Figure 2.10). If we compare the protein concentration and transduction efficiency of VLPs after each step of purification, we find that after ultrafiltration, the protein concentration is increased. However, the transduction efficiency is reduced. Conversely, the concentration of proteins in the size exclusion chromatography purified VLPs is negligible. The transduction efficiency is still reduced. To justify this effect, we compared data obtained from BCA assay, infection analysis and DLS analysis.

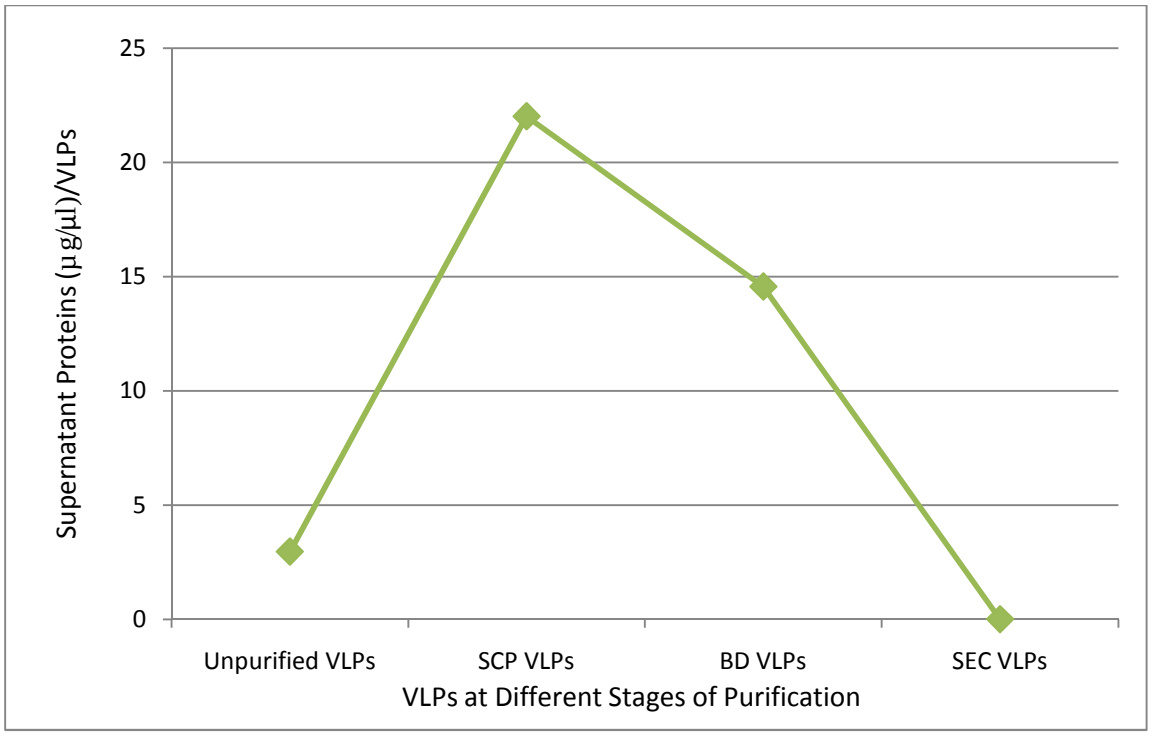

Figure 2.10: Amount of Supernatant Proteins per amount of VLPs after each step of purification

The DLS data showed that the size of complex formed from purified VLPs and PEI was smaller than the size of complex formed from unpurified VLPs and PEI. However, the complex of purified VLP-PEI was less infective as compared to complex formed from unpurified VLP-PEI. The size of complex formed could be one of the factors that could affect the transduction of VLP-PEI complex on cells. Landazuri *et al* studied the factors affecting the transduction of retrovirus-polymer complex. In their study, they added oppositely charged polymers such as polybrene and chondroitin sulfate C (CSC) to retrovirus stocks and studied their transfection on cells. They observed that the complexes formed from polymer and viruses tend to sediment rapidly on the cells as compared to adding viruses alone. With increase in concentration of polymer, the size of complexes increased and so did the infection which was attributed to increase in the rate of sedimentation of the complexes on the cells[20].

The same scenario may apply to our hybrid vector. In place of the negatively charged polymer, however, we have negatively charged proteins. The negatively charged proteins help in formation of large size of complex which rapidly sediments on the cells. Absence of proteins, leads to formation of small sized complex which does not settle rapidly on the cells. Presence of too low amounts of proteins could lead to formation of smaller complexes between VLPs and PEI and this could lead to diffusion of complex before it reaches the cell surface. Presence proteins help in formation of large complexes which tend to sediment rapidly on the cell surface and thus improve the transduction efficiency of the complex.

2.4 CONCLUSION

Our findings show that the serum and cellular proteins present during a complex formation have a significant impact on the transduction efficiency of the resulting complex. The infectivity of the final purified VLPs is less than the infectivity of unpurified VLPs. An excess of proteins also hinders the transduction efficiency of the complex.

We concluded that there is some amount of proteins required for the formation of an infective VLP-PEI complex.

2.5 MATERIALS AND METHODS

Cell Lines: Human embryonic kidney (HEK) cells, HEK-293, and a murine leukemia virus packaging cell line, GP-293Luc, were used for our experiments. HEK-293 cells were purchased from American Type Culture Collection, Manassas, VA. GP-293Luc cells were purchased from Clontech Laboratories Inc., Mountain View, CA. Both cell

lines were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum and cultured at 37 ^{o}C in 5 % CO_2.

Virus and Virus-Like Particles: Envelope-free MLV-VLPs with a *luc* reporter gene were produced from the GP-293Luc cell line. GP-293Luc cells were seeded at 1.5×10^6 in 12 ml of medium on a 10 cm dish. The cells were cultured for three days before the supernatant was collected and filtered through a 0.45 µm polyethersulfone syringe filter. Filtered virus-like particles were used immediately.

Vesicular Stomatitis Virus – Glycoprotein (VSV-G) pseudotyped viruses were produced by transfecting the GP-293 Luc cells with the plasmid pVSV-G. The cells were seeded in a 10 cm dish 18 – 24 hours prior to transfection so as to be ~90 % confluent at the time of transfection. The cells were transfected with 24 µg of envelope plasmid (pVSV-G) using Lipofectamine 2000 and following the protocol supplied with the transfection reagent. The transfection medium was replaced after 6 hours. Viruses were collected 48 hours later and filtered through 0.45 µm polyethersulfone syringe filter.

Polymer: Polyethylenimine was purchased through Sigma-Aldrich, St. Louis, MO. Stock solutions of the polymer were prepared in ultrapure water at a concentration of 10 mg/ml and stored at 4 ^{o}C.

Hybrid Vector Formation: Polymer/VLP complexes were formed through drop-wise addition of stock polymer solution to VLP supernatant while vortexing. Polymer from the stock solution was added to VLPs in a 1.5 ml ultracentrifuge tube and vortexed. The polymer/VLP solution was incubated at room temperature for two hours before infecting the cells.

Cell Transduction: HEK-293 target cells were seeded in 6-well plates 18 – 24 hours before transduction at a seeding density of 1×10^6 cells/well. Immediately before addition of hybrid vector complexes the cell growth medium was replaced with serum-free DMEM. Hybrid vector complexes were then added to each well and incubated at 37 ^{o}C. After four hours, the medium was replaced with serum medium.

Gene Expression Assay: Cells infected with polymer/VLP complexes were assayed after 48 hours after the addition of the vector. For detection of luciferase protein the growth medium was aspirated and 200 µl of cell culture lysis reagent (CCLR), Promega Inc., Madison, WI, was added to the cells. The cells were lysed at room temperature for 10 minutes followed by a 5 minutes freeze-thaw cycle at -80 ^{o}C. Luciferase activity for 20 µl of the cell lysate was measured using Promega's Luciferase Assay System and a Lumat LB9507 luminometer, EG&G Berthold, Bundoora, Australia.

Protein Assay: Protein analysis was carried out using the BicinChoninic Acid (BCA) assay from Pierce, Rockford, IL. The microplate procedure for 96-well plate was followed. Working Reagent from BCA reagent A and BCA reagent B was prepared. The sample addition and working reagent addition was done as per the manufacturers' protocol. Plate reader from Packard Biosciences, Illinois, was used to take absorbance readings at 560 nm.

Transmission Electron Microscopy: VLP and VLP/polymer complexes formed form unpurified and purified VLPs were fixed using 50 µl of Karnovsky's fixative to 100 µl of sample. A carbon formvar grid was held in a tweezers and a few drops of sample were added to the grid in such a way that small amount of sample was allowed to overflow the

grid. The grid was exposed to the sample for three minutes. The grid was then rinsed with PBS. Two drops of Uranyl Acetate (UA) stain were added dropwise on the grid in such a way that the stain replaced the excess of sample on the grid. The grid was stained for two minutes. The excess UA was wicked off using filter paper. The grid was dried for 10 minutes before viewing using JEOL JEM-2100 Scanning Transmission Electron Microscope System.

Ultrafiltration: A stirred cell ultrafiltration unit, Millipore Inc., Bedford, MA, was used for concentrating the VLPs. An ultra-filtration membrane, Millipore Inc., Bedford, MA, with a molecular weight cut-off (MWCO) of 300,000 was used. A steady operating pressure of 45 PSI was used to pressurize the ultrafiltration chamber. The stirrer speed was maintained at 60 rpm.

Benzonase Digestion: Benzonase nuclease was obtained from Novagen. Concentrated solution with an activity of 10,000 units/µl was diluted down to an activity of 250 units/µl using the Tris-EDTA-NaCl (TEN) buffer. The diluted solution was stored at -20 ^{o}C.

Size Exclusion Chromatography: Protein A sepharose CL-4B, Amersham Biosciences, Piscataway, NJ, was packed in a glass column XK16/40 (Fisher Scientific). The gel was packed using a continuous flow of ethanol and then further flushed with (TEN) buffer before running the sample. After running each cycle, the column is flushed with 50 ml 1N NaOH (as per manufacturer's instructions) and then flushed with 150 ml of TEN buffer to clean the column of NaOH.

Absorbance Reader: The fraction analysis was carried out using Cary UV-Visible Spectrophotometer. The analysis was carried out using an un-built filter measuring for a wavelength of 280 nm.

Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE): The SDS-PAGE analysis was carried out using XCell II Blot Module, Invitrogen, Carlsbad, CA. The gels used were 4 – 20 % gradient gels obtained from Pierce Biotechnology and Electrophoresis, Rockford, IL. Tris-HEPES-SDS running buffer was prepared from 100 mM Tris, 100 mM HEPES and 3 mM SDS. One liter of running buffer is sufficient to run 2 gels. Sample buffer was obtained from Expedeon, San Deigo,CA. Sample buffer was diluted to 1X concentration using DI water. Sample and sample buffer were mixed in a ratio of 4:1 and the mixture was boiled for 5 minutes at 100 ^{o}C. A volume of 5 – 10 µl of sample was added to each well of the gel. Protein ladder was obtained from Expedeon, San Deigo, CA. The gels were run at 125 volts for 45 minutes as per manufacturers' instructions.

For detection, we used Pro Sieve Blue Protein Staining Solution obtained from Lonza, Rockland, ME. Microwave instructions mentioned on the bottle of the staining solution were followed.

Western Blot Analysis: Producer cells for rat IgG1 monoclonal antibody were obtained from American Type Culture Collection, Manassa, VA. The cells were grown in RPMI 1640 medium supplemented with 10 % fetal bovine serum and 0.05 mM 2-β-mercaptoethanol. Antibodies were secreted into the medium and the medium collected off the cells after 96 hours. The antibodies were purified using Montage

Antibody Purification Prosep-G Kit, Millipore Corporation, Billerica, MA, following the manufacturer's protocol.

Horseradish Peroxidase Conjugated anti-rat secondary antibody was obtained from Jackson Immunoresearch, West Groove, PA. The antibody was diluted to a concentration of 1:50,000 in DI water per the manufacturer's instructions.

Immobilon transfer membranes were obtained from Millipore Corporation, Bedford, MA. Transfer buffer was made from 125 mM Tris and 960 mM glycine. Wash buffer was made from Tween20 (Fisher Scientific, NJ) in PBS. Stock solution was made of 25 % Tween20. Wash buffer was made by adding 8 ml from stock solution to 2,000 ml of PBS.

SDS-PAGE analysis of samples was carried out. The samples were transferred from the acrylamide gel to a PVDF membrane using the Transblot semi-dry transfer cell at 0.45 volts for 37 minutes. The PVDF membrane was washed with wash buffer and was exposed to primary antibody with continuous stirring for two hours. The membrane was blocked using a blocking reagent from Li-Cor. A preset program on the Li-Cor blot washer was used to perform two cycles involving exposure to secondary antibody followed by washing. The membrane was subsequently exposed to 3,3',5,5'-tetramethylbenzidine (TMB) substrate and colored bands appeared. When the required band intensity was achieved, the membrane was washed with DI water for 5 minutes.

CHAPTER III

ADDITION OF PROTEINS TO IMPROVE TRANSDUCTION EFFICIENCY

3.1 INTRODUCTION

The reduced transduction efficiency of the purified VLPs led to further questions about the role of proteins on complex formation and transduction efficiency. It was also noted that complexes formed from purified VLPs and PEI were much smaller in size compared to those formed from unpurified VLPs and PEI. To study these effects, we added proteins to the purified VLPs and formed complexes. A comparison was performed between complexes formed using unpurified VLPs and purified VLPs and purified VLPs with increasing amounts of protein.

3.2 ADDITION OF PROTEINS

We added both cellular and serum proteins to purified VLPs in order to make the conditions similar to the ones in which VLPs are present before purification. It is however difficult to quantify the exact amount of serum and cellular proteins present in the supernatant. We approximated the conditions by forming a stock solution containing nearly 90 % serum proteins and 10 % cellular proteins by weight. Fetal bovine serum was used as a source of serum proteins. The protein concentration in FBS was found to be 57 µg/µl. HEK-293 cell supernatant was collected and concentration of proteins in the

supernatant was calculated to be 5.3 µg/µl. From the stock solution, we added different amounts of proteins to the purified VLPs. The purified VLPs were diluted to bring the concentration same as that of unpurified VLPs using TEN buffer and proteins from the stock solution, the dilution factor for which was based on the percent recovery of the VSV-G pseudotyped VLPs as described in chapter 2. The amount of proteins and TEN buffer used in the experiment is described below.

3.2.1 Sample Calculations

The concentration of proteins in the final purified VLP solution is considered negligible (< 0.02 µg/µl). Therefore, the amount of serum and cellular proteins added can be used in calculating the final concentration of proteins in the sample (Table 3.2). A stock protein solution was used to prepare the protein/VLP samples. The stock solution was prepared from equal volumes of serum proteins (57 µg/µl) and cell culture supernatant (5.3 µg/µl) to give a stock protein concentration of 31.15 µg/µl.

Different samples containing different volumes of stock protein solution were prepared. The amount of TEN buffer added to the final solution was adjusted so that the final volume is 500 µl after adding the protein stock solution. The concentration of VLPs in the solution after forming the above mentioned solution is same as concentration of VLPs in the unpurified sample. The concentration of proteins in the final solution was calculated for different volumes of stock solution using a simple material balance equation (Equation i) where C_1 is the concentration of proteins in the stock solution and V_1 is the volume of proteins in the stock solution. The term C_2 is the concentration of proteins in the final solution and V_2 be the volume of final solution.

$$C_1V_1 = C_2V_2 \qquad \text{Equation i)}$$

For example, our first sample combines 166 µl VLPs, 332 µl of TEN buffer and 2 µl of the stock protein solution. Using material balance shown in equation i) the final protein concentration is

$$31.15\ \mu g/\mu l \times 2\ \mu l = C_2 \times 500\ \mu l$$

$$C_2 = 0.12\ \mu g/\mu l$$

Similarly, protein concentrations for different amounts of stock solution added to the final solution were calculated as shown in the table below.

Table 3.1: Calculation of protein concentration added to the purified VLPs to form complex.

Amount of VLPs (µl)	**Amount of TEN buffer (µl)**	**Amount of Protein from stock solution (µl)**	**Concentration of protein in final solution (µg/µl)**
166	334	0	0
166	332	2	0.12
166	324	10	0.62
166	314	20	1.24
166	304	30	1.86
166	294	40	2.50
166	284	50	3.11
166	274	60	3.73

3.3 RESULTS AND DISCUSSIONS

Adding proteins before polymer/VLP complex formation had an effect on the transduction efficiency. The protein concentration in unpurified VLP solution was 2.95 µg/µl. The protein concentration in purified VLPs was below the limit of the BCA assay (< 0.02 µg/µl). As proteins were added initially, the transduction efficiency

decreased. For a concentration of 0.62 µg/µl, however, the transduction efficiency increased substantially to a level (Figure 3.1) greater than that of unpurified VLPs (i.e., when the VLPs contained serum and cellular proteins in unknown proportion). As the protein concentration was increased further however the transduction efficiency decreased gradually until the concentration exceeded 2.5 µg/µl. The transduction efficiency did not change to a large extent. As the protein concentration increased above 3.1 µg/µl the hybrid vector transduction efficiency essentially reached zero. At a protein concentration 3.7 µg/µl, no transduction of complex was observed.

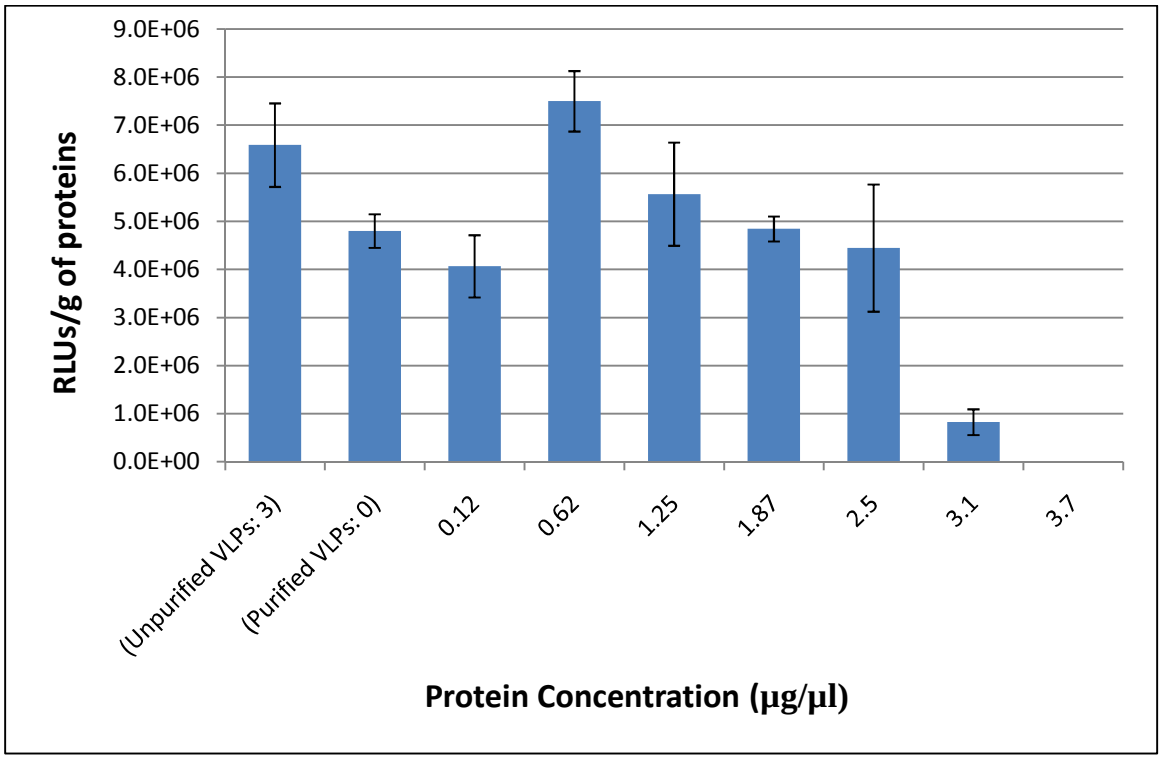

Figure 3.1: Effect on transduction efficiency of purified VLPs after adding proteins

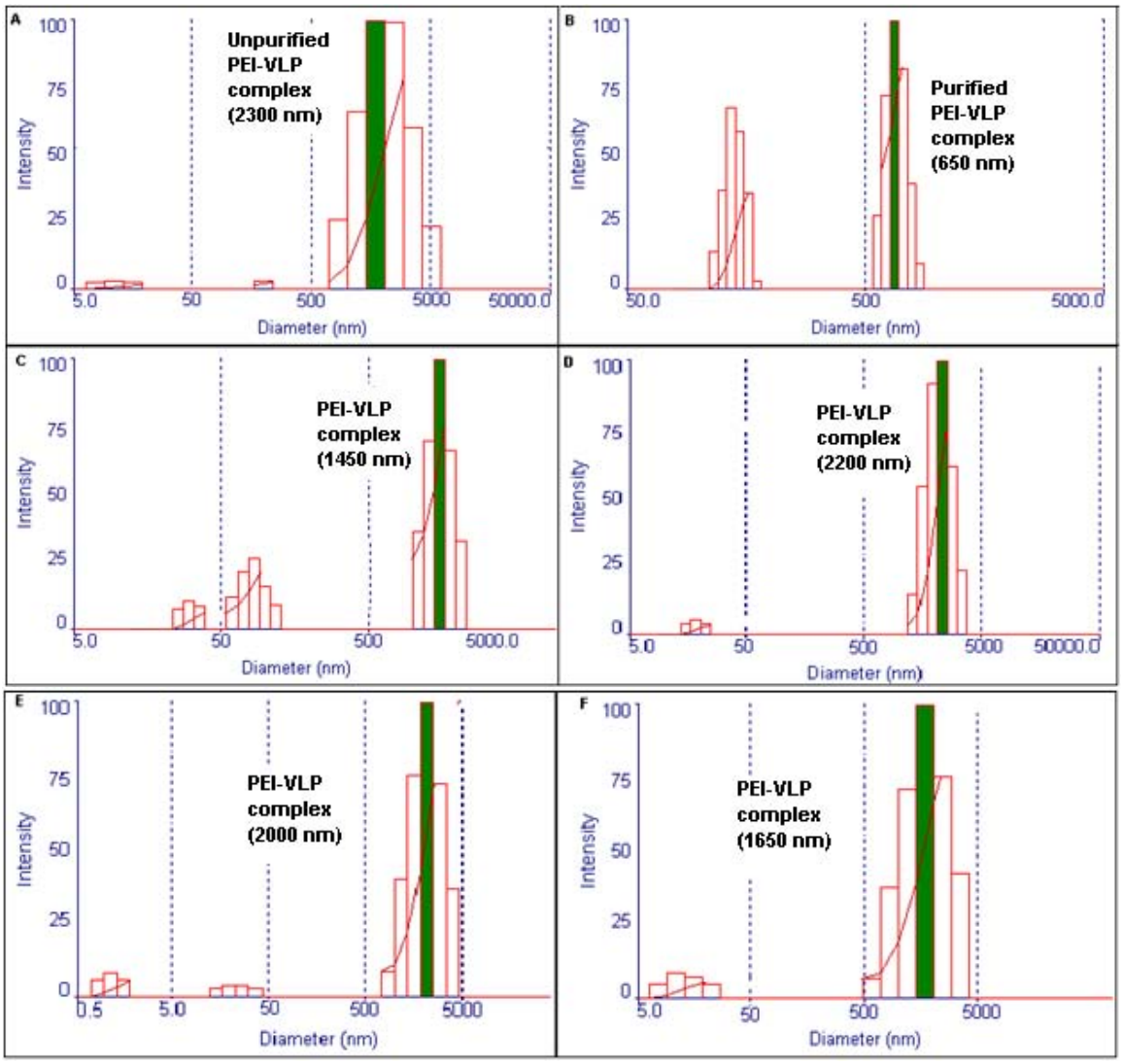

Figure 3.2: DLS data showing Unpurified VLP-PEI complex (A), Purified VLP-PEI complex (B), Purified VLPs + 0.12µg/µl proteins – PEI complex (C), Purified VLPs + 0.62 µg/µl proteins – PEI complex (D), Purified VLPs + 1.24 µg/µl proteins – PEI complex (E), Purified VLPs + 1.86 µg/µl proteins – PEI complex (F)

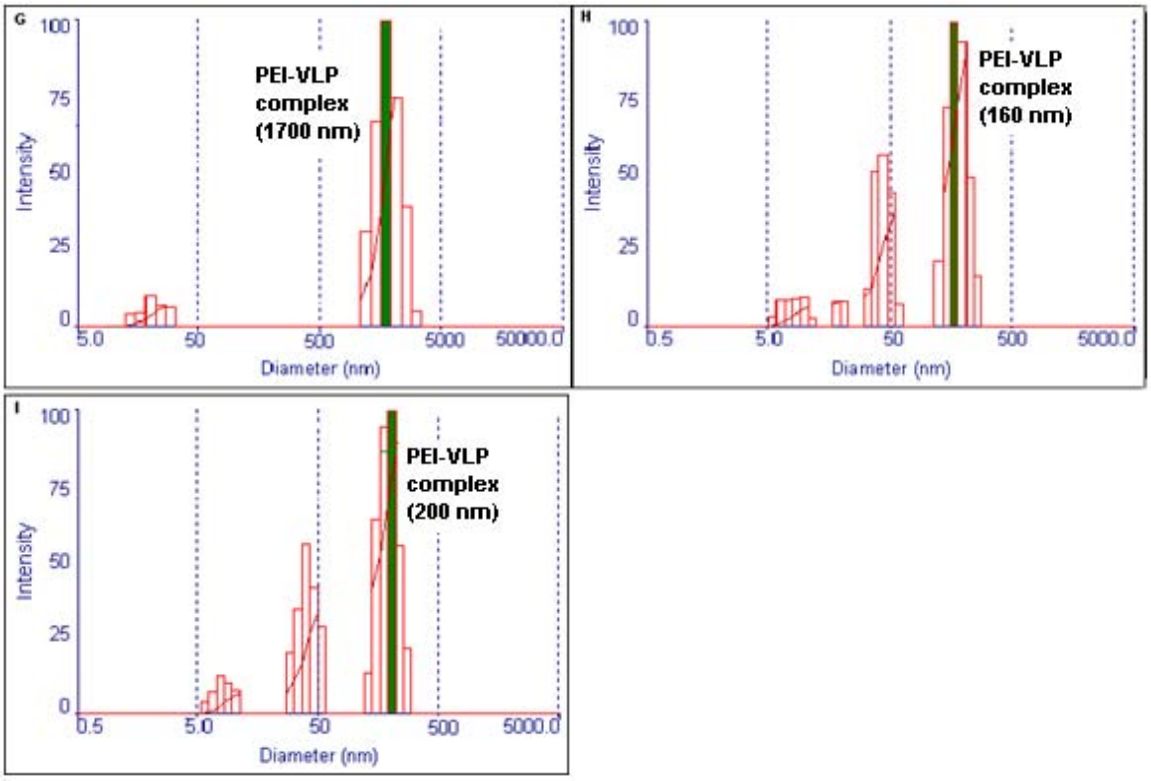

Figure 3.2 - continued: DLS data showing Purified VLPs + 2.5 µg/µl proteins – PEI complex (G), Purified VLPs + 3.11 µg/µl proteins + VLPs (H), Purified VLPs + 3.73 µg/µl proteins – PEI complex (I)

The DLS was used to analyze size of complexes after adding proteins to purified VLPs and PEI. The data shows that increasing the protein concentration increases the size of the complex. The size of the complex formed from purified VLPs with 0.62 µg/µl of protein is same as that formed from unpurified VLPs. The transduction efficiency of the complexes formed from purified VLPs with 0.62 µg/µl of proteins and unpurified VLPs was also found to be same. As we go on adding more proteins however the size of complex goes is reduced. For a concentration of 3.11 µg/µl and 3.73 µg/µl of proteins, very little complex is formed. We see a large number of particles formed which have an average size of 50 nm. These particles could be the protein-PEI complexes which are not infective.

Table 3.2: Size analysis and transduction efficiency of complexes formed with different amount of proteins

Type of Complex	Size (nm)	RLUs
Unpurified VLP-PEI Complex	2300	6.5×10^6
Purified VLP-PEI Complex	650	4.8×10^6
Purified VLP-PEI Complex + 0.12 µg/µl proteins	1400	4.0×10^6
Purified VLP-PEI Complex + 0.62 µg/µl proteins	2200	7.4×10^6
Purified VLP-PEI Complex + 1.24 µg/µl proteins	2000	5.5×10^6
Purified VLP-PEI Complex + 1.86 µg/µl proteins	1650	4.8×10^6
Purified VLP-PEI Complex + 2.5 µg/µl proteins	1700	4.4×10^6
Purified VLP-PEI Complex + 3.11 µg/µl proteins	160	0.8×10^6
Purified VLP-PEI Complex + 3.73 µg/µl proteins	200	0

Another factor that may be contributing to reduced infectivity is charge difference generated in the complex due to proteins. In their study, Landazuri *et al* added a cationic and anionic polymer to retrovirus and studied their transduction in comparison with normal retrovirus. Apart from the fact that size plays an important role in rapid sedimentation of virus-polymer complex on the surface of the cells, they concluded that the presence of negatively charged polymer helps in protecting the cells from what would otherwise be cytotoxic effect of the cationic polymer. They observed that when the dose of cationic polymer was higher than that of anionic polymer, it affected the transduction efficiency because of cytotoxic effect of excess of cationic polymer[21]. Extending the same theory to our PEI-VLP complex, when the complexes are formed from purified VLPs and PEI, we find that the transduction efficiency is reduced which could be due to

the presence of excess of cationic polymer. When we start adding proteins, the proteins start taking part in the complex formation. This leads to reduction of presence of excess of cationic polymer surrounding the cells and as such reduces the cytotoxic effect of the polymer.

3.4 CONCLUSION

VLPs require the presence of proteins to form infective complex with PEI. Presence of proteins increases the size of the complex which leads to faster sedimentation of the complex on the cells and may also decrease the cytotoxic effect of the cationic polymer.

3.5 MATERIALS AND METHODS

Cell Lines: Human embryonic kidney (HEK) cells, HEK-293, and a murine leukemia virus packaging cell line, GP-293Luc, were used for our experiments. HEK-293 cells were purchased from American Type Culture Collection, Manassa, VA. GP-293Luc cells were purchased from Clontech Laboratories Inc., Mountain View, CA. Both cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum and cultured at 37 ^{o}C in 5 % CO_2.

Virus and Virus-Like Particles: Envelope-free MLV-VLPs with a *luc* reporter gene were produced from the GP-293Luc cell line. GP-293Luc cells were seeded at 1.5×10^6 in 12 ml of medium on a 10 cm dish. The cells were cultured for three days before the supernatant was collected and filtered through a 0.45 µm polyethersulfone syringe filter. Filtered virus-like particles were used immediately.

Vesicular Stomatitis Virus – Glycoprotein (VSV-G) pseudotyped viruses were produced by transfecting the GP-293 Luc cells with the plasmid pVSV-G. The cells were seeded in a 10 cm dish 18 – 24 hours prior to transfection so as to be ~90 % confluent at the time of transfection. The cells were transfected with 24 µg of envelope plasmid (pVSV-G) using Lipofectamine 2000 and following the protocol supplied with the transfection reagent. The transfection medium was replaced after 6 hours. Viruses were collected 48 hours later and filtered through 0.45 µm polyethersulfone syringe filter.

Polymer: Polyethylenimine was purchased through Sigma-Aldrich, St. Louis, MO. Stock solutions of the polymer were prepared in ultrapure water at a concentration of 10 mg/ml and stored at 4 ^{o}C.

Hybrid Vector Formation: Polymer/VLP complexes were formed through drop-wise addition of stock polymer solution to VLP supernatant while vortexing. Polymer from the stock solution was added to VLPs in a 1.5 ml ultracentrifuge tube and vortexed. The polymer/VLP solution was incubated at room temperature for two hours before infecting the cells.

Cell Transduction: HEK-293 target cells were seeded in 6-well plates 18 – 24 hours before transduction at a seeding density of 1×10^6 cells/well. Immediately before addition of hybrid vector complexes the cell growth medium was replaced with serum-free DMEM. Hybrid vector complexes were then added to each well and incubated at 37 ^{o}C. After four hours, the medium was replaced with serum medium.

Gene Expression Assay: Cells infected with polymer/VLP complexes were assayed after 48 hours after the addition of the vector. For detection of luciferase protein the growth

medium was aspirated and 200 µl of cell culture lysis reagent (CCLR), Promega Inc., Madison, WI, was added to the cells. The cells were lysed at room temperature for 10 minutes followed by a 5 minutes freeze-thaw cycle at -80 ^{o}C. Luciferase activity for 20 µl of the cell lysate was measured using Promega's Luciferase Assay System and a Lumat LB9507 luminometer, EG&G Berthold, Bundoora, Australia.

Protein Assay: Protein analysis was carried out using the BicinChoninic Acid (BCA) assay from Pierce, Rockford, IL. The microplate procedure for 96-well plate was followed. Working Reagent from BCA reagent A and BCA reagent B was prepared. The sample addition and working reagent addition was done as per the manufacturers' protocol. Plate reader from Packard Biosciences, Illinois, was used to take absorbance readings at 560 nm.

Ultrafiltration: A stirred cell ultrafiltration unit, Millipore Inc., Bedford, MA, was used for concentrating the VLPs. An ultra-filtration membrane, Millipore Inc., Bedford, MA, with a molecular weight cut-off (MWCO) of 300,000 Da was used. A steady operating pressure of 45 PSI was used to pressurize the ultrafiltration chamber. The stirrer speed was maintained at 60 rpm.

Benzonase Digestion: Benzonase nuclease was obtained from Novagen. Concentrated solution with an activity of 10,000 units/µl was diluted down to an activity of 250 units/µl using the TEN buffer. The diluted solution was stored at -20 ^{o}C.

Size Exclusion Chromatography: Protein A sepharose CL-4B (Amersham Biosciences) was packed in a glass column XK16/40 (Fisher Scientific). The gel was packed using a continuous flow of ethanol and then further flushed with Tris-EDTA-NaCl (TEN) buffer

before running the sample. After running each cycle, the column is flushed with 50 ml 1N NaOH (as per manufacturer's instructions) and then flushed with 150 ml of TEN buffer to clean the column of NaOH.

Absorbance Reader: The fraction analysis was carried out using Cary 50 UV-Visible Spectrophotometer. Absorbance was measured at a wavelength of 280 nm.

CHAPTER IV

FUTURE WORK

The unpurified VLPs contained unspecified amount of serum and cellular proteins. However, when we added back proteins, we added equivalent amount of serum and cellular proteins. Hence, it becomes necessary to analyze the effect of adding only serum proteins and only cellular proteins to the VLPs and as such their effect on complex formation and transduction.

We formed complexes between VLPs and PEI. PEI is the most widely used polymer in gene therapy. However, toxicity has been a major issue in the use of PEI. Previous efforts have shown use of poly-L-lysine (PLL) and co-polymers for gene delivery. Using of PLL and co-polymers for delivery of purified VLPs would be an attractive alternative.

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VITA

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Pages in Study: 39 Candidate for the Degree of Master of Science

Major Field: Chemical Engineering

Scope and Method of Study: Our study involved formation of hybrid vector complex from viral and synthetic parts and studying the effect of presence of proteins on transduction of the hybrid vector. We separated proteins from virus-like particles and formed complexes to study their transduction. We added proteins to our viral part and formed complexes and studied the transduction efficiency of the hybrid vector.

Findings and Conclusions: Our experiments showed that removal of proteins decreased the transduction efficiency of the hybrid vector. We also observed changes in the morphology of complex formed from purified VLPs and PEI compared to that formed from unpurified VLPs and PEI.

We concluded that proteins have an impact on hybrid vector complex formation and transduction efficiency. Removal of proteins decreased the transduction efficiency of the hybrid vector and changes the morphology of the hybrid vector.

ADVISER'S APPROVAL: Dr. Joshua D. Ramsey