# EVALUATION OF CELL PENETRATING PEPTIDE/ADENOVIRUS PARTICLES FOR TRANSDUCTION OF CAR-NEGATIVE CELLS

By

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Abstract:

Adenovirus (Ad) is a promising gene therapy vector and is used currently in more than 23% of clinical gene therapy trials. The viral vector, however, has drawbacks such as immunogenicity, promiscuous tropism, and the inability to infect certain types of cells. The focus of this work was to develop an improved vector through electrostatic formation of a complex between negatively charged adenovirus and positively charged cell-penetrating peptides (CPPs), including Tat, Penetratin, polyarginine and Pep1. The resulting complexes were demonstrated to be capable of transducing cells that lack the coxsackie-adenovirus receptor (CAR) and are otherwise difficult to infect with native Ad. The transduction efficiency of the complexes was optimized by varying the multiplicity of infection, complex formation time and ratio of CPPs to Ad. The complexes improved the transduction efficiency on CAR-negative cells by more than 100-fold compared to unmodified Ad. Physicochemical characterization, including measurements of the size and zeta-potential of the complex, was performed to determine the suitability of the complex for *in vivo* gene delivery studies and investigate correlations between physicochemical properties and gene delivery efficiency. The size of CPP/Ad complex is initially less than 300 nm, but stability studies performed in the presence of serum indicate that the complex aggregates with serum after an extended time. The results of the present study indicate electrostatic modification of Ad with CPPs provides a relevant platform for developing effective Ad-based gene therapy vectors.

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# CHAPTER 1

# INTRODUCTION

During the last three decades, progression in the field of genetics made it clear that many diseases which affect humans are caused by genetic malfunctions. These malfunctions lead to abnormal production of cellular proteins causing disease. The ability to correct these genetic defects would provide an advancement in the treatment of diseases caused by genetic malfunctions (Friedmann and Roblin 1972). Gene therapy is a promising strategy of treating diseases at a genetic level by introducing genes to a target cell in order to correct, restore, modify or enhance cellular activities (Mountain 2000; Nathwani, Benjamin et al. 2004). Gene therapy employs nucleic acid to prevent or treat acquired diseases such as cancer (McNeish, Bell et al. 2004), neurological diseases (Ribotta 2001), cardiovascular diseases (Katz, Swain et al. 2010) or inherited diseases such as muscular dystrophy (Inui, Okada et al. 1996) or cystic fibrosis (Griesenbach, Geddes et al. 2006). To implement gene therapy it is vital to understand the pathogenesis of the disease, genes that induce desired genetic modification and the delivery mechanism of the genes to the target tissues (Zaia 2007).

Based on the nature of the disease, gene therapy materials can be therapeutic genes, suicide genes, gene silencing materials or DNA vaccines (Hwang 2006). Therapeutic genes can be delivered to the nucleus of cells to repair or substitute defective genes andproduce therapeutic proteins (Ouma, Jonas et al. 2012). Suicide genes can be delivered to the nucleus of cancer cells where the gene encodes a protein product that causes cellular apoptosis (Mitry, Sarraf et al. 2000; Fillat, Carrio et al. 2003). Antisense oligonucleotides or siRNA can be delivered to the nucleus of a specific target cell to block the gene expression and silence a problematic gene (Kang, Kim et al. 2000; Li, Fu et al. 2005). DNA vaccines can be delivered into dendritic cells or muscle cells to be converted into a protein vaccine and manipulate the immune system (Donnelly, Wahren et al. 2005).

The use of gene transfer to cure diseases started in the 1990s. Since then over 1,843 human gene therapy clinical trials have been performed. In the last 5 years, over 413 clinical gene trials have been carried out (Wiley 2012). The first human gene therapy clinical trial was conducted to cure a patient with Adenosine deaminase (ADA) deficiency that damages the immune system (Sheridan 2011). The trial involved the treatment of T-cells extracted from the patient by introducing genes that correctly encode ADA. The treated cells were then administered back to the patient. After the clinical trial the patient exhibited a provisional response and was further treated with enzyme replacement therapy.

The first clear-cut success in the field of gene therapy was treatment of two children with X-SCID, an immunodeficiency disease caused by the inability of T-cells to differentiate. The patients were treated with murine leukemia virus carrying complimentary DNA

capable of encoding the cytokine receptor essential in the delivery of differentiation and growth signals to progenitor cells (Cavazzana-Calvo, Hacein-Bey et al. 2000). The trial was successful in correcting the disease, but out of the 20 patients who received the treatment, five developed leukemia and one died (Hollon 2000). Studies suggested the development of leukemia was attributed to retroviral gene insertion to host cell genome (Couzin and Kaiser 2005). These side effects put treatment of diseases using gene therapy in jeopardy. The practicability of gene therapy was questioned earlier when a trial involving 18 year old patient with ornithine transcarbamylase deficiency (OTC) died after being treated with the pilot version of a gene vector based on that used human adenovirus (Raper, Chirmule et al. 2003). The cause of death was later associated with the patient's immune response to the adenoviral vector (Hollon 2000).

Studies have concluded that the most difficult challenge in the field of gene therapy is developing the right vector for gene delivery (Verma and Somia 1997). Depending upon the vector used to deliver the genetic materials, gene therapy can be *ex vivo* or *in vivo* (Figure 1.1). In e*x vivo* gene therapy, cells extracted from a patient are treated outside the body and administered back to the patient (Naldini 2011). The treated cells induce the desired therapeutic change. *Ex vivo* gene therapy has to be tailored to a patient. Hence, it incurs high manufacturing costs and quality-control difficulties. In *In vivo* gene therapy, gene therapy materials are directly administered to the patients using gene delivery vectors. With this technique the gene delivery vector can be used for different patients. Hence, *in vivo* gene therapy has reduced application costs but requires a more sophisticated vector. Systematic *in vivo* gene therapy usually causes the wide

dissemination of particles leading to the transduction of undesired tissues and complicating immune responses (Mountain 2000).

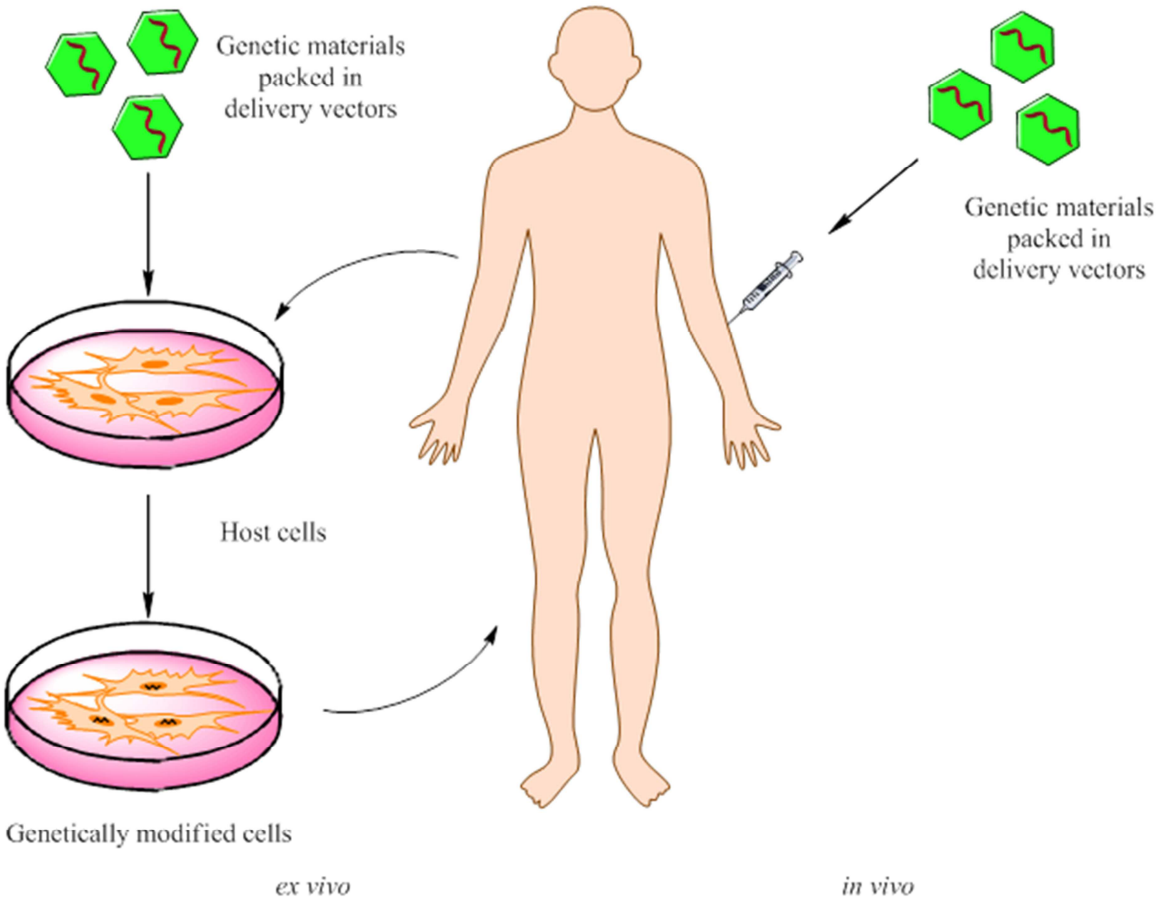


Figure 1.1: *Ex-vivo* or *in-vivo gene therapy.*

For effective *in vivo* gene delivery, gene delivery vectors are required to efficiently maneuver through a number of barriers. These barriers can be divided into extracellular and intracellular (Figure 1.2). The vector has to first overcome extracellular barriers to reach the surface of the target cell. Depending on the type of administration, gene delivery vectors have to escape the vascular system and local tissue matrices. Gene

delivery vectors have to avoid degradation by enzymes and neutralization by antibodies (Lechardeur, Sohn et al. 1999). Then, the vector has to associate with the target cell by binding to specific cellular receptors which facilitate recognition and internalization through endocytosis (Wu, Wilson et al. 1989).

Once associated with the target cells, the vector has to overcome intracellular barriers. The gene delivery vector has to go through the endolysosomal network if the vector avoids exocytosis (Luzio, Mullock et al. 2001). Vectors have to escape the endolysosomal network at optimum location within close vicinity to the nucleus before being degraded by the harsh environment (Cho, Kim et al. 2003). After the vector reaches the nucleus the genetic material has to be unpacked. The unpacking has to be done at a close proximity to the nucleus or after entering the nucleus to avoid interaction with degradative enzymes. The genes unpacked outside the nucleus have to passively diffuse through the nuclear pore to enter the nucleus. Vectors can also utilize the vulnerability of the nucleus membrane during mitosis to gain access to nucleus for gene transcription (Wilke, Fortunati et al. 1996).

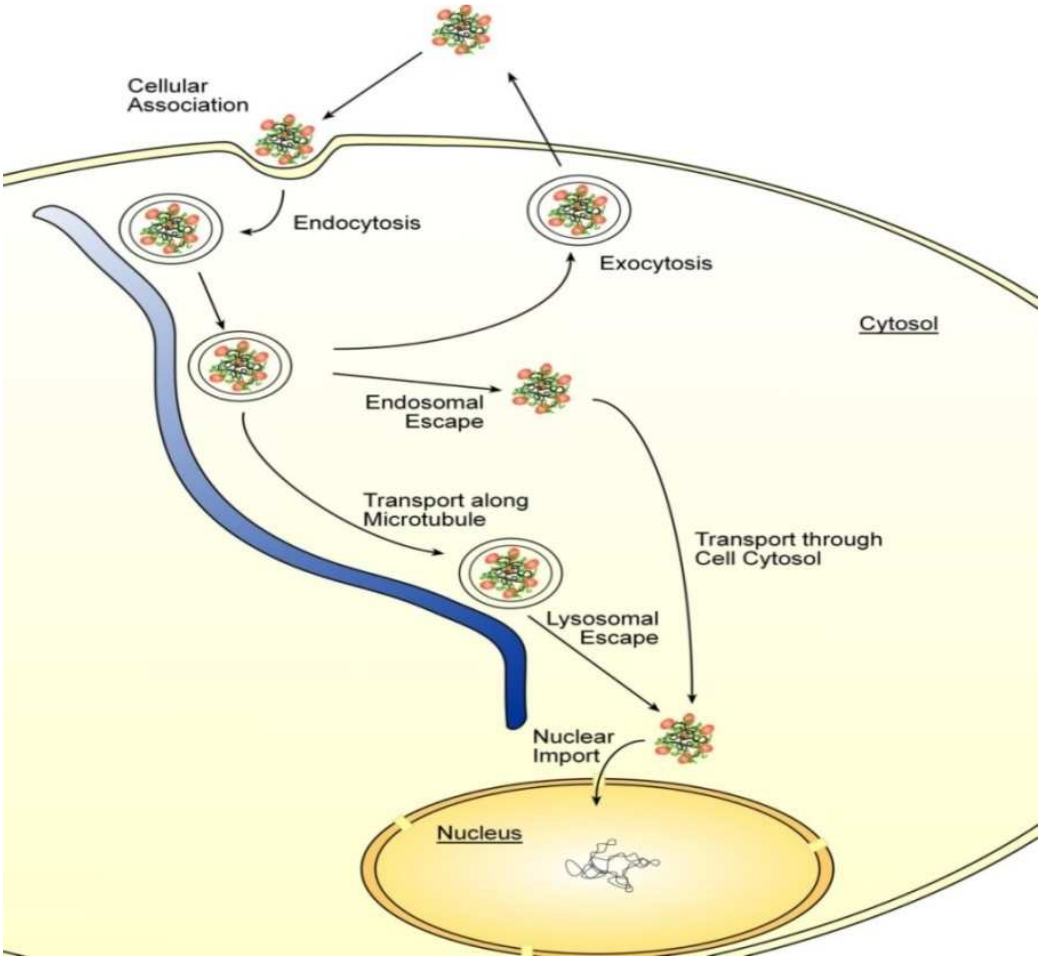


Figure: 1.2 Stages of internalization and transport of gene delivery vehicle (adapted from (Ramsey 2006))

In addition to efficiency, gene delivery vectors have to be safe. Gene delivery vectors should not cause undesired innate and adaptive immune responses. Innate responses will elicit elimination of transduced target cells while adaptive responses developed by the patient will neutralize re-administrated vectors. Since target cells are found in a heterogeneous surrounding or distributed within different parts of the body, vectors should have specificity to target cells. The vector should accommodate large sized

genetic materials for delivery. Once delivered, the vector should not promote random integration of genetic materials into the host chromosome which may cause integrational mutagenesis. The gene should reside as an episome or integrate into the chromosome at the desired site. The vector has to fulfill the desired level of gene expression, whether it is transient expression in cases of vaccines, regulated expression in cases such as diabetes or life time expression in cases such as hemophilia. Finally, the vector should be easy to produce in high titer and production should be inexpensive.

Currently gene delivery vectors are divided into two categories: viral and non-viral. Viruses have the natural ability of introducing their own gene into cells for reproduction and cause an array of diseases. Viruses are often used, however, as vehicles to deliver genes to treat disease in a patient. These viruses are replication defective and carry therapeutic genetic materials instead of their own gene. Of the current ongoing and completed gene therapy clinical trials 70% used viral vectors (Wiley 2012). Various viruses such as retrovirus, adenovirus, lentivirus, herpes simplex virus and adeno-associated virus (AAV) have shown promise as gene delivery vectors. Adenovirus is the leading viral vector in gene therapy clinical trials (used in 23% of trials performed so far) followed by retrovirus (Figure 1.3).

Adenovirus (Ad)-based gene delivery vectors have a number of favorable futures and are being applied widely in clinical gene therapy trials (Benihoud, Yeh et al. 1999; Mizuguchi and Hayakawa 2004). Ad has transient gene expressions and can transduce dividing and non-dividing cells. Unlike retrovirus, Ad does not integrate into the host genome, thus does not lead to undesired integrational mutation. Ad can accommodate large transgenes. Ad however, induces inflammatory immune responses and can be

neutralized by preexisting host immunity. In addition, Ad has native promiscuous tropism. Ad employs the coxsackievirus and adenovirus receptor (CAR) to target and infect cells (Bergelson, Cunningham et al. 1997). Dependency on the receptor prevents gene transfer into cells lacking CAR (CAR-negative), which includes many advanced tumor cells, peripheral blood cells, hematopoietic stem cells, vascular smooth muscle cells (SMCs) and dendritic cells (Wickham 2000; Mizuguchi and Hayakawa 2004). Adenovirus is discussed in detail in the next section.

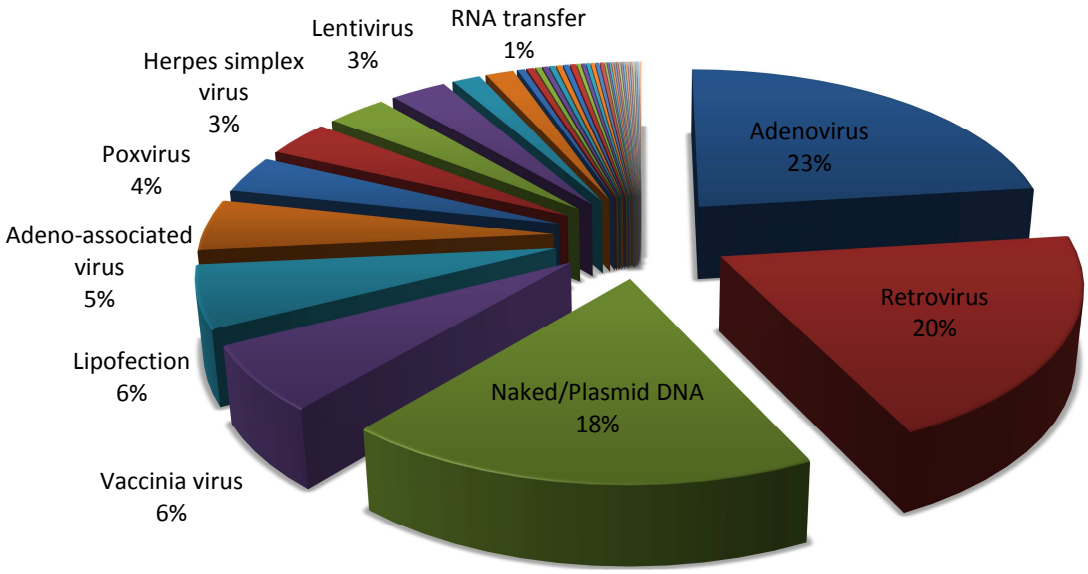


Figure 1.3: Gene delivery vectors used for gene therapy clinical trial (Wiley 2012).

Genetic and chemical modification strategies have been used to broaden transduction efficiency of Ad in a receptor-independent manner. One promising approach involves genetic modification of the fiber/knob and capsid proteins to insert small peptides, such as RGD, that enhance CAR-independent translocation (Vigne, Mahfouz et al. 1999; Ogawara, Rots et al. 2004; Kreppel, Gackowski et al. 2005). Chemical modification is an

alternative approach to genetic modification that allows a wide range of amino acids in the capsid to be modified with polymers (e.g., PEG or PLL) or peptides after conventional production and purification of the virus (Kaplan, Pennington et al. 1998).

One approach to chemically modifying the virus is the use of cell penetrating peptides (CPPs) to increase transduction efficiency of Ad. The CPPs electrostatically bind the surface of the virus and introduce an alternative pathway for transporting the virus into the cell by functioning in place of the fiber/CAR interaction. CPPs are peptides that typically contain less than 30 amino acids and have been shown to possess the ability to translocate peptides, oligonucleotides, plasmid DNA and large proteins into a wide range of cell types (Lewin, Carlesso et al. 2000; Torchilin, Rammohan et al. 2001; Tseng, Liu et al. 2002; Snyder, Saenz et al. 2005; Mae and Langel 2006). In addition, CPPs have been shown to have low toxicity. The peptides are categorized typically as either polycationic or amphipathic. Polycationic CPPs are highly cationic peptides with high isoelectric points mainly composed of arginine or lysine. In comparison, amphipathic CPPs have basic and hydrophobic amino acid clusters which interact with the lipophilic regions of the cell membrane. CPPs are discussed in detail in section 3.

Due to these described characteristics, CPPs are an attractive option for increasing the transduction efficiency of Ad. Forming complexes between CPPs and Ad may be achieved through either covalent or noncovalent attachment of CPPs to the Ad capsid. In fact, Gratton *et al.* (Gratton, Yu et al. 2003) and Lehmusvaara *et al.* (Lehmusvaara, Rautsi et al. 2006) reported that pre-incubation of CPPs with Ad derived from *Drosophila* Antennapedia homeodomain (pen) or human immunodeficiency virus type 1 transcription transactivation (Tat) protein with adenovirus improved adenoviral transduction of cancer

and endothelial cells. In the case of covalent complex formation, specific linker molecules are needed to facilitate the association between CPPs and the cargo; however, this approach limits complex formation flexibility. Noncovalent complex formation, which is simpler from a technological standpoint, involves electrostatic binding between positively charged CPPs and negatively charged cargo. Chapter two will discuss materials and method used by our study in detail.

The objective of this study was to evaluate the potential of the non-covalently formed CPP/Ad complexes to transduce cells the virus would not infect normally. In the study, four CPPs with different futures were used to form the complexes. The first two CPPs are polycationic Tat and amphipatic Pen which have been used by Gratton *et al.* and Lehmusvaara *et al.* for intracellular delivery of adenovirus (Gratton, Yu et al. 2003; Lehmusvaara, Rautsi et al. 2006). The other two CPPs are a polyarginine(pArg), with nine argine residues and Pep1. The pArg peptide has led to improved translocation of proteins and liposomes (Tseng, Liu et al. 2002), and Pep1 has been shown to carry cargo into cells after non-covalent attachment (Morris, Depollier et al. 2001; Gros, Deshayes et al. 2006). These four CPPs were selected to find the best working CPP to translocate Ad into CAR-negative cells. The resulting CPP/Ad complexes were compared against native Ad alone and were characterized physically and chemically. Chapter 3 and chapter 4 discuss my findings in detail.

## 1.1 Adenovirus

### 1.1.1 An overview of Adenovirus

Adenovirus was first isolated from human adenoid tissues in 1953 (Rowe, Huebner et al. 1953). Adenoviruses are responsible for acute respiratory diseases, urinary tract infections and gastrointestinal infections. Currently more than 50 adenovirus serotypes have been isolated from different animals. Out of the characterized adenovirus serotypes, type 2 and 5 serotypes have been studied in great detail and are being used as gene transfer vectors.

### 1.1.2 Structure of Adenovirus

Adenovirus is a 36 kbp linear double-stranded DNA molecule non-enveloped virus with an icosahedral shaped capsid protein. The virus has a particle size of between 70 to 100 nm in diameter and mass of 150 MDa. The capsid is responsible to protect and preserve the genome. The capsid is composed mainly of 20 hexon capsid faces, 12 penton-base vertices, and 12 fiber/knob proteins emanating from each of the 12 vertices (Rux and Burnett 2004). Each capsid face is made up of 12 hexon capsomeres. The hexon faces are glued to each other with adhesive protein IX (pIX) (Furcinitti, van Oostrum et al. 1989). Penton base is found at the vertices of each hexon. The fibers protrude from each penton-base have three distinct regions. These are N-terminal tails that fasten to the penton base, a shaft domain and a C-terminus knob. Fiber proteins and penton-bases have motifs that bind to cell surface integrin and receptors to facilitate endocytic uptake (Zubieta, Schoehn et al. 2005).

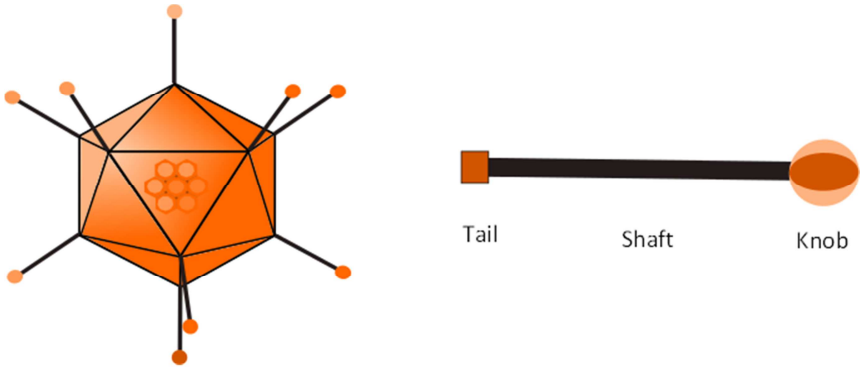


Figure 1.4: Structure of adenovirus. Fiber protein emanate out of the surface of the capsid.

### 1.1.3 Generations of Adenovirus

The adenovirus genome encodes five early transcription units (E1A, E1B, E2, E3 and E4), three delayed early transcription units (pIX, IVa2 and E2 late) and one major late transcription unit (L1 to L5) (McConnell and Imperiale 2004). The E1A gene products activate DNA transcription while the E1B gene products disengage apoptosis. The E2 gene encodes viral DNA replication while E3 gene products regulate cellular immune responses of viral infection and aid in release of the virus. Modulation of transcription and translation are among the tasks of E4 gene products and delayed early transcription units. The late gene products are used to generate the capsid of the virus (McConnell and Imperiale 2004).

Adenovirus vectors are grouped into three generations based on early gene modifications (McConnell and Imperiale 2004). The first generation vectors have E1 and/or E3 gene deletions. E1 gene deletion makes the vector replication deficient. Complimentary cells such as the 293 cell line are needed for vector production of this generation. These cells possess an adenoviral genome containing the E1 genes. Moreover E3 genes encode

proteins that are responsible for protecting infected cells from the immune system. This generation is capable of carrying up to 8.2 Kbp of gene (Danthinne and Imperiale 2000). The second generation vectors are created by removing E2 and E4 genes in addition to E1 and E3 genes. These deletions allow a maximum of 14 Kbp foreign DNA insertions (Danthinne and Imperiale 2000). Like the first-generation vectors, the second-generation vectors are produced with a complimentary cell line. The first and second generation adenovirus vectors trigger an immune response in the hosts making these generation good vaccine vectors (Imler 1995; Tatsis and Ertl 2004). However, immune response impedes the application of these viruses as gene delivery vectors. Helper-dependent generations have complete deletion of adenovirus genome except the ITR and the encapsulation signal. This gutless high capacity generation can carry up to 30 Kbp of foreign genetic materials (Parks and Graham 1997). The high capacity allows expression of larger genes with reduced cellular immunogenicity associated with the viral genes.

### 1.1.4 Infection Pathway of Adenovirus

The adenovirus infection pathway involves a series of steps. In the first step the virus binds to the cell membrane. This step is mainly mediated by the fiber protein. The knob domain of the fiber protein binds to a particular plasma membrane protein called the coxsackie B virus and adenovirus receptor(CAR) (Howitt, Anderson et al. 2003). CAR is an extracellular transmembrane protein that belongs to the immunoglobulin super family (Bergelson, Cunningham et al. 1997). Next, the penton base links with integrin receptors on the cell through the argentine-glycine-aspartic acid (RGD) motif (Wickham, Mathias et al. 1993). In addition to CAR and integrin, lysine-lysine-threonine-lysine (KKTK) motifs found in the shaft domain of the fiber protein and heparin sulphate proteoglycans

found on the cell plasma membrane aid cell attachment (Smith, Idamakanti et al. 2003). These interactions between the virus and cell surface matrices lead to receptor-mediated endocytosis (Meier and Greber 2003). After endocytosis, the virus enters the cytosol encapsulated with in an endosomal membrane. As the endosome acidifies capsid proteins undergo conformational change (Seth, Willingham et al. 1985; Furcinitti, van Oostrum et al. 1989; Greber, Willetts et al. 1993), where the virus partially disassembles, upsets the endosomal membrane and escapes into the cytoplasm (Graham, Smiley et al. 1977). Upon escape from the endosome, the virus particles associate with the dynein/dynactin motor complexes, which guide the particles along microtubules to the nucleus (Kelkar, Pfister et al. 2004). Once in the proximity of the nucleus, microtubules release the virus particles, which bind to the nuclear pore complex (Greber, Suomalainen et al. 1997). Virus particles disassemble and further viral DNA is transferred into the nucleus to complete infection of the cells.

### 1.1.5 Adenovirus as a Gene Delivery Vector

Adenovirus has been utilized as a gene delivery vehicle (Benihoud, Yeh et al. 1999). Thus far, adenovirus has been used for more than 23% of gene delivery clinical trials (Wiley 2012). The capability to carry up to ~30Kb of genetic material to both dividing and non-dividing cells with high transgene expression made the viral vector appealing. Adenovirus can generate high levels of short term transgene expression in most tissues except hematopoietic cells, adenovirus receptor deficient cells cancerous cells and muscle cells. Adenovirus does not integrate genetic materials into the host genome. Hence it eliminates insertional mutagenesis. Most people have preexisting immunity to the virus. Patients will also develop neutralizing antibodies after administration. The adaptive

immunity to the virus hampers the efficacy of the vector upon readministration. Systematically administered adenovirus accumulates in the liver and induces an inflammatory response. The death of Jesse Gelsinger, a patient suffering from Ornithine Transcarbamylase (OTC) deficiency and enrolled in a gene therapy clinical trial that used an adenoviral vector, is a sad reminder to how adverse the immune response to the virus can be (Marshall 1999). These drawbacks complicate the development of adenoviral vectors.

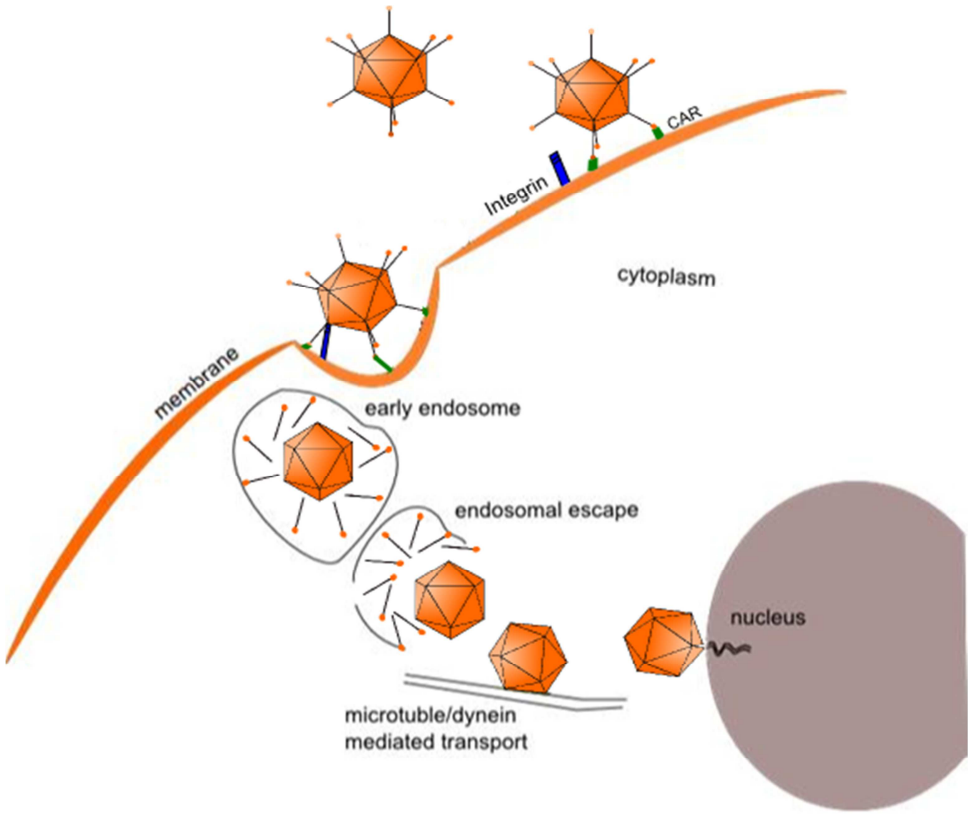


Figure 1.5: Cellular attachment and internalization of adenovirus. Fiber and penton base proteins interact with cell receptors to allow cellular entry and intracellular trafficking.

## 1.2 Cell-Penetrating Peptides

### 1.2.1 Definition and Properties of CPPs

Cell-penetrating peptides (CPPs) are short residue peptides which are capable of crossing a biological membrane. These peptides consist of less than 30 amino acids and can mediate movements across a cellular membrane into the cytoplasm and progress into intracellular pathways. CPPs translocate into cells without cytotoxic effects (Saar, Lindgren et al. 2005). CPPs include protein-transduction domains (PTD), membrane-translocating sequences (MTS) and synthetic cell-permeable peptides which can overcome extra and/or intracellular restrictions of biomolecules to be internalized by cells. CPPs induce internalization activity and rapid endosomal release of many molecules. Cargos such as plasmid DNA (Ignatovich, Dizhe et al. 2003), nucleic acid (Meade and Dowdy 2007; Crombez, Aldrian-Herrada et al. 2009), oligonucleotide (Mae and Langel 2006), liposomes (Kale and Torchilin 2007), peptides (Yang, Wang et al. 2006) and proteins (Matsushita, Tomizawa et al. 2001) can be attached to a CPP covalently or non-covalently, forming nanoparticles that cells can internalize in a receptor-independent manner.

### 1.2.2 Discovery of CPPs

The first CPP was discovered by Frankel and Pabo in 1988 (Frankel and Pabo 1988). They observed that the human immunodeficiency virus transactivating regulatory protein (Tat) could be internalized by cells. In 1991 Joliot *et al*. discovered that Drosophila Antennapedia homeodomain could translocate into neuronal cells (Joliot, Pernelle et al. 1991). A short while later in 1996, the Derossi *et al.* demonstrated that the 16 amino acid

residue peptide pAntennapedia, commonly called penetratin,(RQIKIYFQNRRMKWKK) can be covalently bound to cargo and translocate into cells (Derossi, Calvet et al. 1996). In 1998, the minimum peptide sequence of Tat (YGRKKRRQRRR) necessary for cellular uptake was identified by Vives *et al.* (Vives, Brodin et al. 1997). In 2001, Wender *et al.* (Wender, Mitchell et al. 2000) and Futaki *et al.* (Futaki, Suzuki et al. 2001) investigated and identified a polyargenine amino acid residue that can initiate internalization of molecules into cells.

### 1.2.3 Classification of CPPs

CPPs can be grouped into three classes based on their origin. The first group is composed of CPPs derived from naturally occurring proteins. This includes Tat, a CPP derived from human immunodeficiency virus trans-activating protein (Tat) (Vives, Brodin et al. 1997) and penetratin derived from Drosophila Antennapedia homeodomain (Derossi, Joliot et al. 1994). The second group is comprised of model CPPs that are developed based on functionality without any homology to natural sequences. These CPPs include polyarginine and polylysine (Mitchell, Kim et al. 2000). The third group of CPPs consists of chimeric sequences. This includes transportan which is composed of the neuropeptide galanin and an amino acid derived from wasp venom, mastoparan (Pooga, Hallbrink et al. 1998) and Pep1 composed of N-terminal hydrophobic motif, a linker/spacer domain, and a hydrophilic lysine rich domain derived from the nuclear localization sequence of simian virus 40 T antigen (Morris, Depollier et al. 2001).

Another method to classify CPPs is by their common properties. CPPs have two common properties, amphipathicity and positive charge. CPPs incorporate positively

charged amino acids (e.g., arginine and lysine) giving them a net positive charge at physiological pH. These CPPs include Tat and polycationic homopolymers such as polyarginines and polylysines peptide. These peptides are called polycationic CPPs. All CPPs other than polycationic homopolymers are amphipathic CPPs. Some amphipathic CPPs have distinct hydrophobic and hydrophilic parts in their sequence. Other amphipathic CPPs have the hydrophobic and hydrophilic amino acids distributed within their sequence.

### 1.2.4 Mechanism of Internalization

#### 1.2.4.1 Early Study

Understanding the internalization mechanism of CPPs is essential for the effective delivery of their cargo. Earlier studies reported that internalization of CPPs occurred in receptor and energy-independent manner (Lundberg, Wikstrom et al. 2003; Vives 2003). Studies showed that internalization occurs at low temperature, which indicated an energy-independent pathway (Derossi, Calvet et al. 1996; Wender, Mitchell et al. 2000). Furthermore, similar translocation efficiency of a CPP and its reverse sequence demonstrated that internalization was independent of specific sequence and receptor (Futaki, Suzuki et al. 2001). It was suggested that the internalization mechanism of a CPP was due to direct interactions of lipid membrane and strong lipid-binding affinity of a CPP (Drin, Mazel et al. 2001). Translocation of penetratin in model membrane systems appeared to support an uptake mechanism in which a CPP with a high number of cationic amino acids can cross the plasma membrane by an energy-independent pathway (Thoren, Persson et al. 2003).The validity of the early studies suggesting an energy-independent translocation of CPPs, however, was later questioned.

#### 1.2.4.2 Current Studies

Numbers of studies were conducted to reevaluate the internalization mechanism of CPPs across the plasma membrane. These studies demonstrated that transmembrane potential is required for the peptides to be absorbed by neutral and charged lipid bilayer systems (Terrone, Sang et al. 2003; Ziegler, Blatter et al. 2003; Henriques and Castanho 2004). Moreover, several investigations suggested that the apparent energy-independent internalization mechanism of CPPs proposed by the earlier studies were due to artifacts in the cell-fixation (Lundberg, Wikstrom et al. 2003; Richard, Melikov et al. 2003). Despite the studies conducted to reevaluate the cellular uptake pathway of CPP across the plasma membrane, detailed understanding remains unclear. Studies suggested several types of translocation mechanisms. For example, Tat-derived peptides and penetratin were shown to internalize by an energy-dependent endocytosis (Drin, Cottin et al. 2003; Vives 2003). Recently, it became evident that these CPP can use multiple pathways for cellular entry, depending on the context of the experimental conditions (Duchardt, Fotin-Mleczek et al. 2007).

The internalization mechanisms of CPPs into cells are debated. Discrepancies between studies are attributed to the use of different cell types, incubation conditions and cargo or label. Properties of CPPs, such as sequence, molecule length, secondary structure, and charge delocalization can influence the uptake mechanism of the peptide. Properties of the associated cargo, such as size, concentration, and ability of the cargo to interact with cell surface can also factor into the uptake mechanism of the peptide. Studies have proposed endocytotic cellular entry pathways (Jones, Christison et al. 2005) such as

macropinocytosis (Nakase, Niwa et al. 2004), caveolae (Fittipaldi, Ferrari et al. 2003) and non endocytotic membrane perturbation (Henriques and Castanho 2004).

#### 1.2.4.3 Factors Affecting Internalization

Cellular uptake of CPPs is determined by the interaction of the CPP with the cell membrane surface. Several studies have reported that the interaction of cationic CPPs with the negatively charged cell membrane is due to the presence of heparan sulphate proteoglycan (Drin, Cottin et al. 2003; Richard, Melikov et al. 2003; Fischer, Kohler et al. 2004; Ziegler and Seelig 2004). This electrostatic interaction leads to binding of CPPs and heparan sulphate on the membrane surface and trigger endocytotic internalization of the complex (Fuchs and Raines 2004). Studies have shown that anti-HS antibodies that inhibit binding of CPP to heparan sulphate and competent polyanionic compounds such as dextran sulphate and heparin weakened the internalization pAntp, Tat, and these CPPs coupled to a cargo (Suzuki, Futaki et al. 2002; Console, Marty et al. 2003). These findings suggested that the role of the peptide may be restricted to aiding the attachment of cargo to the cell membrane.

In addition to the cationic properties of CPPs studies showed that other properties influence translocation. Studies have shown some hydrophobic residues contributed to the membrane binding and translocation (Derossi, Calvet et al. 1996; Fischer, Zhelev et al. 2000). Derossi et al. showed hydrophobic residues played a role in membrane binding and translocation (Derossi, Joliot et al. 1994). Furthermore, Mitchell et al. and Wender et al. proved that a guanidinium group of arginine facilitated cellular uptake of arginine rich CPPs (Mitchell, Kim et al. 2000; Wender, Mitchell et al. 2000). Another noteworthy

finding is that CPPs of similarly high (pI 10-13) isoelectric points (pI) showed dissimilar internalization activities and some CPPs with low pI such as integrin (pI 5.49) showed good cellular uptake (Mueller, Kretzschmar et al. 2008). In addition, homopeptides of similar length polymers of arginine showed higher uptake activity compared to lysine, histidine or ornithine (Mitchell, Kim et al. 2000). The length of the sequence and the conformation of the CPP are proposed factors that play important roles in the cellular uptake of CPPs (Fischer, Zhelev et al. 2000). Further studies showed concentration of CPPs attaching to the membrane affects the translocation mechanism. Above certain concentrations, cellular uptake for Tat and nanoarginine occurred through energy-independent direct uptake instead of endocytosis (Duchardt, Fotin-Mleczek et al. 2007).

A factor that may influence the translocation mechanism is the nature of the cargo coupled to the CPPs. In cases of CPPs coupled to cargo, the internalization mechanism was influenced by the size of the conjugate (Zuhorn, Visser et al. 2002). Despite the fact that different CPPs are internalized into cells through endocytosis or energy-independent mechanisms, cellular uptake of these CPPs coupled with high molecular weight cargo occurred via endocytosis (Console et al. 2003; Lundberg et al. 2003; Takeshima et al. 2003). Studies have shown that CPPs coupled to high-molecular-weight maleimide-derivatized phospholipid are internalized by endocytosis (Console et al. 2003).

### 1.2.5 CPPs of Interest

#### 1.2.5.1 Penetratin

Derived from the highly conserved 60-residue *Drosophila melanogaster* Antennapedia homeodomain protein, Penetratin is one of the most investigated CPPs (Derossi, Calvet et

al. 1996). It was determined that the third helix 16-residue peptide (residues 43–58) was responsible for translocation (Derossi, Joliot et al. 1994). The penetratin sequence is composed of several positively charged basic amino acids interspersed throughout the hydrophobic residue. Hence, Penetratin has a low amphipathicity. Penetratin has low toxicity in cells and did not show membrane perturbation in model membrane systems (Magzoub, Eriksson et al. 2003). Even though the process involved in internalization is still controversial, recent studies suggest that cellular uptake of penetratin is largely due to an endocytotic mechanism. Penetratin promotes endocytosis by binding to cell surface glycosaminoglycans (Drin, Cottin et al. 2003; Fischer, Kohler et al. 2004) Studies have shown that Penetratin has successfully facilitated cellular delivery of oligonucleotides (Astriab-Fisher, Sergueev et al. 2002), siRNA (Lundberg, El-Andaloussi et al. 2007) and PNA (Chaubey, Tripathi et al. 2008) *in vitro* and *in vivo.*

1.2.5.2 Tat

In the 1980s, Frankel & Pabo reported that HIV 1 trans-activating protein (Tat) was able to penetrate cells (Frankel and Pabo 1988). Later, it was proven that a shorter truncated Tat solely composed of basic amino acid and nuclear localization sequence (NLS), Tat (48-60) promotes effective internalization (Vives, Brodin et al. 1997). The initialization mechanism that Tat takes has been broadly debated. Recent studies however, suggested that Tat uses macropinocytosis and/or Clathrin-dependent endocytotic mechanism (Richard, Melikov et al. 2003; Wadia, Stan et al. 2004; Ziegler and Seelig 2004). The mechanism is initiated by the binding of the peptide to anionic glycosaminoglycans on the plasma membrane (Richard, Melikov et al. 2003; Fischer, Kohler et al. 2004) A similar uptake mechanism was reported for Tat/cargo conjugates (Lundberg, Wikstrom et

al. 2003; Wadia, Stan et al. 2004). Tat has shown the ability to carry a wide range of macromolecules into cells without compromising cell viability (Zorko and Langel 2005). Recently, macromolecules such as liposomes (Torchilin and Levchenko 2003), oligonucleotides (Astriab-Fisher, Sergueev et al. 2002), and plasmid DNA (Rudolph, Plank et al. 2003) (Rudolph et al. 2003) conjugated with Tat showed effective translocation into cells.

#### 1.2.5.3 Polyarginines

In the early 2000s, homopeptides attracted the interest of investigators as candidate CPPs. Polymers of arginine, lysine, histidine or ornithine of similar length were studied and polyarginine demonstrated superior cellular uptake compared to other homopeptides (Mitchell, Kim et al. 2000). Studies showed polyarginine with 8 to 10 arginine molecules internalized into cells most efficiently (Futaki, Suzuki et al. 2001). Efficiency of polyarginine compared to other homopeptides suggested that the cationic property of the amino acid residues was not the only factor affecting internalization. In fact, the guanidinium group of arginine was mainly responsible for cellular uptake (Wender, Mitchell et al. 2000). A study by Mitchell *et al*. supported this argument by showing that cellular uptake of polyarginine failed when the nitrogen of the guanidine was replaced by oxygen (Mitchell, Kim et al. 2000). Polyarginine binds to the cell surface heparan sulphate to facilitate uptake by endocytosis (Fuchs and Raines 2004). Nakase el al. reported macropinocytosis pathway as an uptake mechanism (Nakase, Niwa et al. 2004). The study further reported that the uptake of polyarginine in the presence of macropinocytosis inhibiters depended on the length of polyarginine which suggests the possibility of an additional pathway contributing to their uptake (Nakase, Niwa et al.

2004). Nonetheless polyarginine has facilitated efficient intercellular delivery of siRNA (Kim, Christensen et al. 2006), plasmid (Kish, Tsume et al. 2007) and different proteins (Futaki, Suzuki et al. 2001; Wright, Rothbard et al. 2003).

#### 1.2.5.4 Pep1

Pep1 is a chimeric and amphipathic peptide composed of N-terminal hydrophobic motif, a linker/spacer domain, and a hydrophilic lysine rich domain derived from the nuclear localization sequence of simian virus 40 T antigens (Morris, Depollier et al. 2001). The hydrophobic motif is required for traversing the plasma membrane and to facilitate complex formation with cargo. The hydrophilic motif is required for intracellular trafficking while linker/spacer domain separates and interlinks the hydrophobic and hydrophilic motif (Morris, Depollier et al. 2001). Pep1 has to first bind to phospholipids on the cell membrane, initiate conformational changes and prompt cellular uptake through direct translocation (Morris, Depollier et al. 2001). Rapid dissociation of Pep1 from its cargo after internalization reduces the influence of the CPP on biochemical activity and the final destination of its cargo (Morris, Depollier et al. 2001). Pep1 has been shown to promote internalization of a wide range of proteins such as protein Kinase (Maron, Folkesson et al. 2005), PNA and antibodies (Morris, Depollier et al. 2001).

# CHAPTER 2

# MATERIALS AND METHODOLOGY

## 2.1 Cell Lines

Human embryonic kidney (HEK293) and mouse fibroblast (NIH/3T3) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The HEK293 cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-BRL, Grand Island, NY) with 10 % fetal bovine serum (FBS), and the NIH/3T3 cell line was cultured in DMEM with 10 % calf serum (CS). The cells were subcultured every 2 to 3 days and stored in a humidified incubator at 37 °C with 5 % $CO_2$.

## 2.2 Production of Adenovirus

Recombinant adenovirus (Ad) with an E1/E3 deletion and packing *lacZ* reporter gene was purchased from Capital Biosciences (Rockville, MD) and amplified by infecting HEK293 cells in 10 cm dishes. The infected cells were incubated until the cytopathic effect was observed after which three freeze/thaw cycles were performed to lyse the virus-producing cells. Ad contained in the cell lysate was isolated and purified using a Vivapure Adenopack purification kit (Sartorius Stedim, Arvada, Colorado) by following

the manufacturer's protocol. The viral titer (cfu/ml) was measured by X-Gal staining of HEK293 cells infected with serially diluted viruses.

### 2.3 Synthesis of Cell Penetrating Peptides

Cell penetrating peptides, Tat (YGRKKRRQRRRC), Pen (RQIKIWFQNRRMKWKKC), pArg (RRRRRRRRRC) and Pep1 (KETWWETWWTEWSQPKKKRKVC), were synthesized by EZBiolab (Westfield, IN). A cysteine residue was added to the C-terminus end of each peptide. The purity and uniformity of the peptides were analyzed using NMR and HPLC. Peptides were then diluted in HEPES buffer (pH 7. 4) at a concentration of 2 μg/μl and aliquoted and stored at -80 °C. The isoelectric point and net charge of CPPs were calculated with a peptide property calculator from GenScript (Table 4.1) (GenScript).

Table 2.1 Selection of CPP sequences.

| CPPs | sequence | CPP charge at pH 7 | Isoelectric Point | Hydrophilic % | Hydrophobic % | MW (g/mol) |
|---|---|---|---|---|---|---|
| Pen | RQIKIWFQNRRMKWKKC | 7 | 12.3 | 41 | 35 | 2350 |
| Tat | YGRKKRRQRRRC | 8 | 12.5 | 67 | 0 | 1663 |
| pArg | RRRRRRRRRC | 9 | 13.0 | 90 | 0 | 1527 |
| Pep1 | KETWWETWWTEWSQPKKKRKVC | 3 | 10.2 | 41 | 32 | 2951 |

## 2.4 Formation of CPP/Ad complexes

Complexes were formed through electrostatic conjugation of Ad and CPPs (Figure4.1). The Ad particles were diluted in HEPES buffer (pH 7.4) at desired multiplicity of infection (MOI) of 5, 10, 30, 40, 50 or 100. CPP/Ad complexes were prepared by adding CPPs to the virus in a drop-wise fashion and vortexing gently. The mixture was then incubated at room temperature for various times to optimize the CPP and Ad interaction. As the CPP was added to the negatively charged Ad, noncovalent electrostatic attraction formed the CPP/Ad complex.

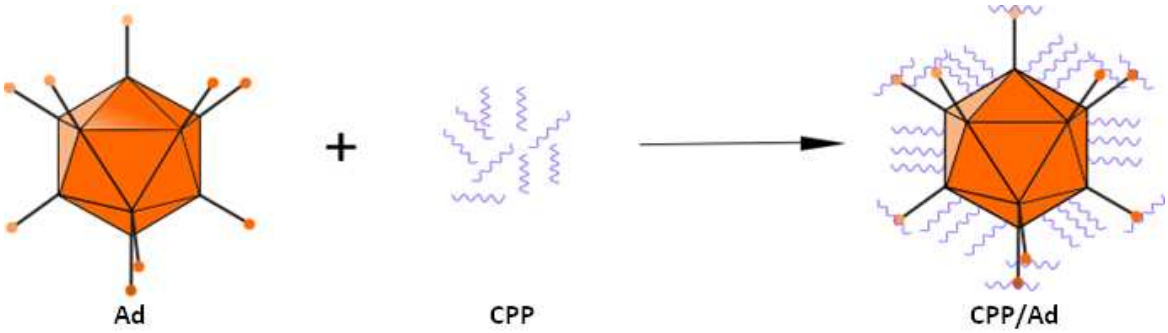


Figure 2.1 Schematic of CPP/Ad complex formation.

## 2.5 Particle Size Measurements

A Brookhaven 90Plus Dynamic Light Scattering (DLS) instrument (Brookhaven Instrument, Inc., Worcestershire, UK) was used to measure the hydrodynamic diameter of the complexes. Complexes were diluted in DMEM with 10 % CS (pH 7.4) to a concentration of $1 \times 10^7$ cfu/ml and were maintained at 25 $^{o}$C. The light scattering was measured at 90° relative to the laser source. The particle size of complexes in each sample was calculated from six repeat measurements of three samples where each measurement acquired data over 30 seconds.

## 2.6 Zeta-Potential Measurements

A Brookhaven 90Plus ZetaPALS (Brookhaven Instrument, Inc., Worcestershire, UK) was used to measure the zeta-potential. Samples were diluted in phosphate buffered saline (PBS, pH 7.4) to a concentration $1 \times 10^7$ cfu/ml and volume of 1,500 µl. Zeta-potential measurements were performed in triplicate, and the zeta potential of each sample was calculated from ten repeat measurements where each measurement acquired data for 30 seconds.

## 2.7 Transduction of CPP/Ad

Transduction of CPP/Ad was studied on CAR-negative NIH/3T3 cells. The cells were seeded 24 hours prior to transduction at $2.5 \times 10^5$ cells per well in a 12 well plate. The cells were then infected with Ad and CPP/Ad at specific MOIs (5, 10, 50, or 100). An MOI of 40 was used for further studies to assure the presence of MOI greater than 30. Quantitative levels of transduction were measured 48 hours post transduction using the chemiluminescence-based, Beta-Glo assay (Promega Inc., Madison, WI), which quantifies β-galactosidase protein expressed from the lacZ reporter gene packaged by the virus. The quantity of β-galactosidase was measured in terms of relative light units (RLUs) with a Lumat LB9507 luminometer (EG&G, Berthold, Bundoora, Australia). Reporter gene expression was normalized to total cellular protein, which was quantified by a bicinchoninic acid (BCA) protein assay (Pierce Inc., Rockford, IL).

## 2.8 Statistical Analysis

Statistical analysis was performed by One Way Analysis of Variance (ANOVA) with Holm-Sidak method to compare the difference between the means of two groups. An

overall significance level of 95% was accepted as significant. Mean values with standard error are reported and all experiments were performed in triplicate.

# CHAPTER 3

# RESULTS

## 3.1 Transduction of CPP/Ad Complex

CPP/Ad complexes were prepared by incubating Ad and CPP for 60 minutes at a concentration of 6.25 µg of CPP per $10^6$ Ad. CAR-negative NIH/3T3 cells were infected with unmodified Ad and CPP/Ad complexes, and the MOI was varied from 5 to 100 to evaluate the effect of MOI on transduction efficiency (Figure 5.1). The results of the gene expression study confirmed that the unmodified Ad, as expected, is unable to infect CAR-negative cells. The virus alone resulted in low infectivity with no notable increase in infectivity as the MOI was increased. All CPP/Ad complexes showed significantly higher levels of gene expression at each MOI compared to native Ad, verifying the ability of the CPPs to efficiently translocate Ad into CAR-negative cells. At an MOI of 50, Tat/Ad, Pen/Ad and pArg/Ad produced a 50-fold improvement in gene expression compared to the unmodified Ad whereas Pep1/Ad showed only a 36-fold improvement. Gene expression resulting from the CPP/Ad complexes did not increase significantly at MOIs greater than 30.

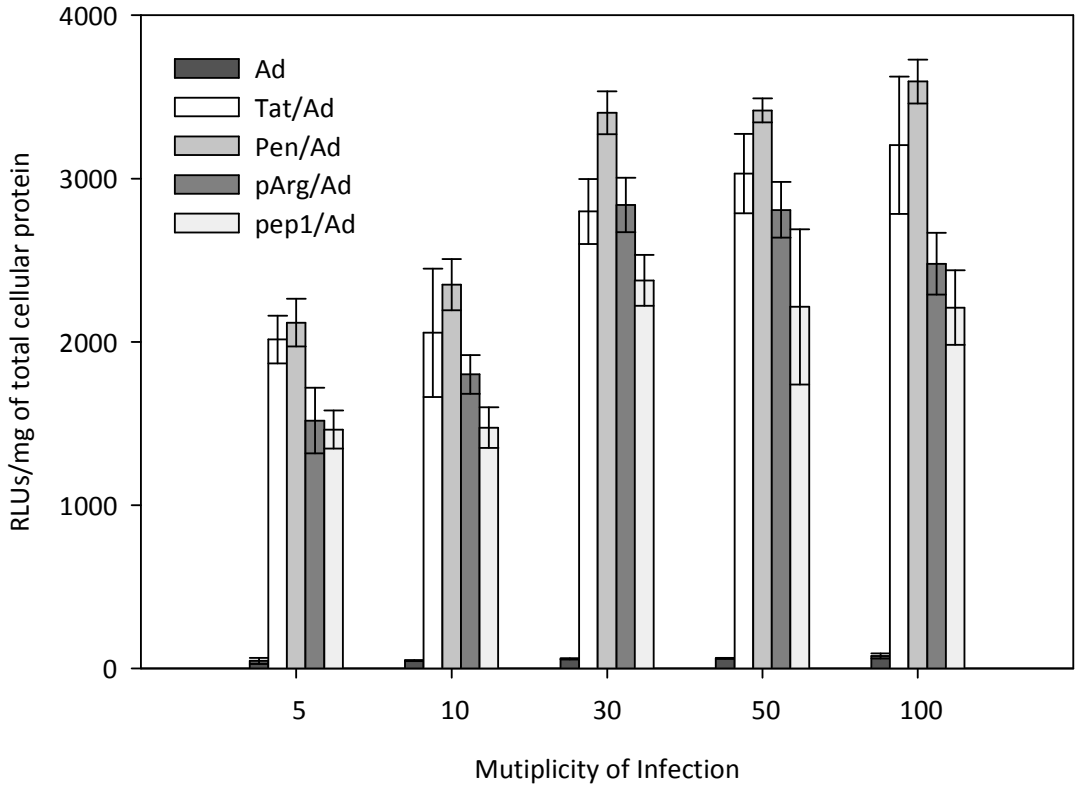


Figure 3.1 Transduction study of CPP/Ad complexes with varied MOI.

3.2 Effect of Incubation Period on Transduction

Formation of CPP/Ad complexes is dependent on electrostatic interaction between positively charged CPPs and negatively charged Ad. Incubation of CPPs and Ad facilitates the interaction and formation of the complex. To evaluate the effect of incubation period on the transduction efficiency, CPP/Ad complexes were prepared by varying the incubation period from 5 to 90 minutes while keeping constant the concentration of CPP (6.25 µg CPP/$10^6$ Ad) and the MOI of the virus. High transduction efficiency was observed for all CPP/Ad complexes compared to unmodified Ad at all

incubation periods. The incubation period at which the optimum infectivity was achieved, however, varied for each individual CPP/Ad complex (Figure 5.2).

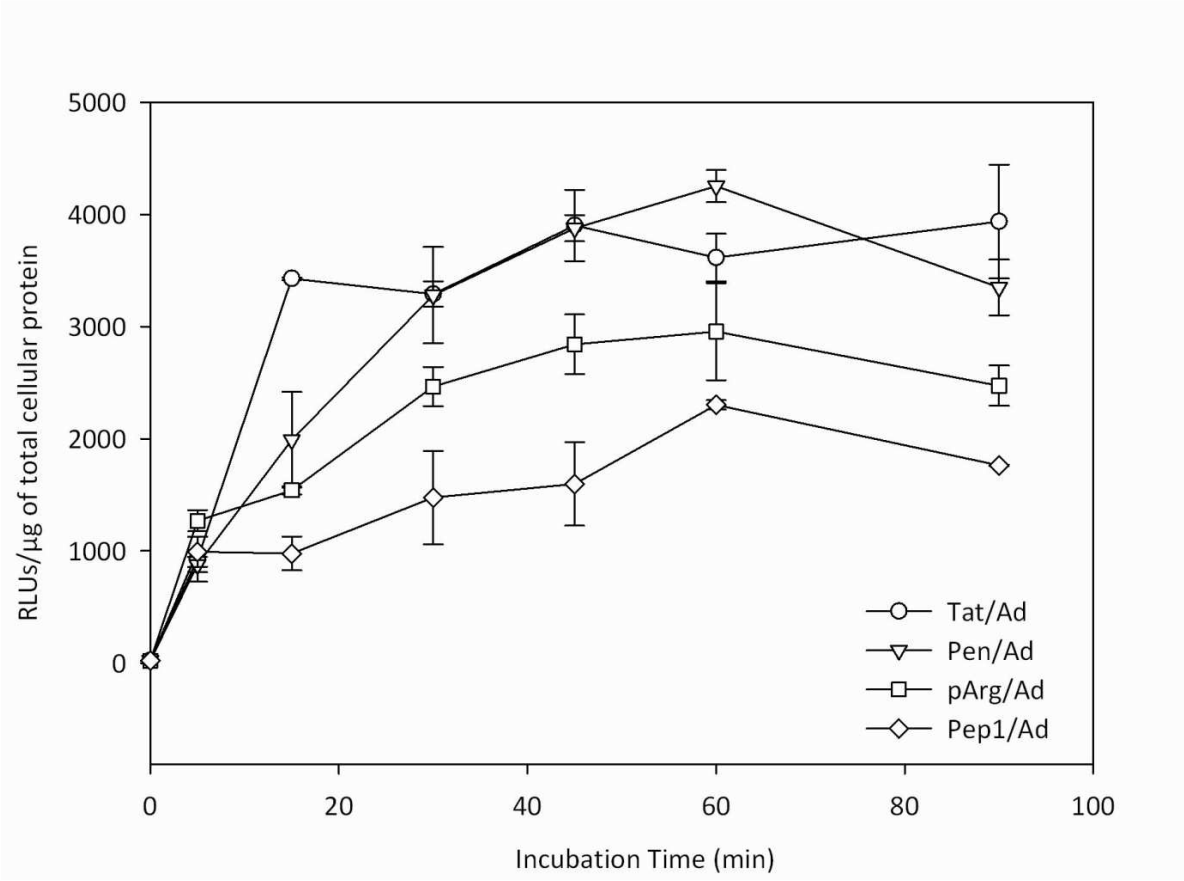


Figure 3.2 Transduction study of CPP/Ad complexes with varied times allowed for CPP/Ad complex formation.

Tat/Ad complexes showed significantly higher transduction efficiency when incubated for 15 minutes compared to 5 minutes. Longer incubation periods produced only minor improvements of gene transduction for the Tat/Ad complex. The transduction efficiency of Pen/Ad, pArg/Ad, and Pep1/Ad was maximized after an incubation period of 60 minutes, and further increase in the incubation time had no improvement on the transduction efficiency of CPP/Ad complexes. Based on these results, an incubation

period of 60 minutes, which gives optimal gene expression for all the CPP/Ad complexes, was used for the remainder of the study.

3.3 Effect of Concentration of CPP and Type of CPP on Transduction

CPP/Ad complexes were formed using CPP concentrations ranging from 0.1 to 50 µg/$10^6$ Ad to determine the effect of concentration of CPP on the formation of CPP/Ad complexes and the transduction of CAR-negative cells. The results show that CPP/Ad complexes formed from high concentrations of CPPs exhibited better efficiency than complexes formed at low concentrations (Figure 5.3). There was a substantial increase in transduction over the CPP concentration range of 0.1 to 6.25 µg/$10^6$ Ad for all four CPPs explored in the study. Transduction levels continued to increase beyond this initial CPP concentration range, but at a much lower rate.

Pen/Ad produced the highest level of cell transduction over the entire concentration range of CPPs. Tat/Ad was the second most effective complex followed closely by pArg/Ad. Pep1/Ad produced the lowest gene expression over the entire concentration range. At the highest CPP concentration, Pen/Ad showed 100-fold higher transduction efficiency compared to unmodified Ad. At this concentration, transduction of Tat/Ad and pArg/Ad were approximately 95-fold higher than unmodified Ad, while the Pep1/Ad transduction was only 66-fold higher compared to unmodified Ad.

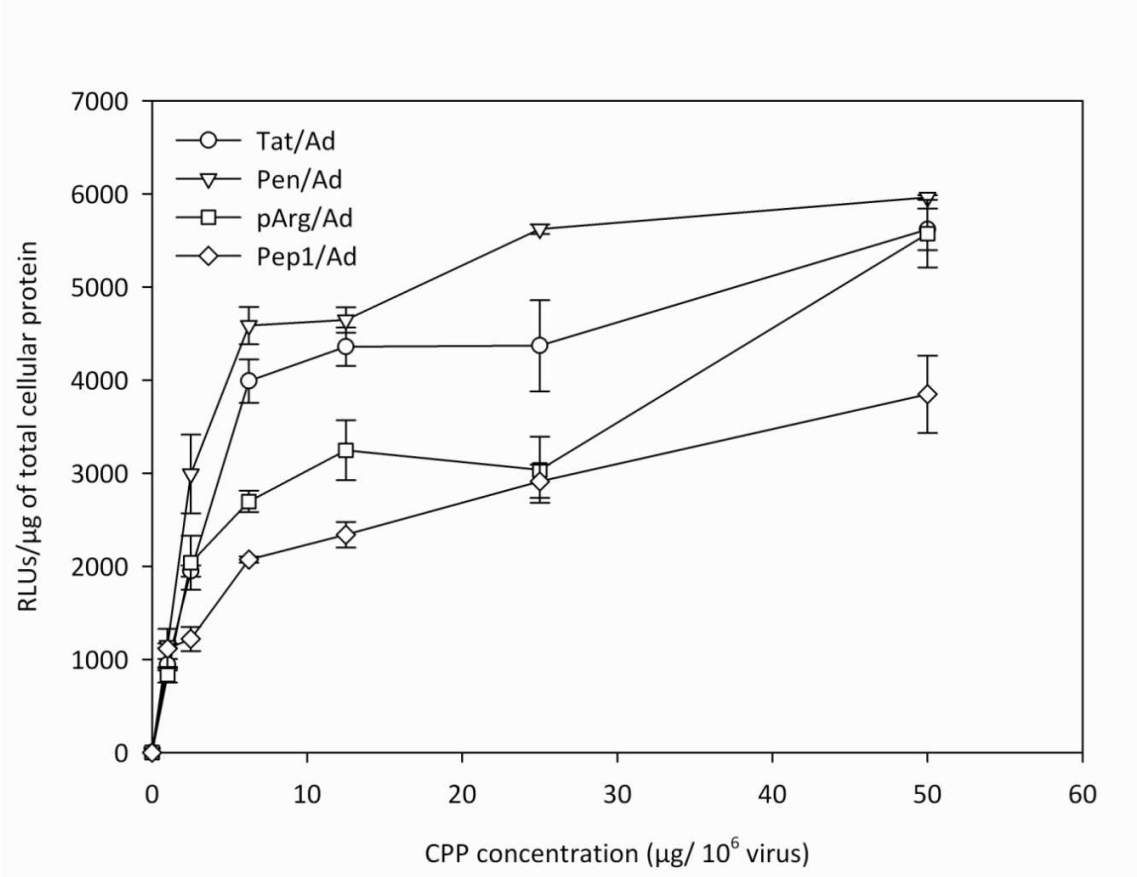


Figure 3.3 Transduction study of CPP/Ad complexes with varied CPP to Ad ratio.

3.4 Effect of Serum on Transduction of CPP/Ad

Serum proteins can have a substantial impact on the transduction efficiency of gene vectors due to electrostatic affinity between positively charged complexes and negatively charged serum proteins. Although an ultimate goal with many gene vectors is systemic administration of the vector in a protein-rich environment, most transduction studies of complexes are performed in serum-free medium. In this study, however, transduction experiments using CPP/Ad complexes at a concentration of 6.25 µg CPP/$10^6$ Ad were performed in the presence and absence of 10 % CS. Based on the results of this comparative study, the serum did not significantly affect the transduction of Tat/Ad or

Pen/Ad complexes (Figure 5.4). In contrast, the transduction efficiency of Pep1/Ad and pArg/Ad complexes decreased by 63 and 30 %, respectively, in the presence of serum.

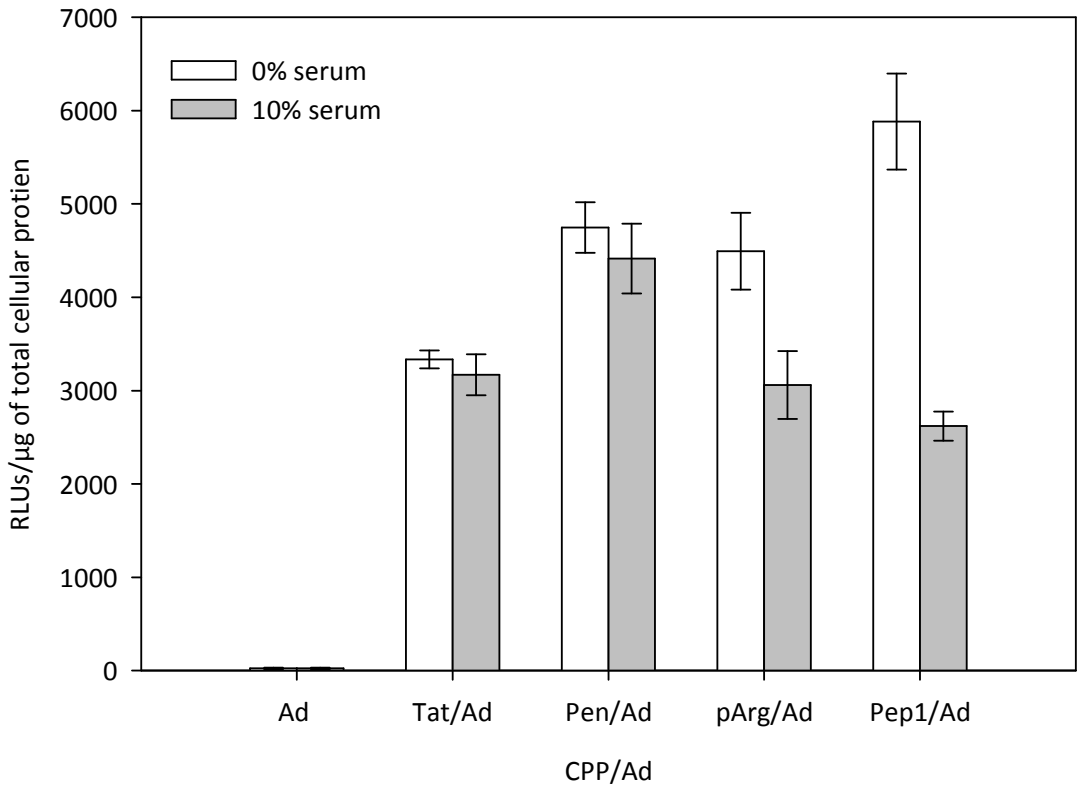


Figure 3.4 Transduction study of CPP/Ad complexes in the presence and absence of serum.

3.5 Significance of Free CPPs as Translocation Agents

The importance of pre-incubating CPPs with Ad to form a CPP/Ad complex was evaluated by comparing CPP/Ad complexes with Ad added directly to cell culture medium containing the same concentration range of CPP (i.e., 2.5 – 50 µg/$10^6$ Ad) without first forming complexes. Ad and free CPPs in solution exhibited greater gene expression than unmodified Ad alone and showed some dependency on the CPP

concentration (Figure 5.5). Compared to the preformed complexes, however, viruses and free CPPs were significantly less efficient than the CPP/Ad complexes, which exhibited 5- to 14-fold higher gene expression than Ad and free CPPs.

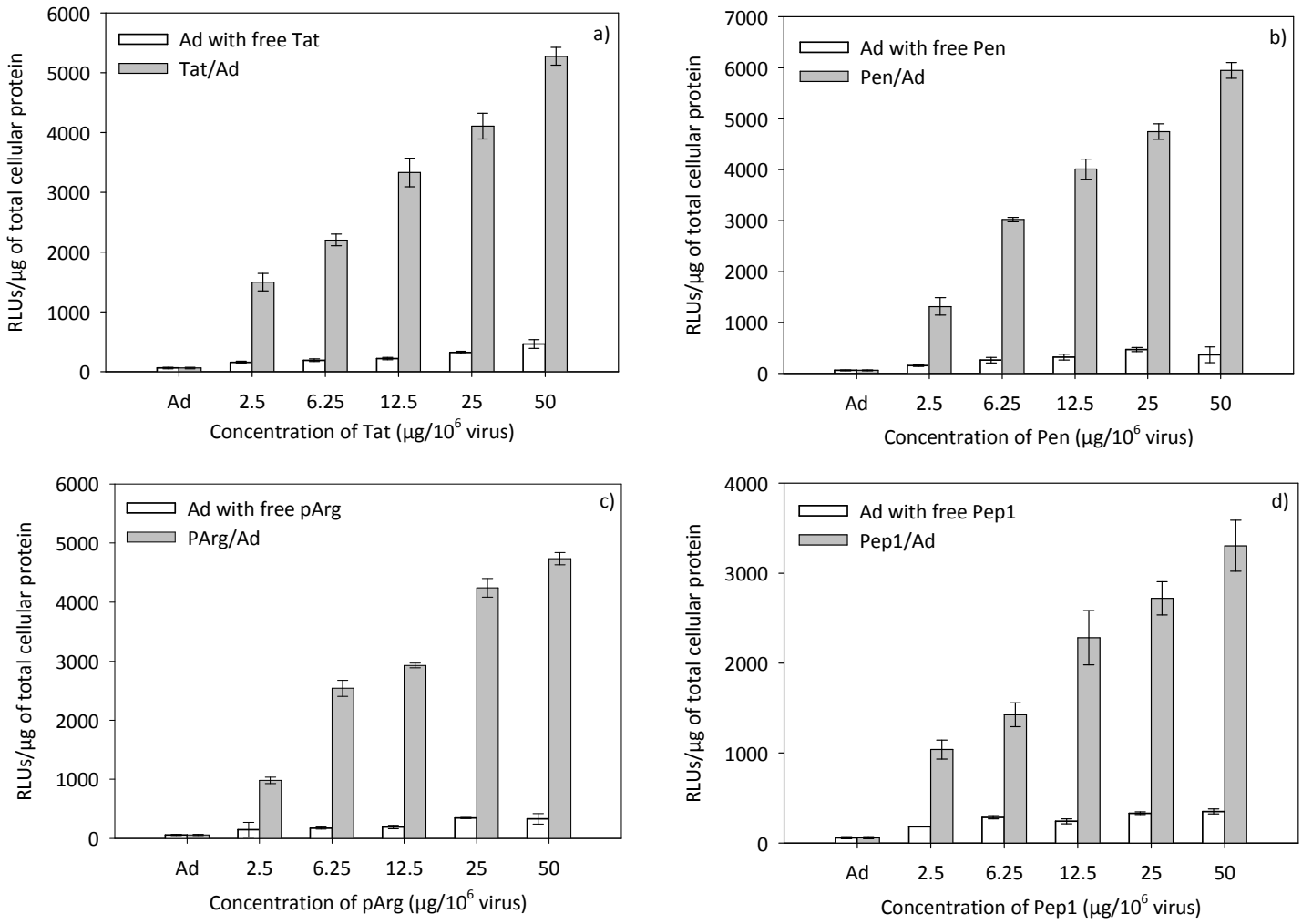


Figure 3.5 Transduction study of CPP/Ad complexes and Ad mixture with free CPP for a) Tat/Ad, b) Pen/Ad, c) pArg/Ad and d) Pep1/Ad.

## 3.6 Physicochemical Properties of CPP/Ad Complexes

The surface charge and hydrodynamic diameter of the CPP/Ad complexes were studied to better understand the physicochemical properties associated with the most efficient complexes. CPP/Ad complexes were prepared with CPP to Ad ratios ranging from 0.1 to 50 µg CPP/$10^6$ Ad. The zeta-potential of the complexes was measured and revealed that binding of CPPs to Ad modifies the overall surface charge of the negatively charged Ad. While the surface charge of the unmodified Ad was measured to be -21 ± 4 mV, the maximum surface charge of the CPP/Ad complexes was measured to be 12 ± 2 mV (Figure 5.6). No significant difference was observed between the surface charges of CPP/Ad complexes composed of different CPPs. As the concentration of CPP increased, however, all CPP/Ad complexes exhibited an increase in surface charge. The increase in charge was sizable from concentration of 0.1 up to 6.25 µg CPP/$10^6$ Ad and only moderate for higher concentrations.

The mean hydrodynamic diameter of the CPP/Ad complexes was also measured as a function of CPP to Ad ratio over the range 0.1 to 50 µg CPP/$10^6$ Ad (Figure 5.7). As CPPs were added to the virus, the size increased from the size of the virus alone (i.e., approximately 100 nm) to more than double the size of the virus. As with the zeta-potential measurement, the greatest change in hydrodynamic diameter occurred within the concentration range 0.1 to 6.25 µg CPP/$10^6$ Ad. Table 5.1 lists the size of the CPP/Ad complexes at this higher concentration of 6.25 µg/$10^6$ Ad. Worth noting is that the hydrodynamic diameter remained below 300 nm for all CPP/Ad complexes as long as the concentration was not increased above 25 µg/$10^6$ Ad. Above this concentration, the

diameter of complexes composed of pArg/Ad or Tat/Ad increased significantly to sizes greater than 300 nm.

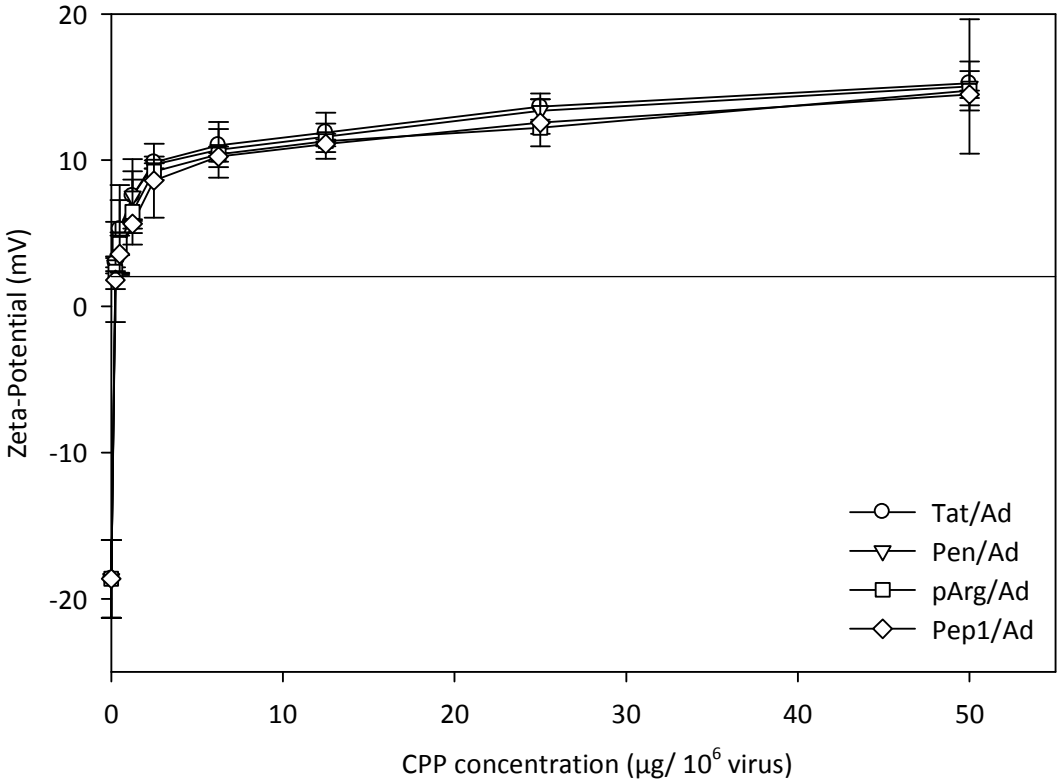


Figure 3.6 surface charge characterizations of CPP/Ad complexes as a function of CPP concentration.

Table 3.1 Size of Particles

| Particle | Hydrodynamic Diameter (nm) |
|---|---|
| Ad | 115 ± 12 |
| Tat/Ad | 226 ± 48 |
| Pen/Ad | 219 ± 54 |
| pArg/Ad | 231 ± 50 |
| Pep1/Ad | 217 ± 52 |

## 3.7 Stability of CPP/Ad Complexes

The stability of the CPP/Ad complexes was evaluated by measuring the size of complexes in the presence and absence of serum as a function of incubation time (Figure 5.8). The complexes were prepared at a concentration of 6.25 µg CPP/$10^6$ viruses. The complexes were then incubated at room temperature in medium with (i.e., 10 % CS) and without serum. For both cases the results showed a trend of increasing particle size with increasing incubation time. The increase in the size of the complex was much greater, however, in the presence of serum than in the absence of serum. The size of each CPP/Ad complex in the presence of serum was approximately four times higher than the size in the absence of serum.

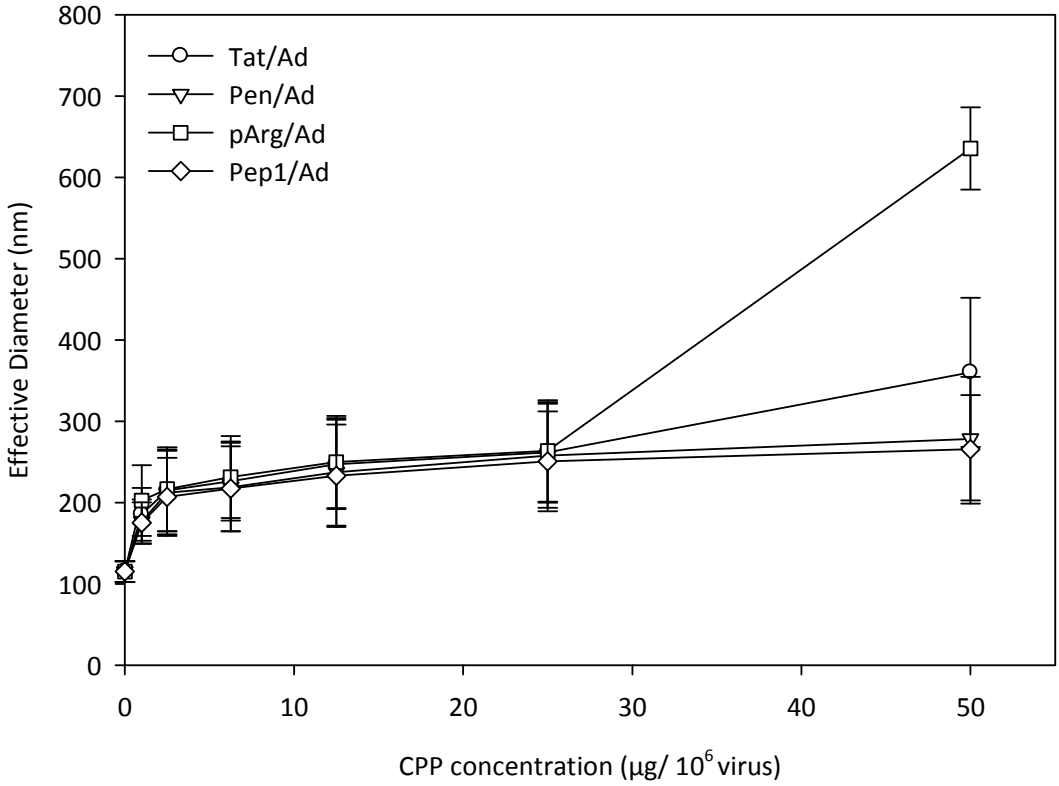


Figure 3.7 Particle size characterizations of CPP/Ad complexes as a function of CPP concentration.

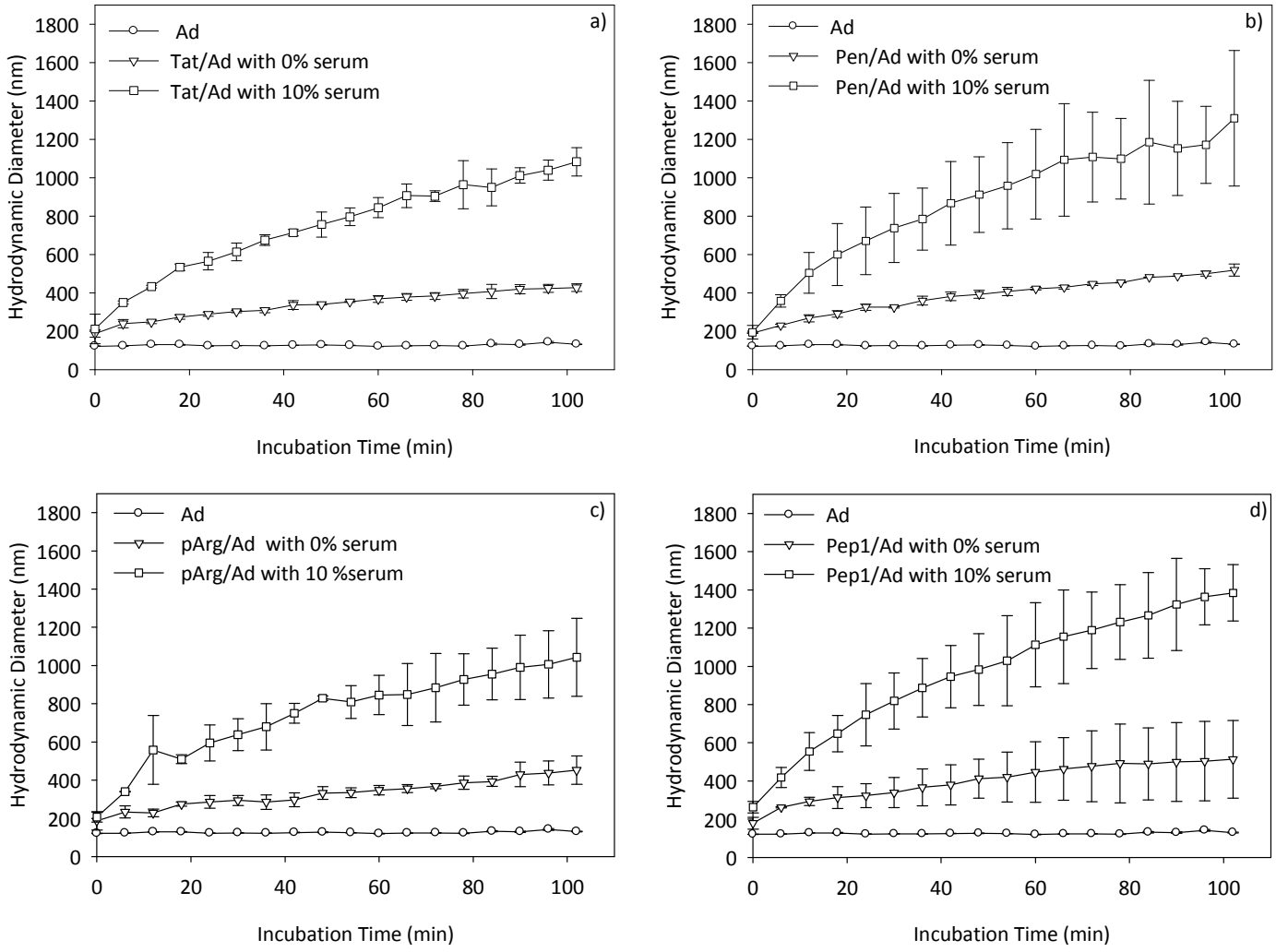


Figure 3.8: Aggregation of CPP/Ad complexes in the presence and absence of serum for a) Tat/Ad, b) Pen/Ad, c) pArg/Ad and d) Pep1/Ad.

# CHAPTER 4

# DICUSSIONS

The ability of CPPs to deliver macromolecules such as peptides (Yang, Wang et al. 2006), liposomes (Pappalardo, Quattrocchi et al. 2009), siRNA, (Crombez, Aldrian-Herrada et al. 2009) and DNA (Ignatovich, Dizhe et al. 2003) across the plasma membrane of a wide range of cell types without causing toxic side effects makes the peptides explored in this study an attractive option for gene delivery. To further demonstrate the usefulness of these CPPs (Tat, pArg, Pen, and Pep1) that have high isoelectric points and a net positive charge at physiological conditions, the peptides were evaluated to determine if they could electrostatically bind Ad and transform cells otherwise hard to infect using Ad alone.

For *in vivo* application, the best way to facilitate the transfer of cargo into a target cell is to simultaneously position both the translocation agent and cargo near the cell. Although studies have shown that CPPs can associate with the plasma membrane of cells and transfer cargo through the membrane without forming a complex with the cargo (Kosuge, Takeuchi et al. 2008) a more efficient approach would utilize complexes formed between the translocation agent and the cargo. In this study, CPP/Ad complexes were formed electrostatically by incubating CPPs (positively charged) and Ad (negatively charged).

The transduction ability of CPP/Ad complexes was compared to Ad in the presence of free CPPs to determine if the improvement in transduction was the result of complexes formed between CPPs and Ad or the result of these CPPs settling onto the surface of the cells. The CPP/Ad complexes exhibited a 14-fold higher gene expression than the virus in the presence of free CPPs, illustrating the importance of complex formation. Thus, the ability of the CPPs to form the CPP/Ad complex is an important criterion that plays a role in the transduction efficiency and was further studied by evaluating incubation time required to form the best working complex.

By knowing the optimum complex formation time, one can avoid inefficient complex formation due to short incubation periods as well as avoid formation of large aggregates resulting from long incubation periods, both of which reduce the transduction efficiency of the complex. As the results of the study show, increasing the incubation time for complex formation improved the gene delivery efficiency of all CPP/Ad complexes studied. Slightly different trends of transduction efficiency were observed, however, for the different kinds of CPP/Ad complexes. For example, Pen/Ad, pArg/Ad and Pep1/Ad showed optimum transduction after an incubation period of 60 minutes. Tat/Ad produced its highest level of gene expression after only a 15 minute incubation period, the lowest incubation period of all the peptides. This level of gene expression after such a short incubation period demonstrates the ability of Tat to quickly associate with Ad and form complexes sooner than the other peptides, even though the charge of the peptide is similar to Pen and pArg (Table 5.1). This result suggests that the overall charge of the CPP is not the only factor that influences the formation of the CPP/Ad complexes. Both

the charge and arrangement of the cationic amino acids plays a role in the formation of CPP/Ad complexes.

The potential of CPPs to efficiently deliver cargo is dependent on the cellular association of the CPP to the target cell and the mechanism by which the CPP/cargo is transported into the cell. The mechanism of membrane translocation of CPPs and cargo, however, has yet to be identified definitively (Zorko and Langel 2005; Duchardt, Fotin-Mleczek et al. 2007; Patel, Zaro et al. 2007) and there are conflicting studies regarding whether the CPP internalization mechanism involves energy-dependent endocytosis or a non-endocytic mechanism (Drin, Cottin et al. 2003; Richard, Melikov et al. 2003; Ziegler, Nervi et al. 2005). Despite the fact that different CPPs internalize into cells through different mechanisms (i.e., endocytosis or energy-independent membrane perturbations), recent observations have indicated that internalization of these CPPs occurs via endocytosis when the CPPs are coupled with high molecular weight cargo (Console, Marty et al. 2003; Lundberg, Wikstrom et al. 2003). These findings suggested that the role of the peptide in this case is may be limited to facilitating attachment of cargo to the cell membrane. Nevertheless, the results of the current study show different levels of transduction induced by different CPPs of similar physicochemical properties, which suggest that it is unlikely the CPP serves only to facilitate attachment with the cell membrane. Instead, the CPP is likely facilitating cellular association and influencing the mechanism of membrane translocation, which others have reported to be dependent on properties of the CPP such as sequence (Mueller, Kretzschmar et al. 2008), net charge (Magzoub and Graslund 2004), number and arrangement of hydrophobic residues (Magzoub and Graslund 2004), numbers and arrangement of arginine residues (Zaro and

Shen 2003), concentration (Duchardt, Fotin-Mleczek et al. 2007) and other physicochemical properties of the complex formed.

Our study showed that each of the four CPPs has the ability to translocate Ad into cells that are hard to infect, albeit with varying degrees of efficiency. This is attributed to the different properties these CPPs possess which influence membrane binding and translocation of CPP/Ad. CPPs used in the study have different sequences, net charges, numbers of arginine residues, and amphipathicity (Table 5.1). For example, Tat (Frankel and Pabo 1988) and pArg (Mitchell, Kim et al. 2000) are highly charged peptides containing cationic, basic amino acids that are highly hydrophilic (e.g., arginine and lysine). In comparison, Pen (Derossi 1995) is a peptide with low amphipathicity consisting of several positively charged basic amino acids interspersed throughout a hydrophobic region of the peptide, and Pep1 (Deshayes, Heitz et al. 2004) is an amphipathic peptide with hydrophilic, hydrophobic and linker domains.

In the gene delivery studies, Pen/Ad showed relatively high gene expression compared to all the other CPP/Ad complexes. Tat/Ad and pArg/Ad (i.e., peptides with a large number of arginine residues) produced lower levels of gene expression compared to Pen/Ad, except at the highest concentration (50 $\mu g/10^6$ Ad). Pep1/Ad, which has distinct cationic and hydrophobic regions, low net charge and few arginine residues, exhibited the lowest efficiency compared to the other CPP/Ad complexes. The fact that Pen/Ad was more effective compared to Tat/Ad and pArg/Ad indicates that the high number of arginine residues does not guarantee a high level of translocation. In fact, studies have shown, in addition to the positively charged amino acids residues, some hydrophobic residues contributed to the membrane binding and translocation (Derossi, Calvet et al. 1996;

Fischer, Zhelev et al. 2000). Also, poor efficiency of Pep1/Ad, relative to the other CPP/Ad complexes, further supports the likelihood that a high net positive charge improves complex formation and the level of cellular uptake, at least to some degree. In addition to the number of arginine residues and peptide charge, some researchers have claimed that amphipathicity affects translocation ability of a CPP since this property is thought to be important in membrane interaction (Drin, Mazel et al. 2001). The results of this study, however, found no direct relationship between amphipathicity and translocation efficiency. In fact, the amphipathic CPPs, Pen and Pep1, were both the best and worst performing peptides, respectively.

Another factor that influenced the transduction efficiency is concentration of the peptide (i.e., the relative ratio of the peptide to virus). In this study, the transduction efficiency of the complex increased by orders of magnitude for all CPP/Ad complexes up to a CPP concentration of 6.25 $\mu g/10^6$ Ad and increased thereafter by only a moderate amount. Based on this observation there is likely an effective amount of CPP that can completely coat the surface of the virus, thereby hindering additional CPPs from binding to the complex. Consequently, further increasing the concentration of CPP only moderately improved the level of transduction. The moderate improvement in transduction was a result of the combined effect of some additional binding between the CPP and CPP/Ad complex and free CPPs that remained unbound to the virus. Physicochemical properties (e.g., size and charge) showed similar trends. As the CPP concentrations increased, size and surface charge increased dramatically for all CPP/Ad complexes up to a CPP concentration of 6.25 $\mu g/10^6$ Ad and leveled off as the concentration increased beyond this point. This observation further supports the suggestion that at these high CPP

concentrations, the peptides may have completely coated the surface of the virus, thereby hindering additional CPPs from binding to the complex.

The goal of the present work was to produce a CPP/Ad complex capable of efficiently transducing cells the virus would not infect on its own. The results show that the best CPP/Ad complex was Pen/Ad, which improved the transduction of CAR-negative NIH/3T3 cells by 100-fold compared to the unmodified Ad. The Pen/Ad complex performed better than similar complexes reported by others. For example, Gratton *et al.* reported that noncovalent modifications of Ad with Pen improved the gene delivery efficiency of the virus to monkey kidney fibroblast cells by 10-fold (Gratton, Yu et al. 2003). Lehmusvaara *et al*., however, reported the same modification only improved gene delivery by 2-fold (Lehmusvaara, Rautsi et al. 2006). These differences in the level of cell transduction can be attributed to several factors, including the cell type, method of complex formation, MOI and virus purity. These groups reported also that Tat is sometimes as effective as Pen (Gratton, Yu et al. 2003; Lehmusvaara, Rautsi et al. 2006). Similarly, the present study found that Tat/Ad and pArg/Ad performed nearly as well as Pen/Ad at high concentrations.

Studies have also shown that the initial electrostatic interaction of CPPs with anionic cell membrane surfaces is an important factor for the uptake of all cationic CPP-cargo complexes (Deshayes, Morris et al. 2005; Duchardt, Fotin-Mleczek et al. 2007; Poon and Gariepy 2007). The presence of competing anionic material can limit the interaction CPPs with an anionic cell membrane surface, restricting cellular uptake (Fischer, Bieber et al. 1999). Most *in vitro* transduction efficiency studies evaluate complexes in the absence of serum. The present study, however, evaluated how adverse the effects of

competing anionic serum were to transduction efficiency. Even though the cellular uptake of CPPs and their cargo is often reduced in the presence of serum, the results of the present study show that serum did not significantly inhibit or promote the transduction efficiency of Tat/Ad or Pen/Ad complexes. Others have reported similar findings where uptake of CPP-cargo complexes by HeLa cells was not affected by the presence of serum (Saalik, Elmquist et al. 2004).

While the performance of Tat/Ad and Pen/Ad was not impacted significantly by serum, Pep1/Ad and pArg/Ad were both negatively affected by the presence of serum. Contrary to these observations, Morris *et al* reported that an advantage of Pep1 is a lack of sensitivity to serum (Morris, Depollier et al. 2001). Morris *et al*., however, observed that the impact of serum on the performance of Pep1 depends on the concentration of the peptide (Morris, Depollier et al. 2001). The apparent discrepancy between these earlier studies and our own can be explained by concentrations that were reported as being either lower or slightly higher than the concentrations used in the present study. Similarly, Kosuge *et al.* explored sensitivity of arginine-based peptides to serum and investigated specifically the impact of the number of arginine residues (Kosuge, Takeuchi et al. 2008). They found that peptides with more than 8 arginine residues became sensitive to serum proteins due to differences in the valency with serum (Kosuge, Takeuchi et al. 2008). The 9-residue pArg peptide used in the present study was sensitive to serum, likely a result of a high capacity to bind to serum. The decrease in transduction efficiency of pArg/Ad and Pep1/Ad illustrates that the sensitivity of complexes to serum is not only dependent on the cationic properties of the peptide, but also potentially dependent on how the cationic basic amino acids are arranged within the peptide.

The physicochemical properties (e.g., size and charge) of the peptide/virus complex were measured to better understand the characteristics associated with highly efficient CPP/Ad complexes and to ensure their suitability for *in vivo* gene delivery. Based on the dynamic light scattering and zeta-potential measurements, the size and surface charge of the different CPP/Ad complexes were similar at a given concentration, independent of the particular CPP. For example, the hydrodynamic diameter of the CPP/Ad complexes at their optimal incubation periods was smaller than 300 nm, a desired range to prevent edema *in vivo*, for concentrations of CPP lower than 25 µg/$10^6$ virus. Although these properties were similar, the transduction efficiency of the different CPP/Ad complexes was quite different, further supporting that the efficiency of each CPP is more closely related to the particular amino acids residue sequence of the peptide than to the overall charge of the peptide or the resulting charge of the CPP/Ad complex.

The surface charge of CPP/Ad complexes is also a concern. A high positive charge adversely affects the stability of the complex and reduces the effectiveness of complexes as a delivery agent (Kwoh, Coffin et al. 1999). While serum had only a minor effect on the transduction efficiency of the CPP/Ad complex, the high surface charge poorly affected the stability of all of the CPP/Ad complexes. The CPP/Ad complexes used in this study formed aggregates when stored beyond the optimal incubation period, regardless of the presence or absence of serum. Although similar trends were observed in both environments due to the high positive charge, complexes incubated in a protein-rich environment exhibited particle sizes four times larger than the corresponding complexes incubated in a protein-free environment. This difference in size indicates that aggregates between oppositely charged CPP/Ad complexes and serum were formed when complexes

were incubated with serum. Clearly improvements need to be made to reduce the surface charge of the CPP/Ad complexes. The use of polymers that would reduce sensitivity to serum (e.g., polyethylene glycol) in combination with CPPs may be a potential route for improving serum stability.

# CHAPTER 5

## CONCLUSIONS AND RECOMMENDATIONS

The focus of the present study was on developing an improved adenoviral based gene delivery vector that can efficiently transform tissues that lack CAR necessary for the infection pathway of the virus. We evaluated if CPPs could form complexes with Ad and function in place of the fiber/CAR interaction to transport the virus into cells lacking the CAR. CPP/Ad complexes were produced using simple electrostatic association. Complex formation and transduction efficiencies of the four CPP/Ad complexes were optimized by varying the incubation period and the ratio of the peptide to virus. The CPP/Ad complexes showed improved transduction efficiency compared to the virus alone, indicating that the CPPs are capable of promoting transduction of cells that are otherwise hard to transform using only the virus. The transduction efficiency of Ad was improved by more than 100-fold. Pen/Ad produced the highest transduction efficiency followed by Tat/Ad, pArg/Ad, and Pep1/Ad. Even though Pep1/Ad was the least efficient, the complex still improved gene expression by 66-fold compared to Ad alone.

In this study, the size of the complexes is appropriate for *in vivo* gene delivery, although in its current state, the high positive surface charge of the complex is likely to result in undesired interactions with serum proteins. In addition, the complexes provide untailored

cell targeting. Surface charge can be reduced by using a low concentration of CPP. In addition, incorporating high molecular weight polyethylene glycol (PEG), a safe and biocompatible polymer commonly used to prolong the circulatory lifetime of proteins, drugs, and nanoparticles (Kochendoerfer 2003; Otsuka, Nagasaki et al. 2003), can provide the desired property. The issue of untailored cell targeting can be tacked by using cell specific ligand in combination with CPP and PEG.

To advance beyond the laboratory stage, it is critical to address the drawbacks associated with adenovirus. In addition to the inability to efficiently infect certain types of cells, adenovirus has serious drawbacks such as immunogenicity and promiscuous tropism. A variety of approaches have been attempted to improve immunogenicity of adenovirus. One of the most common attempts used to diminish the susceptibility of the virus to immune inactivation and inflammatory immune responses has been to coat the surface of the virus with PEG. This process called PEGylation, reduces susceptibility of the virus to immune inactivation and initiation of inflammatory immune response (Aliabadi, Brocks et al. 2005). Adding the element of PEGylation of strategy to CPP/Ad conjugate will improve the stability and reduce susceptibility to immune response of the complex.

Genetic modification of Ad may reduce promiscuous tropism and immune response associated with the virus. The crucial role played by fiber and knob proteins of the virus in transduction prompts the genetic modification of these proteins. Studies have shown that transduction efficiency of the virus has shown dependency on the length of the fiber protein (Legrand, Spehner et al. 1999; Shayakhmetov and Lieber 2000; Vigne, Dedieu et al. 2003). Further, complete removal of the knob domain has been established as a way to

ablate tropism (Magnusson, Hong et al. 2001). Genetic modifications and replacement of the fiber and knob protein will further overcome the drawbacks associated with the virus.

# CHAPTER 6

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