Nonviral Vectors for Gene Delivery

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Abstract

The development of nonviral vectors for safe and efficient gene delivery has been gaining considerable attention recently. An ideal nonviral vector must protect the gene against degradation by nuclease in the extracellular matrix, internalize the plasma membrane, escape from the endosomal compartment, unpackage the gene at some point and have no detrimental effects. In comparison to viruses, nonviral vectors are relatively easy to synthesize, less immunogenic, low in cost, and have no limitation in the size of a gene that can be delivered. Significant progress has been made in the basic science and applications of various nonviral gene delivery vectors; however, the majority of nonviral approaches are still inefficient and often toxic. To this end, two nonviral gene delivery systems using either biodegradable poly(D,L-lactide-*co*-glycolide) (PLG) nanoparticles or cell penetrating peptide (CPP) complexes have been designed and studied using A549 human lung epithelial cells.

PLG nanoparticles were optimized for gene delivery by varying particle surface chemistry using different coating materials that adsorb to the particle surface during formation. A variety of cationic coating materials were studied and compared to more conventional surfactants used for PLG nanoparticle fabrication. Nanoparticles (~200 nm) efficiently encapsulated plasmids encoding for luciferase (80-90%) and slowly released the same for two weeks. After a delay, moderate levels of gene expression appeared at day 5 for certain positively charged PLG particles and gene expression was maintained for at least two weeks. In contrast, gene expression mediated by polyethyleneimine (PEI) ended at day 5. PLG particles were also significantly less

cytotoxic than PEI suggesting the use of these vehicles for localized, sustained gene delivery to the pulmonary epithelium.

On the other hand, a more simple method to synthesize 50-200 nm complexes capable of high transfection efficiency or high gene knockdown was also explored. Positively charged CPPs were complexed with pDNA or siRNA, which resulted in 'loose' (~1 micron) particles. These were then condensed into small nanoparticles by using calcium, which formed "soft" crosslinks by interacting with both phosphates on nucleic acids and amines on CPPs. An optimal amount of $CaCl_2$ produced stable, ~100 nm complexes that exhibited higher transfection efficiency and gene silencing than PEI polyplexes. CPPs also displayed negligible cytotoxicity up to 5 mg/mL. Biophysical studies of the pDNA structure within complexes suggested that pDNA within CPP complexes (condensed with calcium) had similar structure, but enhanced thermal stability compared to PEI complexes. Thus, CPP complexes emerged as simple, attractive candidates for future studies on nonviral gene delivery *in vivo*.

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Chapter 1. Gene Delivery

1.1. Introduction

Gene therapy is a potential application to correct genetic deficiencies or treat acquired diseases by altering or manipulating genes. Gene therapy as a modern molecular medicine is a relatively young subject that was born in 1989 with the transfer of a drug resistance gene marker to a patient's lymphocytes in the first gene transfer clinical trial [1]. Gene therapies are being developed for cardiovascular [2], neurological [3-5] and infectious diseases [6], wound healing [7] and cancer [8-10]. They promise a great future and in the meantime offer new and distinct challenges ranging from delivery to production. To date, nearly 1560 gene therapy clinical trials have been conducted (http://www.wiley.com/legacy/wileychi/genmed/clinical/).

Gene therapies can be categorized on the basis of therapy as gene inhibitors, gene vaccines and gene substitutes. Gene inhibitors (i.e. siRNA) are potent therapies that silence or alter defective genes [11]. Gene vaccines include antigens of specific pathogens and often include the genes or RNA that activate cell-mediated and humoral immune response [12]. Gene substitutes are transcriptionally fully competent genes introduced into cells to reimburse deficiency of a specific protein.

Since its inception the biggest challenge in the field has revolved around the word "delivery". Nucleic acids are labile macromolecules that are challenging to deliver intracellularly owing to their size and hydrophilic nature, which includes a high density of negatively charged phosphate groups. In addition, they are very susceptible to degradation by nucleases. Depending on how nucleic acids are introduced, gene therapy can be divided into *ex vivo* and *in vivo*. In ex vivo gene therapy, cells are removed from

the patient, genetically modified and reintroduced into the patient while in vivo gene therapy aims to deliver genes directly to the site of action.

Exogenous genetic materials must be transferred into cells by passing through a series of hurdles in gene therapy, for the production of therapeutic proteins [13,14]. To realize gene therapy, a vehicle that satisfies the requirements for both a high transfection efficiency and low toxicity is essential [15].

1.2. Methods of gene delivery

Gene delivery vehicles can be classified into two categories; viral and nonviral vectors. Recombinant viruses have had their genome altered to prevent viral replication and allow incorporation of therapeutic transgene. Many viruses have been used for the production of viral vectors including retrovirus, adenovirus, adeno-associated virus, herpes simplex virus and vaccinia virus. In theory, viral carriers can provide a high transfection rate and rapid transcription of the foreign material inserted in the viral genome [16,17]. However, many clinical trials utilizing viral vectors have been interrupted since the application of these vectors induced unexpected adverse effects such immunogenicity, pathogenicity and toxicity [18,19]. Jesse Gelsinger was the first patient to die in a clinical trial for gene therapy in 1999. Gelsinger suffered from ornithine transcarbamylase deficiency, an X-linked genetic non-life-threatening disease of the liver. After injections of adenovirus carrying the corrected genes, the patient died from a massive immune response. Other challenges include limitations in target cell specificity and high cost associated with the vector production.

On the other hand, nonviral vectors are potentially less immunogenic, relatively easy to produce, flexible in formulation design and associated with fewer safety concerns

[20,21]. Furthermore, this category of gene delivery may enhance delivery to specific cells through incorporation of a targeting ligand [22-27]. In general, synthetic nonviral vectors are materials that electrostatically bind nucleic acids (i.e., DNA or RNA), condense them into particles a few tens to several hundred nanometers in diameter, confer protection from degrading factors that exist in the extracellular and intracellular environment and mediate cellular internalization. The functionalities of these nonviral systems stem from the materials used for the construction of the vectors. Among the many types of materials used, lipids and polymers represent the two major classes.

Gene delivery vectors must be taken up by cells direct the nucleic acids to an appropriate intracellular destination (cytoplasm for siRNA and nucleus for pDNA), release the nucleic acids to allow their action and exhibit minimal toxicity. Up to now, many efforts have addressed these objectives in a serial fashion [28]. It is not surprising that the efficiency of gene delivery by nonviral vectors has yet to rival viruses as gene delivery agents as evidenced by their limited clinical application. A key issue impeding the development of nonviral vectors is thus the need to overcome various physiological barriers.

Compared to viral and nonviral gene delivery, methods employing external energy or force offer some advantages for gene transfer. Enhanced gene transfer can be obtained with naked pDNA, actively facilitating the passage of the gene across various barriers and eliminating some problems associated with the formulation, toxicity and immunogenicity. Based on the type of the physical methods employed, these can be categorized as direct injection hydrodynamic, electroporation, ultrasound-mediated delivery, laser irradiation, magnetic force mediated delivery and electrical field induced

molecular vibration [29]. Even though these methods are relatively independent of cell type and gene size, the efficiency is typically low. The major drawbacks include the small amount of cells transfected per treatment, surgical procedures required for transfecting internal tissues and high energy (i.e. voltage) that must be applied to tissues which can result in irreversible tissue damage. Moreover, most physical techniques are at a rudimentary stage of development and much work must be done before reaching human clinical trials.

1.3. Barriers to nonviral gene delivery

Successful gene delivery systems must navigate a series of obstacles, both extracellular and intracellular, from the point of administration to the site of action (cytoplasm for siRNA and nucleus for pDNA) (Figure 1). Overcoming these barriers is the main design goal of nonviral vectors.

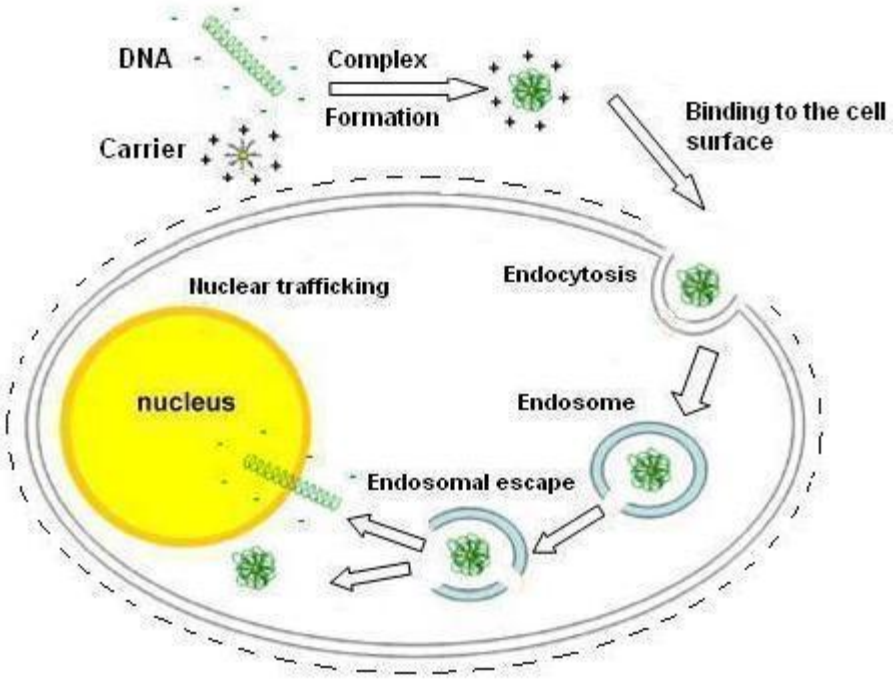

Figure 1. Barriers to nonviral gene delivery

1.3.1. Extracellular barriers

The first major barrier to gene delivery is the extracellular milieu, a space which surrounds the cells that contains a set of macromolecules, polysaccharides or glycosaminoglycans, fibrous proteins, salts and water [30]. This space can compromise the chemical stability of the nucleic acids as well as the physical stability of the delivery vehicle. To reach the plasma membrane of target cells, the nucleic acids must diffuse through the extracellular matrix without being degraded by the extracellular nucleases. Previous work showed that the delivery of macromolecules such as pDNA to cells is affected by the amount of nucleases and by the amount of extracellular environment components [31-37]. One strategy to increase the diffusion and distribution of pDNA into target tissue consists of controlled and partial degradation of extracellular matrix using enzymes such as hyaluronidase and collagenase [35,38-45]. Results showed that the tissues pretreated by these enzymes showed more gene expression than others without enzymatic treatment.

Numerous subtle improvements have been made to overcome nuclease degradation within extracellular matrix by either condensing the nucleic acids with a variety of polycations or by complexing with polymers that bind to but do not condense nucleic acids [46-48]. The condensation process is believed to be kinetic [49] and is highly dependent on the properties of polycations, the nucleic acids concentration, the order of mixing, and the ionic strength of the buffer [49-51]. It is important to realize, however, that excessively high affinity of nucleic acid with vector components may hinder intracellular release of the gene, which is required for its action to take place. Charge-neutral polyelectrolyte complexes of nucleic acid with a polycation tend to exhibit rapid aggregation upon

exposure to physiological levels of salt. Further, the colloidal stability of positively charged nonviral gene vectors may be disrupted in the presence of excess positive charge owing to possible interactions with exracellular matrix components that effectively compete for binding sites of nucleic acid to the cationic nonviral vehicle [47,52-54]. Some studies have shown that the aggregate size of positive complexes is time dependent [49]. Several hydrophilic, nonionic polymers, such as polyethylene glycol (PEG) [55,56], N-(2-hydroxypropyl) methacrylamide (PHPMA) [56-59], oligosaccharides [56,60], and sugars [61,62], have been used to modify the surface of the nonviral vectors in order to decrease potential destabilizing particle interactions. PEGylation, for example, has been incorporated covalently [55,63-65] and noncovalenty [66,67] into nonviral gene vectors and has been found to increase the colloidal stability of formulations. However, this modification may minimize the efficiency of gene delivery by reducing internalization or by inhibiting endosomal escape of the vector [68].

One more obstacle to a nonviral gene delivery system in the extracellular milieu is the lack of target cell selectivity. The requirement for targeting in gene therapy varies by the therapeutic applications. In the case of cancer therapies for example, gene delivery to cells of interest might be required for destroying specific cells while maintaining viability of healthy tissue. An efficient targeting strategy is thought to depend on factors such as, the conjugation chemistry of the ligand to the vehicle, the molecular weight and the size of the ligand, the distance between the ligand and its vehicle, the number of targeting ligands per vector and the ligand-receptor binding affinity [67,69-71].

Several strategies have been reported to increase cellular uptake and selectivity. Polycations have been shown to essentially increase the cellular association of nucleic

acids by mediating the repulsion between the nucleic acid and cell surface through electrostatic interaction with cell surface heparin sulfate proteoglycans, a protein core with one or more glycosaminoglycans attached, (HSPGs) [72,73]. Targeting ligands have been incorporated within cationic lipids (Lipoplexes) or polymers (Polyplexes) and, upon binding to the receptor on the target cell surface, induce cellular uptake *in vitro* [74-76] and *in vivo* [48,77-81]. Moreover, direct covalent conjugation of targeting ligands to the nucleic acid has been investigated [82].

1.3.2. Intracellular barriers

Nonviral gene delivery systems need to provide mechanisms to overcome numerous intracellular obstacles. Cellular internalization, endosomal escape, unpackaging of DNA, persistence within the cytoplasm and delivery to the cell nucleus are the major barriers for effective nonviral gene delivery. Comprehensive efforts have been made to address these perceived barriers.

Untargeted cationic nonviral vectors bind electrostatically to the surface of cells (proteoglycans) [68,73] and are internalized via endocytosis [68,83-86]. Clathrin-dependent endocytosis has been reported in the internalization of ~ 200 nm cationic nonviral complexes [87-90]. Phagocytosis and macropinocytosis are other possible pathways of internalization that have been reported [84,91,92]. Alternatively, delivery systems with targeting ligands are often internalized by receptor-mediated endocytosis [74,79,93]. Following internalization, the vectors are engulfed by membrane vesicles [68,88,94-98] and often merge with other vesicles en route to lysosomes filled with degradative enzymes. It has been generally assumed that avoidance of lysosomes can give rise to improved gene delivery with nonviral systems. Therefore, the escape of gene vector (or the nucleic acid

itself) from the endosome has been demonstrated in several studies as one of the fundamental hurdles [99-103].

Initially the vectors become localized within early endosomes which are divided into two types, sorting endosomes from which the internalized material can be redistributed and recycling endosomes which are thought to be responsible for returning internalized material to the membrane and out of the cell [104]. The inability of vectors to escape the endosomal compartment and enter the cytoplasm results in trafficking via the late endosome to lysosomes where the nucleic acid is eventually degraded.

Several designs have been applied for engineering endosomal escape in nonviral gene delivery. Incorporation of a helper lipid, such as dioleoyl phosphatidylethanolamine (DOPE) in the vehicle formulation may promote nucleic acid release by facilitating endosomal disruption [105-109]. The cationic polymer polyethyleneimine (PEI) is thought to facilitate endosomal release by buffering the acidification of the endosomal compartment ("proton sponge" hypothesis). Accumulation of protons, counterions and water eventually leads to osmotic swelling of the endosome and, ultimately, osmotic lysis [13,110-115]. In addition, fusogenic viral [116-118] or synthetic peptides [76,119-122] have been attached to vectors to provide endosomal escape. Some fusogenic liposomes and pH-sensitive liposomes have also been designed for destabilizing endosomal compartment [123]. Transfection efficiency is also potentiated by utilizing nonspecific lysomotropic agents, such as chloroquine, polyvinylpyrolidone or sucrose [91,124-126].

Once released from the endosomes, the vector or freed nucleic acids must traverse through the cytoplasm and gain access to the nucleus (e.g., pDNA). The presence of high protein concentration, microtubules and many organelles imposes an intensive molecular

crowding of the cytoplasm which limits the diffusion of the vehicle or nucleic acid [127]. The diffusion coefficient of pDNA in the cytoplasm is size dependent with pDNA < 3000 base pairs in length is less than 1% of the diffusivity in water, and pDNA > 3000 base pairs is essentially immobilized [128]. To date, no evidence for active transport of pDNA in the cytoplasm has been reported. In addition to the physical diffusion barrier, the known degradation of pDNA in the cytosol due to the presence of nucleases poses a significant barrier to efficient gene delivery as well [129,130]. Microinjection of pDNA into the cytoplasm resulted in degradation with a half-life of 50-90 min. Some studies have provided evidence that pDNA was still partially complexed to cationic polymers (e.g., PEI) in the cytoplasm. PEI gene delivery systems are purported to overcome these obstacles by actively transporting pDNA along microtubules to the nuclear region which resulted in increased gene expression [98,131]. The mechanism by which polycations remain bound to the nucleic acid in the cytoplasm to facilitate gene expression need to be well characterized to improve the design of polymeric vectors.

The ultimate destination for pDNA delivered inside the cell is the nucleus where the gene can be transcribed. Nuclear transport of pDNA alone or complexed to the nonviral vector is still not fully clear. There are three possible routes for pDNA to gain entry into the nucleus; (i) during mitosis, the nuclear membrane is temporarily disrupted which allows pDNA to diffuse into the nucleus, (ii) molecules of < 9 nm can passively diffuse through nuclear pores and (iii) particles < 26 nm can be actively transported through nuclear pore complexes (NPCs) [132-136]. The transfection efficiency of polymer and lipid based systems in cells undergoing division was 30 to 500-fold more effective than transfection of cells at the beginning of their cell cycle [137]. Nuclear localization

sequences (NLSs), short cationic peptides that are recognized by importins, have been demonstrated to increase nuclear translocation of pDNA [138]. Covalently linking NLS to pDNA or noncovalent association of NLS to pDNA complexes has been reported to enhance nuclear translocation [139]. It is quite possible that polycation-based vectors may mediate an additional role in the nuclear delivery of pDNA [131,140] but evidence also points to poor nuclear transport [141-143].

1.4. Nonviral carriers

Several nonviral vehicles have been proposed for safe delivery of therapeutic nucleic acids. Lipids and polymers can form effective pharmaceutical delivery systems for nucleic acids; however, many formulations still suffer from relatively low efficiency of gene delivery as well as the toxicity, particularly for *in vivo* applications. The use of cationic lipids and polymers for gene delivery was introduced by Felgner *et al*. (1987) and Wu and Wu (1987), respectively [106,144]. Their use has progressed rapidly from the transfection of cell cultures to clinical gene therapy applications. Three of the major gene delivery systems are described below.

1.4.1. Lipid-based vectors

Cationic lipids (liposomes, micelles, etc.) are one of the most attractive gene delivery vectors. These are amphiphilic molecules composed of a hydrophobic lipid anchor group, linker group and a positively charged head group. The nature of each group affects the gene delivery efficiency. The positive charge of cationic lipids entraps or condenses the nucleic acid to form a complex, commonly known as a lipoplex. The ability of this system to mediate transfection was attributed to its spontaneous

electrostatic interaction with genes. A net positive charge exhibited by the system can promote its association with the negatively charged cell surface and its fusogenic properties that can induce fusion and/or destabilization of the plasma membrane facilitating the intracellular release of complexed or encapsulated gene.

The size and the charge of the cationic head group are important for transfection [145]. Different types of cationic head groups have been used such as quaternary ammonium, primary, secondary or tertiary amines, guanidinium, heterocyclics, amino acids, pyridinium, amidine and peptides [106,146-152]. Many cationic lipids showed excellent transfection activity in cell culture, but most did not perform well in the presence of serum and only a few were active *in vivo* [153]. A large excess of cationic lipids was needed to mediate optimal gene delivery.

A dramatic change in size, surface charge and lipid composition occurs when lipoplexes are exposed to the negatively charged and often amphipathic proteins and polysaccharides that are present in blood, mucus, epithelial lining fluid and tissue matrix. Despite these undesirable characteristics, lipoplexes have been used for *in vivo* gene delivery to lungs by intravenous [154-156] and airway [157-159] administration. In these studies, gene expression was detectable but in most cases was very low due to fast clearance of lipoplexes. Surface shielding through the use of the hydrophilic and charge neutral polymers such as polyethylene glycol (PEG) to reduce excessive charge-charge interactions appeared very effective in prolonging the circulation half life of lipoplexes [160]. Unfortunately, inclusion of PEG into lipoplexes caused a concentration-dependent inhibition in transfection activity. For this reason, PEGs were made detachable using an acid-sensitive linkage through which the PEG moiety can be removed when lipoplexes

are inside the endocytic compartment [161,162]. Cholesterol and dioleoylphosphatidylethanolamine (DOPE) were synergistic with cationic lipids in mediating the transfection [47,154,163]. In most cases, DOPE lead to reduced charge density of lipoplexes, thus lowering toxicity. The fusogenic property of DOPE also facilitated the endosomal escape of lipoplexes. The presence of cholesterol was reported to stabilize the cationic lipidic membrane structures against the destructive activity of serum components and was proposed to provide better activity for *in vivo* transfection [163].

Major obstacles for practical use of lipoplex-mediated transfection are acute toxicity and short duration of gene expression [154,164,165]. Moreover, cationic lipids can stimulate potent inflammatory responses in host cells. Rapid production of cytokines followed by clearance of transfected cells after administration of uncoated lipoplexes compounded these problems. Performance was improved by designing lipids with increased biodegradability [166-168] or by shielding lipoplexes using PEG [160,169]. In addition, cationic lipids that are charged only at mildly acidic but not at neutral pH [170] and those whose charge groups can be eliminated after lipoplex formation [166] may provide potential solutions

1.4.2. Polymer-based vectors

Many types of polymers have been used for gene delivery. Among these polymers, cationic polymers have garnered attention due to their abilities to form polyelectrolyte complexes between nucleic acids and polycations, protect genes from enzymatic degradation and facilitate cell uptake and endolysosomal escape [171]. Cationic polymers are usually classified in two main groups; natural polymers, such as peptides, proteins, or polysaccharides and synthetic polymers, such as polyethyleneimine (PEI), biodegradable

polymers or dendrimers [172]. Natural polymers generally have the advantage of being nontoxic even in large doses. In addition, some offer attributes such as good mucoadhesion and biodegradability [173]. On the other hand, synthetic polymers can provide flexibility in formulation design and can be tailored to fit the size and topology of the gene.

1.4.2.1. Natural polymers

Over the years, a significant number of natural polymers, such as chitosan [174], cationic proteins (e.g., polylysine, protamine and histones) [175-178] and cationic peptides (e.g., arginine-rich and lysine-rich peptides) [179-187] have been explored as carriers for gene delivery. In general, natural polymers have been used much less in gene delivery than synthetic polymers. Among natural polymers, the cationic polysaccharide chitosan has attracted considerable attention as a nonviral gene delivery vector. Although chitosan showed effective nucleic acid binding and compaction, the transfection efficiency was significantly lower compared to other cationic gene delivery agents. This problem may be attributed to retarded endosomal escape of chitosan complexes.

Gene delivery efficiency when employing chitosan is affected by a lot of factors including chitosan molecular weight, salt form, degree of deacetylation and the pH of the culture medium [188]. The latest strategies to improve its transfection efficiency comprise the synthesis of various chitosan derivatives, such as aminoethylchitin (AEC) [189], thiolated chitosan [190], chitosan methoxy polyethylene glycol cholesterol (LCP-Ch) [191] and low molecular weight alkylated chitosans [192]. Incorporation of negatively charged compounds such as hyaluronic acid or poly (γ-glutamic acid) with chitosan has been reported to increase transfection efficiency significantly [193,194].

Peptide-based vectors are advantageous over other nonviral gene delivery systems in that they have the potential to protect nucleic acids, target specific cell surface receptors, disrupt the endosomal membrane and deliver the gene to the nucleus [195]. Cationic peptides rich in basic residues such as arginine and/or lysine have been explored in recent years as a carrier for gene delivery. They have been used in gene delivery either by covalent binding to the vectors [180,185,187] or by simple complexing with the gene through electrostatic interactions [181,182,184,186,196]. Such peptides were able to efficiently condense the gene into particles with a net positive charge that is able to interact with cell membranes and facilitate internalization. Cationic peptide complexes were also shown to be stable in serum [46,197,198].

Peptide sequences derived from protein transduction domains (PTD) were identified as potentially useful agents for intracellular delivery of nucleic acids [199-203]. These peptides were able to selectively lyse the endosomal membrane under acidic conditions leading to cytoplasmic release of the complex [204,205]. Short peptide sequences taken from longer viral proteins, such as the TAT peptide from the HIV transactivator protein TAT, can provide nuclear localization of condensates once they are in the cytoplasm [206-210]. Lysine-rich peptides derived from histone H1, H2A and anti-DNA antibody can also transfer genes into cells [179,211]. The toxicity of the cationic peptides are typically low compared to high molecular cationic polymers, however, the gene expression levels of their complexes require improvement [212-214]. The explanation for the poor transfection efficiency may emanate from the fact that the cationic peptides and their nucleic acids for the most part remain entrapped in endocytic vesicles [215,216].

Poly-L-lysine (PLL) is a polypeptide that offers a natural and biodegradable vector for nucleic acids [217]. The primary amine groups of lysine in PLL are protonated at neutral pH and electrostatically interact with negatively charged phosphate groups of the nucleic acid to form nanoparticles. PLL generally has poor transfection efficiency when applied alone or without modification [218,219]. PLL complexes using high molecular weight of PLL showed a relatively high cytotoxicity [220] and a tendency to aggregate and precipitate depending on the ionic strength [221]. In order to increase the transfection efficiency of PLL, histidine residues have been introduced to PLL to facilitate endosomal escape [222]. Conjugating PLL with chitosan or lipids such as myristic and stearic acids has also been reported to modify the transfection efficiency of PLL [223,224]. Another useful modification was the attachment of polyethylene glycol (PEG) to the PLL to prevent plasma protein binding and increase circulation half-life of the complex [225-227]. In addition, PLL complexes have been targeted to specific cells through conjugation of folate [228], sugars [134,229,230], antibodies [231] and RGD-displaying peptides [91].

1.4.2.2. Synthetic polymers

Polyethyleneimine (PEI) is one of the most effective polymer transfection agents due to its ability to tightly compact nucleic acids and buffer pH (presumed to facilitate endosomal escape). The transfection efficiency of certain formulations has been comparable to those of viral vectors [112,232]. PEI has primary (25%), secondary (50%) and tertiary amines (25%), of which the majority of the amines are not protonated in physiological milieu [115,233]. The primary amines are mainly responsible for the high degree of gene binding, but they also contribute to the toxicity during transfection. The

secondary and the tertiary amines provide good buffering capacity to the system [234]. The buffering property gives PEI an opportunity to escape from endosome [112].

PEI exists in either branched or linear structures. A study of different cationic densities of linear PEI showed that cell cytotoxicity and transfection efficiency were dependent not only on cationic density but also on the molecular weight of the polymer [235]. High molecular weight PEI (e.g., 25 kDa) is highly efficient for gene delivery, but very toxic to many cell lines; however, PEI of 2 kDa or smaller is essentially nontoxic but very ineffective for gene delivery [236,237]. The clinical development of PEI has been halted due to acute toxicity [232,234].

PEI has been extensively modified in an effort to reduce toxicity and improve transfection efficiency. PEGylation of PEI induced a substantial decrease in cytotoxicity and reduced the extent of aggregation of the complexes [65,68,119]. Cross-linking of low molecular weight PEI with a biodegradable bond such as disulfide or ester bond resulted in oligomers that were as active as PEI 25 kDa but significantly less toxic to cells [238,239]. Conjugation (e.g., sugar, small polymers and antibodies) has yielded PEI derivatives that were more efficient in transfection and less toxic [240-243]; however, modification or elimination of too many amine groups, in general, leads to reduction or loss of the transfection activities of PEI.

Biodegradable polymers were designed to address cytotoxicity and gene delivery barriers. Non-degradable polymers are difficult to eliminate and, therefore, they can potentially accumulate within tissues or cells to elicit further toxicity. A class of biodegradable polymers was reported for gene delivery based on cationic polyesters, including poly(α-[4-aminobutyl]-$_L$-glycolic acid) (PAGA) [244], poly(4-hydroxyl-1-proline

ester) [245], hyperbranched poly(amino ester) [246] and poly(β-amino esters) [247-249]. Generally, these polymers showed much less cytotoxicity and higher transfection efficiency compared to unmodified PLL, especially in the presence of chloroquine.

Poly(lactic-*co*-glycolic acid) (PLGA) is another biocompatible and biodegradable polymer that has been investigated as a carrier for controlled release of nucleic acids [250-252]. The release rate could be controlled by the molecular weight and copolymer composition of PLGA [253]. PLGA has the advantage of lower toxicity compared to cationic lipids and cationic polymers, however, its transfection efficiency requires significant improvement. Recent efforts have focused on modifying PLGA to yield positively charged vectors in order to increase cellular uptake [254,255]. PLGA nanoparticles that contain a hydrophobic core and a hydrophilic lipid/PEG/folate shell were developed for co-delivery of drug and gene to folate receptors [256]. Incorporation of PEI or cationic polyamines or polysaccharides (e.g., chitosan) into PLGA nanoparticles improved gene loading and gene expression [257-262]. In addition, dimethyldidodecylammonium bromide (DMAB) coated PLGA nanoparticles loaded with GFP reporter plasmid exhibited significant improvements in transfection efficiencies with comparison to non-modified PLGA nanoparticles [263].

Several dendrimers such as polyamidoamine (PAMAM) [264], poly(propylene imine) (PPI) [265], poly(L-lysine) (PLL) [266] and poly(2,2-bis(hydroxymethyl) propionic acid (bis-MPA) [267] have been studied as drug delivery systems. Dendrimers with positively charged surface groups are used to deliver nucleic acids where their core-shell nanostructures enable gene loading by encapsulation, surface adsorption, or chemical conjugation [268-271]. The biocompatibility (e.g., cytotoxicity and immunogenicity) of the

dendrimers is related to their structure, molecular weight and surface charge [272,273]. These synthetic macromolecules have also been chemically modified to mask the cationic groups. PEGylation has increased the gene expression [274,275]. The transfection efficiency is generally a function of the generation of the dendrimers [276-278]. Luteinizing hormone-releasing hormone (LHRH), galactose, TAT and arginine peptides have been conjugated to various dendrimers to improve cell targeting and membrane permeation of dendriplexes [279-281]. The difficulties encountered with the synthesis of the dendrimers have limited their clinical applications.

1.5. Lipid/polymer-based vectors

Another unique nonviral vector consists of nucleic acid precondensed with polycations, then coated with cationic lipids [282,283], anionic lipids [284], or amphiphilic polymers with helper lipids [285]. Linear poly-L-lysine, protamine, histone and several polypeptides have been used as nucleic acid condensation component. Lipid/polymer system has been reported to be more efficient in transfection than lipid-based vector *in vitro* [282,283] and is equally active *in vivo* [286]. The cytotoxicity of such vector was reduced and the application of receptor-mediated targeting became possible without interference of nonspecific charge-charge interaction. In addition, several aspects related to lipid composition, the presence of shielding PEG-lipid conjugates and the nature of chemical bonding that contributes to the biodegradability of PEG-lipid conjugates in cells have been studied [287,288].

1.6. Hybrid vectors

Many ongoing efforts have emphasized new ways of better utilizing the advantages of both viral and nonviral vectors for gene delivery. This approach, called a 'hybrid' system, aims to overcome the limitations of individual vectors by combining them. Cationic lipids and polymers may help in improving the transduction efficiency of viral vectors by engaging the cell surface and allowing internalization of the viral vectors, while possibly reducing immunostimulation. Cationic lipids have been shown to promote the delivery of adenovectors into target cells resulting in improved gene expression [289,290]. Adenovirus protein was reported to enhance the nuclear translocation and increase the transfection efficiency of PEI/pDNA complexes [291]. Furthermore, polycations and PEG have been conjugated to adenoviral vector to prolong circulation half-life, enhance gene expression and prevent immune response [292,293].

1.7. Thesis overview

This thesis work aimed to identify simple, safe and effective gene vectors. Biodegradable polyester (poly($_{D,L}$-lactide-*co*-glycolide) (PLG) and short cationic peptides (cell penetrating peptides) (CPPs) have been explored as carriers for gene delivery. In chapter 2, PLG nanoparticles encapsulating pDNA were coated with different surface modifiers and examined for the ability to transfect A549 lung epithelial cells. Cationic surface modifiers were confirmed to increase pDNA encapsulation efficiency and enhance uptake by A549 cells. PLG nanoparticles exhibited sustained low-level gene expression for at least 14 days and negligible cytotoxicity.

In chapter 3, the addition of $CaCl_2$ to TAT/pDNA complexes yielded a network of "soft" crosslinks leading to sustained gene expression levels higher than observed for

control branched PEI gene vectors in A549. TAT-Ca complexes were stable, maintaining particle size and transfection efficiency in the absence and presence of 10% of fetal bovine serum (FBS). In addition, TAT peptide showed no evidence of cytotoxicity and A549 cells maintained high viability, while branched PEI induced substantial cytotoxicity (IC_{50} ~ 35 μg/mL). In chapter 4, four representative CPPs were studied as DNA complexing agents for gene delivery; Arginine 7 (Arg7), Arginine 9 (Arg9), Antennapedia Heptapeptide (Ahp) and Antennapedia Leader Peptide (Alp). A $CaCl_2$ concentration of 113 mM produced small (100-140 nm) and stable CPP polyplexes with a single particle population (polydispersity < 0.23). Cytotoxicity profiles of Arg7, Arg9 and Ahp peptides revealed no evidence of cytotoxicity and cells maintained high viability, while Alp peptide showed moderate cytotoxicity (IC_{50} ~2144 μg/mL). CPP polyplexes showed the highest level of gene expression at 113 mM of added $CaCl_2$ for the various N/P ratios when compared to branched PEI, which had excellent transfection efficiency only in the absence of $CaCl_2$. A slight decrease in transfection efficiency of CPP polyplexes was observed in the presence of serum (10% FBS) and CPP polyplexes exerted a similar reduction in gene expression as that observed for PEI polyplexes.

In chapter 5, pDNA within TAT complexes was characterized with the objective of contrasting physical properties with PEI polyplexes. Extrinsic fluorescence studies provided evidence of compaction of pDNA, with PEI seemingly inducing tighter complexes. Second derivative UV analysis demonstrated that the pDNA maintained the B form when complexed with various N/P ratios of TAT and PEI. FTIR and CD spectroscopies indicated that the secondary structure of pDNA was stabilized within

complexes. TAT complexes including calcium improved the thermal stability of pDNA compared to PEI.

Finally, in chapter 6, TAT and dTAT complexes including different concentrations of $CaCl_2$ were tested to see if these carriers could perform as safe, effective siRNA vectors. The efficacy of siRNA delivery was studied as a function of CPP/siRNA charge ratio and siRNA concentration. We found that 23.1, 34.6, and 69.2 mM $CaCl_2$ produced small TAT and dTAT complexes via "soft" crosslinks leading to knockdown efficiencies higher than observed for PEI vectors in A549-luc-C8 lung epithelial cells. Only a small siRNA dose of 10 nM was necessary to achieve a luciferase knockdown of 80-87%. The TAT and dTAT peptide also showed negligible cytotoxicity up to 5 mg/mL. In comparison, PEI was very cytotoxic (IC_{50} 22 μg/mL).

1.8. References

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Chapter 2.

Cationic Surface Modification of PLG Nanoparticles Offers Sustained Gene Delivery to Pulmonary Epithelial Cells

2.1. Introduction

Biodegradable nanoparticles have received considerable attention in recent years as a possible means of delivering drugs and genes through multiple routes of administration. Various biodegradable polyesters have been used in drug delivery, such as poly(D,L-lactide-*co*-glycolide) (PLG) and poly(lactic acid) (PLA) [1,2] as they may control drug release and thus increase the therapeutic benefit, while minimizing side-effects [3-6]. Many techniques are available for producing nanoparticles from these or other materials including double-emulsion solvent evaporation, interfacial polymerization, solvent diffusion, nanoprecipitation, and salting-out emulsification methods [7]; however, PLG may be of special interest since it is FDA approved.

Pharmaceutical nanoparticles generally range in size from 10 to 1000 nm and the drug is encapsulated or attached to the nanoparticle matrix [8]. The nanometer size-ranges of such systems offer certain distinct advantages for drug delivery. Due to their size, nanoparticles can penetrate into tissues through fine capillaries, cross the fenestrations present in the endothelial lining (e.g., liver), and are generally taken up efficiently by many types of cells [9]. Also, by modulating polymer chemistry or molecular weight, one can control the release of a therapeutic agent from nanoparticles to achieve a desired drug level in the target tissue for a required duration to optimize therapeutic efficacy. Further, nanoparticles may be delivered to target sites either by localized delivery using a catheter-based approach with a minimally invasive procedure [10] or by conjugating a bio-specific ligand, which could direct them to the target tissue or organ [11].

Indeed, biodegradable PLG nanoparticles are extensively investigated for drug delivery [12-15] and, to a limited extent, gene delivery [16,17]. Robust and scalable methods are

still needed to overcome the difficulties in formulation reproducibility, particle size, and surface chemistry. The size and surface chemistry of nanoparticles have been identified as the most significant factors in determining pharmacokinetics and biodistribution [9,18,19]. The particle size and charge of nanoparticles depend upon some preparative variables such as the type and concentration of stabilizer or coating material, biodegradable polymer used, time and mechanism of shear (e.g., stirring, sonication or homogenization), diffusion rate of organic solvent, ratio between aqueous and organic phases, etc. [7,20]. Creating the proper particle size is a key to effective drug delivery. For example, it has been shown that the efficiency of cellular uptake of 100-200 nm size particles was 15-250 fold greater in various cells than larger sized microparticles [21,22].

Apart from the size of nanoparticles, surface modification of particles is desired since the surface charge and relative hydrophobicity determine the amount of adsorbed components, mainly proteins, which qualifies the *in vivo* fate of nanoparticles [23,24]. This modification has been achieved by two methods; (1) reactions to coat the particle surface or (2) adsorption of surfactants or other coating materials [6]. Some of the widely used surface coating materials possess a hydrophilic segment such as poly(ethylene glycol) (PEG) [25-30]. Block co-polymers such as poloxamine and poloxamer [31-36] have been used as well with fewer examples of coatings such as polyethyleneimine (PEI) [37-40]. Previous reports show that the type of coating material has important effects on drug loading into nanoparticles [41] and on the cellular uptake of nanoparticles [42,43]. Hydrophilic coating materials such as PEG have been effective for increasing the circulation half-life of nanoparticles delivered by IV administration; however, this study is primarily interested in the potential of direct application of nanoparticles to the pulmonary epithelium.

Controlling particle size and surface chemistry dramatically affect endocytic uptake and intracellular distribution of the vehicles, which in turn will affect the delivery of therapeutics such as DNA.

In this study, PLG nanoparticles (~200 nm) encapsulating pDNA encoding for firefly luciferase were prepared via the solvent diffusion method in the presence of selected coating materials. Surface modifications using a variety of cationic materials were compared to more conventional surfactants for the ability to transfect cells. Certain cationic PLG nanoparticles enhanced gene expression for at least 14 days in A549 cells (Type II pneumocytes), which serve as a model of the alveolar epithelium. Perhaps most interestingly, PLG particles did not exhibit detectable gene expression until day 5 whereas gene expression achieved using PEI ended on the same day. Reports in the field of gene typically assay for gene expression at short times (24-48 hours), which has led to the potential of PLG for gene delivery going largely unnoticed. Here, the features of sustained, low-level gene expression and negligible toxicity suggest potential value for localized, PLG-based gene delivery to the pulmonary epithelium.

2.2. Materials and methods

2.2.1. Materials

Poly(D,L-lactide-*co*-glycolide) (PLG) 50:50 with molecular weight of 20 kDa was purchased from LACTEL Absorbable Polymers. 2,2,2-trifluoroethanol and dimethyldioctadecylammonium bromide (DODAB) were obtained from Fisher Scientific (Pittsburgh, PA). 3β-[N-(Dimethylaminoethane)carbamoyl]cholesterol (DC-Chol), hexadecyl trimethyl ammonium bromide (Cetrimide), chitosan, and protamine, were purchased from Sigma-Aldrich (St. Louis, MO). Pluronic F-127 was obtained from

Invitrogen (Carlsbad, CA). Poly(vinyl alcohol) (PVA) and Coumarin 6, laser grade, were obtained from Polysciences, Inc (Warrington, PA). The plasmid DNA encoding firefly luciferase (pGL3, 4.8 kbp) was obtained from Promega (San Luis Obispo, CA) and transformed into *Escherichia coli* DH5α™ (Invitrogen). A single transformed colony picked from an agar plate was cultured in LB Broth Base (Invitrogen) liquid for plasmid DNA preparation. Plasmid DNA was purified with Plasmid Giga Kit 5 (Qiagen, Valencia, CA) following the manufacturer's instructions. All pDNA had purity levels of 1.8 or greater by UV/Vis (A_{260}/A_{280}). A549 cells were obtained from the American Type Culture Collection (ATCC, Manassa, VA). The cell culture medium (Ham's F-12 Nutrient Mixture, Kaighn's modified with L-glutamine) was purchased through Fisher Scientic. Fetal bovine serum (FBS) was purchased from Hyclone (Waltham, MA). Penicillin-streptomycin was purchased from MB Biomedical, LLC (Solon, OH). Trypsin-EDTA was purchased through Gibco® Invitrogen. MTS reagent [tetrazolium compound; 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] was purchased from Promega.

2.2.2. Encapsulation of pDNA in PLG nanoparticles

PLG nanoparticles were prepared using the solvent diffusion method. In brief, aqueous solution (0.2 mL) containing pGL3 (100-200 μg) in Tris-EDTA (TE) buffer (pH 8) was mixed with 1 mL of PLG (50:50 lactide to glycolide ratio, 20 kDa) solution (15 mg/mL) in 2,2,2-trifluoroethanol (TFE)[44]. The resultant mixture was added dropwise through a syringe pump (17.5 mL/h) to an aqueous solution (10 mL) containing various coating materials stirring at 100-250 rpm at room temperature. The formed nanoparticles were dialyzed using a 1,000,000 Da MWCO Dialysis Cassette (Spectrum Laboratories,

Inc., Rancho Oominguez, CA) for 6-16 h against distilled deionized water (DI) to remove excess coating materials and unentrapped pGL3. The mean particle size and zeta potential of the PLG nanoparticles encapsulating pGL3 were measured using a ZetaPALS dynamic light scattering instrument (Brookhaven Instruments Corporation, Holtsville, NY). Zeta potential was determined in 1 mM KCl solution.

2.2.3. Plasmid DNA analysis

The integrity of pGL3 extracted from PLG nanoparticles was studied. Plasmid DNA was extracted from lyophilized particles using a 2,2,2-trifluoroethanol and Tris-EDTA buffer (TFE-TE) extraction method. Briefly, PLG nanoparticles (20 mg) were dissolved in 150 μL TFE into which 850 μL of TE buffer were added and the tube was rotated for 1 h at room temperature. The aqueous layer was removed after centrifugation at 10,000 rpm for 10 minutes. The pGL3 from the extraction was analyzed for its integrity using UV spectrophotometry and agarose gel electrophoresis.

2.2.4. Release and stability studies of PLG nanoparticles

To study the pGL3 release and the change in particle size and zeta potential of incubated nanoparticles over a period of time, some preparations of PLG nanoparticles of different coating materials; PVAm, chitosan, cetrimide, PVA and Pluronic F-127 were used. Each batch was suspended in 7 mL of PBS and the suspension was split into 7 tubes. The tubes were maintained in a shaker incubator (150 rpm) at 37°C. At predetermined time points, the tubes were centrifuged at 12,000 rpm for 8 minutes and the nanoparticles were then washed twice with double-distilled water and resuspended in water. Study of the size stability of PLG nanoparticles in cell culture media in the

absence and presence of 10% FBS was achieved by suspending 200 µL of fresh sample in 800 µL media and the suspension was incubated at 37°C for 5 h and 12 h. The particles size and zeta potential of each sample were measured using the ZetaPALS dynamic light scattering system. On the other hand, for the release study the tubes were centrifuged and then the supernatant was collected into a new tube after a period of time and the nanoparticles were resuspended in fresh PBS and placed back in the incubator. Plasmid DNA concentration was measured in the supernatant using UV spectrophotometry.

2.2.5. Determination of pDNA loading and encapsulation efficiency

An accurately weighed amount of nanoparticles (20 mg), freeze-dried without lyoprotectant, was dissolved in 150 µL TFE into which 850 µL of TE buffer were added and the tube was rotated for 1-2 h at room temperature. The amount of DNA extracted was assayed using UV spectroscopy at 260 nm. The DNA loading and the encapsulation efficiency were calculated in the following manner.

$$\text{pDNA loading} = \frac{\text{amount of pDNA in particles}}{\text{weight of particles}} \times 100$$

$$\text{Encapsulation efficiency} = \frac{\text{pDNA loading}}{\text{theoretical pDNA loading}} \times 100$$

The pDNA loading studies were performed in triplicate.

2.2.6. Cell culture

Culturing of human epithelial lung cell line A549 was performed according to the protocol given by the American Type Culture Collection. A549 cells were grown in F-

12K supplemented with 10% (v/v) FBS and 1% (v/v) Penicillin-streptomycin at 37º C in a humidified air atmosphere containing 5% CO_2.

2.2.7. *In vitro* cell transfection studies

A549 cells were trypsinized, counted and diluted to a concentration of approximately 80,000 cells/ mL. Then 0.1 mL of that dilution was added to each well of a 96-well plate and the cells were incubated in a humid 5% CO_2 incubator at 37°C for 24h. Immediately before transfection, the cells were washed once with PBS and a 100 µL sample (15% of fresh PLG nanoparticles (1.5 mg/mL, 273 ng of DNA) to 85% of serum free cell culture media) was added to each well. Cells were incubated with the PLG nanoparticles for 5 h. The media containing nanoparticles was then removed and 100 µL of fresh serum media was added followed by further incubation. A luciferase expression assay was performed using the Luciferase Assay System from Promega following the manufacturer's recommended protocol. The light units were normalized against protein concentration in the same cells extracts, which were measured using Coomassie Plus™ Protein Assay (Thermo Scientific, Waltham, MA). The transfection results were expressed as Relative Light Units (RLU) per mg of cellular protein.

2.2.8. Assessment of cytotoxicity (MTS Assay)

Cytotoxicity of lyophilized PLG nanoparticles with 5% w/v mannitol was determined by the CellTiter 96® Aqueous Cell Proliferation Assay. A549 cells were grown as described in the transfection experiments. Cells were treated with the samples for 24 h. The media were then removed and replaced with a mixture of 100 µL fresh culture medium and 20 µL MTS reagent solution. The cells were incubated for 3 h at

37°C in a 5% CO_2 incubator. The absorbance of each well was then measured at 490 nm using a microtiter plate reader (SpectraMax, M25, Molecular Devices Corp., CA) to determine cell viability.

2.2.9. Cell uptake studies

A549 cells were seeded and incubated for 1 h with PLG nanoparticles encapsulating coumarin 6 (Polysciences, Inc., Warrington, PA), using a solvent diffusion method**,** in 96-well plates as described above for transfection studies. Cells were then washed thrice with ice-cold PBS and solubilized (80 μL/well) with 0.5% Triton X-100 in 0.2 M NaOH for 30 minutes. Cell-associated nanoparticles were quantified by analyzing the cell lysate using a fluorescence plate reader (SpectraMax, M25, Molecular Devices Corp., CA, λ_{ex} 458 nm, λ_{em} 505 nm). The fluorescent intensity was normalized against protein concentration in the same cells lysate, which were measured using a BCA protein assay kit (Pierce Biotechnology).

2.2.10. Statistical analysis

Statistical evaluation of data was performed using an analysis of variance (one-way ANOVA). Newman–Keuls was used as a post-hoc test to assess the significance of differences. To compare the significance of the difference between the means of two groups, a *t*-test was performed; in all cases, a value of $p < 0.05$ was accepted as significant.

2.3. Results

2.3.1. Preparation and characterization of PLG nanoparticles

Different coating materials were used for the preparation of PLG/DNA nanoparticles by the solvent diffusion method. Transmission electron micrographs of PLG nanoparticles showed that the nanoparticles were spherical and discrete particles without agglomeration and that they were smooth in surface morphology (Figure 1). The average particle size of the PLG nanospheres encapsulating DNA was ~200 nm with relatively narrow polydispersity regardless of coating material. A variety of zeta potentials were observed with the different types of coating materials. The values ranged from 32 to 40 mV for cationic surface modifiers and from -11 to -24 mV for weakly anionic surfactants (Table 1).

Table 1. Characteristics of PLG nanoparticles coated with cationic and anionic materials.

	PVAm	Chitosan	DC-Chol	Cetrimide	Protamine	DODAB	PVA	Pluronic F-127
% (w/v)*	0.03	0.03	0.02	0.1	0.02	0.02	2.0	0.1
Size ± SD (nm)	196±4	201±1	167±1	197±2	204±3	163±4	137±2	169±2
Polydispersity	0.145	0.168	0.068	0.145	0.022	0.074	0.099	0.122
Zeta potential ± SD (mV)	+37.5±0.8	+36.8±0.4	+33.2±0.7	+35.4±1.6	+17.7±1.4	+40.9±0.9	-11.0±2.0	-23.2±1.1
pDNA loading (%)	1.08	1.06	1.10	1.14	1.09	1.12	1.00	1.03
Encapsulation efficiency (%)	83.1±1.2	81.5±2.3	84.6±0.7	87.7±0.5	83.8±1.2	86.2±0.2	77.0±1.1	79.2±0.5

* Weight/ volume percent (the concentration of the coating materials).

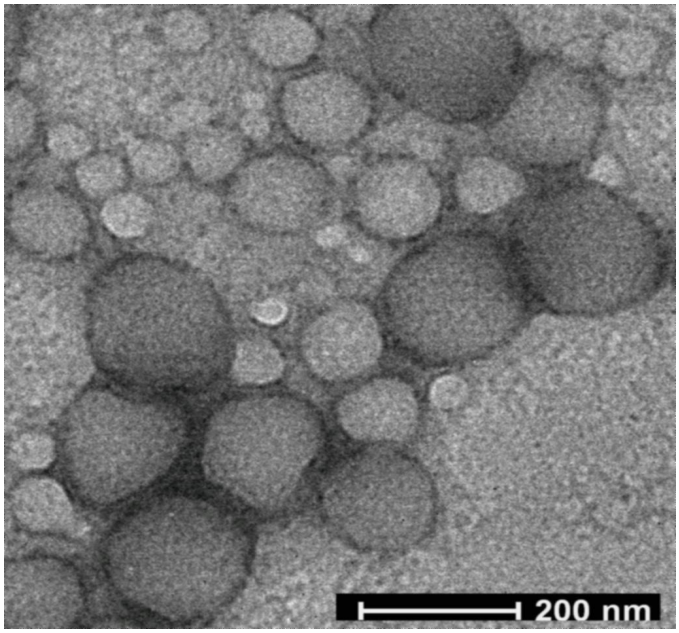

Figure 1. Transmission electron micrograph (TEM) of PLG nanoparticles.

2.3.2. Stability and encapsulation efficiency of DNA

Maximum extraction of pGL3 from the PLG/TFE phase into the aqueous phase was achieved within 60 min. The plasmid DNA structure was determined by agarose gel electrophoresis. The distinct bands present on the gel showed that the encapsulated DNA retained its structural integrity regardless of coating material when compared with the original DNA (Figure 2A). Aqueous extracts from various nanoparticles were analyzed by UV spectrophotometry to determine DNA loading. The efficiency of pGL3 encapsulation in the PLG nanoparticles did not significantly depend on the type of cationic coating materials. Various cationic coating materials exhibited encapsulation efficiencies as high as ~88% (Table 1). Nanoparticles made using weakly anionic coating materials showed lower efficiencies for the encapsulation of DNA, reaching only ~79 %. These results suggested that the presence of cationic coating materials could improve the entrapment of the anionic DNA molecule within the PLG nanoparticles.

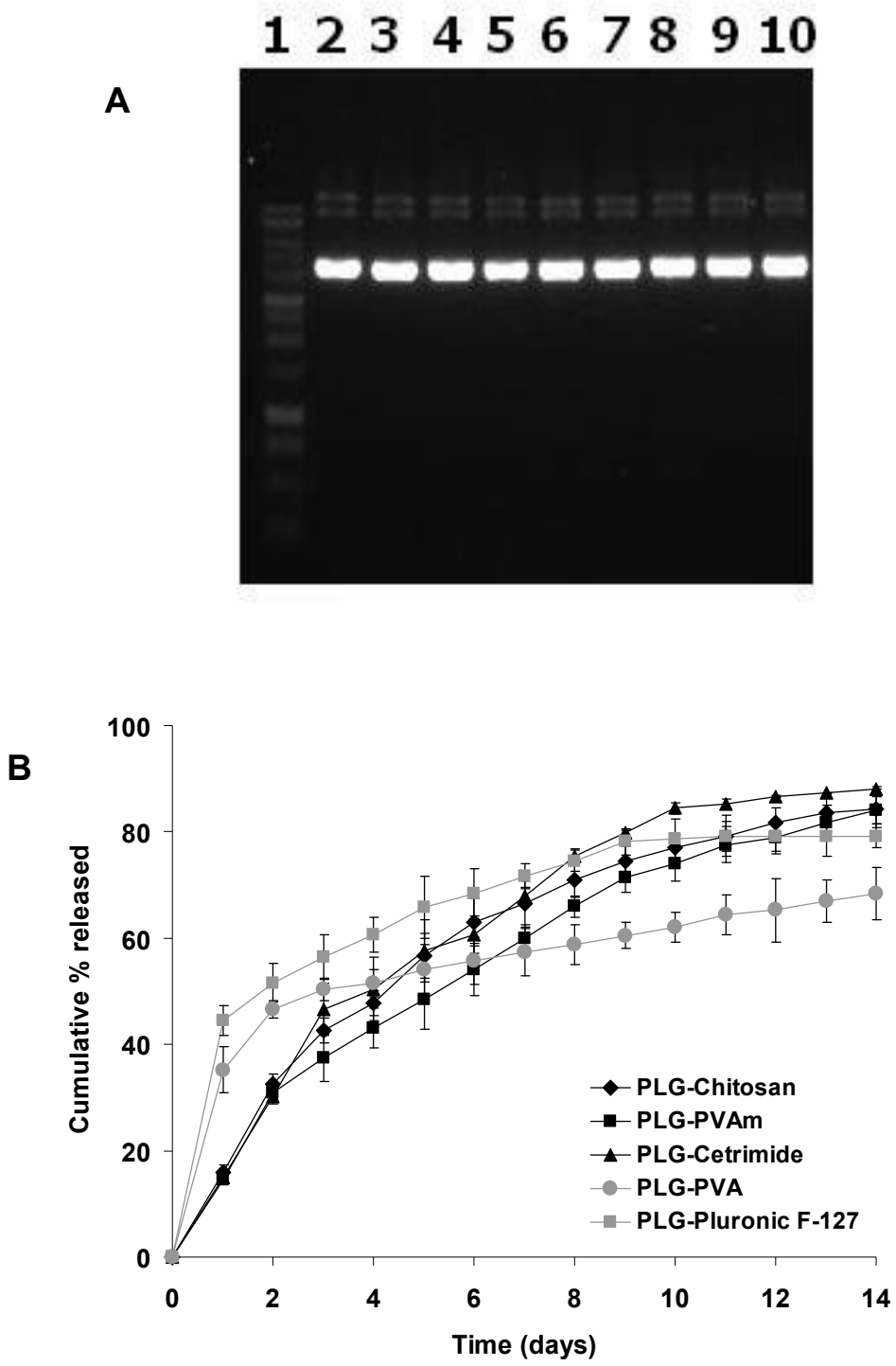

Figure 2. (A) Gel electrophoresis of plasmid DNA extracted from PLG nanoparticles, (1) marker, (2) naked pDNA, (3) PLG-PVA, (4) PLG-PluronicF-127, (5) PLG-PVAm, (6) PLG-Chitosan, (7) PLG-DC-Chol, (8) PLG-Cetrimide, (9) PLG-Protamine, and (10) PLG-DODAB nanoparticles. (B) Release profile of pDNA from PLG nanoparticles in PBS at 37°C.

2.3.3. *In vitro* release and stability studies of PLG nanoparticles

In vitro profiles of plasmid DNA release were compared for five different PLG coating materials, PVAm, chitosan**,** cetrimide, PVA and Pluronic F-127 in PBS at 37°C. Irrespective of the type of coating material, the DNA was released in a sustained manner for 14 days (Figure 2B). Using cationic coating materials significantly decreased the initial burst release of the DNA from the nanoparticles. At the end of the experiment, more than 84% of the total DNA encapsulated in the cationic PLG nanoparticles was released.

Apart from the release study, the stability of the size and the zeta potential of PVAm**,** DC-Chol, cetrimide, DODAB, PVA and Pluronic F-127 coated PLG nanoparticles was investigated as a function of time. Zeta potential and size of gene delivery vehicles have been reported to be important factors in facilitating gene transfer in cells [46,47]. The endocytotic uptake of particles is clearly affected by their size and tends to increase with decreasing diameters and increasing zeta potential [48,49]. The PLG nanoparticles remained stable in PBS over a period of 3 days and retained their size and surface charge. Exemplary data are shown (Figures 3A and B). The initial change in the particle size and the zeta potential over the first five hours of this study may be attributable to a reduction in agglomeration as the resuspended nanoparticle/mannitol powders slowly hydrated and dispersed.

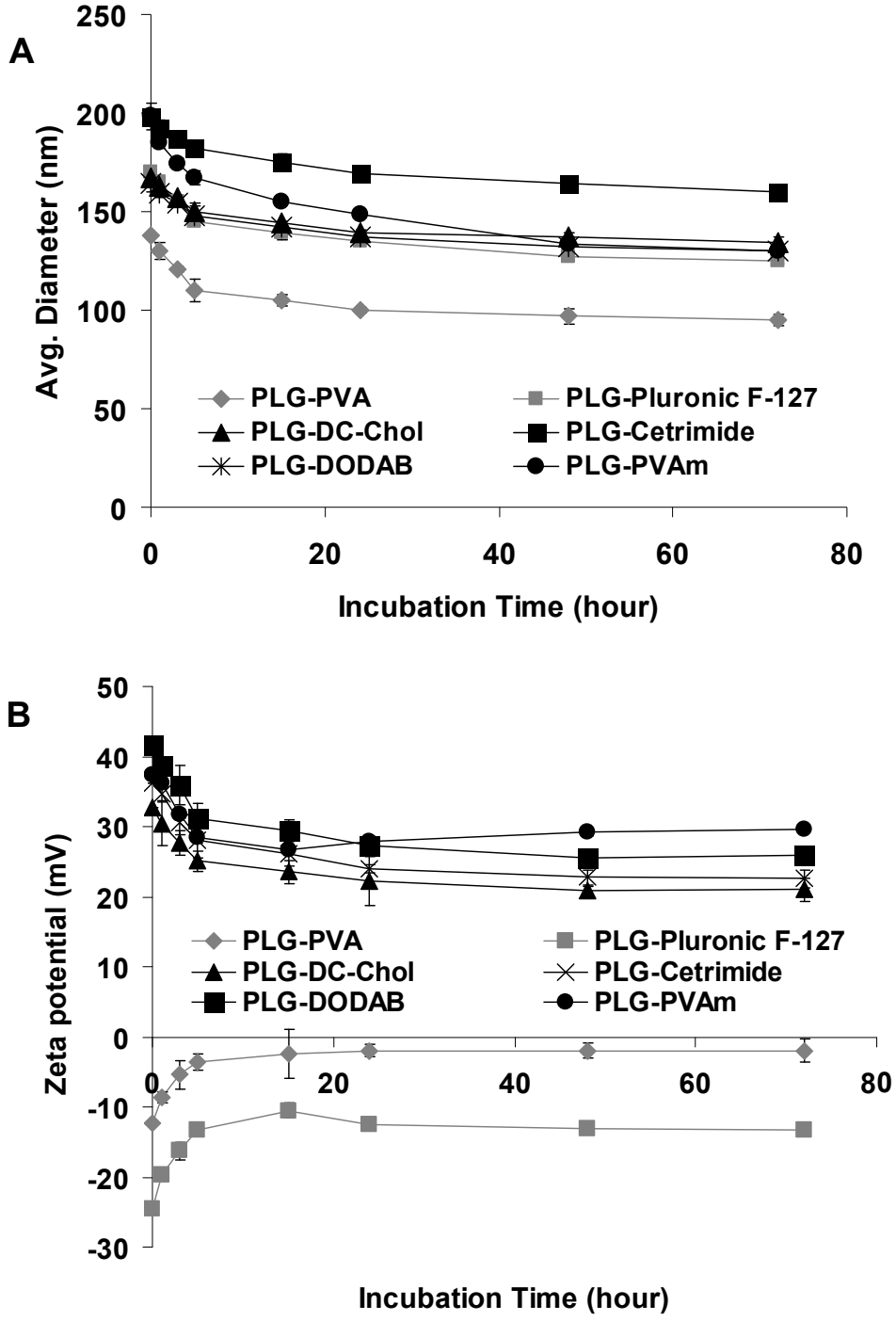

Figure 3. (A) Size of PLG nanoparticles coated with various materials as a function of time. (B) Zeta potential of PLG nanoparticles coated with various materials as a function of time. Data are presented as mean ± SD (n = 3).

On the other hand, PLG nanoparticles coated with PVA and Pluronic F-127 showed good stability in serum-free culture media over a period of 5 and 12 h. PLG nanoparticles with cationic coating materials showed some agglomeration. In the presence of 10% FBS, only PLG-PVA nanoparticles retained their size (Figures 4A and B). Precipitation was not noted in any of the samples over the course of 5 h, which was an analogous time to the transfection experiments. The colloidal stability of the PLG particles over this time suggested that any differences in gene transfection were not attributable to particles precipitating onto the cell monolayers.

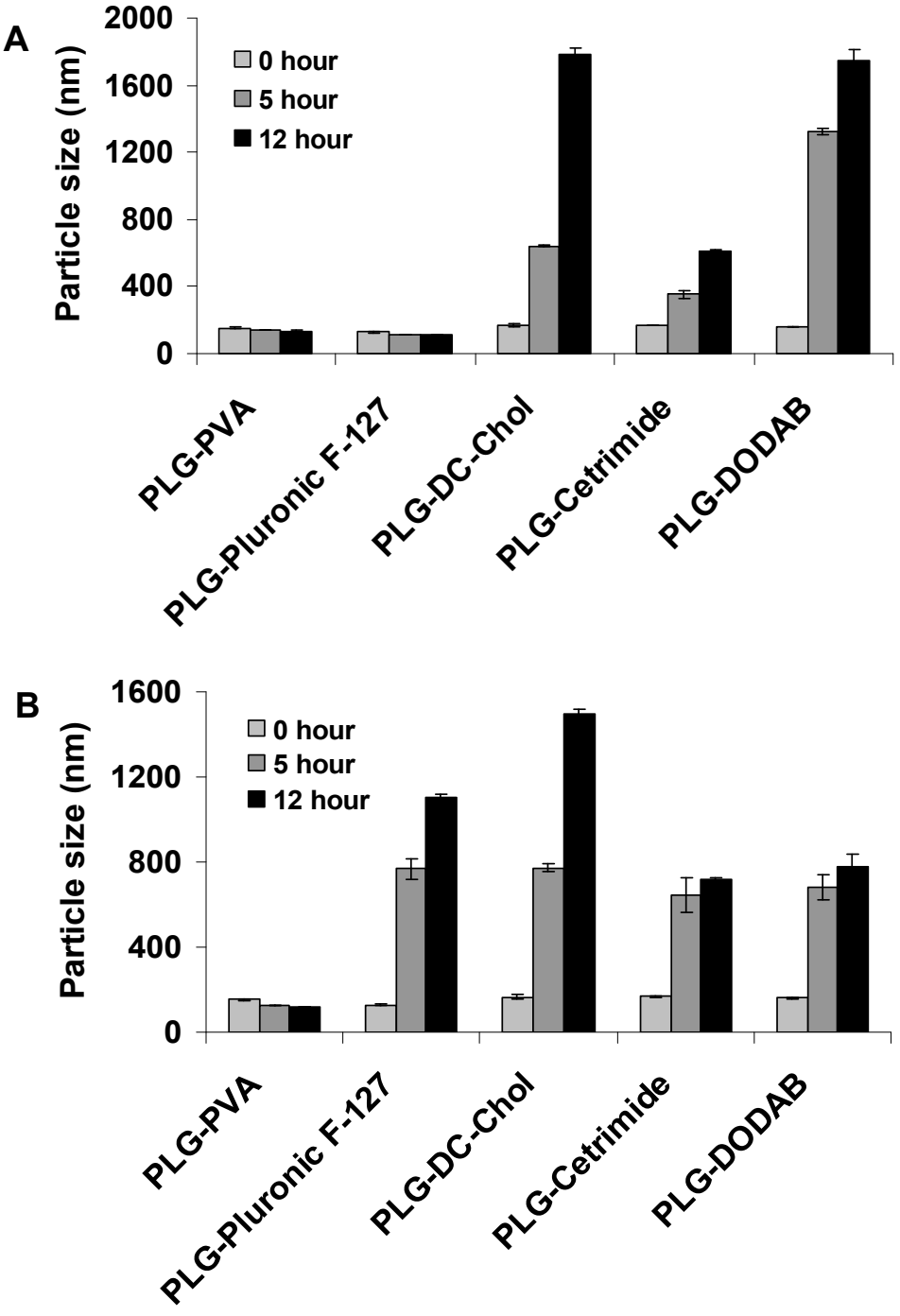

Figure 4. Stability of PLG nanoparticles coated with various materials over time in (A) the absence and (B) presence of 10%FBS. Data are presented as mean ± SD (n = 3).

2.3.4. Effect of PLG nanoparticles on cell viability

The effect of the PLG nanoparticles on the viability of A549 cells was studied by incubating the cells up to 5 mg/mL of lyophilized PLG/DNA nanoparticles with 5% w/v mannitol. Minimal cytotoxic effects were evident and cells maintained a high viability (Figure 5) even for cationic coating materials (IC_{50} ~1190-2450 µg/mL). In contrast, branched PEI complexes showed significant cytotoxicity at low concentration (IC_{50} ~35 µg/mL).

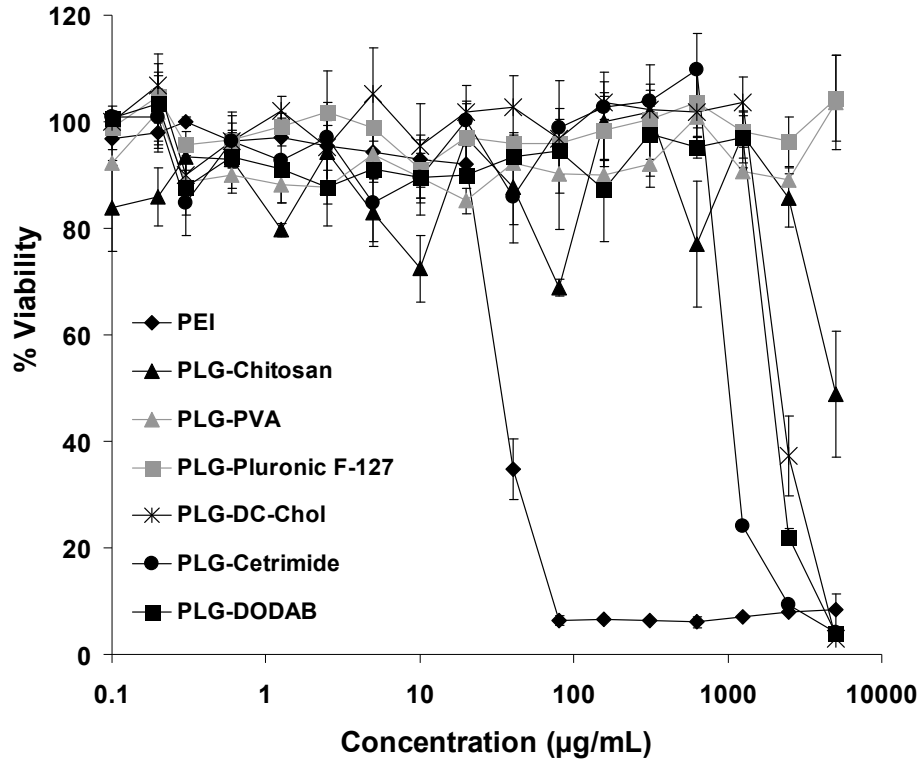

Figure 5. Cell viability of PLG nanoparticles in A549 cells in comparison with branched PEI (25k). Data are presented as mean ± SD (n = 3).

2.3.5. Nanoparticle uptake by A549 cells

PLG nanoparticles coated with different materials were used to study cellular uptake by A549 lung epithelial cells. In these studies, PLG nanoparticles were stained with the fluorescent dye, coumarin 6. All coating materials showed uptake of nanoparticles within 1 h of incubation but with different magnitudes (Figure 6). The nanoparticles formulated from PLG with cationic coatings, especially chitosan, showed high levels of nanoparticle uptake compared to weakly anionic coatings as expected. No correlation of the magnitude of zeta potential and nanoparticle uptake was observed. These results support previous research indicating that the nanoparticle surface can be modified to provide enhanced uptake by cells [25]. To exclude the possibility of non-specific staining of cells by coumarin 6, control experiments were performed. PLG-coumarin 6 nanoparticles were shaken in serum free cell culture media for 3 h and centrifuged. The supernatant was incubated with A549 cells to check for any leakage of dye from nanoparticles, which might stain cells. No fluorescence was detected in the cells.

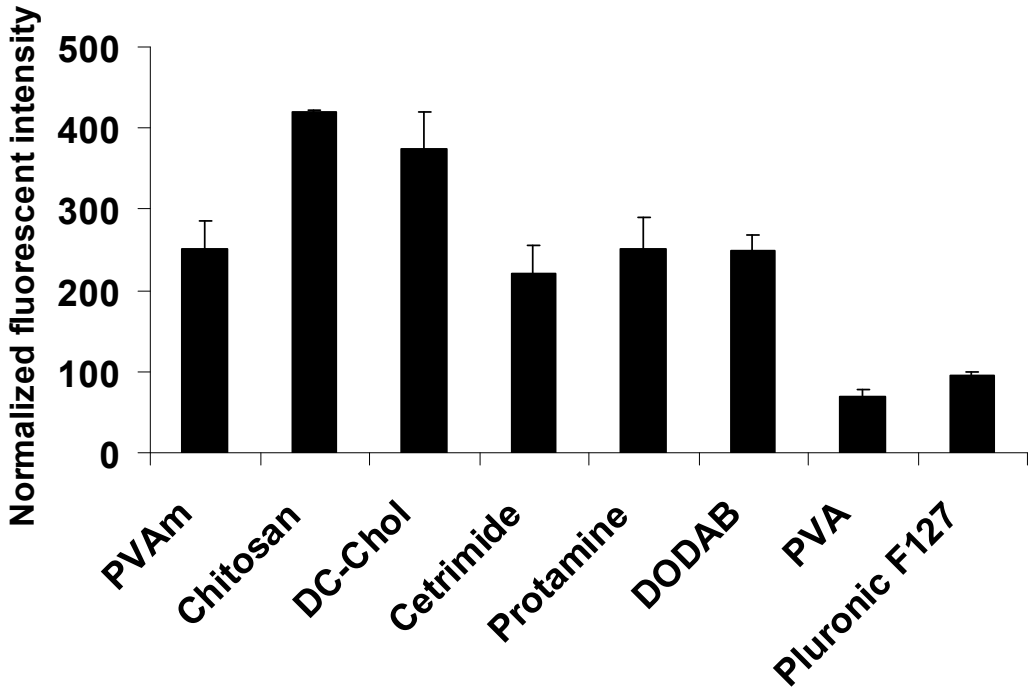

Figure 6. Cellular uptake of PLG nanoparticles coated with various materials. Cationic coating materials enhanced the level of nanoparticle uptake compared to anionic coatings. Data are presented as mean ± SD (n = 3).

2.3.6. *In vitro* transfection efficiency of PLG nanoparticles

To assess the feasibility of PLG nanoparticles for gene delivery, *in vitro* transfection was performed using the human lung carcinoma cell line A549. The luciferase-encoding plasmid pGL3 was used as a reporter. On day 2 after transfection, PLG nanoparticles did not show gene expression. In comparison, branched PEI showed excellent transfection efficiency on day 2 (Figure 7). Gene expression mediated by certain cationic PLG nanoparticles suddenly appeared on day 5, whereas the gene expression of branched PEI showed a marked decrease during the same time frame. The enhanced gene expression from certain cationic PLG nanoparticles was sustained for at least 14 days. The transfection efficiency of PLG with PVAm, DC-Chol, cetrimide and DODAB coatings was almost similar to the transfection efficiency of branched PEI at day 5, but tended to increase at day 7 and 14. On the other hand, no expression was observed for PLG nanoparticles coated with chitosan, protamine and Pluronic F-127 despite the fact that these coating materials showed relatively efficient cellular uptake. These results support the findings reported in the literature that nanoparticle uptake is not indicative of gene expression [50]. Importantly, however, transfection performance may be the result of the fact that the type of coating materials can ultimately affect uptake pathways and nanoparticle fate within the cell, which remains to be investigated.

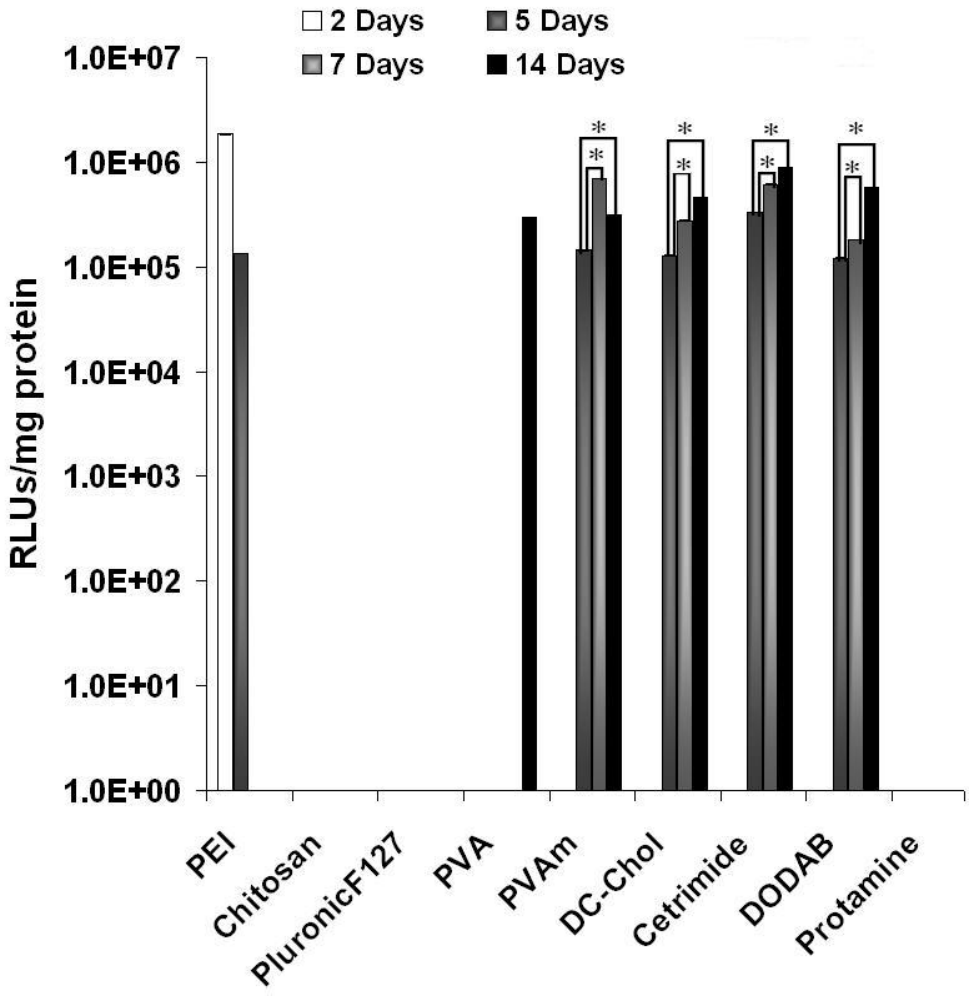

Figure 7. Transfection efficiency of PLG nanoparticles in A549 cells varied with different coating materials. Most PLG nanoparticles exhibited long-term expression that tended to increase over time. PEI complexes N/P 10 showed higher gene expression that dropped to negligible levels by day 5. Expression of day 14 was significantly higher than day 7 in A549 cells. Data are presented as mean ± SD (n = 3), * $p < 0.001$ when compared to day 5 for the same sample.

2.4. Discussion

Nanoparticles of PLG have been widely studied for their use in drug delivery [51]. Among non-viral vehicles, these particulates may offer a non-toxic and efficient carrier for sustained intracellular delivery of genes. However, PLG nanoparticles encapsulating DNA need to be carefully designed to achieve efficient gene delivery. In the present study, PLG/DNA nanoparticles were fabricated using a solvent diffusion method. The protocol was developed to avoid shear and centrifugation. These processes may not only affect the integrity of DNA encapsulated in nanoparticles but may also diminish DNA encapsulation efficiency. In addition, the nanoparticle stability may be compromised by centrifugation procedures, which can lead to irreversible agglomeration. The methods reported here resulted in high encapsulation efficiencies (~88%) and retention of DNA structure as indicated by gel electrophoresis of the pDNA extracted from various PLG nanoparticles.

Positively charged nanoparticles have been shown to bind to the negatively charged cell surface, and thereby facilitate their uptake [52]. Higher levels of cellular uptake of PLG nanoparticles were achieved using cationic coating materials compared to the weakly anionic PVA and Pluronic F-127 coatings. Unlike PEI, PLG nanoparticles degrade by hydrolysis, and were expected to sustain delivery of DNA; however, a cationic coating material was desired to improve cellular uptake of the particles. In the present study, the release of DNA from PLG nanoparticles was sustained for approximately 14 days and release was found to be more constant for nanoparticles with cationic coatings. After observing the slow release of DNA, gene transfection levels were studied for 2 weeks in A549 cells.

The level of gene expression in the A549 lung epithelial cells was higher in the branched PEI/DNA complexes than in the PLG nanoparticles for the initial 4 days, but the trend was reversed after day five. This was perhaps a result of the sustained DNA release from the PLG nanoparticles. Thus, modified PLG/DNA nanoparticles appeared to be superior to PEI/DNA complexes for the long-term expression of DNA. Typical gene transfection studies reported in the literature assess gene expression at 24 or 48 h, which may explain the low level of previous reports on PLG nanoparticles for gene delivery. At this point, it is difficult to speculate if the differences in gene expression observed for different cationic PLG nanoparticles were caused by endosomal escape, differences in the release of DNA within cells, or other factors. Perhaps most importantly, modified PLG nanoparticles showed little to no cytotoxicity after 24 h incubation with A549 cells compared to branched PEI complexes.

2.5. Conclusion

In this study, PLG nanoparticles coated with different surface modifiers were examined for the ability to transfect A549 lung epithelial cells. A series of physicochemical studies demonstrated that the PLG nanoparticles coated with cationic materials increased DNA encapsulation efficiency and enhanced uptake by A549 lung epithelial cells. PLG nanoparticles exhibited excellent particle size and surface charge stability during three days incubation in PBS at 37°C, however, some increase in particle size was noted in media with serum at longer incubation times (>5 h). Gene expression was sustained for at least 14 days for several PLG nanoparticle formulations and gene expression levels tended to increase over the duration of the study. Conversely, gene expression levels for branched PEI/DNA complexes showed high initial gene expression

that dropped to negligible levels by day 5. *In vitro* cytotoxicity studies showed that cationic PLG nanoparticles exhibited much lower cytotoxicity than branched PEI (25 kDa). Taken cumulatively, these data suggest that cationic PLG nanoparticles may be an effective vehicle for delivering DNA to the pulmonary epithelium.

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Chapter 3.

"Soft" Calcium Crosslinks Enable Highly Efficient Gene Transfection Using TAT Peptide

3.1. Introduction

Intensive effort has been devoted to develop gene therapy systems capable of overcoming a variety of limitations including low gene transfection efficiency, toxicity, and in vivo instability [1-7]. Nonviral vectors have been given considerable attention as gene delivery vehicles because of their presumed safety, ease of synthesis, relatively unrestricted vector size, low cost, and low degree of immunogenicity in comparison to viral vectors [8]. Plasmid DNA complexed with cationic lipids (lipoplexes) and polymers (polyplexes) are the most commonly employed nonviral gene delivery vehicles [9-17]. Because naked plasmid DNA does not easily penetrate cellular membranes [18], nonviral gene delivery systems may include agents to improve intracellular delivery in an effort to promote transfection. Finally, gene delivery vehicles are subject to dilution, degradation and elimination in vivo, which amplify the need for safe and efficient gene delivery at high doses of DNA.

New strategies have been put forward to enhance cellular uptake of gene delivery vehicles, among which peptides enhancing cell adhesion and internalization have reached a prominent position [19]. Peptide sequences, also designated as protein transduction domains (PTD) or membrane translocalization signals (MTS), were identified as potentially useful labels for intracellular delivery of peptides, proteins, oligonucleotides, and plasmid DNA [20-24]. By modifying the surface of gene delivery vectors with cell penetrating peptides (CPPs), vectors have been shown to traverse the membranes of biological cells within seconds to minutes [25]. Polycationic CPPs have even been reported to enhance cell permeability and facilitate the intracellular delivery of nanoparticles [26].

The mechanism of cell entry of CPPs alone or with their cargoes still remains somewhat of an enigma. Some reports indicating that cellular translocation of CPPs is energy as well as endocytosis independent and that there is direct transfer of the peptides through the lipid bilayer by inverted micelle formation [27-32]. Another report, however, proposed an energy dependent mechanism of cell entry of CPPs [33], which may also involve extracellular heparan sulfate and various endocytosis and macropinocytosis pathways [34-41]. It was also suggested that classical and nonclassical endocytosis pathways may be associated simultaneously with CPP translocation depending upon the biophysical properties of CPPs and their cargo [32,42,43].

One particular CPP of interest is the HIV-1 TAT peptide. This peptide, which represents a protein transduction domain [44,45] and a nuclear localization sequence (NLS) [46], has been reported to show unusual translocation abilities by directly crossing biological membranes independent of receptors and temperature [47]. In addition, the NLS function of TAT peptide could facilitate nuclear localization of a therapeutic agent due to interaction with the endogenous cytoplasmic nuclear transport machinery. The cationic nature of the TAT sequence arising from several arginine residues has already been utilized in gene delivery either by covalent coupling of this CPP to the gene delivery vehicle [48-50] or by simple mixing of plasmid DNA with TAT to form TAT/pDNA complexes via noncovalent electrostatic interactions [51-54]. However, the transfection efficiency of such complexes remains quite low and requires improvement.

Obviously, a strong affinity between TAT peptide and plasmid DNA is required to stabilize the resulting polyplex and to achieve an optimized transfection yield. On the other hand, a sufficiently low affinity between TAT peptide and plasmid DNA is desired

to facilitate the release of the cargo after cellular uptake. Thus, the critical balance between the TAT/pDNA binding affinities has important consequences for enhancing the transfection efficiency. Our objective was to design a more efficient and less toxic means of gene delivery using TAT/pDNA complexes containing "soft" crosslinks. Calcium was found to control the delicate balance between binding affinities within TAT/pDNA polyplexes. The addition of $CaCl_2$ to TAT/pDNA complexes directly affected particle size and transfection efficiency in a concentration-dependent manner. The optimum calcium concentration (113 mM final $CaCl_2$ concentration) resulted in a 1000-fold enhancement in TAT/pDNA polyplex transfection efficiency and showed no detectable cytotoxicity. Gene transfection levels were as high as that observed for PEI polyplexes suggesting the possible translation of the TAT-Ca/pDNA complexes.

3.2. Materials and methods

3.2.1. Materials

Plasmid DNA encoding firefly luciferase (pGL3, 4.8 kbp) was obtained from Promega (Madison, WI, USA) and transformed into *Escherichia coli* DH5ά (Invitrogen, Carlsbad, CA). A single transformed colony picked from an agar plate was cultured in LB Broth Base (Invitrogen) liquid for plasmid DNA preparation. Plasmid DNA was purified with Plasmid Giga Kit 5 (Qiagen, Germantown, MD) following the manufacturer's instructions. All pDNA had purity levels of 1.8 or greater as determined by UV/Vis inspection (A_{260}/A_{280}). TAT peptide (RKKRRQRRR; Mw = 1338.85 Da, TFA salt = 2632.9 Da) was synthesized in house. Branched polyethyleneimine (PEI, 25 kDa) was obtained from Aldrich (Milwaukee, WI). Calcium chloride ($CaCl_2$. $2H_2O$) and agarose medium were purchased from Fisher Scientific (Pittsburgh, PA). A549 cells were

obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cell culture medium (Ham's F-12 Nutrient Mixture, Kaighn's modified with L-glutamine) was purchased through Fisher Scientic. Fetal bovine serum (FBS) was purchased from Hyclone. Penicillin-streptomycin was purchased from MB Biomedical, LLC. Trypsin-EDTA was purchased through Gibco. MTS reagent [tetrazolium compound; 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] was purchased from Promega. Heparin sodium was obtained from Spectrum (Gardena, CA).

3.2.2. Preparation of TAT-Ca/pDNA complexes

TAT/pDNA complexes were synthesized by rapidly adding 10 µL (0.1 µg/µL) of pDNA to 15 µL (N/P ratio of 25) TAT solution while pipetting. To this solution, 15 µL of known molarity (e.g. 113 mM final $CaCl_2$ concentration) $CaCl_2$ was added and mixed by vigorous pipetting followed by 20 min incubation at 4º C prior to use.

3.2.3. Formation of PEI/pDNA complexes

PEI-DNA complexes were prepared by adding 10 µl (0.1 µg/µL) of pDNA solution to 15 µL (N/P ratio of 5 or 10) PEI solution dropwise while stirring. Complexes were incubated at 4º C for 20 min before dilution 1.7 times (15 µL) with the appropriate buffer (e.g. nuclease-free water or $CaCl_2$ solution). Complexes were freshly prepared before each individual experiment.

3.2.4. Size and Zeta potential measurement

Suspensions containing complexes with TAT or PEI were prepared as described earlier using a DNA concentration of 0.1 µg/µL. All samples intended for light scattering

analyses were prepared using 10 mM Tris buffer, pH 7.4, which was prefiltered with a 0.22 μm filter to remove any trace particulates. Particle sizes were measured by dynamic light scattering (DLS) using a Brookhaven (Holtsville, NY) instrument equipped with a 9000AT autocorrelator, a 50 mW HeNe laser operating at 532 nm (JDS Uniphase), an EMI 9863 photomultiplier tube, and a BI 200M goniometer. The light scattered at 90º from the incident light was fit to an autocorrelation function using the method of cumulants. Zeta potential measurements were obtained by phase analysis light scattering using a Brookhaven Zeta PALS instrument. The electrophoretic mobility of the samples was determined from the average of 10 cycles of an applied electric field. The zeta potential was determined from the electrophoretic mobility from the Smoluchowski approximation. Zeta potential was determined in 1 mM KCl solution.

3.2.5. Agarose gel electrophoresis assays

The pDNA binding ability of the TAT, TAT-Ca, $CaCl_2$ and PEI complexes was analyzed by agarose gel electrophoresis. The TAT-Ca/pDNA and PEI/DNA complexes containing 1 μg luciferase reporter gene were prepared as described at various N/P ratios. The N/P ratio refers to the molar ratio of amine groups in the cationic polymer, which represent the positive charges, to phosphate groups in the plasmid DNA, which represent the negative charges. The DNA complex suspensions (i.e. 25 μL) at various N/P ratios were diluted by adding 4 μL of 10X Tris-acetate-EDTA (TAE) gel running buffer (Promega) and 4 μL of 100X SYBR Green (Invitrogen) solutions. The DNA loading buffer (7 μL of 6X) was added to the complex suspensions. The mixtures were allowed to incubate at room temperature for 40 min to ensure labeling of the DNA with the SYBR Green dye. Thereafter, the complexes were loaded into individual wells of 1% agarose/

1X TAE gel buffer, and subjected to electrophoresis at 110 V for 30 min. Uncomplexed DNA diluted with an identical volume of solution was used as a control. The resulting DNA migration patterns were revealed using an AlphaImager® Imaging System (Alpha Innotech, San Leandro, CA).

3.2.6. Cell culture

Culturing of human epithelial lung cell line A549 was performed according to the protocol provided by the American Type Culture Collection. A549 cells were grown in F-12K supplemented with 10% (v/v) FBS and 1% (v/v) Penicillin/streptomycin at 37º C in a humidified air atmosphere containing 5% CO_2.

3.2.7. *In vitro* cell transfection studies

A549 cells were trypsinized, counted and diluted to a concentration of approximately 80,000 cells/ mL. Then 0.1 mL of that dilution was added to each well of a 96-well plate and the cells were incubated in a humidified atmosphere at 5% CO_2 and 37°C for 24 h. Immediately before transfection, the cells were washed once with PBS and 100 µl sample (20% of complex to 80% of serum free cell culture medium) was added to each well. Cells were incubated with the complexes for 5 h. The transfection agent was then removed by aspiration and 100 µL of fresh serum medium was added followed by further incubation. The Luciferase Assay System from Promega was used to determine gene expression following the manufacturer's recommended protocol. The light units were normalized against protein concentration in the cells extracts, which were measured using the Coomassie Plus™ Protein Assay (Thermo Scientific). The transfection results were expressed as Relative Light Units (RLU) per mg of cellular protein.

3.2.8. Assessment of cytotoxicity (MTS Assay)

Cytotoxicity of polymers was determined by the CellTiter 96® Aqueous Cell Proliferation Assay. A549 cells were grown as described in the transfection experiments. Cells were treated with the samples for ~24 h. The media were then removed and replaced with a mixture of 100 μL fresh culture media and 20 μL MTS reagent solution. The cells were incubated for 3 h at 37°C in the 5% CO_2 incubator. The absorbance of each well was then measured at 490 nm using a microtiter plate reader (SpectraMax, M25, Molecular Devices Corp., CA) to determine cell viability.

3.2.9. SYBR Green assay of TAT and PEI complexes

The degree of pDNA accessibility following complexation with TAT or PEI was assessed by the double-stranded-DNA-binding reagent SYBR Green (Invitrogen). Briefly, 10 μL (0.1 mg/mL) of pDNA was mixed with 15 μL of TAT or PEI solution, then 15 μL deionized water or metal solution was added. Complexes were allowed to form for 30 min at room temperature prior to use. After incubation, 120 μL deionized water and 160 μL 10X SYBR Green solutions were added. And then 80 μL of sample was added to three wells of a 96-well cell culture plate. The fluorescence was measured using a fluorescence plate reader (SpectraMax M5; Ex., 497 nm; Em, 520 nm).

3.2.10. TNBS assay of TAT and PEI complexes

The accessibility of free amine groups of TAT or PEI following complexation with pDNA was measured using a colorimetric assay with 2,4,6-trinitro-benzenesulphonic acid (TNBS) as an assay reagent (Pierce). Briefly, 10 μL of complex solution was added to 190 μL deionized water and then 200 μL of 0.02 % TNBS solution in 0.1 M sodium

bicarbonate buffer (pH 8.5) was added. The solution was rapidly mixed. After incubation at 37 °C for 2 hours, 80 μL of sample was added to three wells of a 96-well cell culture plate. The absorption at 335 nm was determined using a plate reader.

3.2.11. The effect of heparin on the stability of TAT and PEI complexes

The effect of heparin on the stability of complexes was evaluated by the means of the change in fluorescence intensity obtained with the fluorescent probe SYBR Green. TAT and PEI complexes were freshly prepared in absence and the presence of $CaCl_2$ (113 mM) as above described. 120 μL of heparin solution was added to these various complexes to yield final diverse heparin concentrations up to 50 μg/μL, incubated for 30 min at room temperature and then 160 μL 10X SYBR Green was added. In triplicate, 80 μL of each sample was added to the well of 96-well plate and the fluorescence was measured as indicated in SYBR Green assay.

3.2.12. Statistical analysis

Statistical evaluation of data was performed using an analysis of variance (one-way ANOVA). Newman–Keuls was used as a post-hoc test to assess the significance of differences. To compare the significance of the difference between the means of two groups, a *t*-test was performed; in all cases, a value of $p < 0.05$ was accepted as significant.

3.3. Results

3.3.1. Formation of TAT-Ca/pDNA and PEI/pDNA complexes

TAT and PEI complexes were prepared by mixing pDNA with each polycation at various N/P ratios as described. In order to demonstrate complex formation, a gel

electrophoresis assay was performed using 1% agarose gel. Uncomplexed pDNA was used as a control. Gel electrophoresis indicated that pDNA complexed with polycations could be retained in the loading wells. Above a certain N/P ratio no bands were observed during electrophoresis indicating that TAT and PEI completely complexed the pDNA. Under these conditions, $CaCl_2$ showed negligible ability to complex the plasmid DNA even at high concentration (375 mM) (Figure 1).

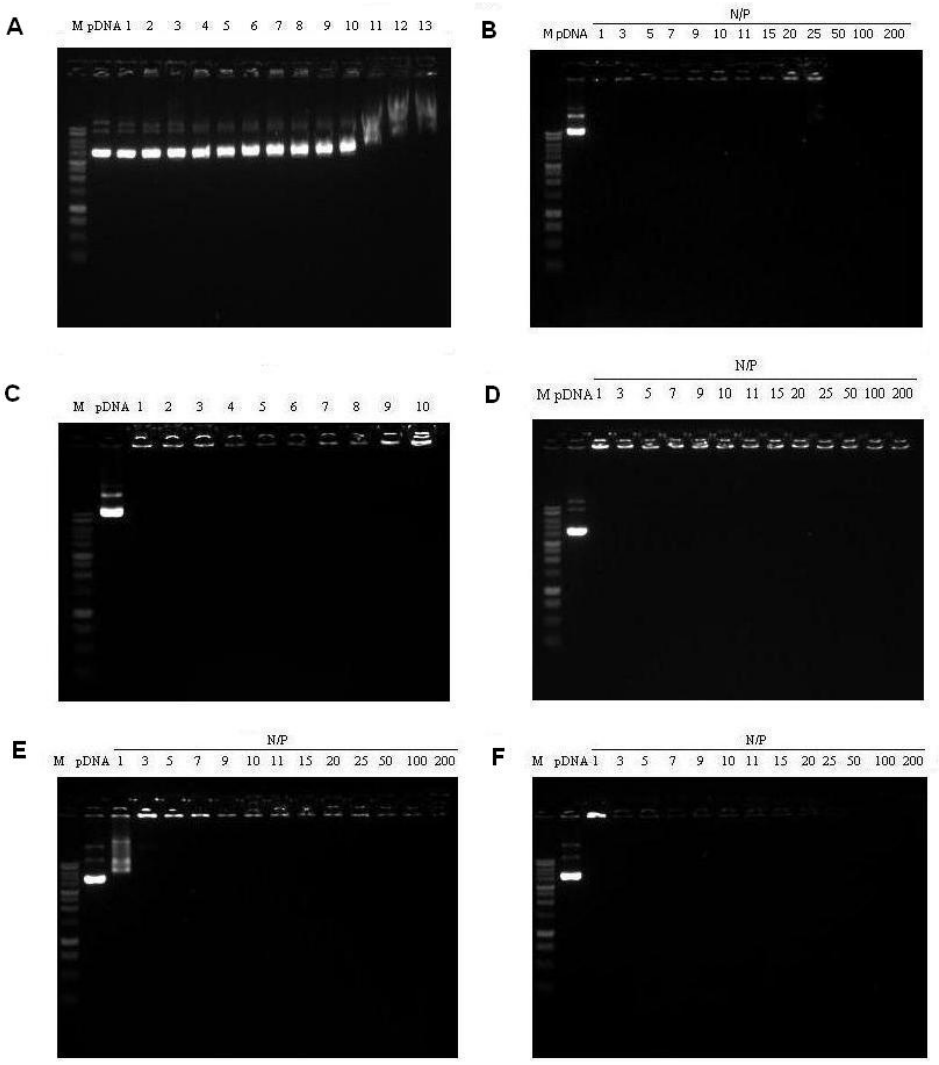

Figure 1. Gel electrophoresis study of (A) $CaCl_2$/pDNA complexes; 1 = 38mM, 2 = 75mM, 3 = 113mM, 4 = 150mM, 5 = 188mM, 6 = 225mM, 7 = 263mM, 8 = 300mM, 9 = 338mM, 10 = 375mM, 11 = 1.13M, 12 = 1.9M and 13 = 2.63M of $CaCl_2$. (B) TAT/pDNA complexes. (C) TAT-Ca/pDNA (N/P 25) complexes; 1 = 0mM, 2 = 23mM, 3 = 47mM, 4 = 94mM, 5 = 113mM, 6 = 131mM, 7 = 150mM, 8 = 188mM, 9 = 375mM and 10 = 750mM of $CaCl_2$. (D) TAT/pDNA complexes with 113mM $CaCl_2$. (E) Branched PEI/pDNA complexes. (F) Linear PEI/pDNA complexes.

3.3.2. Morphological and physical characterization of TAT-Ca/pDNA and PEI/pDNA complexes

The effect of calcium chloride concentration on the particle size and surface charge of TAT/pDNA and PEI/pDNA complexes was investigated. Over a certain concentration range, calcium addition to TAT/pDNA complexes induced a substantial decrease in the particle size. In comparison, PEI/pDNA complexes showed an increase in particle size (Figure 2A). The added $CaCl_2$ concentration range of 113-188 mM (final $CaCl_2$ concentration) produced small (50-100 nm) and stable TAT/pDNA complexes with relatively narrow polydispersity (< 0.15). In general, the zeta potential of TAT and PEI complexes increased significantly from 11 to 27 mV with increasing concentration of $CaCl_2$ (Figure 2B).

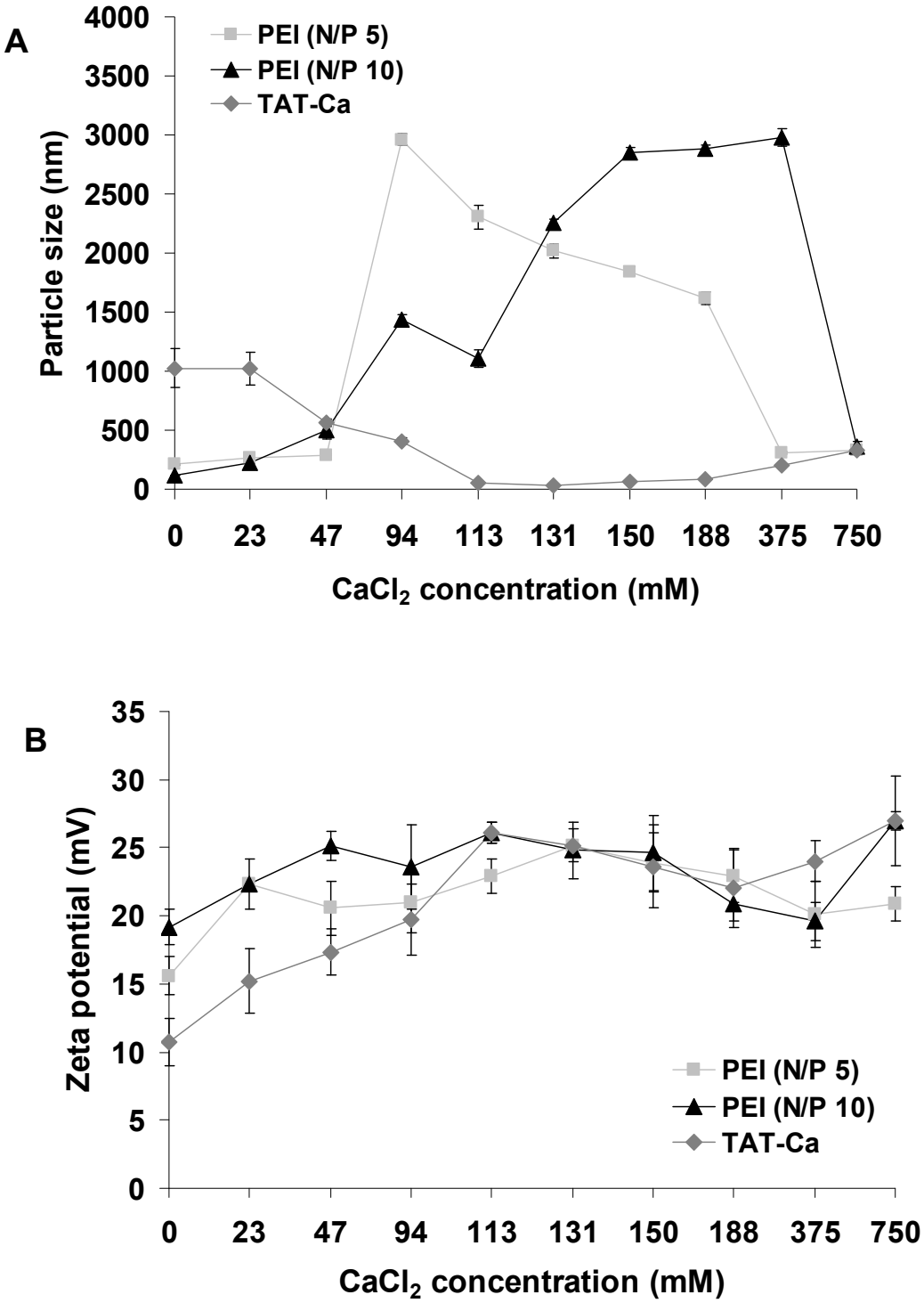

Figure 2. The effect of $CaCl_2$ concentration on (A) particle size and (B) charge of TAT-Ca/pDNA (N/P 25) complexes and PEI/pDNA complexes (N/P 5 and 10). Data are presented as mean ± SD (n = 3).

A transmission electron microscope (FEI field emission transmission electron microscope, Tecnai G^2 at 200 kV) equipped with an energy-dispersive analytical X-ray (EDAX) was used to image the morphology of the TAT/pDNA and TAT-Ca/pDNA complexes and to characterize elements in these complexes, respectively. TEM samples were prepared by depositing a drop of the complex suspension on a copper carbon grid and allowing it to dry in a dessicater overnight. Transmission electron micrographs of TAT/pDNA complexes revealed an asymmetric morphology. The darker areas in TAT-Ca/pDNA seemed to suggest calcium trapped inside the particles (Figure 3A and B).

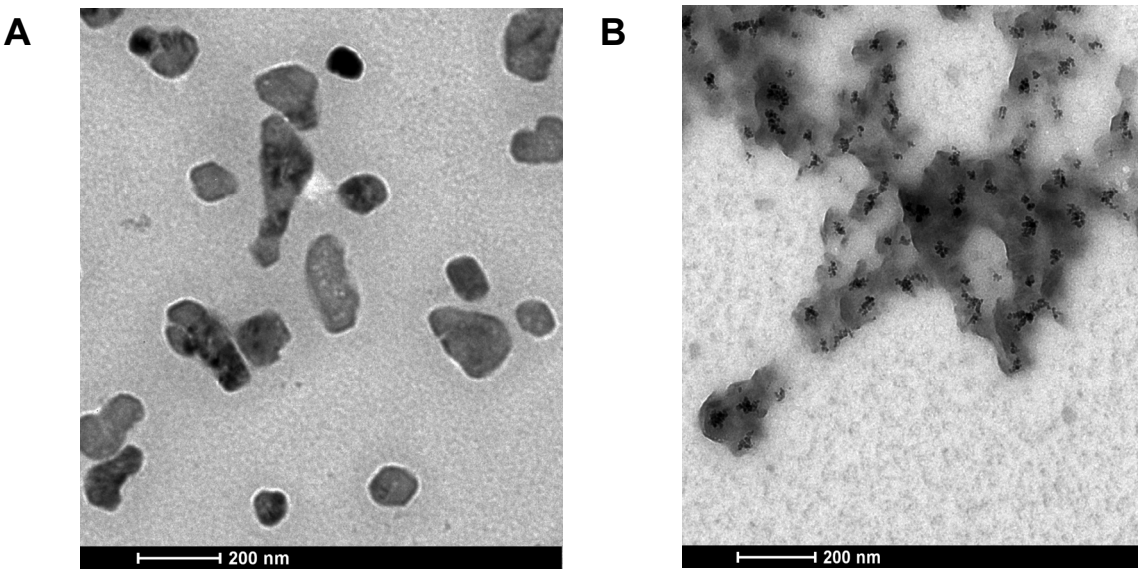

Figure 3. Transmission electron micrograph (TEM) of (A) TAT/pDNA and (B) TAT-Ca/pDNA complexes.

Samples were imaged using scanning transmission electron microscopy and analyzed using EDAX spectroscopy to detect the location of calcium. EDAX spectrum for two labeled areas in the scanning transmission electron micrograph (on the particles and substrate) revealed significantly higher calcium concentration in the particles compared to free calcium that would have dried on the substrate (Figure 4A, B and C).

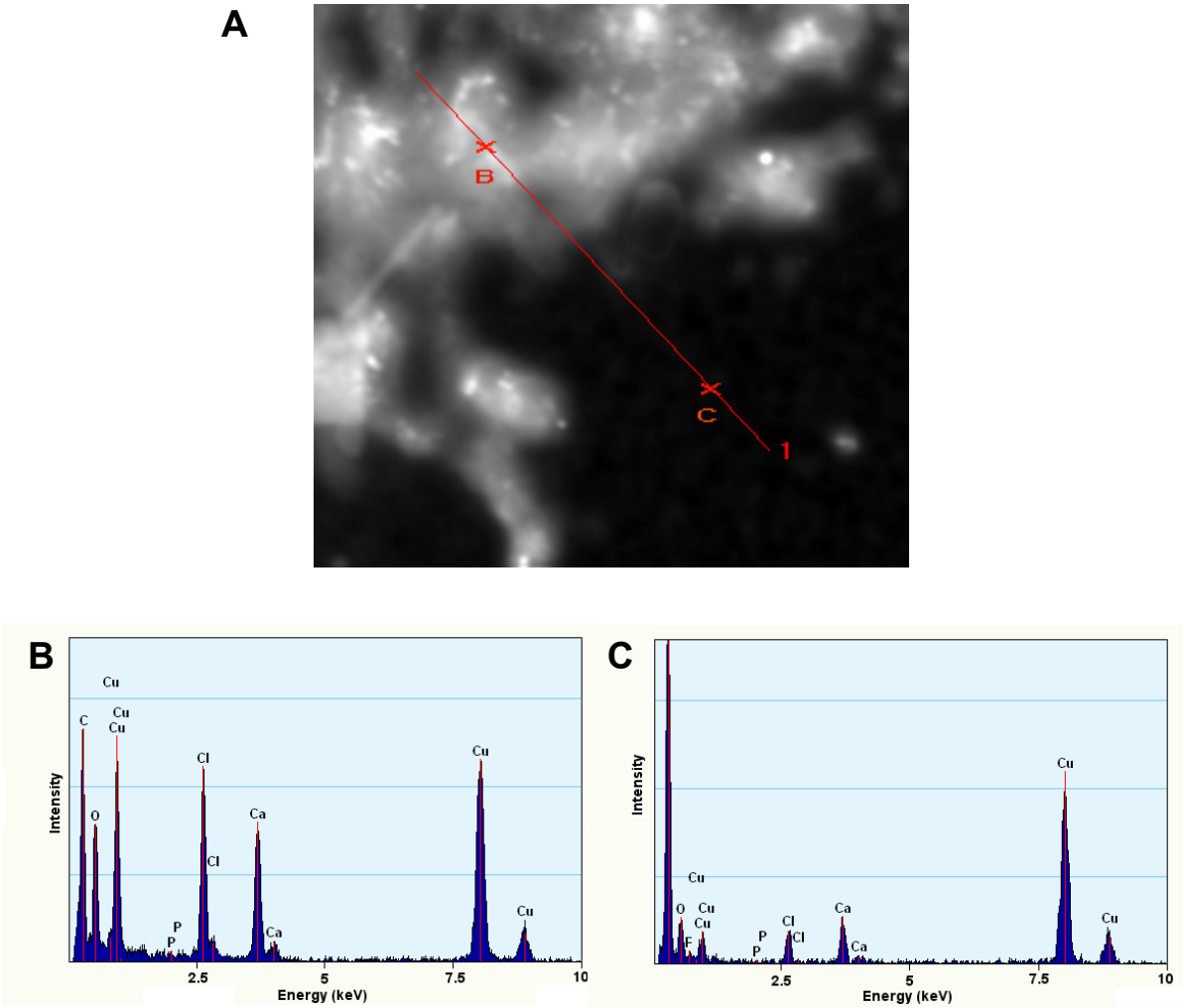

Figure 4. (A) Scanning transmission electron micrograph and EDAX spectrum of TAT-Ca/pDNA. The characterized areas (B) on particles and (C) on substrate are indicated in the scanning transmission electron micrograph. EDAX spectra showed the elemental composition of particles and substrate (Cu peaks result from the copper grid).

The stability of TAT and PEI complexes was investigated as a function of time in the absence and presence of 113 mM $CaCl_2$ and 10% FBS (Figure 5A and B). TAT/pDNA complexes including 113 mM $CaCl_2$ remained stable in the absence and presence of 10% FBS over a period of 8 days. Conversely, PEI/pDNA complexes showed a marked increase in size.

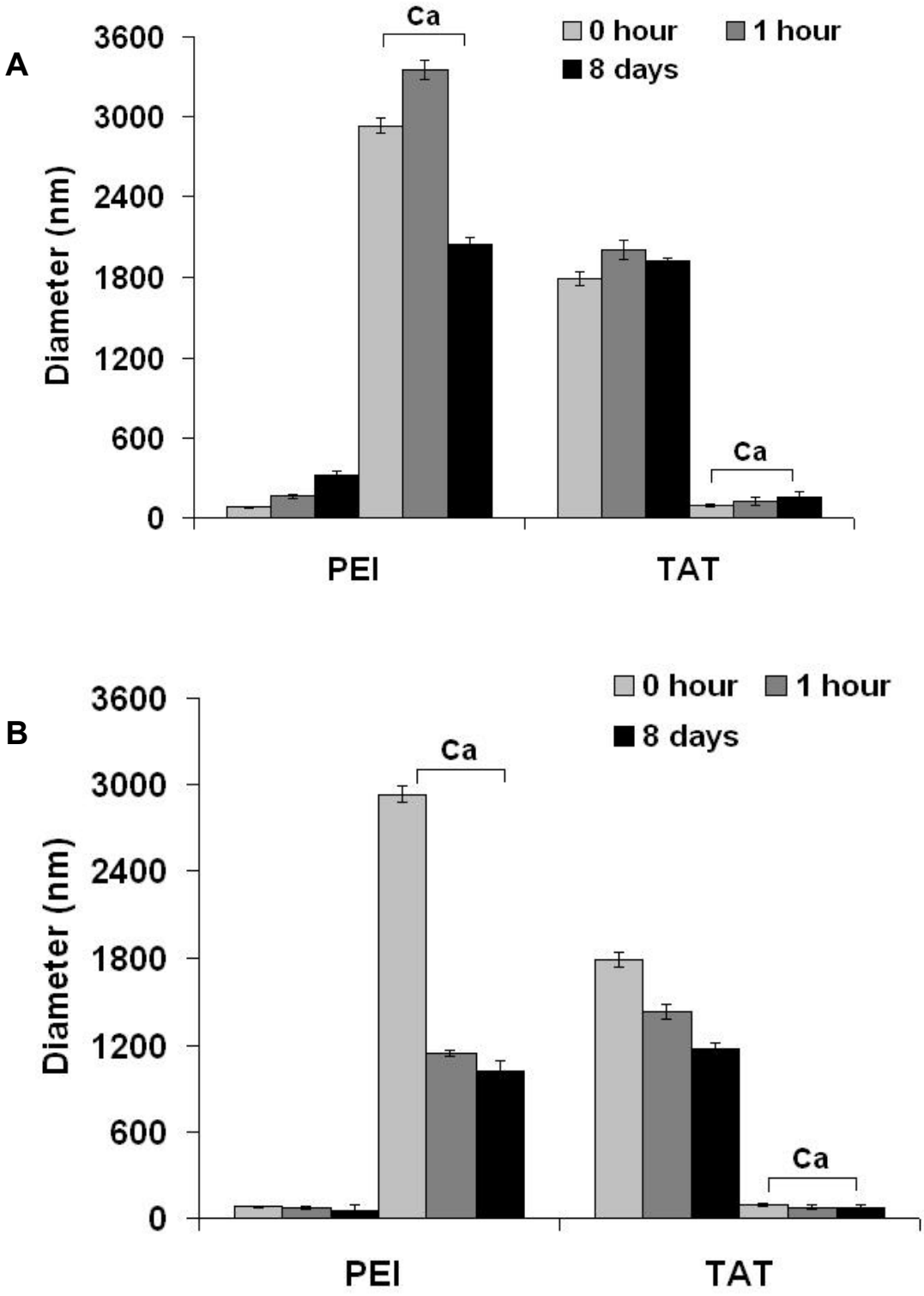

Figure 5. The stability of TAT-Ca/pDNA and PEI/pDNA complexes over time in (A) the absence and (B) presence of 10%FBS. Results are presented as mean ± SD (n = 3).

3.3.3. Cytotoxicity of TAT and PEI complexes

Low cytotoxicity together with high transfection efficiency are extremely important attributes for nonviral gene vectors. The cytotoxicity of free TAT, PEI, and $CaCl_2$ was studied by incubating A549 cells with up to 5 mg/mL of TAT or PEI and with up to 5 M $CaCl_2$ (Figure 6A and B). TAT peptide revealed no evidence of cytotoxicity and cells maintained high viability. Branched PEI induced significant cytotoxicity (IC_{50} ~35 μg/mL). $CaCl_2$ alone showed modest cytotoxicity (IC_{50} ~210 mM).

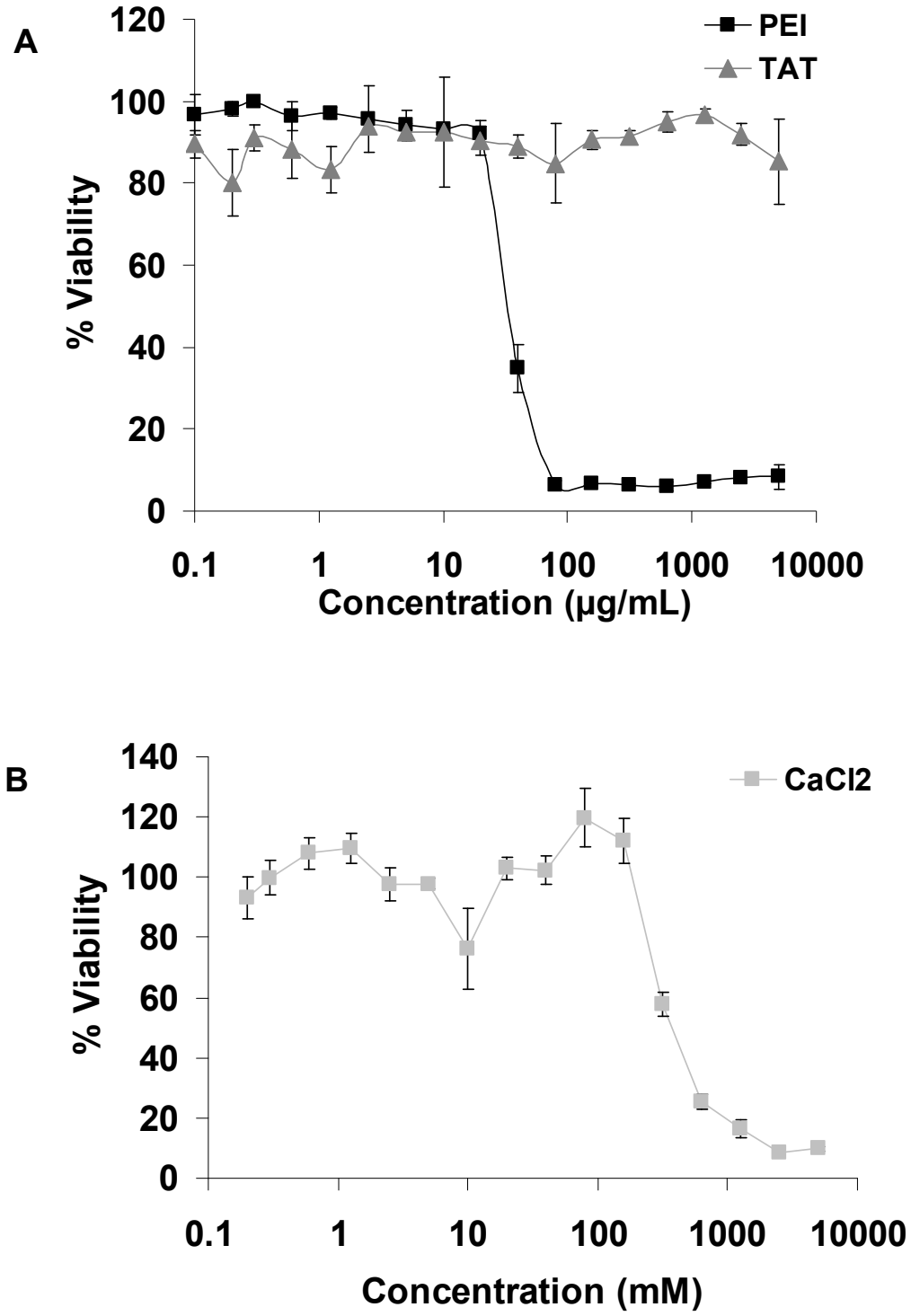

Figure 6. Cytotoxicity profiles of (A) TAT, PEI and (B) $CaCl_2$ in A549 cells. Viability is expressed as a function of polymer concentration. Results are presented as mean ± SD (n = 3).

3.3.4. *In vitro* transfection efficiency of TAT-Ca/pDNA and PEI/pDNA complexes

The *in vitro* transfection efficiency of these complexes was studied using the human lung carcinoma cell line A549. Luciferase gene expression was evaluated on day 1 of transfection using the TAT or PEI polyplexes including different concentrations of $CaCl_2$ during the complex formation. TAT complexes showed a higher level of gene expression at 113 mM $CaCl_2$ when compared to PEI, which had excellent transfection efficiency in the absence of $CaCl_2$ (Figure 7). It was interesting that the optimized level of gene expression of TAT-Ca/pDNA complexes was similar to the transfection efficiency of branched PEI and increased over the first four days. Conversely, the gene expression of PEI/pDNA complexes showed a marked decrease during the same time frame. The gene expression was sustained for at least 10 days and TAT-Ca/pDNA complexes appeared to be superior to PEI/DNA complexes at day 8 and 10 (Figure 8). Strikingly, no expression was observed for TAT/pDNA complexes without $CaCl_2$. In addition, PEI-Ca/pDNA complexes exhibited lower levels of gene expression compared to PEI/pDNA complexes. It is important to note that the optimum gene transfection for TAT-Ca/pDNA complexes occurred when 113 mM of $CaCl_2$ was used, well below the $CaCl_2$ IC_{50} value of 210 mM.

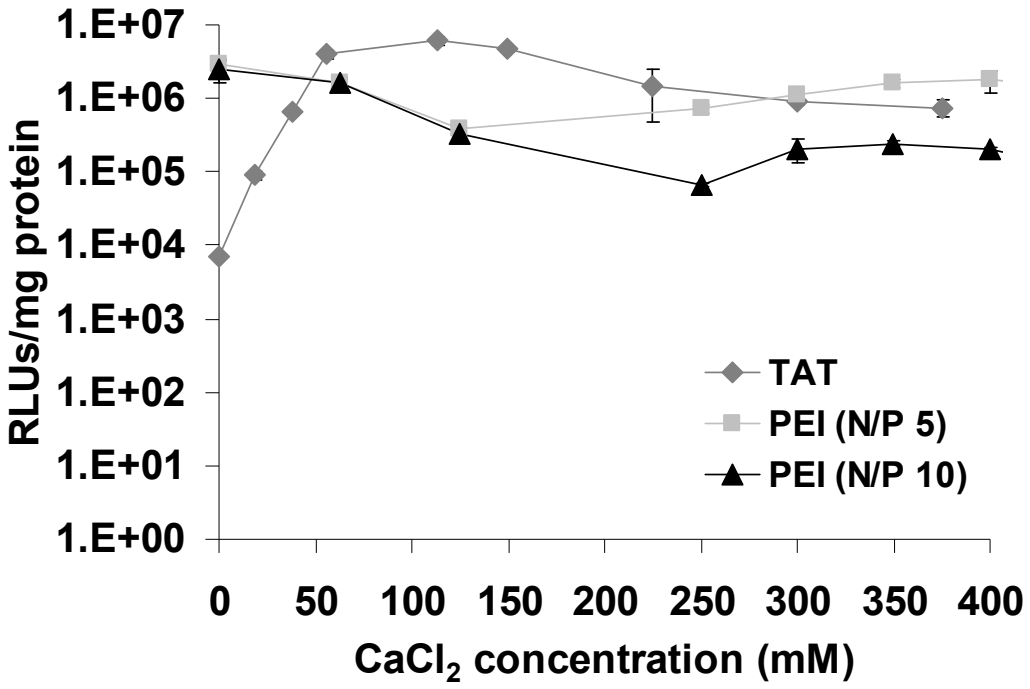

Figure 7. The transfection efficiency of TAT-Ca/pDNA and PEI/pDNA complexes with different concentration of $CaCl_2$. Results are presented as mean ± SD (n = 3).

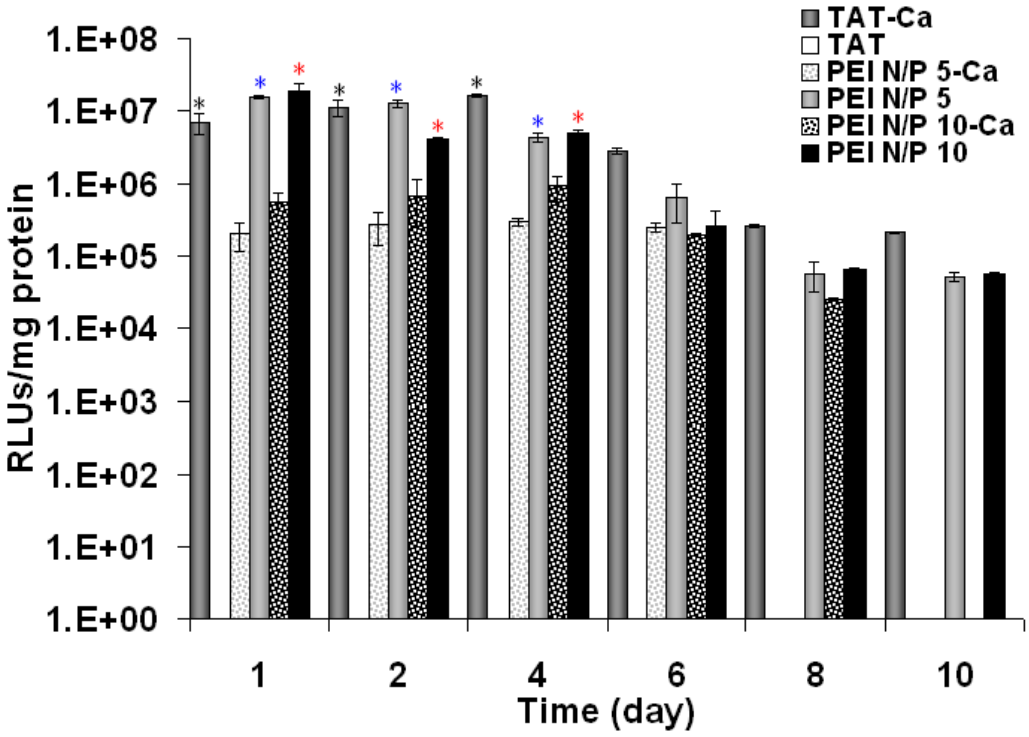

Figure 8. The transfection efficiency of TAT-Ca/pDNA (N/P 25) and PEI/pDNA polyplexes with and without 113 mM $CaCl_2$. Results are presented as mean ± SD (n = 3). *** $p < 0.001$.

3.3.5. The effect of serum on transfection efficiency and particle size

It has been previously reported that the presence of serum may have a significant influence on the transfection efficiency of nonviral gene delivery vehicles [55-57]. To determine the effect of serum on gene expression, A549 cells were transfected with optimized TAT-Ca/pDNA (113 mM $CaCl_2$) or PEI/pDNA complexes in the absence and presence of 10% FBS. Results indicated that serum did not significantly inhibit the transfection efficiency mediated by TAT-Ca/pDNA complexes. In contrast, PEI complexes showed slightly decreased transfection efficiency in the presence of 10% FBS (Figure 9). The effect of serum on transfection efficiency should be considered in light of the stability of the size of TAT and PEI complexes which were investigated as a function of time. TAT/pDNA complexes without $CaCl_2$ exhibited some agglomeration behavior in the absence and presence of 10% of FBS after 8 days (Figure 5A and B). However, TAT-Ca/pDNA complexes showed good stability in serum-free and supplemented culture media during the same time frame. On the other hand, PEI/pDNA complexes remained stable in the absence of serum and $CaCl_2$ over a period of 8 days and retained their size, whereas the particle size of PEI-Ca/pDNA showed a marked decrease during the same time frame in the presence of serum.

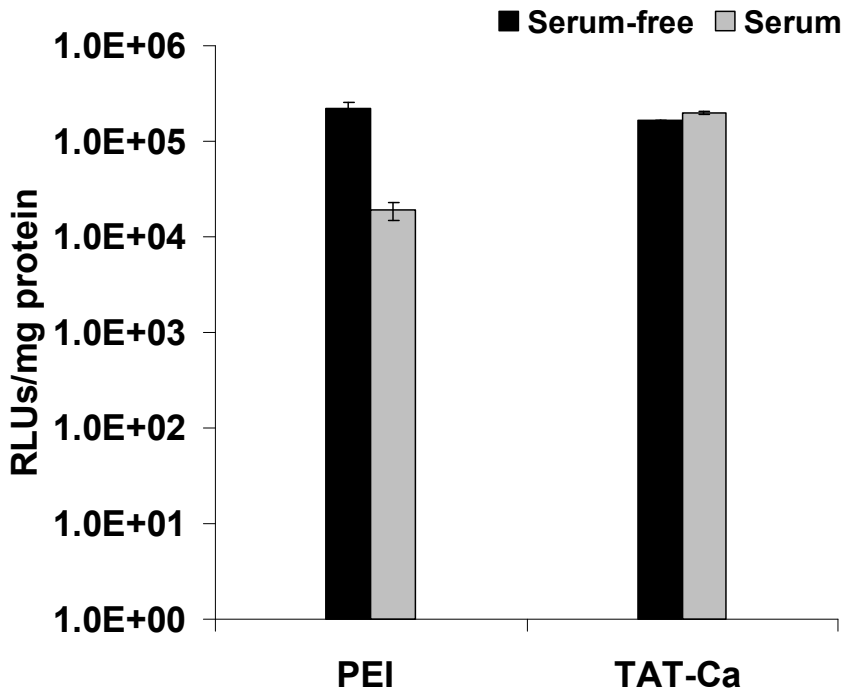

Figure 9. Transfection efficiency of TAT-Ca/pDNA (N/P 25) and PEI/pDNA (N/P 10) complexes in A549 cells in the absence or presence of 10% FBS. Results are presented as mean ± SD (n = 3).

3.3.6. SYBR Green assay

Dye displacement assays provide a simple, nondestructive, and high throughput method for investigating the pDNA accessibility within complexes. Various concentrations of $CaCl_2$ were examined to identify the effect on pDNA packaging. SYBR green, which binds double-stranded DNA, was used for the assay [58]. TAT/pDNA complexes showed weak fluorescence intensity. The addition of different $CaCl_2$ concentrations up to 113 mM likely condensed the TAT complexes, which resulted in lowering the accessibility of pDNA (Figure 10A). The fluorescence intensity transitioned into a gradual increase with increasing concentration of $CaCl_2$. In contrast, PEI/pDNA complexes exhibited a negligible fluorescence for all concentrations of $CaCl_2$ except at 0 and 750 mM $CaCl_2$ where slight fluorescence intensities were observed.

3.3.7. TNBS assay

A TNBS assay was also used to determine the accessibility of the primary amine groups within the TAT/pDNA or PEI/pDNA complexes. The TNBS assay revealed that the free TAT had more primary amine groups than PEI, which decreased to negligible levels when adding the designated volume of 113 mM $CaCl_2$ to them (Figure 10B). Control studies indicated that adding 23 mM $CaCl_2$ decreased the absorption intensity of TNBS by less than 10%. Increasing $CaCl_2$ concentration did not affect the absorption intensity further. These results supported the hypothesis that the efficiency of DNA compaction was increased by the introduction of $CaCl_2$. Therefore, calcium interaction with amines also facilitated the observed stabilization and decrease in particle size for TAT/pDNA complexes. PEI complexes including $CaCl_2$ showed low binding of the dye as well, but this may result from particle agglomeration or precipitation as suggested by the particle size studies.

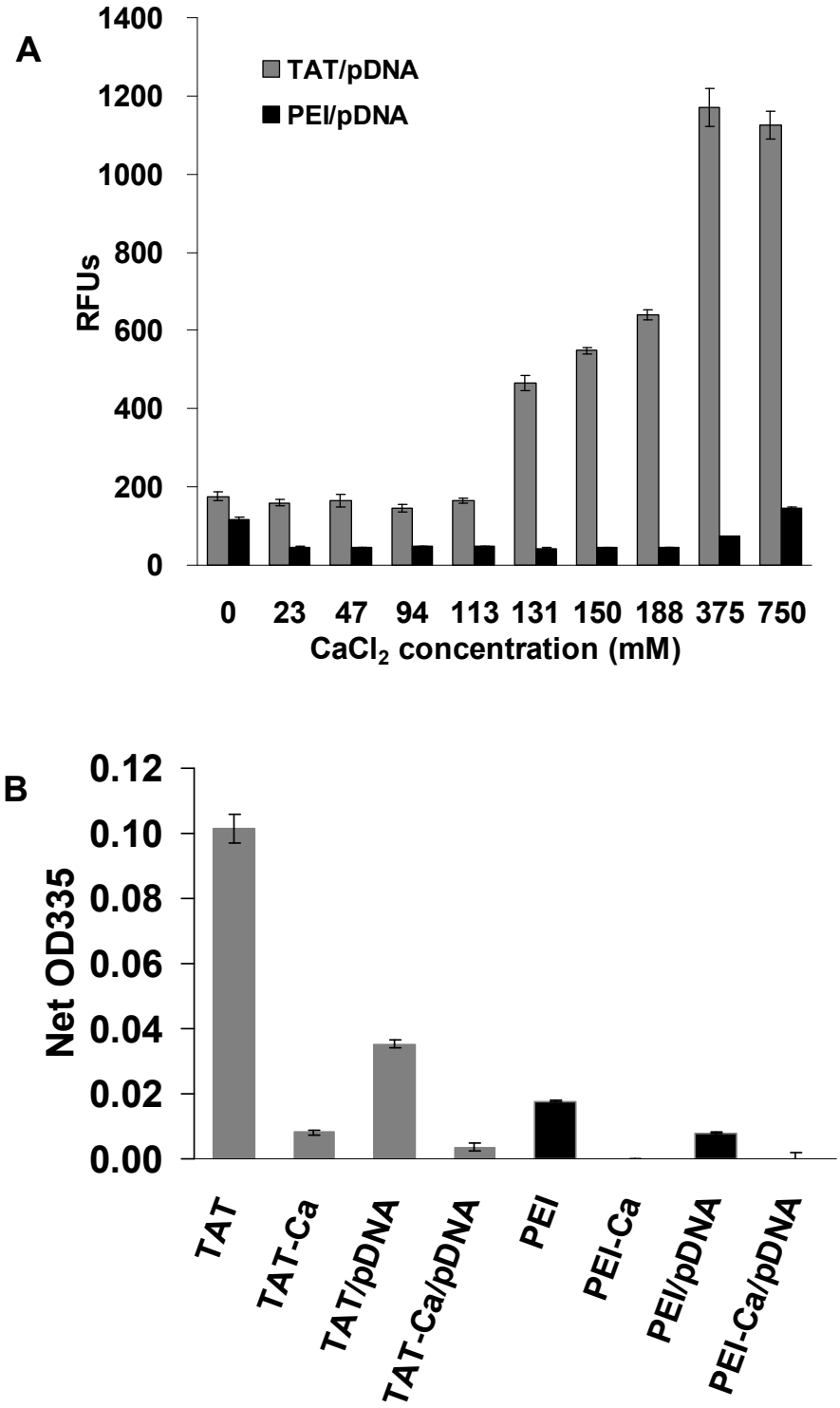

Figure 10. (A) Effect of $CaCl_2$ concentration on the TAT/pDNA and PEI/pDNA complexes by using the SYBR Green assay to assess DNA accessibility and (B) condensation of TAT and PEI complexes assessed using the TNBS assay as a probe for amine accessibility. Results are presented as mean ± SD (n = 3).

3.3.8. Stability of complexes exposed to heparin

The stability of TAT and PEI complexes when exposed to the biological polyanion heparin was studied by determining changes in the SYBR Green-pDNA fluorescence. Exposing the complexes to heparin yielded an increase in the fluorescence signal as the heparin concentration increased (Figure 11). TAT and PEI complexes including 113 mM $CaCl_2$ showed release of the plasmid DNA from the complexes at lower heparin concentrations than without calcium. These results indicated that calcium may control the delicate balance between binding affinities within polycations and pDNA complexes.

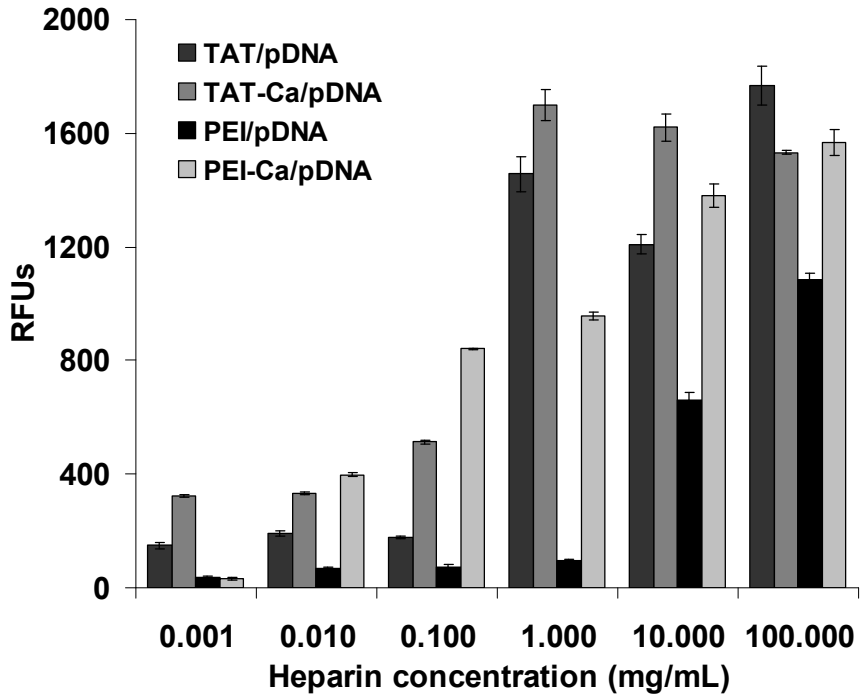

Figure 11. Unpackaging of TAT and PEI complexes by heparin displacement of DNA. Results are presented as mean ± SD (n = 3).

3.4. Discussion

Improvement in the field of gene therapy is currently hindered by the lack of translatable gene delivery vectors. Synthetic, nonviral, vehicles based on polycations are promising vectors for gene delivery [59-64]. The toxicity of these materials may be reduced or eliminated by reducing polycation molecular weight. However, the levels of gene expression mediated by low molecular weight polycations are typically low compared to higher molecular weight polycations (e.g. 25 kDa PEI) [65,66]. As a result, many researchers have aimed to improve upon the deficiency of either system.

Among nonviral vehicles, cell penetrating peptides (CPPs) have been used to deliver a variety of therapeutics. For example, experimental anti-cancer cargo including small molecules, proteins and nucleic acids, were delivered into cells *in vitro* using CPPs and have been explored to treat pre-clinical tumor models *in vivo* [67,68]. Complexes of plasmid DNA with TAT peptides have been used in gene delivery either by covalent coupling of the peptide to the vectors, or by simple mixing of the plasmid DNA with the TAT peptide to form TAT/pDNA complexes via electrostatic interactions. However, the transfection efficiency for these complexes was quite low in comparison to PEI.

In our studies, TAT/pDNA complexes alone also exhibited no gene expression. The size of these complexes was quite large (~1000 nm) for gene delivery. Therefore, calcium was explored as a condensing agent to reduce the size of TAT/pDNA complexes and to improve transfection efficiency. Calcium was found to form tight and compact complexes (~60 nm) through "soft" crosslinks that could be competitively disrupted in order to increase transfection efficiency. The precise balance between binding affinities within TAT/pDNA polyplexes was adjusted by controlling $CaCl_2$ concentration as a

means to optimize transfection efficiency. Previous studies have shown that both small and larges particles may provide efficient levels of gene expression [69-72]. However, the condensed size of TAT-Ca/pDNA complexes in this study appeared to correlate with enhanced transfection efficiency.

The ability of calcium to condense TAT/pDNA complexes occurred as a result of calcium interactions with both amines (polycations) and phosphates (DNA). The binding affinity of calcium to double-stranded DNA was observed as indicated by gel electrophoresis of the TAT-Ca/pDNA complexes. A specific $CaCl_2$ concentration range was necessary to achieve small particles. Dye displacement studies provided some indication of the extent of compaction of DNA when bound to TAT or PEI. Nearly complete exclusion of TNBS from both TAT/pDNA and PEI/pDNA complexes was achieved when 113 mM calcium was added to the polyplexes suggesting that the primary amine groups within these complexes were blocked. Moreover, pDNA was mostly inaccessible to SYBR green when TAT/pDNA complexes were formulated with up to 113 mM $CaCl_2$ suggesting that pDNA was also extensively condensed within the complexes. The accessibility of pDNA increased at higher concentrations of $CaCl_2$ which demonstrated that calcium may also competitively inhibit the amine/phosphate interaction.

The stability of TAT and PEI complexes upon exposure to the polyanion heparin revealed that complex dissociation depended on the inclusion of calcium as well. Heparin and heparan sulfate have been previously reported to bind to PEI and release pDNA from complexes [73,74]. Complex stability is significant because sufficiently low affinity between polycation and plasmid DNA may be desirable to facilitate the release of the DNA after

cellular uptake. The inclusion of calcium in the formulation of TAT and PEI complexes facilitated the dissociation of these complexes by heparin. Therefore, the release of pDNA from the complexes suggested that a critical concentration of calcium can condense the complexes into small particles yet facilitate the release of DNA.

A successful gene delivery system should be able to deliver DNA to the cell without negatively affecting the viability of the host cell. A cytotoxicity study in A549 human lung carcinoma cells indicated that TAT peptide provided substantially higher cell viability than PEI. Further studies will be necessary to determine if this gene vector is benign in primary human cells.

3.5. Conclusion

Drug delivery strategies using CPPs such as TAT have been widely explored to improve the intracellular delivery of a large number of cargo molecules. Electrostatic complexation of pDNA using TAT has been less explored due to the relatively low levels of gene expression observed when using such low molecular weight polycations as DNA condensing agents. We have found that the binding affinity of calcium for TAT peptide and pDNA can be used to effectively mediate the charge balance within these complexes. In this study, it was shown that 113 mM $CaCl_2$ produced small and stable TAT/pDNA complexes via "soft" crosslinks leading to gene expression levels higher than observed for control PEI gene vectors in A549 lung epithelial cells. TAT-Ca/pDNA complexes were stable, maintaining particle size in the absence and presence of 10% of FBS over a period of 8 days. Gene expression of TAT-Ca/pDNA complexes was sustained for at least 10 days and tended to increase over the first four days of the study. Conversely, gene expression levels for PEI/pDNA complexes showed high initial gene expression that

dropped to low levels after day 4. Moreover, the transfection efficiency of TAT-Ca/pDNA complexes was not significantly influenced by the presence of serum. The TAT peptide also showed negligible cytotoxicity up to 5 mg/mL. In comparison, PEI was very cytotoxic (IC_{50} ~35 μg/mL). Thus, these data suggest that TAT-Ca complexes are a novel and effective vehicle offering some potential for translatable gene delivery.

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Chapter 4.

Calcium Condensation of DNA Complexed with Cell Penetrating Peptides Offers Efficient, Noncytotoxic Gene Delivery

4.1. Introduction

Nucleic acid therapeutics continue to offer promise for the treatment of both acquired and inherited diseases. One major obstacle impeding the successful application of nucleic acid drugs is the difficulty to develop a simple, safe, and efficacious delivery system [1-3]. Gene delivery vectors must compact genetic material into nanoparticles that are colloidally stable, protect nucleic acids from enzymatic degradation, effectively transit nucleic acids to target cells, and achieve a significant transfection yield. Viral vectors remain the most effective method of gene delivery even though problems such as immunogenicity remain a concern.

Polymer-mediated gene delivery has emerged as a viable alternative to viral transfection due to potential attributes such as low immunogenicity, low toxicity, ease of synthesis and low cost [4-6]. Many studies have shown that DNA complexes electrostatically with polycations to form "polyplexes" that are endocytosed by many cell types and deliver DNA with varying degrees of efficiency and toxicity [7-16]. Frequently, the most effective polyplexes are also the most toxic, thus hampering clinical translation [1-6]. As a primary example, polyethyleneimine (PEI) exhibits efficient gene delivery but is also very cytotoxic [17,18]. Cell penetrating peptides (CPPs) offer a potential alternative to PEI. These short polycations achieve intracellular access by crossing the plasma membrane directly or by endocytosis [19-25] while typically exhibiting low cytotoxicity [26,27]. Covalently conjugating CPPs to gene vectors (e.g. liposomes, polymer nanoparticle, etc.) has shown some promise, but polyplexes of CPPs and DNA have proven to be relatively inefficient and require improvement [28,29].

To provide a simple method for improving the gene delivery of CPP polyplexes, calcium was used to condense large and inefficient CPP polyplexes. The resulting nanoparticles transfected human lung carcinoma cell line A549 more efficiently than PEI and exhibited very low cytotoxicity. Four representative CPPs were studied; Arginine 7 (Arg7), Arginine 9 (Arg9), Antennapedia Heptapeptide (Ahp) and Antennapedia Leader Peptide (Alp) (Table 1). Plasmid DNA encoding firefly luciferase (pGL3, 4.8 kbp) was used as a reporter.

Table 1. Structure of CPPs, Arginine 7 (Arg7), Arginine 9 (Arg9), Antennapedia Heptapeptide (Ahp) and Antennapedia Leader Peptide (Alp).

CPP	Sequence	Molecular weight (Da)
Arg7	RRRRRRR	1,111.3
Arg9	RRRRRRRRR	1,423.3
Ahp	RRMKWKK	1,032.6
Alp	KKWKMRRNQFWVKVQRG	2,276.2

4.2. Experimental Section

4.2.1. Materials

Plasmid DNA encoding firefly luciferase (pGL3, 4.8 kbp) was obtained from Promega (Madison, WI). Cell penetrating peptides (CPPs) were purchased from Pi Proteomics (Huntsville, AL). Branched polyethyleneimine (PEI, 25 kDa) was obtained from Aldrich (Milwaukee, WI). Calcium chloride ($CaCl_2$. $2H_2O$) was purchased from Fisher Scientific (Pittsburgh, PA). A549 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cell culture medium (Ham's F-12 Nutrient Mixture, Kaighn's modified with L-glutamine) was purchased through Fisher Scientic. Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Penicillin-

streptomycin was purchased from MB Biomedical, LLC (Solon, OH). Trypsin-EDTA was purchased through Gibco. MTS reagent [tetrazolium compound; 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] was purchased from Promega (Madison, WI).

4.2.2. Preparation of CPP/pGL3 complexes

CPP/pGL3 complexes were prepared by adding 10 µL (0.1 µg/µL) of pGL3 to 15 µL CPP solution while pipetting. To this solution, 15 µL of known molarity (e.g. 113 mM) $CaCl_2$ was added and mixed by vigorous pipetting. Before performing experiments, the complexes were allowed to equilibrate for 20 min at 4º C.

4.2.3. Preparation of PEI/pGL3 complexes

PEI/pGL3 complexes were prepared by adding 10 µl (0.1 µg/µL) of pGL3 solution to 15 µL (N/P ratio 10) PEI solution while pipetting followed by 20 min incubation at 4º C. Complexes were freshly prepared before each individual experiment.

4.2.4. Characterization of CPPs and PEI complexes

The size and zeta potential of the different complexes were measured by using ZetaPALS dynamic light scattering (DLS) (Brookhaven, Holtsville, NY).

4.2.5. Cell culture

Culturing of human epithelial lung cell line A549 was performed according to the protocol provided by the American Type Culture Collection. A549 cells were grown in F-12K supplemented with 10% (v/v) FBS and 1% (v/v) Penicillin/streptomycin at 37º C in a humidified air atmosphere containing 5% CO_2.

4.2.6. *In vitro* cell transfection studies

A549 cells were trypsinized, counted and diluted to a concentration of approximately 80,000 cells/ mL. Then 0.1 mL of that dilution was added to each well of a 96-well plate and the cells were incubated in a humidified atmosphere at 5% CO_2 and 37°C for 24 h. Immediately before transfection, the cells were washed once with PBS and 100 µl sample (20% of complex to 80% of serum free cell culture medium) was added to each well. Cells were incubated with the complexes for 5 h. The transfection agent was then removed by aspiration and 100 µL of fresh serum medium was added followed by further incubation. The Luciferase Assay System from Promega was used to determine gene expression following the manufacturer's recommended protocol. The light units were normalized against protein concentration in the cells extracts, which were measured using the BCA™ Protein Assay (Thermo Scientific, Rockford, IL). The transfection results were expressed as Relative Light Units (RLU) per mg of cellular protein.

4.2.7. Assessment of cytotoxicity (MTS Assay)

Cytotoxicity of polymers was determined by the CellTiter 96® Aqueous Cell Proliferation Assay. A549 cells were grown as described in the transfection experiments. Cells were treated with the samples for ~24 h. The media were then removed and replaced with a mixture of 100 µL fresh culture media and 20 µL MTS reagent solution. The cells were incubated for 3 h at 37°C in the 5% CO_2 incubator. The absorbance of each well was then measured at 490 nm using a microtiter plate reader (SpectraMax, M25, Molecular Devices Corp., CA) to determine cell viability.

4.3. Results and Discussion

Peptides offer a highly attractive feature of incorporating various biological activities required for biomedical applications [30-34]. Cell penetrating peptides, a group of short peptides with the potent ability to translocate across the plasma membrane of the cells, have been reported to mediate plasmid DNA delivery into cells [35-37]; however, improving the transfection efficiency of their DNA complexes remains a major challenge. The relatively low transfection level of certain complexes may be due to inadequate escape from endosomes or the inefficient release of DNA from the complexes [38]. Previous studies demonstrated that the HIV-1 TAT peptide could only provide a high level of gene expression when chloroquine (an endosomolytic agent) was added, which is not feasible for gene delivery *in vivo* [28,39-41]. One approach for overcoming this limitation was to link the CPPs to produce high molecular weight polypeptides [42-44] or to directly conjugate the CPPs using histidine or cysteine residues[38,45]. Others have tried a low molecular weight PEI with covalently linked TAT to overcome the poor transfection efficiency of the CPP alone [39]. Targeting studies have also been explored. For example, the YIGSR pentapeptide, known to target cell surface laminin receptors, or the LK15 peptide was conjugated to TAT. In each case, the transfection efficiency improved, but gene expression levels were still low compared to PEI [46-48].

In studies reported here, CPP/pGL3 complexes were synthesized by rapidly adding pGL3 to CPP. These complexes were thoroughly mixed by pipetting and then $CaCl_2$ was added to decrease the large size of these complexes. Calcium was previously reported to interact with both amines (e.g. on CPPs) and pDNA within polyplexes to form compact complexes through "soft" crosslinks. Here, the reduction in the size of CPP/pGL3

complexes likely led to some of the noted increase in transfection. A $CaCl_2$ concentration of 113 mM consistently produced small (100-140 nm) and stable CPP polyplexes with a single particle population (polydispersity < 0.23). In general, the zeta potential of CPP polyplexes increased significantly from 8 to 26 mV with increasing concentration of $CaCl_2$ (Figure 1). The CPP polyplexes synthesized with $CaCl_2$ remained stable in the absence and presence of 10% FBS at 37º C for 1 hr. Conversely, CPP polyplexes without added calcium remained large (Figure 2).

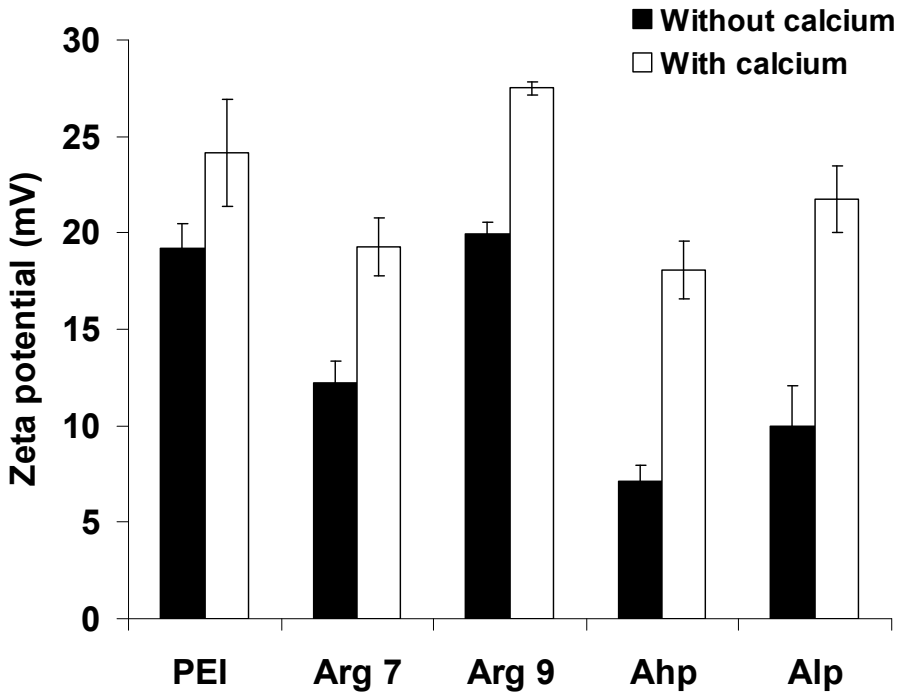


Figure 1. The effect of $CaCl_2$ (113 mM) on the charge of CPPs and PEI complexes. Results are presented as mean ± SD (n = 3).

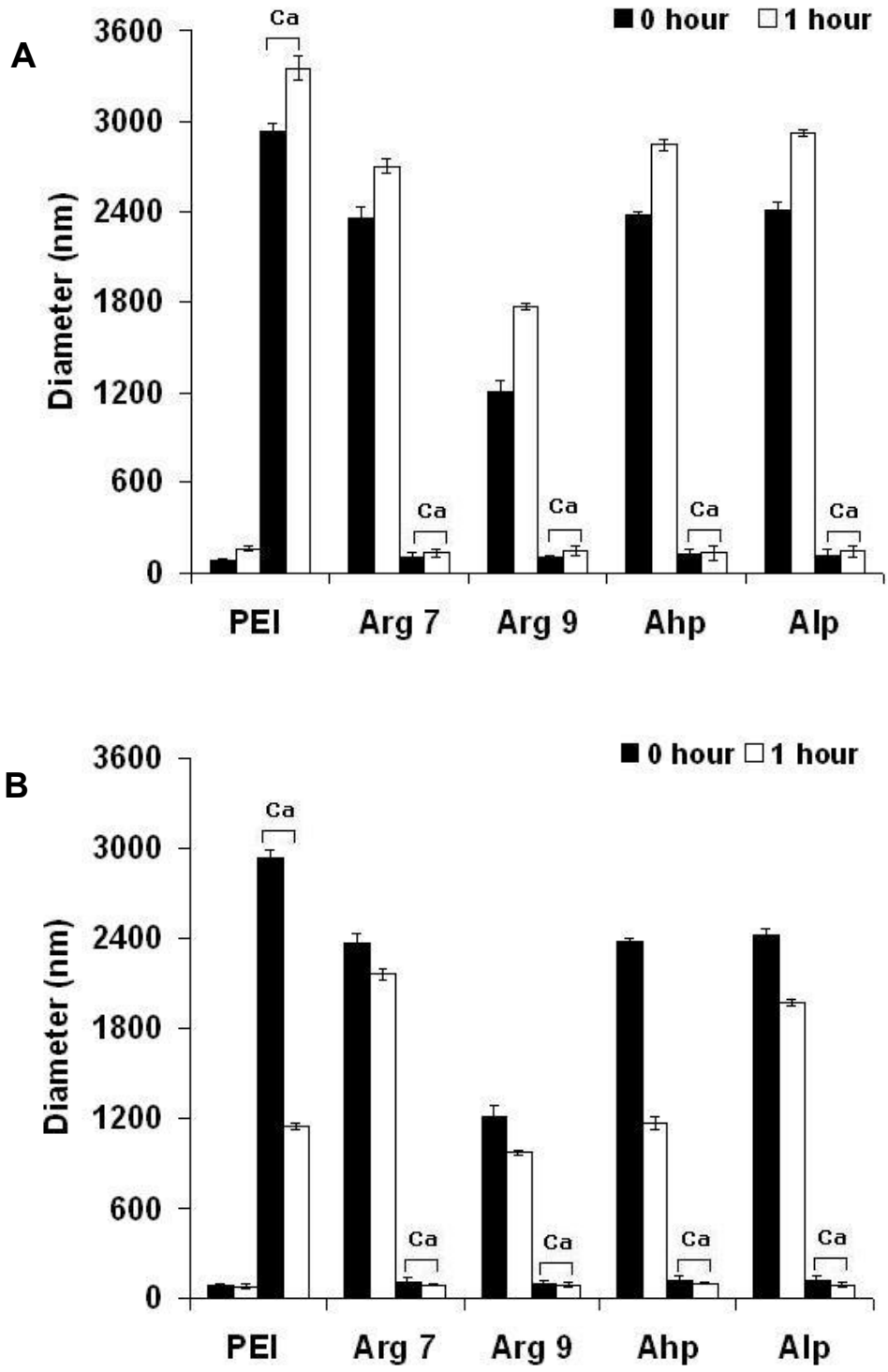


Figure 2. The diameter of CPPs-Ca/pGL3 and PEI complexes (without and with 113 mM $CaCl_2$) in (A) the absence and (B) presence of 10% FBS. Results are presented as mean ± SD (n = 3).

A successful gene delivery vector should be able to deliver gene to the cell without negatively affecting the viability of the host cell. To investigate whether the CPPs affected the viability of A549 human lung carcinoma cells, an MTS cytotoxicity assay of free CPP or branched PEI (25 kDa) was conducted. A549 cells were incubated with up to 5 mg/mL of CPPs or PEI for ~24 hrs. Cytotoxicity profiles of Arg7, Arg9 and Ahp peptides revealed no evidence of cytotoxicity and cells maintained high viability (Figure 3), while Alp peptide showed moderate cytotoxicity (IC_{50} ~2144 μg/mL). Branched PEI induced substantial cytotoxicity (IC_{50} ~35 μg/mL).

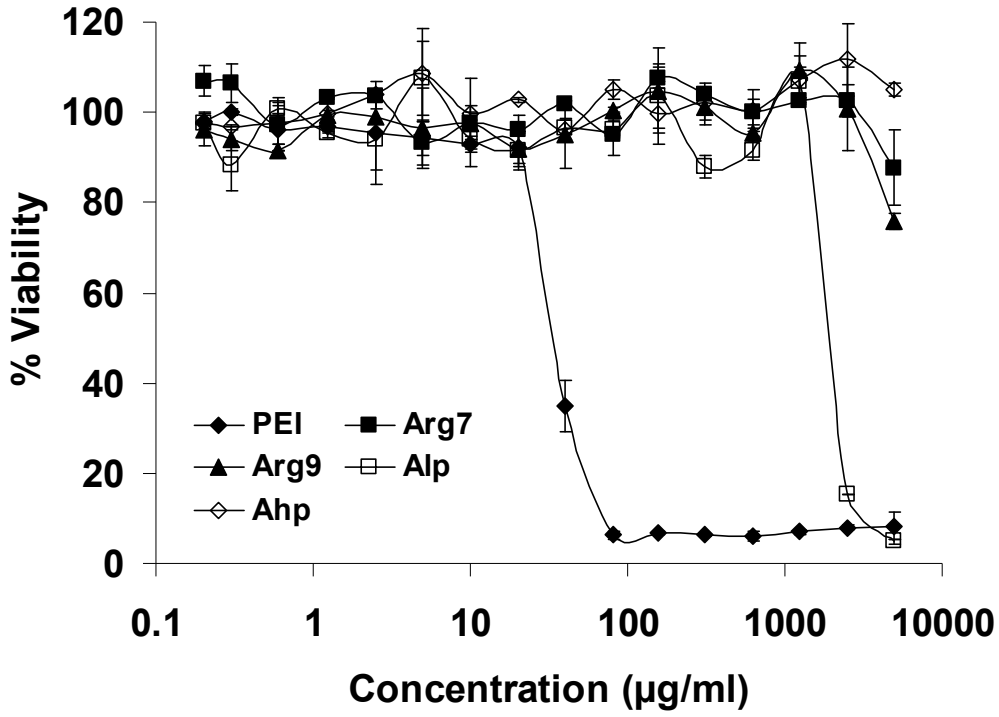
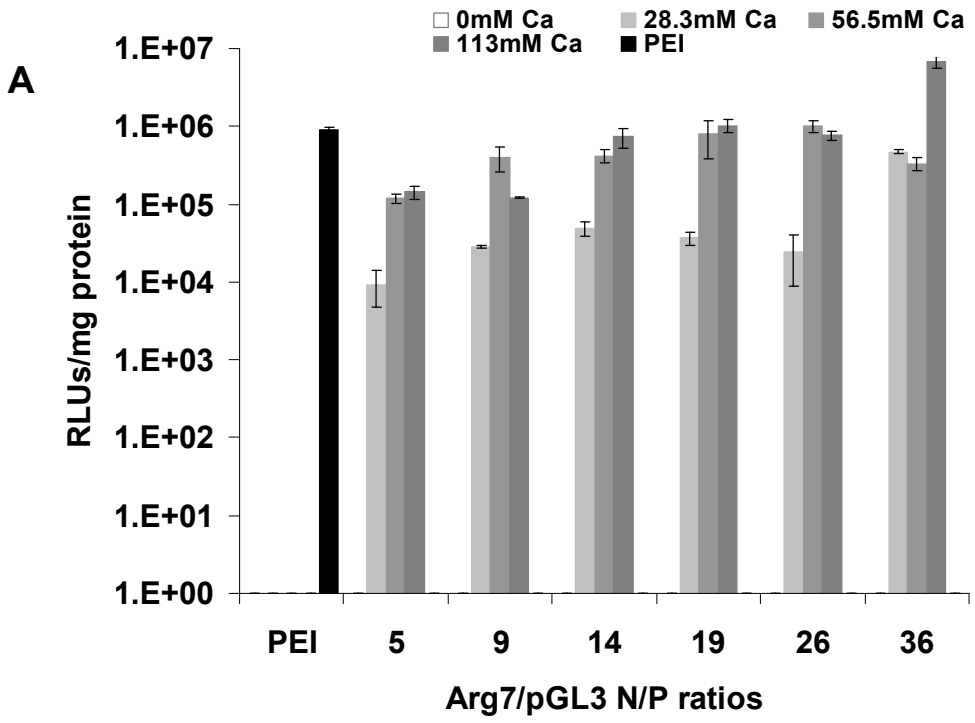
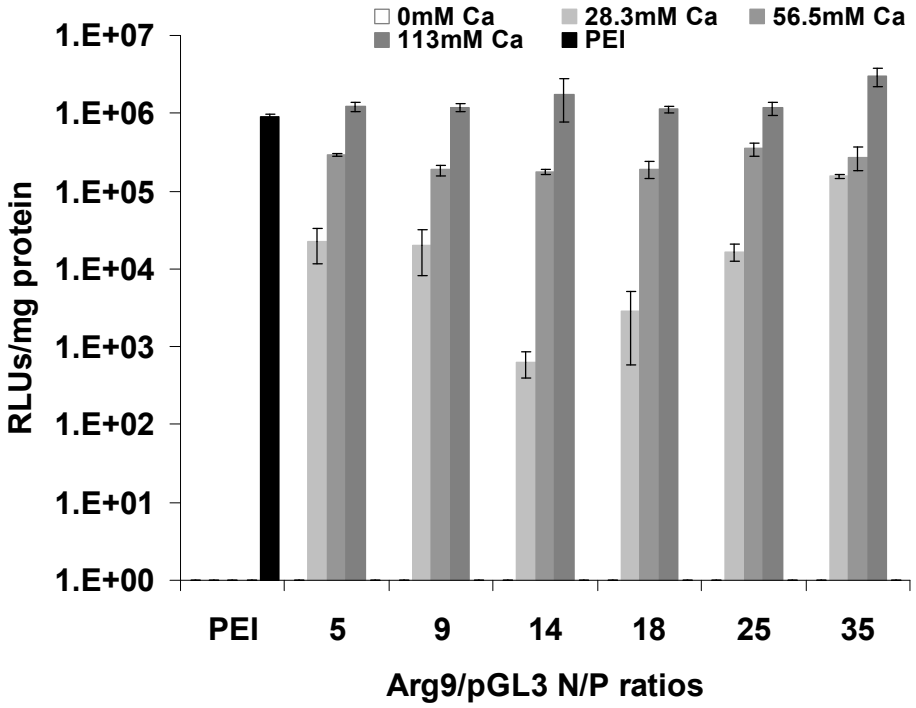


Figure 3. Cytotoxicity profiles of CPPs and PEI in A549 cells. Viability is expressed as a function of polymer concentration. Results are presented as mean ± SD (n = 3).

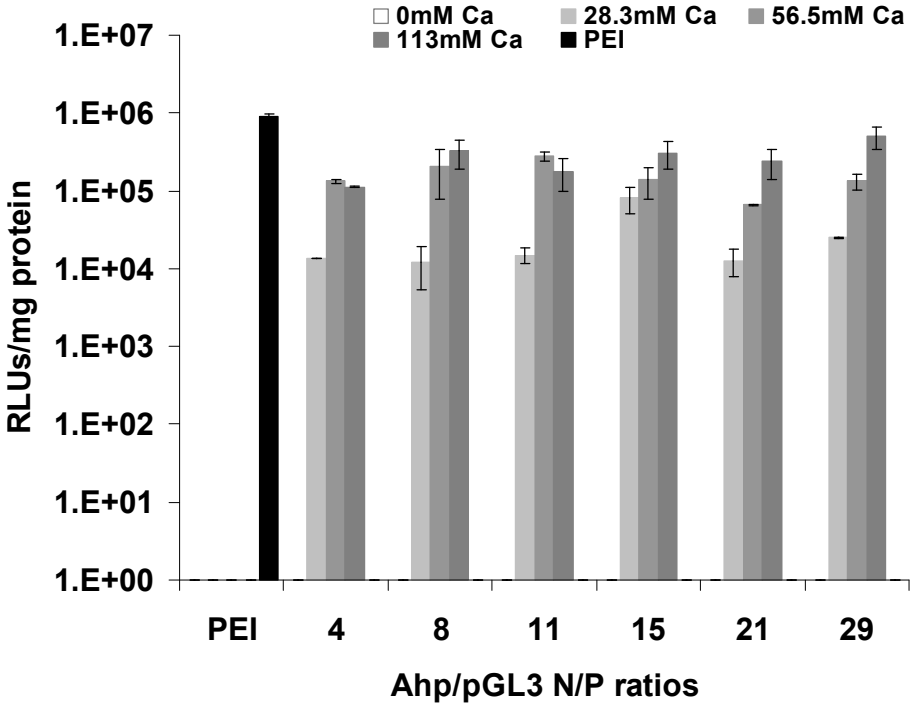
Luciferase gene expression was measured 24 h after transfection in order to study the ability of CPP polyplexes to transfect A549 cells. Different N/P ratios of the CPP or branched PEI (N/P 10) polyplexes were studied using different concentrations of $CaCl_2$; 0, 28.3, 56.5, and 113 mM as a condensing agent after complex formation. Most CPP polyplexes showed the highest level of gene expression at 113 mM of added $CaCl_2$ for the various N/P ratios when compared to branched PEI, which had excellent transfection efficiency only in the absence of $CaCl_2$ (Figure 4). Arg7, Arg9, Ahp and Alp revealed the greatest transfection efficiency with 113 mM $CaCl_2$ at N/P ratios of 36, 35, 29 and 15 respectively. It is important to note that gene expression was not detectable for CPPs/pDNA complexes without $CaCl_2$.



B


0mM Ca
28.3mM Ca
56.5mM Ca
113mM Ca
PEI
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
RLUs/mg protein
PEI
5
9
14
18
25
35
Arg9/pGL3 N/P ratios

C


0mM Ca
28.3mM Ca
56.5mM Ca
113mM Ca
PEI
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
RLUs/mg protein
PEI
4
8
11
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21
29
Ahp/pGL3 N/P ratios

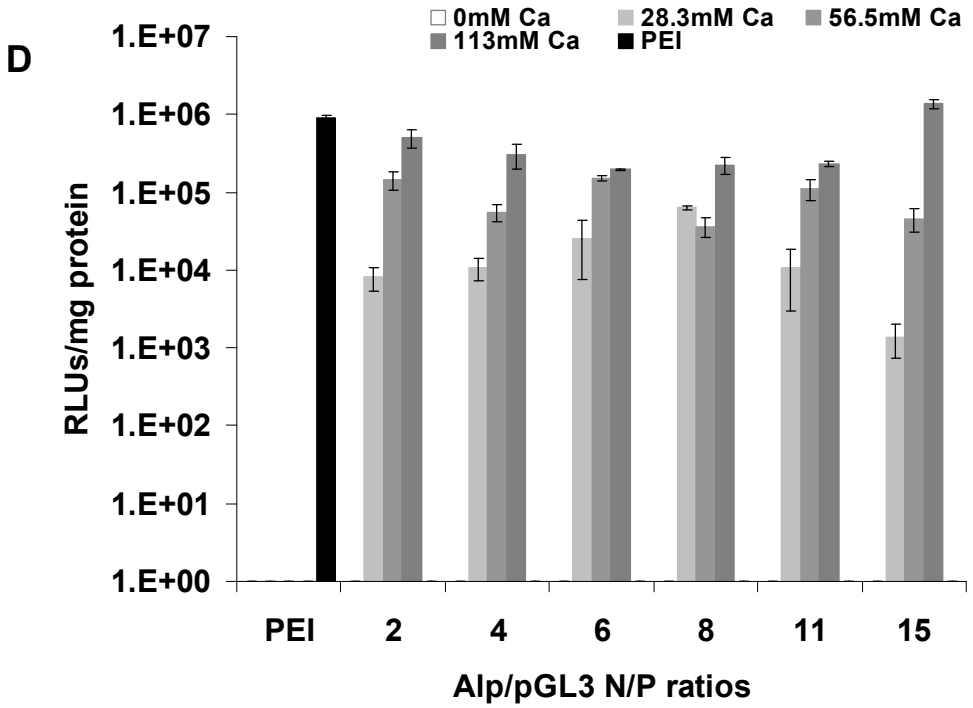

Figure 4. The transfection efficiency of CPP and PEI complexes using (A) Arg7, (B) Arg9, (C) Ahp or (D) Alp with different concentrations of added $CaCl_2$. Results are presented as mean ± SD (n = 3).

To gain insight into the potential of utilizing calcium condensed CPP polyplexes as delivery vectors in vivo, A549 cells were transfected in the presence of serum (Figure 5). A slight decrease in transfection efficiency was observed and CPP polyplexes exerted a similar reduction in gene expression as that observed for PEI polyplexes. PEI polyplexes have shown effective gene transfection *in vivo*, but is dose limited due to toxicity.

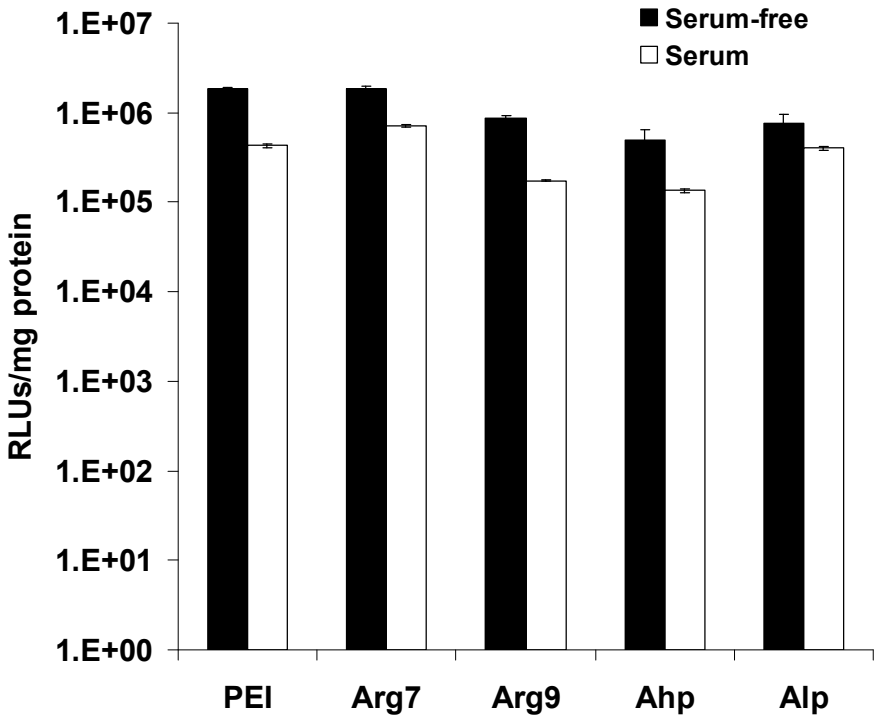

Figure 5. The transfection efficiency of CPP and PEI polyplexes in the absence and presence of 10% FBS. Results are presented as mean ± SD (n = 3).

4.4. Conclusion

The delivery of therapeutic nucleic acids by CPP polyplexes condensed with calcium may offer a simple and effective gene delivery method with potential for clinical translation. Adding $CaCl_2$ to CPP polyplexes produced small nanoparticles leading to gene expression levels higher than observed for optimized PEI gene vectors in A549 lung epithelial cells. CPP polyplexes were stable, maintaining particle size in the absence and presence of 10% of FBS over a period of 1 h. The CPPs generally showed negligible cytotoxicity up to 5 mg/mL, which may offer an opportunity to increase the dose of nucleic acid therapeutics to achieve a desired therapeutic effect. The simplicity of the formulation in combination with the efficacy and low cytotoxicity of CPP polyplexes makes them highly interesting vectors for future studies *in vivo*.

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Chapter 5.

DNA Complexed with TAT and Condensed Using Calcium Possesses Unique Structural Features Compared to PEI Polyplexes

5.1. Introduction

While a variety of vectors currently exist for gene translocation, cell penetrating peptides (CPPs) have become one of the most popular and efficient agents for intracellular delivery of genetic material. CPPs have successfully achieved intracellular delivery of proteins [1], nucleic acids [2], small molecule therapeutics [3], quantum dots [4], and even MRI contrast agents [5]. Highly efficient translocation mediated by CPPs has been confirmed in a variety of cell lines with minimal toxicity, overcoming challenges often faced with other delivery methods [6]. Additionally, CPPs can be covalently or noncovalently attached to drugs effectively towing their cargo into cells with no loss of biological activity. Once inside the cell, many of these CPPs are also able to localize to the nucleus, with or without their cargo [7]. A major problem with the use of CPPs as nonviral gene vectors, however, is their low level of induction of gene transfection compared to viral vectors. The problem is compounded by the lack of structural information for these complexes. Previous studies have defined the structure of nonviral gene delivery complexes by a combination of methods such as dynamic light scattering (DLS), fluorescence, Fourier transform infrared spectroscopy (FTIR), circular dichroism (CD) and differential scanning calorimetry (DSC) [8-17]. These methods potentially provide information-rich data that may be used to describe gene delivery complexes and serve as a basis to design next generation gene vectors.

CPPs possess the physical and chemical characteristics desired of gene delivery agents [18]. For example, their polycationic, sometimes amphiphilic, nature has been shown to overcome one or more of the major biological barriers to transfection (e.g. cell entry, endosome escape, and nuclear localization). In addition, many of these peptides can

complex pDNA, although somewhat inefficiently. Cationic lipoplex vectors have been extensively investigated by various physicochemical methods in previous studies, but detailed characterization studies of CPP containing "polyplex" vectors remain scarce.

TAT complexes containing "soft" calcium cross links have recently shown promise by our group as efficient gene delivery agents [19]. A method to synthesize small (60-175 nm) and stable TAT complexes was achieved by the formation of a 'loose' complex between TAT peptide and pDNA that was then condensed by calcium. The TAT-Ca system was shown to display high transfection efficiency even in the presence of serum, and negligible toxicity *in vitro* and *in vivo* compared to branched PEI polyplexes. The transfection efficiency of TAT complexes depends on several factors including the nitrogen/phosphate (N/P) ratio and the concentration of $CaCl_2$.

Despite extensive previous efforts investigating the potential of polycations to deliver plasmid DNA, correlations between the properties of polyplexes and their ability to transfect cells are still poorly understood [20-24]. Thus, we have conducted a thorough physical characterization of TAT complexes with the objective of establishing a relationship between their physical properties by comparison to PEI polyplexes. The secondary structure of pDNA upon complexation with TAT was investigated by using FTIR, UV second derivative absorption and CD spectroscopies. The thermal stability of pDNA complexed to TAT was studied using DSC. In addition, fluorescence spectroscopy was used to probe the extent of pDNA condensation in complexes. Finally, light scattering studies were performed to assess the particle size of the complexes.

5.2. Materials and methods

5.2.1. Materials

Plasmid DNA encoding firefly luciferase (pGL3, 4.8 kbp) was obtained from Promega (Madison, WI, USA) and transformed into *Escherichia coli* DH5ά (Invitrogen, Carls-bad, CA). A single transformed colony picked from an agar plate was cultured in LB Broth Base (Invitrogen) liquid for plasmid DNA preparation. Plasmid DNA was purified with Plasmid Giga Kit 5 (Qiagen, Germantown, MD) following the manufacturer's instructions. All pDNA had purity levels of 1.8 or greater as determined by UV/Vis inspection (A_{260}/A_{280}). HIV-1 TAT (49-57) peptide (RKKRRQRRR; Mw = 1338.85 Da) was purchased from Biomatik (Cambridge, Ontario, Canada). Branched polyethylenimine (PEI, 25 kDa) was obtained from Aldrich (Milwaukee, WI). Calcium chloride ($CaCl_2$. $2H_2O$) and agarose medium were purchased from Fisher Scientific (Pittsburgh, PA). A549 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cell culture medium (Ham's F-12 Nutrient Mixture, Kaighn's modified with L-glutamine) and BCA™ Protein Assay were purchased through Fisher Scientic (Pittsburgh, PA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Penicillin-streptomycin was purchased from MB Biomedical, LLC (Solon, OH) and Trypsin-EDTA from Gibco (Carlsbad, CA). The Luciferase Assay System was obtained from Promega (Madison, WI).

5.2.2. Preparation of complexes

Complexes were formed by addition of appropriate volumes of pDNA and TAT or PEI solutions calculated to produce the desired molar ratio, followed by a 20 min

incubation at 4º C prior to use [25]. Complexes were freshly prepared before each individual analysis.

5.2.3. Particle Size and Zeta potential measurement

The effective hydrodynamic diameter of the complexes was analyzed using DLS employing a Brookhaven Instrument (Holtsville, NY) equipped with a 50 mW HeNe laser operating at 532 nm. The complexes were prepared at a constant pDNA concentration of 100 μg/mL while the N/P ratios of the complexes were varied. The scattered light was monitored at 90º to the incident beam. For each sample the data were collected continuously for three 1-min intervals. The mean diameter of the complexes was obtained from the diffusion coefficient by the Stokes-Einstein equation using the method of cumulants. Zeta potential measurements were obtained by phase analysis light scattering using a Brookhaven Zeta PALS instrument. The electrophoretic mobility of the samples was determined from the average of 10 cycles of an applied electric field. The zeta potential was determined from the electrophoretic mobility from the Smoluchowski approximation.

5.2.4. UV Absorption Spectroscopy

UV absorption spectra of pDNA were obtained employing a Hewlett-Packard 8453 UV-visible diode-array spectrophotometer (Agilent, Palo Alto, CA). A pDNA concentration of 0.025 μg/μL was used. Second derivatives of the absorption spectra from 240 nm to 300 nm were generated using a 9-point filter and third degree polynomial then fit to a spline function with 99 points of interpolation using software supplied with the instrument. Peak positions were determined using Origin® software from Microcal™.

5.2.5. Transfection Studies

The transfection efficiencies of the complexes were assessed using plasmid DNA encoding firefly luciferase (pGL3, 4.8 kbp) in A549 human lung carcinoma epithelial cells. Cells were trypsinized, counted and diluted to a concentration of approximately 80,000 cells/ mL. Then 0.1 mL of that preparation was added to each well of a 96-well plate and the cells were incubated in a humidified atmosphere at 5% CO_2 and 37°C for 24 h. Immediately before transfection, the cells were washed once with PBS and 100 µl of sample (20% of complex to 80% of serum free cell culture medium) was added to each well. Cells were incubated with the complexes for 5 h. The transfection agent was then removed by aspiration and 100 µL of fresh serum medium was added followed by 24 h of incubation. The Luciferase Assay System from Promega was used to determine gene expression following the manufacturer's recommended protocol. The light units were normalized against protein concentration in the cellular extracts, which were measured using the BCA™ Protein Assay. The transfection results were expressed as Relative Light Units (RLU) per mg of cellular protein.

5.2.6. Fluorescence Spectroscopy

The dye SYBR Green (Invitrogen, Carls-bad, CA) with an excitation wavelength of 497 nm and emission wavelength of 520 nm was used in fluorescence studies. SYBR Green solution (concentration of 1 X dye) was added to the pDNA before complex formation. After a 30 min incubation, the complexes were prepared as described above. Fluorescence measurements were performed with a QuantaMaster™ spectrofluorometer (PTI, Monmouth, NJ). The excitation and emission slits were set at 4 nm and spectra from 480 to 590 nm were obtained by scanning at 1 nm intervals using a 1 s integration

time. The fluorescence intensity of dye alone in buffer was subtracted from raw sample spectra.

5.2.7. Fourier Transform Infrared (FTIR) spectroscopy

FTIR spectra were obtained using a Nicolet Magna-IR 560 spectrometer equipped with a mercury cadmium telluride detector (Nicolet, Madison, WI). Samples were measured by an attenuated total internal reflectance (ATR) method in which the sample in solution was placed directly in a zinc selenide trough. Spectra were obtained under dry air purge by accumulation of 256 interferograms at a final resolution of 4 cm^{-1}. For ATR experiments, the association band of water near 2200 cm^{-1} was used as a reference for subtraction of water from the spectra. Base-line correction (1804 to 904 cm^{-1}) and seven point Satvitsky-Golay smoothing were applied to all spectra. The final pDNA concentration of the samples was 0.5 μg/μL while the polycations concentration was varied.

5.2.8. Circular Dichroism (CD)

For CD measurements, complexes were formed at a final pDNA concentration of 50 μg/mL**.** CD spectra were obtained using a Jasco J-720 spectropolarimeter (Easton, MD) in a 0.1 cm pathlength quartz cuvette at 25°C. Spectra were recorded from 350 to 200 nm at a scan rate of 20 nm/min and were analyzed as an average of three scans. The CD signal was converted to molar ellipticity [θ] based on the molar base concentration of the pDNA, smoothed with a Jasco Fast Fourier transform method, and then baseline adjusted to zero at 345 nm to correct for a small contribution by differential light scattering.

5.2.9. Differential scanning calorimetry (DSC)

Differential scanning calorimetry thermograms were obtained with a VP-DSC (Microcal, Northampton, MA). Measurements consisted of a single scan from 0 to 120°C at 1°C/min under 3 atm of pressure. Samples were examined in triplicate and degassed before measurement. Baselines were obtained by scanning with buffer in both the sample and reference cells. Data were analyzed by subtracting the baseline from the sample thermogram, and converting the differential heat to molar heat capacity using the molecular weight and concentration of pDNA (0.4 μg/μL).

5.2.10. Statistical Analysis

Statistical evaluation of data was performed using an analysis of variance (one-way ANOVA). Newman–Keuls was used as a post-hoc test to assess the significance of differences. To compare the significance of the difference between the means of two groups, a *t*-test was performed; in all cases, a value of $p < 0.05$ was accepted as significant.

5.3. Results

5.3.1. Particle Sizes and Zeta potentials of TAT and PEI complexes

The mean hydrodynamic diameter and zeta potential of the complexes were determined by DLS and PALS, respectively (Table 1). In general, adding 113 mM $CaCl_2$ to TAT complexes at N/P ratios between 4 and 33 induced a substantial decrease in the particle size (60-175 nm) with relatively narrow polydispersity. In comparison, PEI complexes possessed a small particle size (~74 nm) with zeta potential (16 mV) in the

absence of calcium. The zeta potential of TAT complexes produced particles with a positive surface charge (> 18 mV).

Table 1. The diameter of TAT complexes as a function of N/P ratio with 113 mM $CaCl_2$ concentration. Results are presented as mean ± SD (n = 3).

N/P ratio	Effective Diameter (nm)	Zeta potentials (mV)
4	171.8±3.9	15.7±0.8
9	165.1±3.7	18.2±1.9
13	133.2±4.3	17.5±2.0
18	121.0±1.9	20.3±0.3
25	77.3±9.6	19.0±1.4
33	55.1±7.5	23.6±1.6

5.3.2. *In vitro* transfection of A549 Cells

The transfection efficiencies of TAT and PEI complexes were evaluated in the human lung carcinoma cell line A549. Gene expression levels were dependent on the N/P ratio of the TAT complex (Figure 1). The gene expression of TAT complexes was enhanced by the inclusion of 113 mM $CaCl_2$ for the various N/P ratios. The highest transfection efficiency of TAT complexes was seen at N/P 25. Conversely, negligible expression was observed for TAT complexes without $CaCl_2$. In comparison, PEI had excellent transfection efficiency in the absence of $CaCl_2$.

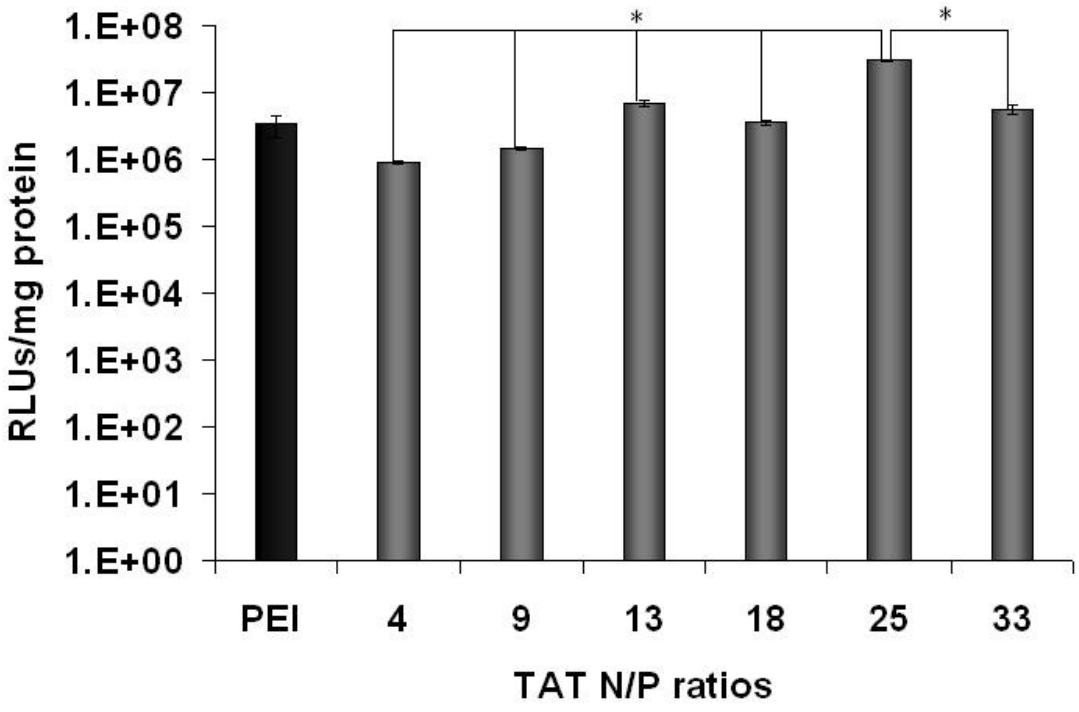
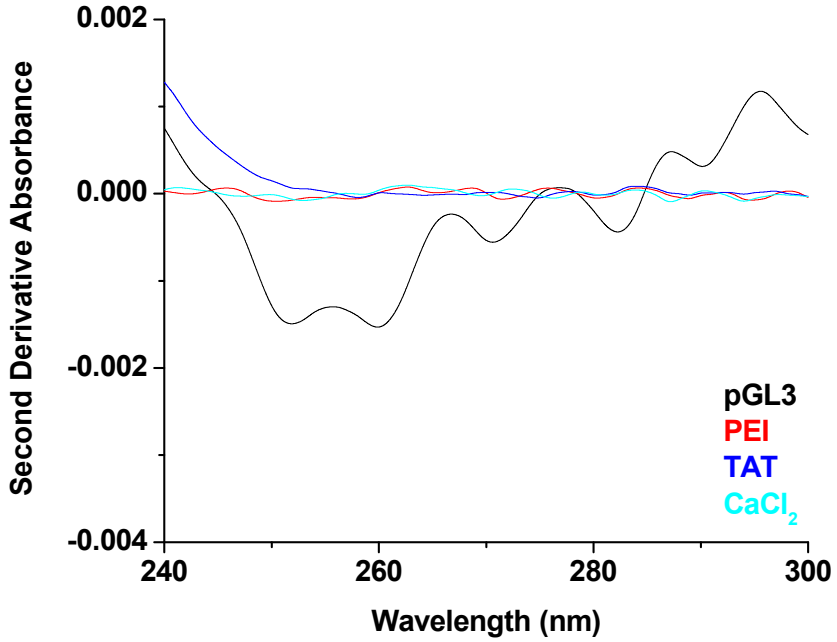


Figure 1. The transfection efficiency of TAT and PEI (N/P 10) complexes as a function of N/P ratio with 113 mM $CaCl_2$ concentration. Results are presented as mean ± SD (n = 3), *$p < 0.001$ for the various N/P ratios of TAT complexes as compared with TAT (N/P 25) complexes.

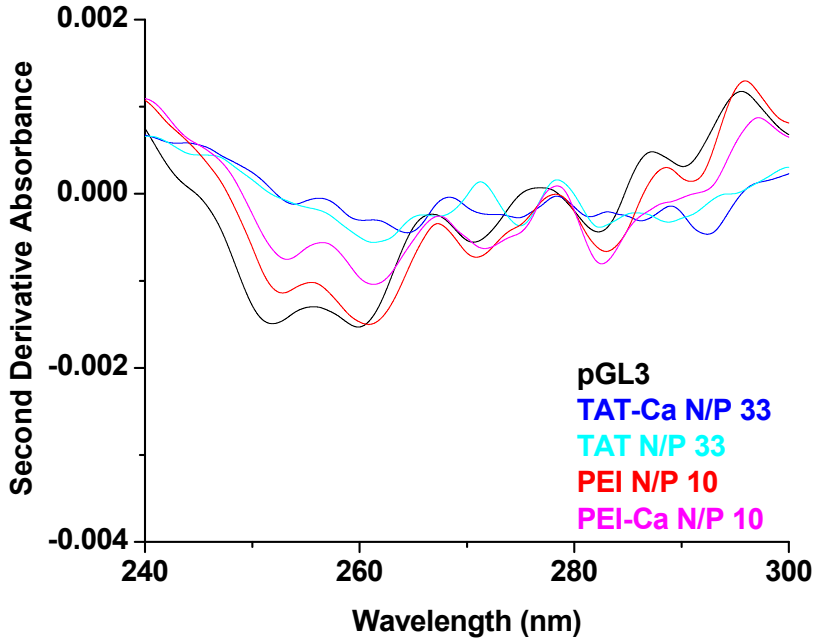
5.3.3. Second Derivative UV Absorption Spectroscopy

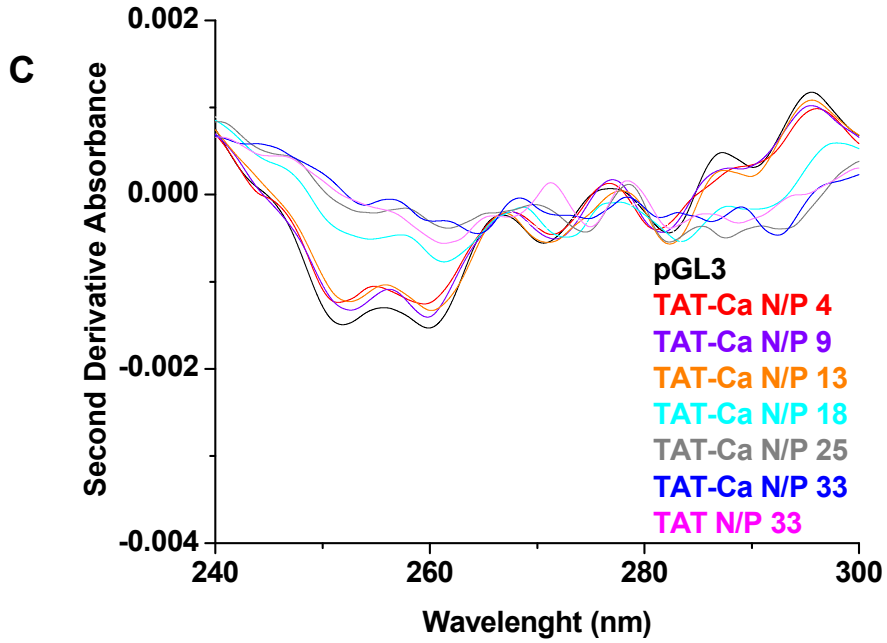
The UV spectrum of pDNA displayed an absorption maximum at 260 nm. Plasmid DNA second derivative analysis revealed numerous absorption peaks between 240 and 300 nm arising from the pDNA bases. Five positive peaks at approximately 256, 268, 277, 287 and 296 nm, and five negative peaks at 253, 259, 271, 282 and 291 nm were found (Figure 2A-E). The positions of these peaks are sensitive to the immediate environment of the corresponding residues. In general, the second derivative peaks of pDNA complexed with polycations display a shift to higher wavelengths when the polycations are in molar excess. TAT complexes in the absence and presence of 113 mM $CaCl_2$ showed a reduction in the absorbance and an increase in the wavelengths of these peaks. Above an N/P of 13, TAT complexes including calcium showed a reduction in the second derivative peaks of pDNA. Conversely, PEI complexes produced only small red shifts in the position of these peaks. Including calcium in the PEI complexes formulation revealed a marked decrease in the absorbance of the peaks. Free TAT, PEI and $CaCl_2$ had no significant peaks over the range of wavelengths examined.

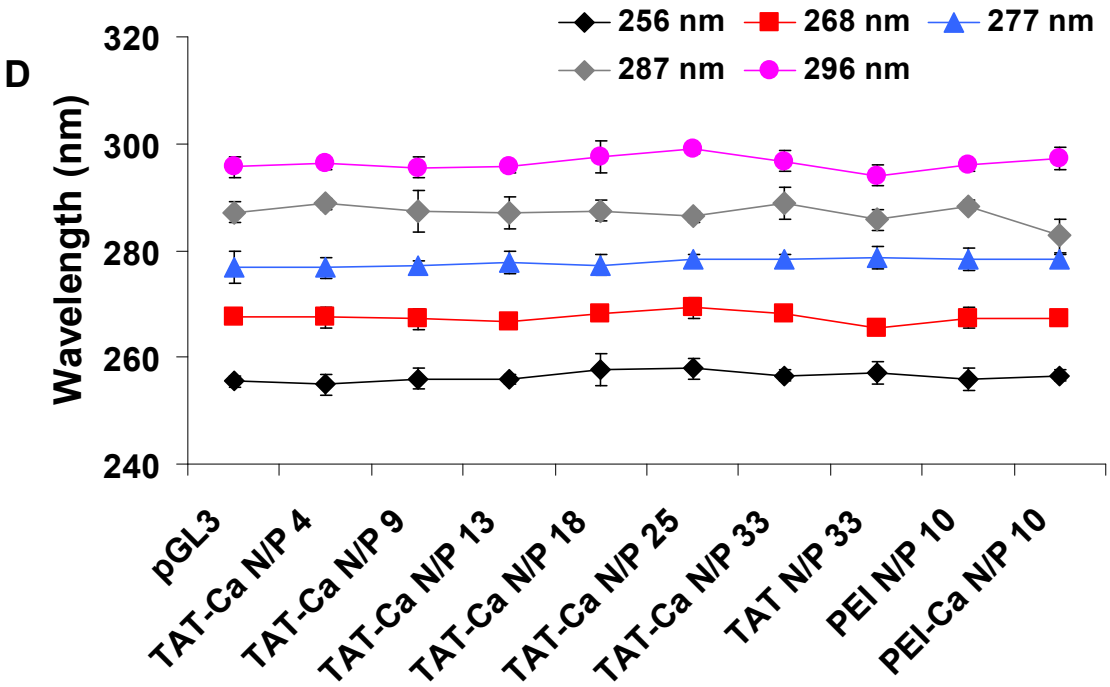
A


0.002
0.000
-0.002
-0.004
240
260
280
300
Second Derivative Absorbance
Wavelength (nm)
pGL3
PEI
TAT
CaCl2

B


0.002
0.000
-0.002
-0.004
240
260
280
300
Second Derivative Absorbance
Wavelength (nm)
pGL3
TAT-Ca N/P 33
TAT N/P 33
PEI N/P 10
PEI-Ca N/P 10


C
0.002
0.000
-0.002
-0.004
Second Derivative Absorbance
240
260
280
300
Wavelenght (nm)
pGL3
TAT-Ca N/P 4
TAT-Ca N/P 9
TAT-Ca N/P 13
TAT-Ca N/P 18
TAT-Ca N/P 25
TAT-Ca N/P 33
TAT N/P 33


D
256 nm
268 nm
277 nm
287 nm
296 nm
320
300
280
260
240
Wavelength (nm)
pGL3
TAT-Ca N/P 4
TAT-Ca N/P 9
TAT-Ca N/P 13
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TAT N/P 33
PEI N/P 10
PEI-Ca N/P 10

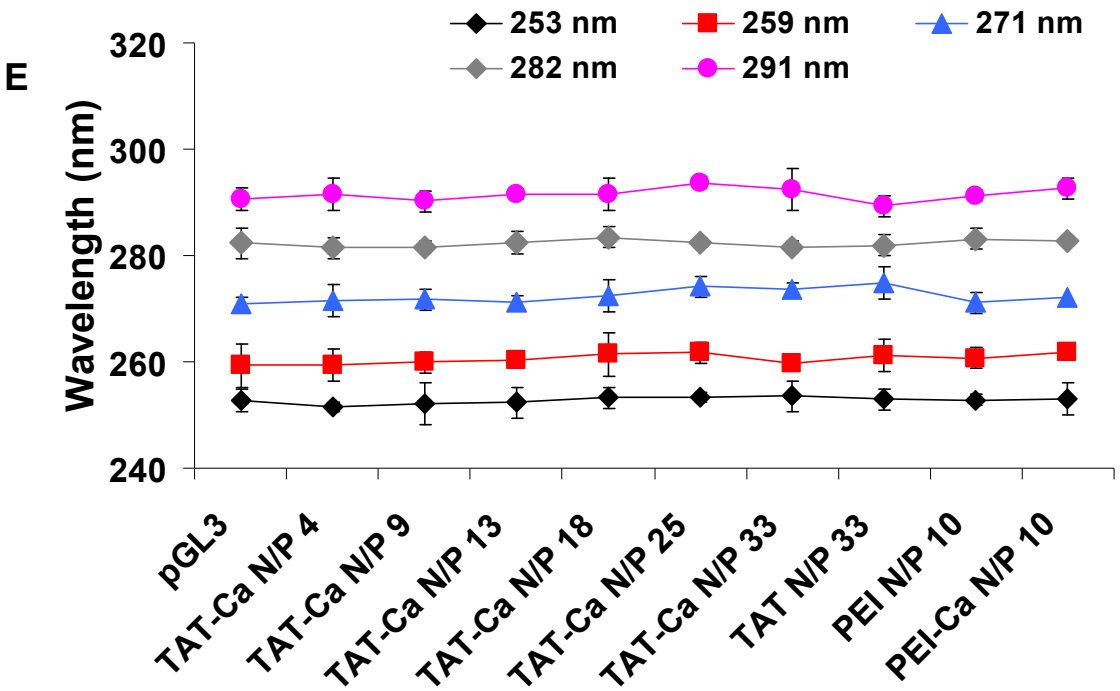


Figure 2. Second derivative UV spectra of (A) free TAT and PEI and (B and C) TAT and PEI complexes as a function of N/P ratio with and without 113 mM $CaCl_2$. The positive peak positions (D) and the negative peak positions (E) of the pDNA bases were plotted against the N/P ratios.

5.3.4. Fluorescence Studies

The condensation of pDNA by the cationic materials in the presence and absence of 113 mM $CaCl_2$ was studied using SYBR green dye [26]. This fluorophore is virtually nonfluorescent in solution but forms highly fluorescent complexes when bound to double-stranded DNA. As expected, the fluorescence intensity of free pDNA probed with SYBR green was significantly higher than when condensed by polycations (Figure 3A and B). It has been shown previously that the addition of cationic carriers to dye labeled DNA quenched the fluorescence of the dye without displacing it from the DNA [27]. The addition of TAT or PEI to pDNA showed a substantial decrease in the SYBR green fluorescence intensity with PEI complexes superior to TAT complexes in quenching SYBR green fluorescence. This implies that the pDNA was more tightly compacted by the PEI. Including 113 mM $CaCl_2$ in the formulation of the complexes resulted in a negligible reduction in the fluorescence of the complexes.

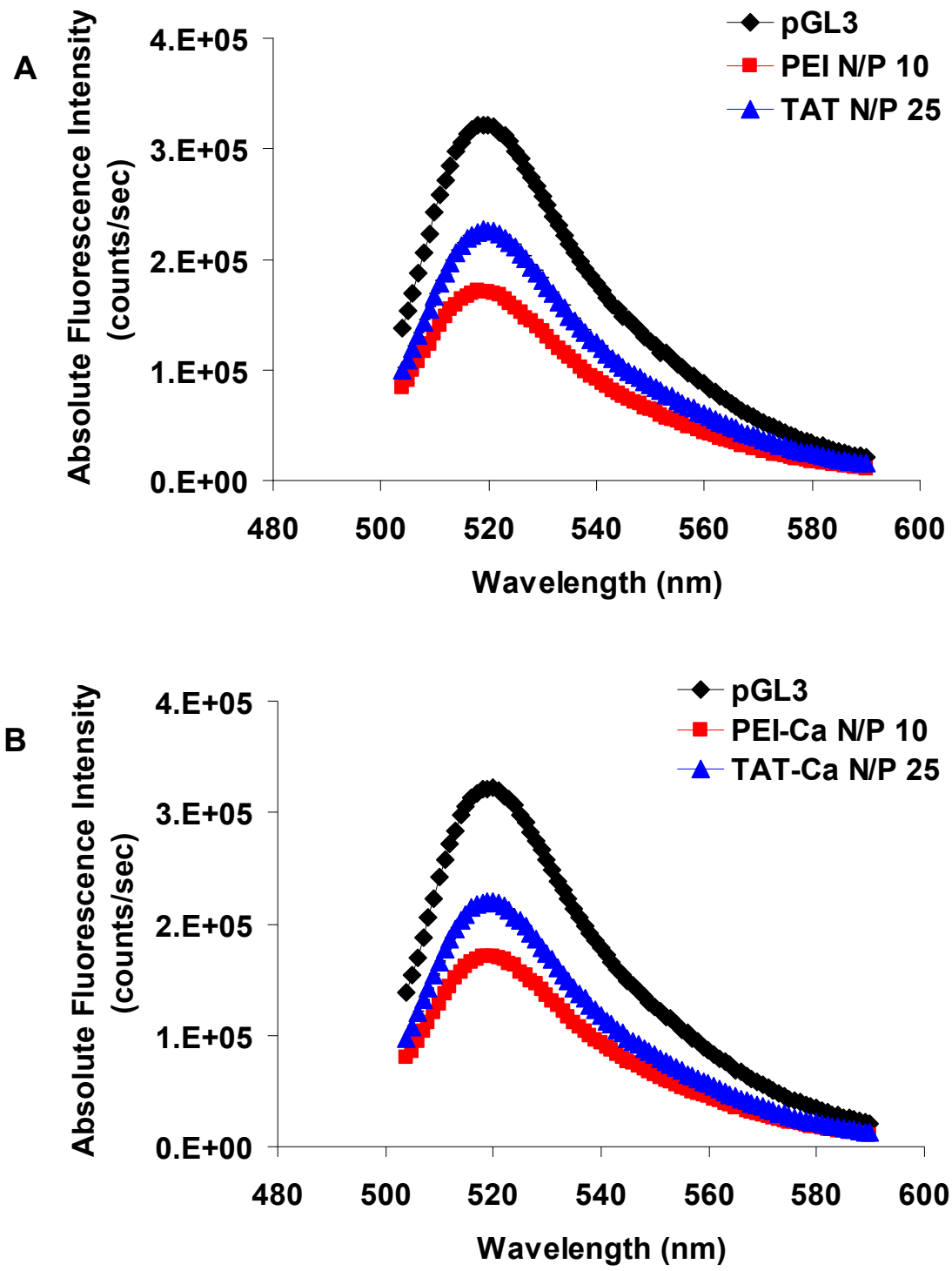
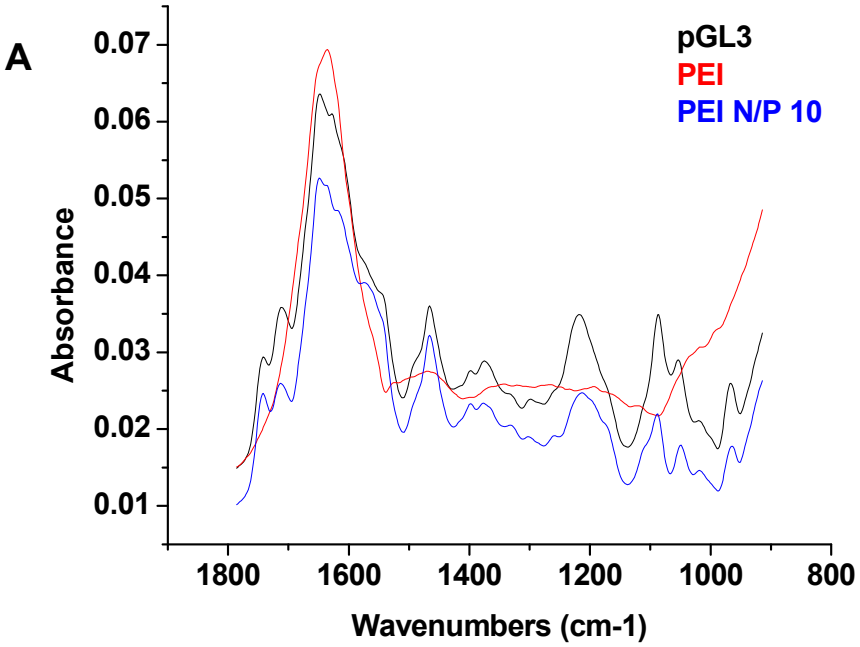
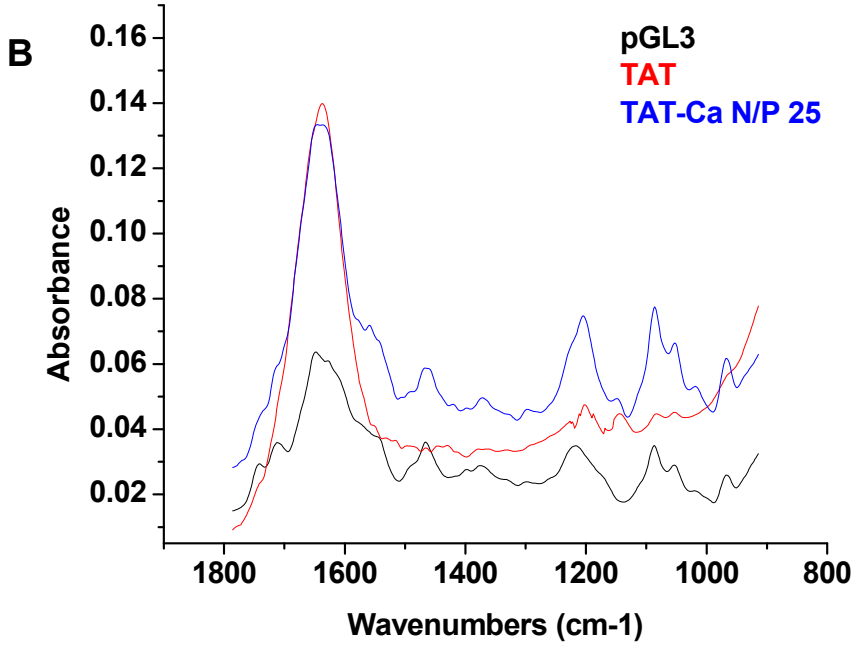


Figure 3. Extrinsic fluorescence spectra of TAT and PEI complexes in (A) the absence and (B) presence of 113 mM $CaCl_2$.

5.3.5. Infrared Spectral Properties of TAT and PEI complexes

FTIR spectroscopy was employed to investigate the secondary structure of the pDNA component of complexes. The FTIR spectra of pDNA and its complexes with TAT (N/P 25) including 113 mM $CaCl_2$ and with PEI (N/P 10) showed clear trends (Figure 4A and B). In the absence of cationic reagent, pDNA was in the B conformation as indicated by the presence of the guanine/thymidine (G/T) carbonyl stretching band at 1711 cm^{-1}, an asymmetric phosphate stretching vibration at 1217 cm^{-1} and a sugar-phosphate coupled vibration at 966 cm^{-1} [8,10,28]. Addition of TAT or PEI to pDNA induced an increase in the peak frequency of the base carbonyl stretching vibration and a decrease in the antisymmetric phosphate stretch (Figure 4C-F). No change in the peak position of the sugar-phosphate coupled vibration was observed upon addition of TAT to pDNA. In comparison, PEI induced a blue shift in the position of the vibration arising from the pDNA backbone. The position of a pDNA ribose sugar vibration in both TAT and PEI complexes (1053 cm^{-1}) shifted to lower frequency. Furthermore, no change in the peak position was observed for the symmetric phosphate vibration (~ 1087 cm^{-1}), which has been found to be independent of pDNA geometry [28].


A
pGL3
PEI
PEI N/P 10
Absorbance
0.07
0.06
0.05
0.04
0.03
0.02
0.01
1800
1600
1400
1200
1000
800
Wavenumbers (cm-1)


B
pGL3
TAT
TAT-Ca N/P 25
Absorbance
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
1800
1600
1400
1200
1000
800
Wavenumbers (cm-1)

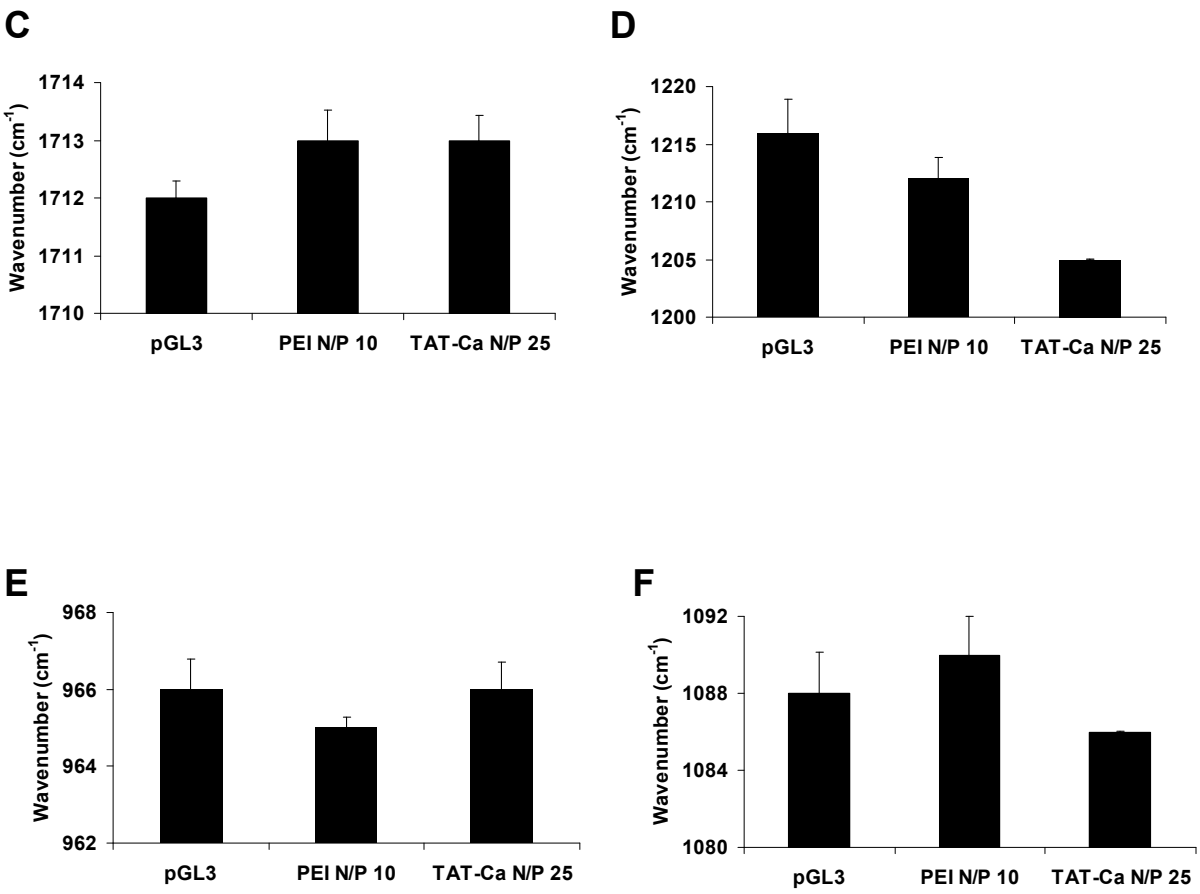


Figure 4. FTIR absorbance spectra of (A) TAT and (B) PEI complexes in solution with and without 113 mM $CaCl_2$. The peak positions of the pDNA base carbonyl (C), pDNA antisymmetric phosphate stretch (D), pDNA sugar-phosphate coupled vibration (E) and pDNA symmetric phosphate vibration (F) were plotted against the TAT and PEI complexes.

5.3.6. CD of TAT and PEI complexes

CD spectroscopy was also used to monitor the secondary structure of pDNA in TAT and PEI complexes. The polycations showed no intrinsic CD signal or ordered secondary structure within the UV region monitored. Therefore, the observed signals arose entirely from the pDNA molecules. The CD spectrum of uncomplexed pDNA demonstrated the characteristics of the native B form conformation, a positive peak at 275 nm and a negative peak at 245 nm (Figure 5). Upon TAT-Ca and PEI complexation, both regions of the pDNA CD spectrum were altered. In general, the spectra of TAT-Ca and PEI complexes showed a decrease in the value of the molar ellipticity of the positive and negative bands, concomitant with a red shift of the peak position of both the 275 nm and 245 nm bands although not to the same degree. A decrease in the ellipticity and a shift in the position to higher wavelengths of both bands were observed after calcium was added to PEI complexes. Exclusion of calcium from the TAT complexes resulted in a change in the overall shape of the spectrum, an increase in the 275 nm peak and a decrease in the 245 nm band. Shifts in the peak position of each band to higher wavelengths were also evident when excluding calcium.

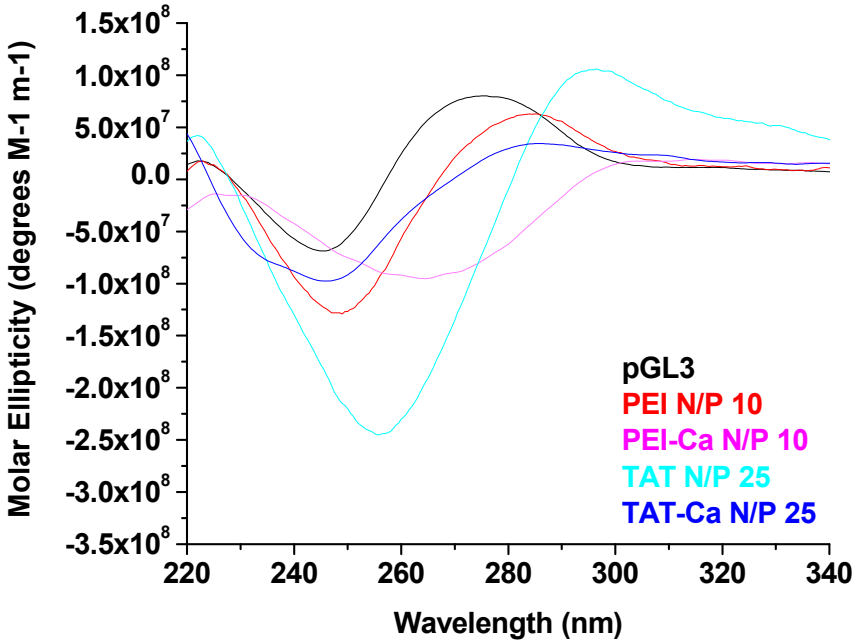


Figure 5. Effect of TAT and PEI in the absence and presence of 113 mM $CaCl_2$ on the CD spectra of pDNA in the complexes.

5.3.7. DSC of TAT and PEI complexes

The thermal stability of pDNA in TAT and PEI complexes was evaluated using DSC (Figure 6). Thermograms of pDNA displayed two major melting transitions. The first originated from supercoiled pDNA as a broad transition at ~ 90º C whereas the second arose from the linear/open circular species as a series of small transitions within the 60º-70º C range [14]. All forms of pDNA were thermally stabilized when complexed to TAT (N/P 25) including113 mM $CaCl_2$ or to PEI (N/P 10). The 25 kDa PEI stabilized both the supercoiled (100º C) and the linear/open circular (78º C) pDNA species at an N/P ratio of 10. The thermogram of TAT complexes demonstrated stabilization of both the supercoiled (~110º C) and linear/open circular form (~95º C) of pDNA with the thermal stability of pDNA in TAT complexes superior to that of PEI complexes.

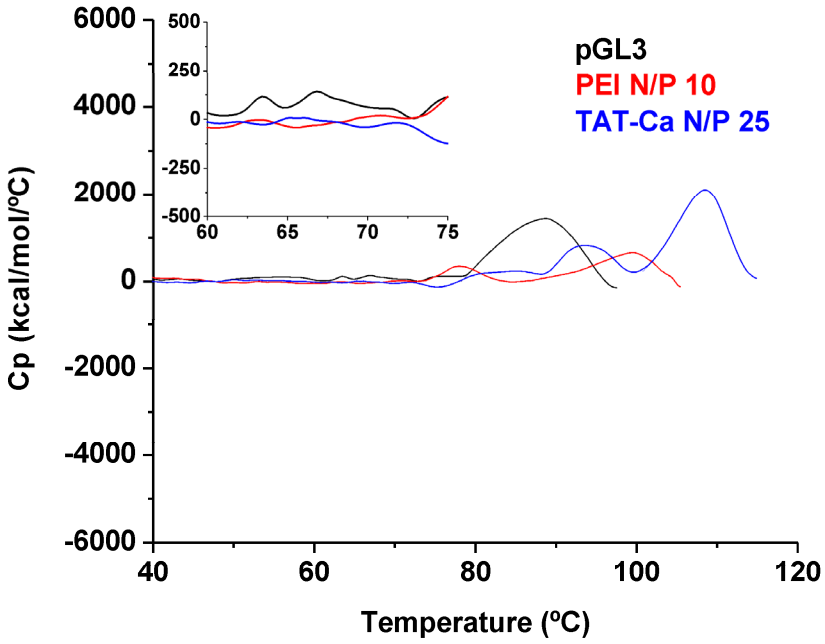


Figure 6. Effect of TAT and PEI in the absence and presence of 113 mM $CaCl_2$ on the thermal stability of pDNA in the complexes.

5.4. Discussion

In our earlier studies, it was established that adding certain concentrations of $CaCl_2$ (e.g., 113 mM) to CPP complexes produced small nanoparticles (60-175 nm), leading to gene expression levels higher than those observed for the more commonly used PEI complexes [19,25]. The transfection efficiency of TAT complexes depended on the N/P molar ratio. Gene expression of TAT complexes formulated with 113 mM $CaCl_2$ was sustained for at least 10 days and was not influenced by the presence of serum. Moreover, CPPs showed negligible cytotoxicity. The main goal of this study was to measure a wide variety of biophysical characteristics of pDNA in TAT complexes to better understand the structure of these particles and compare them to pDNA in PEI complexes.

As observed in previous reports, pDNA remains in the B form when complexed with different amounts, molecular weights and forms of PEI [21]. The thermal stability of the supercoiled and the linear/open circular forms of pDNA, however, was observed to increase in the presence of a charge excess of branched PEI (25 kDa). A direct correlation between the biophysical properties of PEI complexes and transfection efficiency was not, however, observed as had been previously reported [21].

Six methods were selected for this study to probe various structural aspects of the TAT and PEI complexes. DLS provided a direct measurement of the size [17]. The condensation of pDNA induced by polycations was evaluated by dye-binding fluorescence spectroscopy [24]. FTIR and CD have proven to be sensitive tools for characterizing the secondary structure of pDNA in complexes [8,10,28-30] with additional structural changes studied using UV second derivative absorption spectroscopy [29]. DSC was used to assess the thermal stability of pDNA within complexes [20].

A previous study has shown that the UV absorption spectra of pDNA originates from the purine and pyrimidine bases since the sugar phosphate backbones of pDNA produce very little contribution to its absorbance spectra above 200 nm [29]. Nucleotide bases have very low symmetry and several unbonded electrons. Due to the many different transitions that occur for each base in the spectral region from 200 to 300 nm [31], the second derivative spectra of pDNA is complex. The UV second derivative spectra of polynucleotides is known to be dependent on the presence of secondary structure resulting in an extensively describe hyper-chromic effect. Since the secondary structure does not change in this case, however, an interaction between TAT or PEI and pDNA or a subtle change in their internal environment is clearly indicated by the changes in the position and the value of the absorbance of the pDNA second derivative peaks. Despite these changes, the CD and FTIR results lead us to conclude that the pDNA is maintained the B form when complexed to various ratios of N/P TAT and PEI. Although the B form was maintained, the pDNA is condensed into small complexes based on the DLS data. Dye-based fluorescence studies also provide some indication that PEI compacted pDNA to a greater extent than TAT. Surprisingly, the SYBR green fluorescence intensities were not perturbed upon the addition of 113 mM $CaCl_2$ to the TAT and PEI complexes although calcium clearly does cause a significant decrease in the size of TAT complexes.

The FTIR spectra of TAT and PEI complexes showed that the pDNA remained in the B form although there were changes in the position of the pDNA carbonyl and asymmetric phosphate vibrations. The increases in frequency of the base carbonyl vibrations suggested altered hydrogen bonding occurred within the bases. Also, the reduced frequency of the antisymmetric phosphate stretching vibration can be directly

attributed to the electrostatic interactions between polycations and pDNA. All these results confirm interaction between polycation and pDNA where the pDNA was maintained in B form within the complexes but suggest induction of small changes in the structure of the pDNA.

CD spectra of the TAT and PEI complexes also suggested some changes in the structure of pDNA upon complexation, as shown by the changes in the pDNA CD peak positions and intensities. Previous studies suggested that these CD changes were best explained by limited local changes in the base/base interactions (20). Thus, the reduction of the value of the molar ellipticity likely indicated a decreased interaction between bases in the complexes. Moreover, such local structural perturbations may be due to a direct interaction between polycation and pDNA bases as observed in the change seen in the carbonyl stretching region with FTIR. The spectrum for TAT complexes including calcium supported such explanations, yielding a reduction in the molar ellipticity compared to the spectrum for TAT complexes. In addition, a previous study has shown that the CD spectra of Ca/pDNA complexes using different concentrations of calcium, general, were similar to the spectrum of pDNA alone with a slight decrease in the ellipticity at 275 nm [32]. Further increase in calcium concentration beyond ~25.9 mM did not affect the spectrum. Comparing these results suggested that calcium interacted with both amines (TAT) and phosphates (pDNA), which we have described previously [19]. The spectral changes seen are also consistent with absorption flattening but since they occur as the complexes are becoming smaller, this does not seem a probable explanation for the results.

DSC studies of TAT and PEI complexes confirmed an effect of complexation on the thermal stability of the supercoiled and linear/open circular pDNA components. Complexation by both polycations increased the melting temperature of all the forms of pDNA suggesting stabilization of the various pDNA structures. TAT complexes stabilized pDNA more than PEI complexes presumably as a result of the ability of calcium to selectively condense the TAT complexes. Thus, all of the analyses suggest some discrete differences in pDNA structure within TAT and PEI complexes.

5.5. Conclusion

Complexes of CPPs such as TAT with pDNA were previously established as a potent gene delivery vector when condensed into small complexes using calcium. The simplicity of the formulation, high levels of transfection efficiency and negligible effect on the viability of host cells inspired us to investigate the structure of pDNA within TAT complexes. Calcium concentration and N/P molar ratio were previously varied to maximize transfection efficiency and an optimized TAT complex was studied here. Extrinsic fluorescence studies provided evidence of compaction of pDNA, with PEI seemingly inducing 'tighter' complexes. FTIR and CD spectroscopies indicated that the secondary structure of pDNA was stabilized within the complexes. Nevertheless, DSC studies of pDNA components in TAT and PEI complexes demonstrated an enhancement in pDNA thermal stability. These data in combination with the transfection efficacy and the low cytotoxicity of TAT complexes support further exploration and perhaps new design schemes based on this novel nonviral gene delivery vector. Nevertheless, the exact relationship between the structure of the complexes and their efficiency as gene delivery vehicles remains elusive.

5.6. References

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Chapter 6.

Calcium Condensed siRNA Complexes with TAT or dTAT Peptides Offers Simple, Highly Effective, and Noncytotoxic Gene Silencing

6.1. Introduction

The discovery and development of small interfering RNA (siRNA) has provided an elegant strategy for disabling specific genes responsible for pathological disorders [1,2]. Since its discovery, siRNA has been developed as a screening tool for cancer studies [3-5], and has been evaluated as a potential therapeutic agent for variety of nucleic acid based diseases such as HIV [6], hepatitis C [7] and cancer [8,9]. Double stranded siRNA is able to silence genes effectively in a sequence specific manner [10,11]. Once in the cytoplasm, siRNA is loaded into RNA-induced silencing complex (RISC) [12-14]. Within RISC, the passenger strand is degraded while the guide strand is loaded into Argonaut 2 (Ago2), the active nuclease component of RISC. The guide strand binds the complementary sequence of the target mRNA and catalyzes the cleavage of this mRNA, thus silencing the translation of the target mRNA into protein.

The major limitation to clinical application of siRNA, like most nucleic acid based therapeutics, is poor cellular uptake due to the low permeability of the cell membrane to negatively charged molecules [15,16]. Also, enzymatic lability, rapid renal clearance, low transfection efficiency and immune stimulation by siRNA have further delayed therapeutic applications [17-20]. Several siRNA delivery systems have been proposed, including chemical modification of siRNA, lipids, cationic polymers, antibody-protamines, RNA-aptamers and cell penetrating peptides (CPPs) [21-28]. In most cases, toxicity of the delivery system and the endosomal escape are key challenges [29-32].

In general, toxicity can be ameliorated by conjugation with biocompatible, hydrophilic polymers such as poly(ethylene glycol) (PEG) or hydroxypropyl methacrylamide (HPMA), or by removing excess (uncomplexed) cationic polymers [33-36].

Biodegradable polymers are attractive candidates for the design of siRNA polyplexes that often show low toxicity [37,38]. The main hurdle for these vectors is endosomal escape. The siRNA vector or at least the siRNA must escape before degradation in the late endolysomal compartment. In order to circumvent this problem, many strategies have been employed to enhance endosomal escape. pH responsive lipid or lipid-like molecules and viral fusogenic proteins and peptides have promoted endosomal escape via membrane destabilization through a pH dependent shift in their conformation [39-48]. Moreover, polymers such as poly(propylacrylic acid) (PPAA) undergo a hydrophilic to hydrophobic transition at endosomal pH offering another mechanism to mediate membrane disruption [49-54].

Cell penetrating peptides (CPPs) have the earmarks of promising delivery vehicles for intracellular delivery of siRNA [55-60]. Two main strategies have been investigated; chemical linkage of CPPs with siRNA [58,61,62] and noncovalent complexes with siRNA. Complexes using either liposomes or polyelectrolytes, in general, yield significant biological responses [24,55,63]. Various CPPs, including TAT and 3-methyladenine-DNA glycosylase (MPG) proteins from HIV-1 [64-67], as well as polyarginine, penetratin and TP10 [68-70] have been reported to improve siRNA delivery into various cell lines. The interaction between the positive charges of CPPs and the negative charges of phospholipids and/or proteoglycans on the extracellular membrane surface is crucial for CPP-mediated uptake [71]. Due to the negative charge of siRNA and the cationic nature of CPPs, it is very cumbersome to generate covalent conjugates and the CPPs can be effectively neutralized [72]. Moreover, both covalent conjugation and electrostatic complexes of siRNA with CPPs were found to be trapped in endosomes even after

efficiently entering cells [73-76] and required additional molecules to facilitate cytosolic release of siRNA [77-80].

We have previously demonstrated that adding calcium to CPP/pDNA complexes can reduce particle size and maximize transfection efficiency [81,82]. This nonviral vector was found to display high transfection efficiency, even in the presence of serum, and negligible toxicity *in vitro* and *in vivo*. In this study, our objective was to investigate the ability of this system using the CPPs TAT and dTAT (a modified form of TAT) for siRNA delivery. The efficacy of siRNA delivery has been studied as a function of CPP/siRNA charge ratio and siRNA dose. The size and charge of TAT and dTAT complexes were examined by using dynamic light scattering analysis and transmission electron microscopy. Finally, the viability of A549 human lung carcinoma cells was investigated by using an MTS cytotoxicity assay of free TAT, dTAT or branched PEI.

6.2. Materials and methods

6.2.1. Materials

Anti-luciferase siRNA-1 (MW 13358 g/mol) and siRNA control (non-targeting) were supplied by Thermo Scientific Dharmacon® (Chicago, IL). TAT peptide (RKKRRQRRR; Mw = 1338.6 Da) and dTAT (RKKRRQRRRHRRKKR; Mw = 2201.7 Da) peptide were purchased from Biomatik Corporation (Cambridge, Ontario, Canada). Branched polyethyleneimine (PEI, 25 kDa) was obtained from Aldrich (Milwaukee, WI). Calcium chloride ($CaCl_2$. $2H_2O$), Nuclease-free water and BCA™ Protein Assay were purchased from Fisher Scientific (Pittsburgh, PA). A549-luc-C8 Bioware® cell line was obtained from Caliper LifeSciences (Hopkinton, MA). The cell culture medium (RPMI-1640) and (Ham's F-12 Nutrient Mixture, Kaighn's modified with L-glutamine) were

purchased from the American Type Culture Collection (ATCC, Rockville, MD). Heat inactivated fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). Penicillin-streptomycin was purchased from MB Biomedical, LLC (Solon, OH). Trypsin-EDTA was purchased through Gibco (Carlsbad, CA). MTS reagent [tetrazolium compound; 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] and Luciferase Assay System were purchased from Promega (Madison, WI).

6.2.2. Preparation of complexes

TAT, dTAT and PEI complexes were prepared essentially as described previously [81,82]. Briefly, various amounts of polycations and siRNA were first dissolved in known volume of nuclease-free water (NFW). Ten microliters (e.g., 10 nM) of siRNA solution was added rapidly to fifteen microliters polycation (TAT, dTAT or PEI) solution while pipetting. To this solution, fifteen microliters (e.g., 23.1 mM) $CaCl_2$ (or NFW in the case of PEI) was added and mixed by vigorous pipetting. This resulted in different N/P ratios of polycation/siRNA complexes. The complexes then were allowed to form during 20 min incubation at 4º C prior to use. Complexes were freshly prepared before each individual analysis.

6.2.3. Size and Zeta potential measurement

The effective hydrodynamic diameter of the complexes was analyzed using a dynamic light scattering (DLS) system (Brookhaven Instrument, Holtsville, NY) equipped with a 50 mW HeNe laser operating at 532 nm. The complexes were prepared at a constant pDNA concentration of 100 μg/mL whereas the N/P ratios of the complexes

were varied. The scattered light was monitored at 90º to the incident beam. For each sample, the data was collected continuously for three 1-min intervals. The diameter of the complexes was obtained from the diffusion coefficient by the Stokes-Einstein equation using the method of cumulants. Zeta potential measurements were obtained by phase analysis light scattering using a Brookhaven Zeta PALS instrument. The electrophoretic mobility of the samples was determined from the average of 10 cycles of an applied electric field. The zeta potential was determined from the electrophoretic mobility from the Smoluchowski approximation.

6.2.4. Cell culture

Culturing of human epithelial lung cell line A549-luc-C8, stably expressing luciferase, was performed according to the protocol provided by Caliper LifeSciences. A549-luc-C8 cells were grown in RPMI-1640 supplemented with 10% (v/v) heat inactivated FBS and 1% (v/v) Penicillin/streptomycin at 37º C in a humidified air atmosphere containing 5% CO_2.

6.2.5. *In vitro* luciferase gene knockdown studies

A549-luc-C8 cells were trypsinized, counted and diluted to a concentration of approximately 100,000 cells/ mL. Then, 0.1 mL of that dilution was added to each well of a 96-well plate and the cells were incubated in a humidified atmosphere at 5% CO_2 and 37°C. Twenty-four hours before transfection, the cells were washed once with PBS and 100 µl of sample at 10, 25 or 50 nM siRNA concentration (20% of complex to 80% of serum-free cell culture medium (Ham's F-12 Nutrient Mixture, Kaighn's modified with L-glutamine)) was added to each well. Cells were incubated with the complexes for

5 h. The media was then removed by aspiration and 100 μL of fresh serum medium (RPMI-1640) was added followed by further incubation (48 h). In addition to the anti-luc siRNA, a non-silencing siRNA sequence was used to ensure that the decrease in luciferase expression was due to the anti-luc siRNA and not to cytotoxicity effects of the vector. The Luciferase Assay System from Promega was used to determine luciferase gene silencing following the manufacturer's recommended protocol. The light units were normalized against protein concentration in the cells extracts, which were measured using the BCA™ Protein Assay. The data were expressed as a percentage of the control (non-specific siRNA control).

6.2.6. Assessment of cytotoxicity (MTS Assay)

The cytotoxicity of polymers was determined by the CellTiter 96® Aqueous Cell Proliferation Assay. A549-luc-C8 cells were grown as described in the transfection experiments. Cells were treated with the TAT, dTAT or PEI for ~24 h. The media were then removed and replaced with a mixture of 100 μL fresh culture media (RPMI-1640) and 20 μL MTS reagent solution. The cells were incubated for 3 h at 37°C in the 5% CO_2 incubator. The absorbance of each well was then measured at 490 nm using a microtiter plate reader (SpectraMax, M25, Molecular Devices Corp., CA) to determine cell viability.

6.3. Results

6.3.1. Particle Sizes and Zeta potentials of TAT, dTAT and PEI complexes

TAT, dTAT and PEI complexes were prepared by mixing siRNA with each polycation at various N/P ratios. These complexes were thoroughly mixed by pipetting

and $CaCl_2$ was added (final concentration 23.1-69.2 mM). The particle size and zeta potential of the complexes prepared by 25 nM of siRNA were determined by DLS and ZetaPALS, respectively (Table 1A and B). In general, the added $CaCl_2$ produced small TAT and dTAT complexes at all N/P ratios (58.5-201.3 nm) with polydispersity (< 0.24). With increasing N/P ratios and using the higher concentrations of calcium, the size of the TAT and dTAT complexes generally decreased. In comparison, PEI complexes showed a small particle size (90 nm) with modest high zeta potential (~20 mV) in the absence of calcium. The zeta potential of TAT and dTAT complexes produced particles with a positive surface charge (> 15 mV) and the charge did not vary much with the N/P ratio.

Table 1. The diameter of (A) TAT and (B) dTAT complexes as a function of N/P ratio and $CaCl_2$ concentrations. Results are presented as mean ± SD (n = 3).

A

N/P ratio & ($CaCl_2$ mM)	Effective Diameter (nm)	Polydispersity
7 (00.0)	1012.0±23.4	0.265
7 (23.1)	139.7±3.9	0.108
7 (34.6)	113.7±3.7	0.211
7 (69.2)	116.6±11.3	0.125
18 (00.0)	1411.3±54.1	0.195
18 (23.1)	112.0±6.9	0.230
18 (34.6)	99.9±10.8	0.134
18 (69.2)	60.6±7.5	0.139
25 (00.0)	2105.0±87.9	0.263
25 (23.1)	144.2±2.1	0.222
25 (34.6)	117.8±9.7	0.178
25 (69.2)	59.3±6.4	0.203
33 (00.0)	2210.0±76.9	0.271
33 (23.1)	152.9±8.1	0.169
33 (34.6)	122.8±4.5	0.225
33 (69.2)	58.5±7.3	0.189

B

N/P ratio & ($CaCl_2$ mM)	Effective Diameter (nm)	Polydispersity
6 (00.0)	1170.4±90.4	0.199
6 (23.1)	124.3±9.4	0.143
6 (34.6)	101.7±15.2	0.179
6 (69.2)	98.5±6.8	0.201
17 (00.0)	2256.5±45.1	0.253
17 (23.1)	189.3±5.1	0.230
17 (34.6)	203.4±9.4	0.177
17 (69.2)	152.0±13.6	0.215
23 (00.0)	1978.2±68.2	0.223
23 (23.1)	155.7±4.2	0.242
23 (34.6)	113.4±7.9	0.196
23 (69.2)	89.7±5.1	0.103
31 (00.0)	2190.5±39.6	0.247
31 (23.1)	197.4±8.3	0.122
31 (34.6)	145.2±1.9	0.108
31 (69.2)	90.1±3.3	0.127

6.3.2. Cytotoxicity of TAT, dTAT and PEI complexes

Efficient delivery together with low cytotoxicity is extremely desirable. To evaluate the cytotoxicity of free TAT, dTAT, and PEI, an MTS assay was performed by incubating A549-luc-C8 cells with up to 5 mg/mL of TAT, dTAT or PEI for 24 h (Figure 1). TAT peptide revealed almost no evidence of cytotoxicity and cells maintained high viability, while dTAT showed modest cytotoxicity (IC_{50} ~ 4000 µg/mL). Branched PEI induced substantial cytotoxicity (IC_{50} 22 µg/mL) as expected.

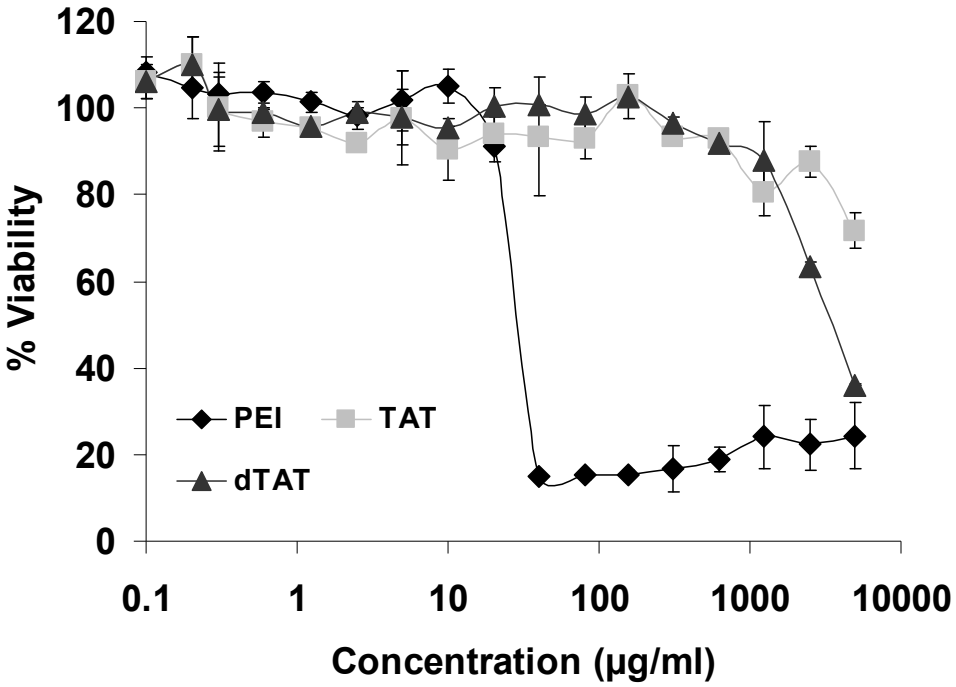
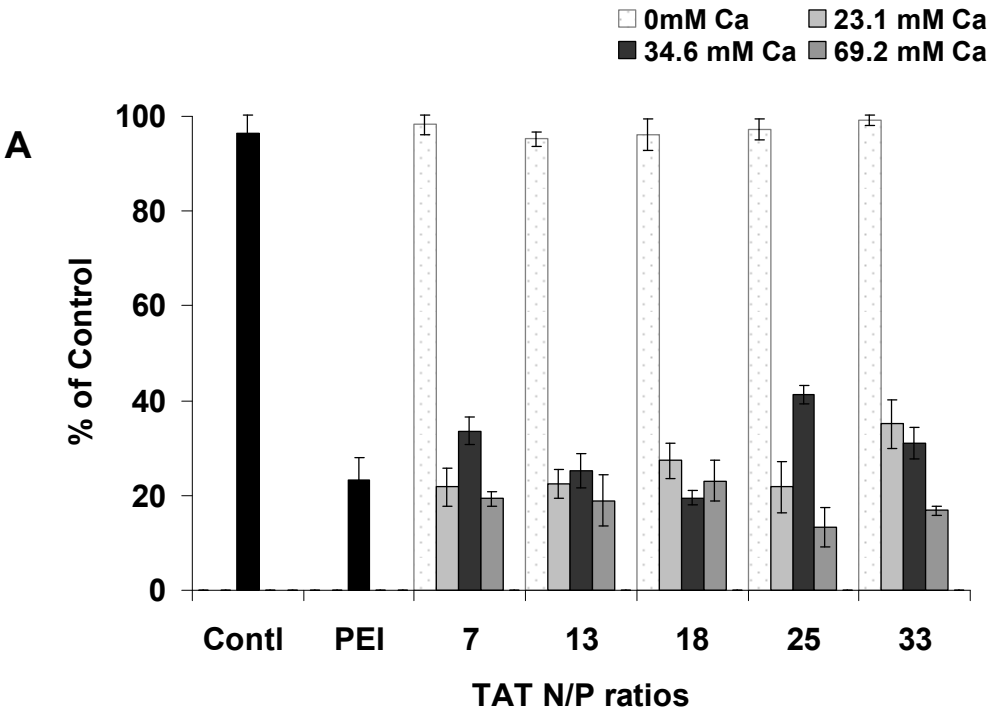


Figure 1. Cytotoxicity profiles of TAT, dTAT and PEI in A549-luc-C8 cells. Viability is expressed as a function of polymer concentration. Results are presented as mean ± SD (n = 3).

6.3.3. *In vitro* luciferase gene knockdown by TAT, dTAT and PEI complexes

The silencing efficiency of TAT and dTAT complexes was investigated using the human lung carcinoma cell line A549-luc-C8. This cell line stably expresses firefly luciferase. Luciferase knockdown was evaluated 48 h after treatment with the TAT, dTAT or PEI polyplexes, and the data were shown relative to the luciferase protein levels of cells treated with control siRNA complexes. Five different N/P ratios of TAT or dTAT complexes were condensed by adding different concentrations of $CaCl_2$ (23.1, 34.6, and 69.2 mM) after complex formation. Different siRNA doses (10, 25 or 50 nM) were studied and compared to PEI polyplexes (N/P 10). In general, TAT and dTAT complexes showed a higher level of luciferase knockdown for the various N/P ratios and $CaCl_2$ concentrations when compared to PEI, which had excellent interference in the absence of $CaCl_2$ (Figure 2 and 3). The level of luciferase knockdown of TAT and dTAT complexes seemed to depend on N/P ratio and $CaCl_2$ concentration. TAT and dTAT typically showed the greatest gene silencing at high calcium concentration (69.2 mM) and moderately high N/P ratios (N/P ratios of 25 and 23, respectively). The luciferase knockdown of PEI complexes was found to be somewhat independent of the siRNA dose. Strikingly, no luciferase knockdown was observed for TAT and dTAT complexes without $CaCl_2$. It is important to note that the TAT and dTAT siRNA control complexes including 69.2 mM $CaCl_2$ did not affect luciferase expression levels, which further indicates that these vectors did not influence the viability of A549-luc-C8 cells.


A
0mM Ca
23.1 mM Ca
34.6 mM Ca
69.2 mM Ca
% of Control
100
80
60
40
20
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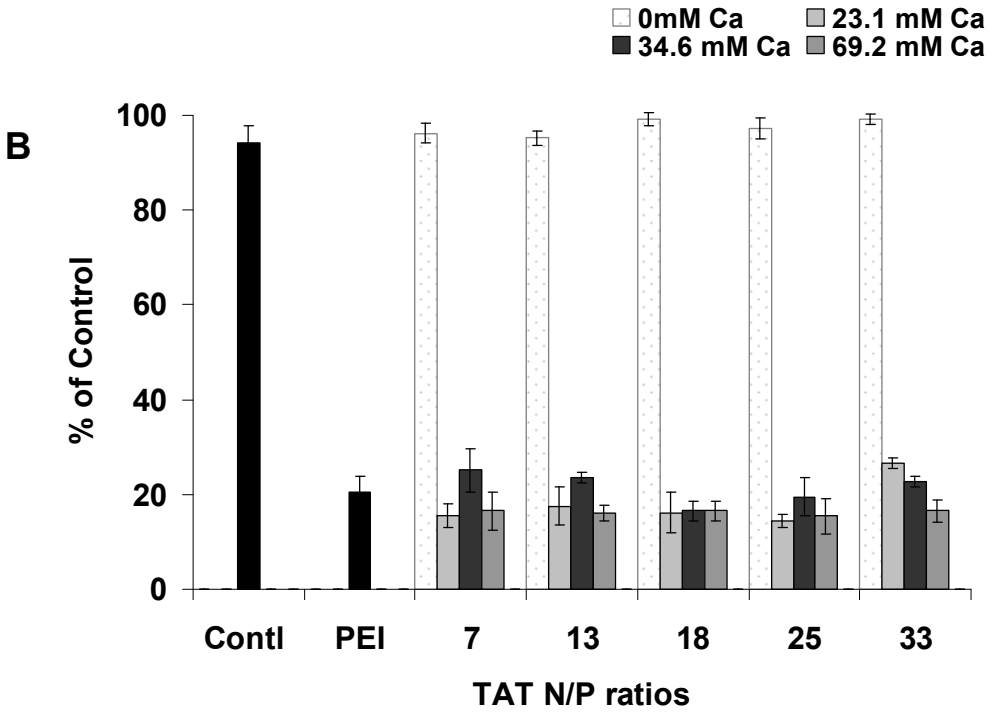
Contl
PEI
7
13
18
25
33
TAT N/P ratios


B
0mM Ca
23.1 mM Ca
34.6 mM Ca
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TAT N/P ratios

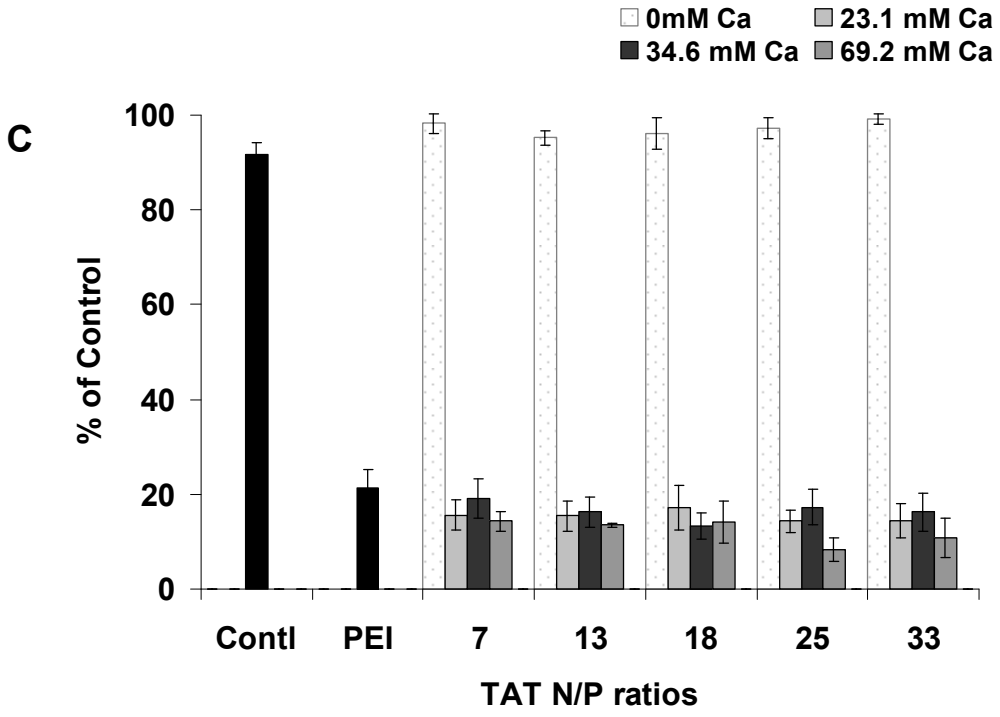
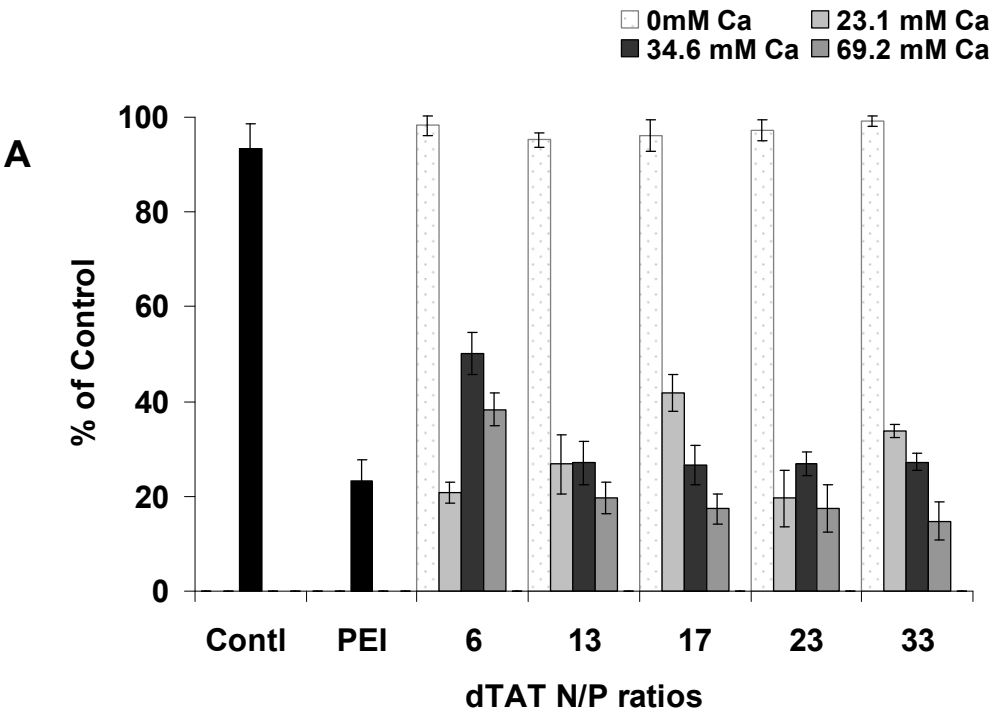


Figure 2. The luciferase knockdown of TAT and PEI complexes as a function of N/P ratio with different concentrations of added $CaCl_2$ using (A) 10, (B) 25 and (C) 50 nM of siRNA. Results are presented as mean ± SD (n = 3).


A
0mM Ca
23.1 mM Ca
34.6 mM Ca
69.2 mM Ca
% of Control
100
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Contl
PEI
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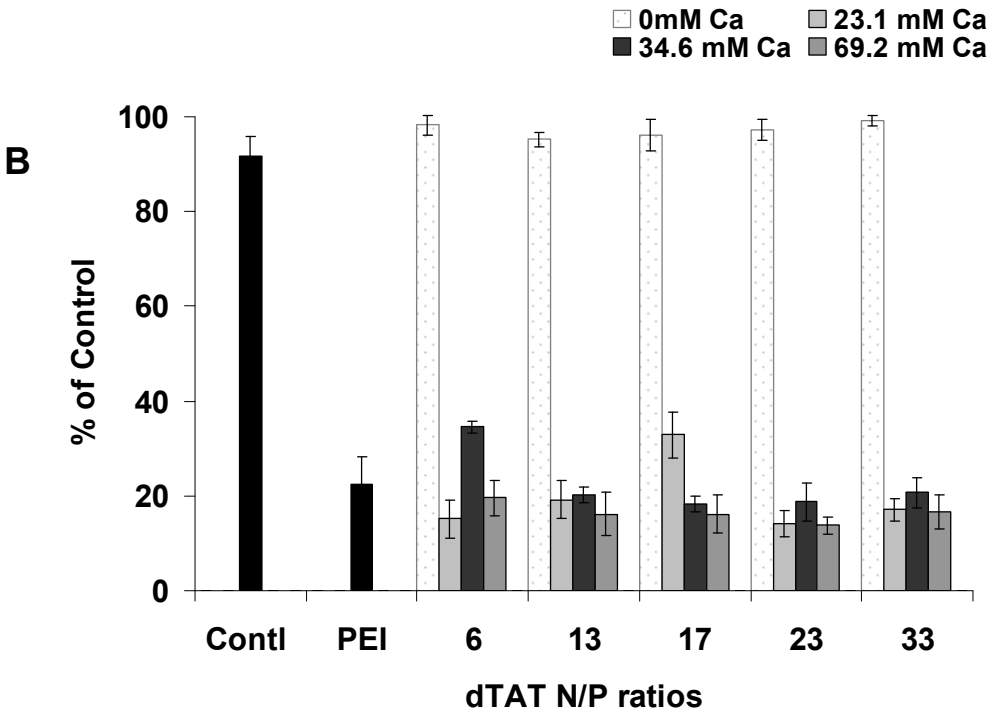
33
dTAT N/P ratios


B
0mM Ca
23.1 mM Ca
34.6 mM Ca
69.2 mM Ca
% of Control
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PEI
6
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17
23
33
dTAT N/P ratios

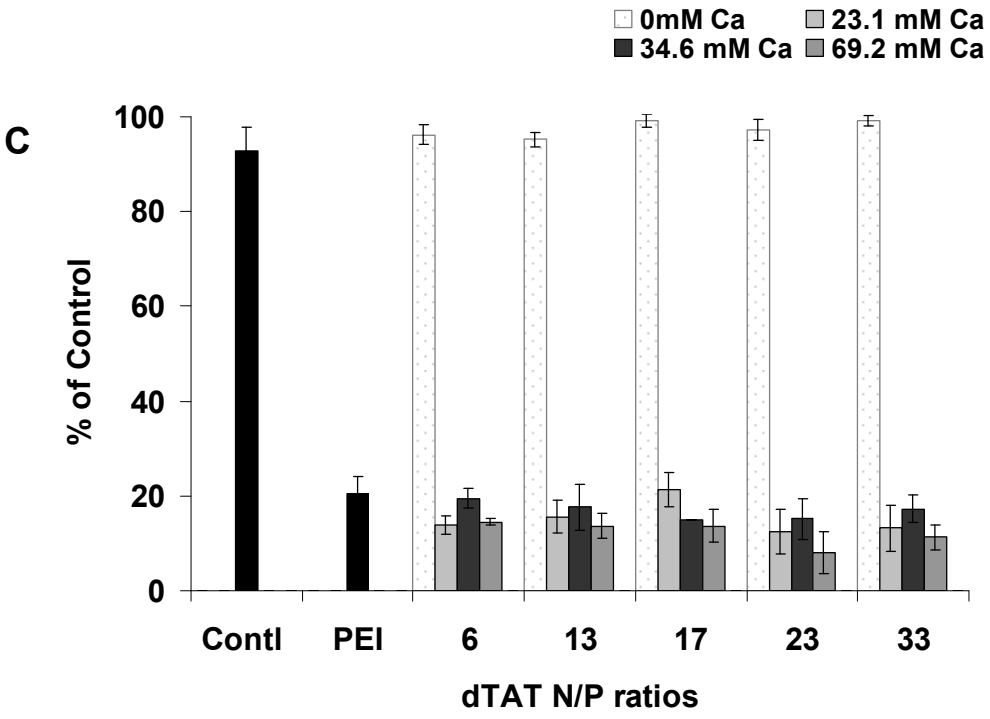

Figure 3. The luciferase knockdown of dTAT and PEI complexes as a function of N/P ratio with different concentrations of added $CaCl_2$ using (A) 10, (B) 25 and (C) 50 nM of siRNA. Results are presented as mean ± SD (n = 3).

6.4. Discussion

Previous studies have shown that liposome mediated delivery may provide efficient knockdown *in vitro* but such systems can be inefficient when applied *in vivo* [83]. Among polymeric vectors, derivatives of PEI such as degradable oligo-ethylenimine, PEGylated PEI and low molecular weight PEI have been used as siRNA carriers [84-86]. It seems, however, that these systems may have limited efficiency in siRNA delivery compared to pDNA delivery and that toxicity may still be a concern [87,88].

Highly efficient intracellular delivery mediated by CPPs has been confirmed in a variety of cell lines with minimal toxicity, overcoming challenges often faced with other delivery methods. We have previously developed CPP polyelectrolyte complexes as a more efficient and less toxic means of gene delivery [81,82]. The use of calcium to condense CPP/pDNA complexes resulted in small particles leading to gene expression levels higher than PEI polyplexes. These complexes were stable, maintaining particle size in the absence and presence of 10% of FBS. Moreover, the transfection efficiency was not significantly affected by the presence of serum. The CPPs also showed negligible cytotoxicity compared with PEI, which was very cytotoxic.

In this study, TAT and dTAT complexes including $CaCl_2$ were investigated to determine whether these vectors could effectively deliver siRNA while minimizing cytotoxicity. Various N/P ratios of TAT and dTAT with different calcium concentrations and different doses of siRNA were used. Calcium was found to form compact TAT and dTAT complexes (58.5-201.3 nm) leading to high knockdown efficiencies in A549-luc-C8 lung epithelial cells. TAT and dTAT showed the smallest particle sizes at high calcium concentration (69.2 mM) across various N/P ratios. The size of these complexes

did not appear to correlate with enhanced gene silencing. A small siRNA dose of 10 nM was enough to knockdown luciferase expression by up to 87%. It is important to note that absolutely no gene knockdown was observed for TAT and dTAT complexes without $CaCl_2$.

6.5. Conclusion

A myriad of design strategies for siRNA vectors in the last few years has led to a broad spectrum of various liposomal and cationic polymer systems. Recently, there have been several advances in the use of complexes using CPPs [62,89-92]. The low levels of gene silencing observed when using these low molecular weight polycations as siRNA condensing agents must to be improved [93]. Here, we show that calcium addition to TAT or dTAT complexed with siRNA induced a substantial decrease in the particle size. As compared to the PEI complexes, the gene silencing of these CPP/siRNA complexes was significantly higher in A549-luc-C8 lung epithelial cells. Furthermore, the TAT and dTAT peptides showed negligible cytotoxicity up to 5 mg/mL. The combined features of high gene silencing with low cytotoxicity of TAT and dTAT complexes appear to make them attractive candidates for nonviral siRNA delivery vectors *in vivo*.

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Chapter 7.

Conclusion and Future Directions

7.1. Conclusion

Gene therapy holds the promise of correcting a genetic defect or altering protein expression to address disease. Various strategies have been explored including replacement of an endogenous gene or inhibition of oncogenes using interference such as small interfering RNA (siRNA). Despite advances in the field, the delivery of nucleic acids to target cells or tissues is still challenging. There are many obstacles to overcome in developing effective nucleic acid therapeutics including tissue and cellular transport barriers, enzymatic degradation, endosomal escape, toxicity, immunogenicity, and rapid clearance after administration.

Successful transfer of nucleic acids into cells usually relies on the use of efficient carriers, commonly viral or nonviral vectors. Viral vectors often show highly effective gene transfer but are deficient in several areas, including the induction of host immune response and challenging, expensive scale-up. Nonviral vectors are potential alternatives to viral vectors that may overcome several problems encountered in viral vector-mediated therapy [1-3]; however, nonviral vectors are often inhibited by poor transfection and suffer from toxicity, particularly for *in vivo* applications [4,5]. Cationic lipids and cationic polymers are the most commonly employed nonviral carriers[6-14].

The objective of this thesis has been to design less toxic vectors for gene delivery that offer high transfection efficiency. Poly(D,L-lactide-*co*-glycolide) (PLG) nanoparticles coated with different surface modifiers were synthesized as a first approach. A variety of cationic coating materials and conventional surfactants were explored to enhance the ability of PLG nanoparticles to transfect A549 lung epithelial cells. High encapsulation efficiencies of pDNA with enhanced cellular uptake of PLG nanoparticles were achieved

using cationic coating materials. Low levels of gene expression were sustained for at least 14 days for several PLG nanoparticle formulations and gene expression levels tended to increase over the duration of the study. *In vitro* cytotoxicity studies showed that cationic PLG nanoparticles exhibited very low cytotoxicity compared to PEI. The complexity and lack of high gene expression compelled us to explore other potential low toxicity vectors.

Cell penetrating peptides (CPPs) were considered as potential polycations to complex and deliver nucleic acids. Calcium was found to compact CPP complexes (~60 nm) through "soft" crosslinks that could be competitively disrupted in order to increase transfection efficiency. The ability of calcium to condense CPP complexes occurred as a result of calcium interactions with both amines (polycations) and phosphates (DNA). CPP-Ca complexes were stable, maintaining particle size in the absence and presence of 10% of FBS. Gene expression of CPP-Ca complexes was higher and more sustained than PEI polyplexes in A549 lung epithelial cells. CPP complexes showed the highest level of gene expression at 113 mM of added $CaCl_2$ for the various N/P ratios considered. Cytotoxicity profiles of most CPPs revealed no evidence of cytotoxicity up to 5 mg/mL, which may offer an opportunity to increase the dose of nucleic acid therapeutics to achieve a desired therapeutic effect. These results encouraged future exploration of the structure of pDNA within complexes.

Plasmid DNA within TAT complexes condensed using $CaCl_2$ showed slight differences in biophysical properties when compared to pDNA within PEI polyplexes. FTIR and CD spectroscopies indicated that the secondary structure of pDNA was stabilized within complexes. DSC studies demonstrated that TAT complexes stabilized

pDNA more than PEI complexes as a result of the ability of calcium to condense TAT complexes.

Finally, TAT or dTAT peptides complexed with anti-luc siRNA and condensed using calcium achieved significantly higher luciferase knockdown in A549-luc-C8 lung epithelial cells. Only a small siRNA dose of 10 nM was necessary to achieve luciferase knockdown as high as 87%. Thus, these data suggest that CPP complexes including calcium are a novel and effective candidate for delivering different types of nucleic acid therapeutics.

7.2. Colloidal stability and calcium condensed complexes

Colloidal particles in a dispersion medium undergo Brownian motion, continuous random movement or diffusion and hence collide with each other frequently. The stability of colloids is thus determined by the interaction between the particles during such a collision. The balance of the repulsive and attractive forces that exist between particles will result in controlling the stability of colloidal system. When attraction dominates, the particles will adhere to each other causing aggregation or precipitation. If repulsion dominates (e.g. electrostatic or steric), the system will be stable and remain in a dispersed state [15]. Therefore, a colloidal dispersion is stable only when a sufficiently strong repulsive force counteracts the attraction.

A very useful tool for understanding the stability of colloids is the theory by Derjaguin, Landua, Verway and Overbeek (DLVO) [16]. The theory is based on a balance of forces between electric double layers, which are repulsive for similarly charged particles, and van der Waals forces, which are usually attractive. In this theory, the potential energy of particles is the sum of these two primary contributions. It is possible

to assess the stability of colloidal system against the aggregation by plotting the potential energy as a function of separation between the surfaces of charged colloidal particles. The minimum of the potential energy depend on the distance between two particles and their corresponding equilibrium state. If an electrolyte is present in a colloidal dispersion (e.g. $CaCl_2$), repulsive forces between electric double layers will be screened. In the latter case, the system will be unstable and aggregation will occur.

Various efforts have been made to obtain stability to colloidal particles including adsorption or attachment of polymeric molecules or surfactants to the particle surface. Galactose [17,18], poly(ethylene glycol) [19,20], poly(vinyl pyrrolidone) [21] and dextran [22] have been attached to the chitosan backbone or to the preformed nanoparticles to improve their physicochemical properties. Phosphorylcholine-substituted chitosan/DNA polyplexes exhibited a higher resistance to aggregation compared to deacetylated chitosan polyplexes [23]. Moreover, PEGylation has proven to improve the stability of PEI/DNA complexes [24]. PEGylation of TAT was also effective for the CPP complexes developed here.

A previous study explored the effect of calcium on the colloidal stability of phosphatidylcholine liposomes in aqueous media. 100-200 mM of calcium induced a slight decrease in the size (from 140 nm to 136 nm) of liposome followed by a slow increase of the size until a maximum of 155 nm in the range of 300-900 mM [25]. Changes in the polydispersity were not observed, suggesting a coalescence mechanism or fusion of liposomes but no aggregation. A calculated stability factor suggested that aggregation/fusion occurred at a critical concentration of 700 mM of calcium. Calcium was found to be highly adsorbed on the liposome surface, which implies an increase in

the surface area per lipid molecules (A_0) in the monolayer. These data indicated that the adsorption of calcium on the liposome improved the colloidal stability by controlling the balance of the repulsive and attractive forces between lipid molecules. Calcium was also induced as part of an electrostatic double layer on the particle surface, which did not adversely affect stability in this case.

In our study, $CaCl_2$ was found to provide unique ability to condense CPP complexes into small size leading to high transfection efficiency or high gene knockdown. It seemed that much of the calcium must have entered the 'loose' complexes, as evidenced by the steady decrease in complex size up to 150 mM of added $CaCl_2$. The hypothesis that calcium interacts with both nucleic acids and polyamines was supported by dye binding assays. Upon further addition of calcium, calcium CPP complexes retained colloidal stability. As with the liposome study mentioned above, calcium likely participated in an electrostatic double layer as the concentration increased. DLS measurements also confirmed stabilization in the size of the CPP complexes in the absence and presence of 10% FBS over a period of time (up to 8 days for TAT complexes). Calcium may interact with CPP complexes via surface association or adsorption as well as some other forces such water polarization as a resulted of their correlated fluctuation on the surface of the complexes. Further studies with other ions may support or refute this hypothesis.

7.3. Future directions

Future work with CPP gene delivery systems should further explore the potential of modified CPPs by engineering features to mediate endosomal escape as a means to optimize transfection. This would include the addition of histidine residues, myristic and

stearic acids to the CPP sequences. For example, TAT peptide contains no acidic residues that can be protonated as pH decreases during endolysosomal trafficking. This strategy has been implicated in endosomal membrane destabilization [26]. For example, attaching histidine residues to the TAT peptide showed much higher levels of transfection efficiency. The imidazole groups of histidine (pKa ~6) likely absorbed protons in the acidic environment (pH 5-6.5) of the endosome, leading to osmotic swelling, membrane disruption and gene escape [27].

The mechanism of a positive effect of calcium on the gene delivery when incorporated in the nonviral vectors should also be determined. It is well known that calcium phosphate forms complexes with nucleic acids, thereby imparting small, stable nanoparticles [28]. Reports also suggest that calcium phosphate may help transport complexes across the cell membrane by enhancing endocytosis or by enhancing plasma membrane permeability [29]. Also, the addition of $CaCl_2$ increased transfection efficiency when added to the cell culture medium during transfection and post-transfection. In the absence of calcium, the complexes were taken up by the cells but remained entrapped at an unknown cellular site. Further emphasis on immunohistochemical and imaging studies of CPP-Ca complexes would provide more information on the exact mechanism of enhanced transfection. The internalization and trafficking should be investigated since it can not be excluded from the effect of calcium on gene transfer when using CPP complexes.

7.4. References

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