NONVIRAL GENE DELIVERY:
USING POLYMER AND PEPTIDE TO DEVELOP SAFE
AND CELL-SPECIFIC GENE VECTORS

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The primary requirements for a clinically effective vehicle for human gene therapy are efficient gene transfer and safety. PEI25kDa has been reported to be one of the most efficient non-viral gene vectors. However, the main obstacle for its use in gene therapy is the cytotoxicity associated with it. In this study, we found that the cytotoxicity is attributed to free PEI present in the gene vector solution, and they must be removed from the solution in order to reduce the toxicity level. Moreover, this study also showed the existence of gaps between molecules on the outer layer of PEI-DNA complexes, and it is possible to improve the transfection efficiencies through better packing of the plasmid DNA by inserting smaller PEI molecules of the appropriate size and charge.

One of the major approaches toward cell-specific gene delivery is to target vehicle binding to a cell-specific receptor through receptor-mediated endocytosis. In this study, we present a non-viral gene transfer vector for targeted gene delivery into TrkA-positive cells. This gene transfer vehicle consists of the hairpin motif of loop 4 linked to 10 lysine residues (NL4-10K) for targeting and nucleic acid binding purposes, respectively, and a low molecular weight polyethylenimine, PEI600 for endosomal escape upon cellular uptake. This gene vector is capable of mediating gene delivery into TrkA-expressing cells only, and the transfection efficiency is dependent on the formulation order of the triplexes as well as the N/P ratios between the plasmid DNA, PEI600 and NL4-10K.
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<th>Description</th>
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<tbody>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>N/P</td>
<td>Amines to phosphates ratio</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffer saline</td>
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<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>PEI25kD</td>
<td>Polyethylenimine of molecular weight 25000 Dalton</td>
</tr>
<tr>
<td>PEI600D</td>
<td>Polyethylenimine of molecular weight 600 Dalton</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-Lysine</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative Light Units</td>
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Chapter 1

Introduction
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1 Introduction

1.1 Overview of Gene Therapy

Somatic gene therapy is based upon the introduction of therapeutically active genes into individual cells. These therapeutic genes encode the genetic information required for producing specific therapeutic proteins to correct or modulate disease. The purpose of somatic gene therapy is to overcome the limitations associated with the direct administration of therapeutic proteins, including low bioavailability, systemic toxicity, in vivo stability, high hepatic and renal clearance rates, and the high cost of manufacturing. Providing a therapeutic gene as a 'predrug' to a patient may not only circumvent some of the limitations associated with the use of recombinant therapeutic proteins (Ledley, 1996), but may also offer some advantages such as specificity to target cells or tissues, and it can eliminate the need for repeated administration of drugs or proteins.

The therapeutic genes can be delivered in vivo or ex vivo. The former approach, whereby the genetic material is transferred directly into the patient's cells, is often the only viable option in tissues for which individual cells cannot be cultured in sufficient quantities, or the cultured cells cannot be re-implanted. In the ex vivo approach (Van Tendeloo et al., 2001; Stephane et al., 2002), the genetic material is first inserted into cells grown in vitro. The transfected cells are then selected, expanded, and introduced into a patient. To avoid rejection by the host's immune system, cells obtained from the same patient (autologous cells) are normally used.

A great variety of effector genes have been shown to hold promise for the treatment of inherited or acquired diseases in humans. Currently however, the greatest hurdle to the actual realization of these therapies is the development of non-toxic and efficacious delivery systems (Anderson, 1998). Ideally, gene delivery
systems should serve to protect a gene expression system from premature degradation in the extracellular milieu and to effect nonspecific or cell-specific delivery to a target cell. Other elements in a gene delivery system may facilitate the intracellular trafficking of a gene expression system (Mahato et al., 1997).

Generally, two different approaches have been utilized for the delivery of nucleic acids in gene therapy, namely that of viral vectors and non-viral vectors. The natural ability of viruses to infect host cells offers an existing class of vectors to introduce foreign DNA sequences into cells for gene therapy. Viral vectors including retroviruses, adenoviruses and adeno-associated viruses are particular attractive as vectors due to ease of production and generally high efficiency of transgene expression (i.e., often 50% or greater). Although their transfection efficiency is impressive, however, several recurring issues have led to a reconsideration of their use in human clinical trial. One of the problems is that they develop a high immunogenicity after repeated administration since the mammalian immune system has developed strategies to eliminate viral invaders. Some viral vectors can also integrate with the host genome and permanently alter its genetic structure, self replicate with a unique possibility of recombination and compliment activation. Another current drawback is that viral vehicles are generally considered to be constrained with respect to the size of DNA they are able to deliver, with complete recombinant adenovirus vectors having a deliverable DNA sequence size of 6-8 kilobase pairs (Berkner, 1988), and ‘gutless’ adenovirus limited at approximately 35 kilobase pairs. Furthermore, the inclusion of a targeting moiety in order to transfect specific cell types or tissues is problematic. Despite these problems, still more than two-thirds of clinical gene therapy trials use viral vectors.

Because of the many intrinsic problems of viral vectors, non-viral vectors emerge as a viable alternative. They are basically synthetic vehicles for the introduction of foreign DNA sequences into target cells. As such, non-viral vectors
have flexibility in design and construction absent from their viral counterparts. Some potential benefits of using non-viral gene transfer formulations include: (a) the capacity to potentially target any cell type via single, specific receptor; (b) an essentially unlimited nucleic acid cargo capacity; (c) plasmid DNA and transfection reagents can be produced at large scale with rather low costs; (d) safety testing of synthetic material is less laborious than testing of recombinant material; (e) the potential for minimizing immune and inflammatory host reactions. This latter advantage is crucial for anticipated treatment repetition of non-viral delivery approaches due to their lack of ability to permanently integrate into host-cell chromosomes and elicit sustained gene expression.

In the last 15 years, several non-viral vector construction approaches have been synthesized and their transfection activity evaluated. This includes the use of naked DNA (Wolff et al., 1990), cationic lipids formulated into liposomes and complexes with DNA (lipoplexes) (Song et al., 1997), cationic polymers complexed with DNA (polyplexes) (Orgris et al., 1999), polymeric vesicles complexed with DNA (polyplexes) (Brown et al., 2000) or a combination of both cationic lipids and cationic polymers complexed with DNA (lipopolyplexes) (Kircheis et al., 1999; Guo and Lee, 2000). There have also been attempts to combine the benefits of viral and non-viral systems into one delivery vehicle (Curiel et al., 1991). Many of these vectors allow efficient transfection of a variety of establish cell lines as well as of primary cells. Moreover, systemic administration of different mono- and polycation/DNA complexes results in a significant transgene expression in the lungs. While these data are encouraging, non-viral vectors suffer from limitations of their own with the increase in flexibility, targeting capabilities, and potentially reduced toxicity are unfortunately accompanied by loss in efficiency for current formulations.
1.2 Naked DNA

The method of gene delivery using naked DNA is via direct delivery of the transgene in the absence of a carrier. Intramuscular application of naked DNA results in efficient gene expression in rodent muscle (Wolff et al., 1990). In addition, conditions have been worked out for the local gene transfer of naked DNA to the skin (Hengge et al., 1996), the liver (Hickman et al., 1994), the lung epithelium (Meyer et al., 1995) and tumors (Vile and Hart, 1993). The transfection efficiency is species-dependent and very large amounts of DNA have to be applied. Nevertheless, there appears to be room for further improvement by optimizing the formulation (Mumper et al., 1996). Alternatives to injection by needle include administration by needle-free injection (Furth et al., 1992) or biolistic delivery of DNA loaded gold microparticles by the gene gun (Yang et al., 1990). Improved local expression was obtained by electroporation of the DNA-loaded area with some few microsecond electrical pulses (Heller et al., 1996). The naked DNA technology has been successfully applied in murine models for genetic vaccinations (Ulmer et al., 1993; Tang et al., 1992). However, this method is ineffective if DNA dosing to anatomically inaccessible sites (e.g. solid tumours in organs) is desired.

1.3 Cationic Polymer-Based Gene Delivery Systems

While the application of naked DNA is restricted to specific circumstances, polymers display striking advantages as vectors for gene delivery. DNA can be condensed into mononuclear or polynuclear particles with an excess of polycations in aqueous solutions (Trubetskoy et al., 1999). The cationic polymer spontaneously forms complexes with DNA because of electrostatic interactions between positively charged amine groups of the polycations and negatively charged phosphate groups
of the DNA. The interaction between cationic polymer/DNA enhance DNA uptake by the cells and thus increases the transfection efficiency. Polymers can also be specifically tailored for the proposed application by choosing appropriate molecular weights, coupling of cell or tissue specific targeting moieties and/or performing other physiological or physicochemical properties. After identifying a suitable polymer structure, a scale-up to the production of large quantities is rather easy as well. A weakness of gene therapy with cationic polymers is our limited knowledge regarding the formation of electrostatic complexes with DNA and their biological effects. Although cellular internalization followed by intracellular transport to the nucleus is not yet clearly understood, there are several interesting polymers that have been widely studied such as poly-L-lysine (PLL), polyethylenimine (PEI), dendrimers, and chitosan. Fig. 1-1 gives an overview of frequently used cationic polymers for non viral nucleic acid delivery. Among these various synthetic vectors, PEIs have shown particularly promising efficacy in transfections in cell culture as well as in a variety of applications in vivo. The following section will discuss PEI in detail.

Fig. 1-1: Cationic polymers most frequently used for nucleic acid delivery (picture adapted from Merdan et al., 2002).
1.4 Polyethylenimine (PEI)

PEI is a cationic polymer composed of 25% primary amines, 50% secondary amines and 25% tertiary amines. It is available in two main forms: linear and branched. The branched form is produced by acid-catalyzed polymerization of aziridine monomers, resulting in random branched polymers. Linear forms of PEI are attainable by a similar process, but performed at lower temperature (Godbey et al., 1999; Tomalia et al., 1985). Both branched (Boussif et al., 1995, 1996) and linear (Ferrari et al., 1997; Chemin et al., 1998) PEI have been introduced as cationic polymers for gene delivery. Unlike PLLs, PEIs show efficient gene transfer without the need for endosomolytic or lysosomotropic agents or indeed any agents facilitating receptor mediated uptake. They also offer a significantly more efficient protection against nuclease degradation than other polycations, possibly due to their higher charge density and more efficient complexation. The huge amount of positive charges, however, results in a rather high toxicity of PEI polymers which is one of the major limiting factors especially for its in vivo use.

PEIs not only exist in linear and branched topology, there are also PEIs of various molecular weights (e.g. 600, 700 Da, 2, 22, 25, 50, 70, 800 kDa) provided by various manufacturers. The influence of molecular weight on the activity of PEI is as yet unclear with some reports detailing an increase in gene transfer activity with a decrease in molecular weight (from 100 to 11,9 kDa) (Fischer et al., 1999) and some reports detailing a decrease in activity on decreasing the molecular weight (from 70kDa to 1.8 kDa) (Godbey et al., 1999).
1.5 Mechanism of Gene Delivery by Polyplexes

The transfer of gene via polycation-mediated gene delivery into eukaryotic cells involves a few distinct steps, namely: condensation of DNA; cellular uptake; release from the endosome; nuclear transport; and vector unpacking and translation (Fig. 1-2). Any of these steps can contribute to the efficiency of gene delivery by polyplexes. Therefore, a thorough understanding of these steps is important for improving polycation-mediated gene delivery. Insights obtained from mechanistic investigations during the past few years are discussed below.

Fig. 1-2: Schematic representation of DNA uptake by mammalian cells. DNA is compacted in the presence of polycations into ordered structures such as toroids, rods, and spheroids. These particles interact with the anionic proteoglycans at the cell surface and are transported by endocytosis. The cationic agents accumulate in the acidic vesicles, increase the pH of the endosomes, and inhibit the degradation of
DNA by lysosomal enzymes. They also sustain a proton influx, which destabilizes the endosome, and release DNA. The DNA then is translocated to the nucleus either through the nuclear pore or with the aid of nuclear localization signals, and decondenses after separation from the cationic delivery vehicle (picture adapted from Wiethoff et al., 2002)

1.5.1 Condensation of DNA

Polycations interact with the polyanion DNA and condense it into compact, ordered particles (20-200 nm in diameter) (Dunlap et al., 1997; Golan et al., 1999; Liu et al., 2001). DNA condensation is a reversible, linear polymer to globule transition favored by the association of the polycation around the DNA phosphate groups. At a certain critical ratio of the nitrogen-to-DNA phosphate (N/P ratio), the latter undergoes localized bending or distortion which facilitates the formation of rods, toroids (Dunlap et al., 1997; Golan et al., 1999), and spheroids (Liu et al., 2001). PEI has been shown to effectively condense plasmids into colloidal particles that effectively transfect plasmid DNA into a variety of cells both in vitro and in vivo (Boussif et al., 1995). Complete condensation of DNA by PEI seems to occur at N/P ratios of 2-3 with the formation of neutral particles. However, at complex neutrality, there is tendency for particle aggregation. Compact particles of small size are usually obtained only at higher polycation/DNA ratios, resulting in complexes with a strong positive net charge.

Particle Size of Polyplexes

The ability of the polycation to condense DNA into nanoparticles is often critical for transfection efficiency. For instance, 2 kDa PEI (PEI2k) condensed DNA into huge aggregates up to 2 µm in size, while 25 kDa PEI (PEI25k) condensed DNA...
into compact particles with diameters of about 80-100 nm (Peterson et al., 2002). PEI2k is a much poorer transfection agent than PEI25k (Han et al., 2001; Thomas and Klibanov, 2002). However, this relationship does not always hold. For branched PEI800/DNA complexes, small particles were found to have transfection efficacy significantly lower than that in larger particles (Ogris et al., 1998; 1999), both in vitro and in vivo. Similar correlations were observed for branched PEI25 (Kircheis et al., 2001; Wightman et al., 2001). A possible explanation could be that the osmolytic endosomal release by the ‘proton sponge’ mechanism (Boussif, 1995) (see section 1.5.3) may work more efficiently when the endosomes are filled with larger PEI/DNA complexes compared to a similar number of small particles. Additionally, a more effective sedimentation of larger particles onto the cells can play a role, particularly for in vitro application (Ogris et al., 1998), an observation which is in good agreement with the reported higher transfection efficacy in vitro if sedimentation of the complexes is enhanced by centrifugation (Boussif et al., 1996).

Beside being a function of the polycation-to-DNA ratio, the size and shape of the polycation/DNA complexes are also dependent on the experimental protocol of complex formation with parameters such as the ionic strength of the solvent, the kinetics of mixing, the DNA and polymer concentration, the ratios of the volumes of the solutions mixed together, the speed of mixing, and the sequence of addition of polycation or DNA. For instance, Boussif and colleagues found that a dropwise addition of the polymer to the plasmid produced polyplexes with 10-fold higher transfection efficiency in vitro compared to those obtained by adding the DNA to the polymer (Boussif et al., 1995; 1996). Therefore, with interplay of such numerous factors, nanoparticle preparation using PEI is still rudimentarily understood.
Surface Charge of Polyplexes

The surface charge of PEI/DNA complexes is another important parameter in determining the transfection efficiency. The polycation-to-DNA ratio used in complex formation can be optimized, as varying this ratio impacts the overall negative, neutral, or positive charge on the complex. Binding of complexes formed with an overall negative charge may be hindered by ionic repulsion due to negatively charged glycosides present on the cell membrane. Conversely, an excess of polycation, as usually used for efficient condensation and tight compact of DNA, leads to a net positive surface charge, resulting in increasing binding to the negatively charged cell surface. Usually, PEI/DNA complexes have zeta potential in the range of +30-35 mV for N/P ratio at complete complexation (Ogris et al., 1999; Kircheis et al., 1999). There seem to be no major differences in zeta potential between DNA using different PEIs (Kircheis et al., 2001).

1.5.2 Cellular Uptake

The positively charge on PEI/DNA polyplexes allows non-specific electrostatic interactions between the polyplexes and negatively charged proteoglycans of the cell membrane (Erbacher et al., 1999). After which, internalization of the polyplexes is mediated by endocytosis, also known as non-specific absorptive endocytosis (Duncan et al., 1979; Leoneti et al., 1990). However, this non-specific, charge-mediated endocytosis poses concerns pertaining to in vivo applications. For example, the injection of positively charged PEI/DNA polyplexes into the tail vein of mice directed gene transfer primarily to the lung (Kircheis et al., 2001). This is because when administered into the blood stream, the polyplexes were being neutralized by the negatively charged plasma proteins and circulating blood cells. As a result, the
neutralized polyplexes aggregated and accumulated in the organs that they were first circulated to, such as lung, liver, and spleen. In contrast, surface-shielded transferrin-PEI-DNA complexes resulted in preferential gene delivery to distantly growing tumors (Kircheis et al., 2001).

1.5.3 Release from the Endosome

Normal cellular trafficking usually directs the endocytosed particles to lysosomes for degradation. The endosomes first mature from the ‘early’ to ‘late’ stage when the pH drops from ~6 to ~5, and the late endosomes fuse with lysosomes (Luzio et al., 2001). Thus, the accumulation of polyplexes in endosomes would eventually lead to their degradation by the lysosomal hydrolytic enzymes, strongly limiting the gene expression. Vectors that possess endosomolytic components should therefore ensure early escape of the polyplexes. For polycations such as PLL, release from the endosomal compartment is therefore a major bottleneck in the transfection process. The addition of endosomal disrupting agent such as chloroquine or glycerol can considerably increase the transfection efficiency of PLL and many other formulations in cell culture conditions (Ciftci et al., 2001; Keil et al., 2001) presumably due to its lysosomotropic activity.

There are only a few polycations which have high transfection potential without the need for additional endosomolytic agents, and PEI is one of them with the highest charge density and a high intrinsic endosomolytic activity. The high charge density is due to every third atom on the PEI backbone being a nitrogen atom. In linear PEI, all of these nitrogen atoms are protonable, whereas in branched PEI, only two-thirds of them can be charged (Garnett, 1999). In the presence of PEI in the endosome, the accumulation of protons brought in by endosomal ATPase is coupled to an influx of chloride anion. This results in large increase in the proton concentration...
within the endosome, resulting in swelling of the polymer by internal charge repulsion, with resultant osmotic swelling of the endosome and subsequent endosome disruption. When PEIs are complexed with plasmid DNA, this 'proton sponge' property (Boussif, 1995) will lead to the release of endocytosed DNA into the cytosol. However, since the exact mechanism of PEI-mediated transfection remains to be elucidated, it is possible that additional properties are required to obtain high transfection efficiencies.

1.5.4 Nuclear Transport

After their release from the endosomes into the cytosol, the polyplexes must enter the nucleus to undergo transcription. Although the precise mechanism of transport of complexes from the cytoplasm to the nucleus is largely unknown, there is evidence that polycations protect DNA from cytosol nucleases (Moret et al. 2001) and thus afford higher probability for nuclear entry. Godbey and colleagues found that intact PEI-DNA polyplexes were found in the nucleus (Godbey et al., 1999), this finding was later supported by Bierber and colleagues (Bieber et al., 2002). Therefore, this suggests that it is not necessary for the polycation to separate from DNA prior to nuclear entry. The transfection efficiency of polyplexes also critically depends on the cell cycle and is enhanced by mitotic activity (Brunner et al. 2000). High transfection efficacy was observed when the complexes were added to be taken up by cells in late S or G2 phase, i.e. in phases preceding mitosis. Thus, gene delivery using non-viral systems is facilitated by nuclear membrane breakdown. However, this notion is not true for all types of PEI-mediated gene delivery. Linear PEI22-mediated transfection was found to be less dependent on the cell cycle, compared to branched PEI25 and LipofectAmine (a widely used liposomal formulation) (Brunner et al., 2002).
1.5.5 Vector Unpacking

Disassembly of the polyplexes to allow the transcription apparatus of the cell to access the DNA efficiency is the final stage in gene expression. Significant gene expression has been observed when polyplexes were injected directly into the nucleus (Pollard et al., 1998) suggests that dissociation of the complex can actually occur there, possibly mediated by DNA polymerase in a manner analogous to the stripping of DNA from histone proteins. Although intact PEI-DNA complexes have been detected in the nucleus (Godbey et al., 1999, 2000; Bieber et al., 2002), cells containing them detached from the surface of the culture dish and were lost upon medium change (Bieber et al., 2002), suggesting that these polyplexes do not contribute to the measured transfection efficiency. Therefore, the high transgene expression may be attributed to high concentration of complexes observed in the perinuclear region for PEI (Bieber et al., 2002). Exactly how the polyplexes dissociate is still unknown.

1.6 Cytotoxicity of Polyplexes

To be useful in gene therapy, polyplexes should be non-cytotoxic. For PEI, transfection efficiency increases with molecular mass up to 25 kDa and then slumps, while the cytotoxicity rises linearly. Free PEI will harm cells, but when bound to DNA the detrimental effects are greatly lessened. A possible reason for toxic effects of PEI on cells is the PEI will permeabilize membrane (Helander et al., 1997, 1998). ‘Lysosomal loading’ could also be another factor contributing to the toxicity of polycations such as PEI25 that have been shown to accumulate in the lysosome for several days (Lecocq et al., 2002). However, Lambert et al. found that PEI did not disrupt neuronal cells caused by transfection process (although PEI concentrations
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above 150 μM were toxic to neuronal cells) (Lambert et al., 1996). All these data collectively suggest that low concentrations of PEI will not harm plasma membranes. Thus, it appears that successful transfection requires the correct balance between gaining adequate access of the complex to the cytoplasm and yet not causing lethal damage to the cell. Recent evidence has shown that low molecular weight preparations of PEI are significantly less toxic than high molecular weight PEI both in cultured cells and in animals (Fischer et al., 1999). Therefore, improving the transfection efficiency of low molecular weight polyations with appropriate chemical modification is also one plausible strategy for reducing cytotoxicity (Thomas and Klibanov, 2002). Alternatively, surface modifications to PEI-DNA complex could also reduce potential immunogenicity and toxicity by shielding the particles from blood components. For example, it was found that PEGylation of PEI resulted in decreased toxicity (Ogris et al., 1999; Kircheis et al., 1999), and glycosylated PEI was also reported to have reduced toxicity (Leclercq et al., 2000).

1.7 Cell Targeting

As mentioned above, the presence of positive charges at the surface of PEI-DNA complexes promotes non-specific interactions with plasma proteins and cell membranes. In order to overcome this problem, efforts have been made to combine or even to exchange the non-specific electrostatic interactions between cells and the transfection complexes with a cell-specific interaction that triggers receptor-mediated endocytosis of the DNA complexes. Fig. 1-3 summarizes the steps involved in receptor-mediated gene delivery.
Fig. 1-3: Schematic diagram of receptor-mediated endocytosis. Receptor-mediated trafficking for ligands and gene delivery vectors begins with binding of the ligand to its receptor followed by internalization, creating early endosomes. Sorting results in recycling to the cell surface or maturation into late endosomes. From late endosomes, transport may occur to and from the trans-Golgi or development into lysosomes where degradation of contents occurs. Gene delivery vectors must escape lysosomal trafficking such that the transgene may eventually reach the nucleus for expression (picture adapted from Varga et al., 2000).

The concept of receptor mediated gene transfer is based on the entry mechanisms widely used by viruses and toxins, but also used for uptake of macromolecules, including nutrients (e.g., LDL, transferrin), growth factors and hormones (e.g., insulin, VEGF, EGF, FGF) into cells. Such an active targeting requires the identification of receptors present at the surface of the target cells and the use targeting ligands such as proteins, peptides, carbohydrates, vitamins, or antibodies that bind with a high specificity and affinity to the recognition sites. The ligands, of natural or synthetic origin, can be conjugated to the polymers by using
different techniques (Kichler et al., 2000). A variety of targeting ligands have been attached covalently or non-covalently to PEI including galactosylated PEI to target hepatocytes (Zanta et al., 1997; Bettinger et al., 1999; Leclercq et al., 2000), RGD-containing peptide conjugated onto PEI to target integrins on cell surface (Erbacher et al., 1999), mannosylated PEI to target dendritic cells (Diebold et al., 1999), transferrin conjugated to PEI to target transferrin receptors (Kircheis et al., 1997, 2001), EGF conjugated to PEI for enhanced uptake into epithelial cells (Blessing et al., 2001), and in our recent publication, NGF hairpin motif combined with PEI600 Da to target TrkA receptors (Ma et al., 2004).

The incorporation of a specific cell binding ligand will allow for specific ligand-receptor-mediated uptake. Nevertheless, additional non-specific electrostatic interactions of the polycation with the negatively charged cell surface may occur. A number of factors determine which type of interaction prevails: (i) the level of receptor expression on the target cells (Cotton et al., 1990, 1993), (ii) the density/amount of negatively charged proteoglycans on the surface of a particular cell type (Mislick and Baldeschwieler, 1996; Ruponen et al., 1999), and (iii) the ratio of polycation to DNA in the transfection complex (Kircheis et al., 1997). In addition, the size of the complex can also be decisive for whether non-specific uptake or receptor-mediated mechanisms prevail. Erbacher and colleagues, using PEI of 25 kDa molecular weight conjugated to integrin RGD peptide, observed receptor-mediated uptake mainly at higher N/P ratios, whereas at neutral charges the formation of large aggregates resulted in a receptor-independent uptake, presumably by phagocytosis (Erbacher et al., 1999). Finally, the amount of DNA complex will be a factor deciding between non-specific vs. receptor-mediated uptake, with the latter having greater importance, particularly at low DNA concentrations (Blessing et al., 2001).
1.8 Nerve Growth Factor (NGF)

Neurotrophins are critical for the development and maintenance of the peripheral and central nervous systems. The neurotrophins represent a family of structurally and functionally related, homodimeric proteins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and neurotrophin-6 (Barde et al., 1982; Ernfors et al., 1990; Hallbook et al., 1991; Gotz et al., 1994).

Nerve growth factor (NGF), discovered 50 years ago (Levi-Montalcini & Hamburger 1951, 1953), is involved in a variety of processes involving cell signaling, such as cell differentiation and survival, vrowth cessation and apoptosis of neurons. These events are mediated through binding to two distinct classes of cell surface receptors, the shared p75 neurotrophin receptor and the TrkA receptors. p75, which is also referred to as the low-affinity neurotrophin receptor, belongs to the tumor necrosis factor receptor family and binds to all neurotrophins with similar, nanomolar affinities (Bothwell, 1995; Yano et al., 1997). Despite this promiscuity, activation of p75 can only be achieved through NGF (Wiesmann et al., 2001). This is followed by apoptosis in the context of Trk-A negative neurons, but promotes survival even at low concentrations of NGF if TrkA is co-expressed on the cell surface (Yano et al., 2000; Dechant et al., 1997; casaccua et al., 1998; Kaplan et al., 2000). TrkA is a receptor with tyrosine kinase activity that forms high-affinity binding site for NGF (Kaplan et al., 1991), it can antagonize NGF in vitro and in vivo (Wiesmann et al., 2001).

The structure of NGF, when determined by X-ray crystallography in 1991 (McDonald et al., 1991), revealed a novel protein fold (Fig. 1-4). NGF is a monomer in elongated shape with the central part of the molecule formed by two pairs of twisted, antiparallel β-strands. Two monomers assemble around a central twofold
axis in a parallel manners to form the physiological dimer. On end of a NGF monomer carries a cysteine-knot motif that stabilizes the fold and locks the molecules in their conformation; while the other end consists of three hairpin loops, the reverse turn (L3) and three $\beta$-hairpin loops (L1, L2 and L4). Subsequent studies on L3 revealed that this region inhibited NGF activity and NGF p74 receptor binding. The NGF sites interacting with TrkA have also been derived via chemical modification (Bradshaw et al., 1994; Woo et al., 1995, 1996), recombinant proteins (Kruttgen et al., 1997; McDonal et al., 1995; Kullander et al., 1997; Drinkwater et al., 1993), and NGF-TrkA co-crystallization approaches (Wiesmann et al., 1999). These studies suggest that TrkA binding sites consists of residues in NGF L2, L4, the N terminus, and the C terminus. In particular, Xie and coworkers (Xie et al., 2000) found that NGF L4 small mimetic molecules function as a partial agonists to activate TrkA-related signaling that in turns regulates both survival and neurite outgrowth. Thus, these studies support the proof of concept that small NGF loop 4 mimetics can mimic death-preventing and neurite-promoting effects of NGF.

Fig. 1-4: Ribbon diagram depicting the structure of the NGF dimer (picture adapted from Wiesmann et al., 2001).
Chapter 2

Objectives
2 Objectives

The ideal non-viral DNA delivery system should have the following properties: (a) structurally well characterized, non-toxic, biodegradable, and non-antigenic systems that protect DNA from degradation and are stable in biofluids; (b) cellular uptake mediated by cell-specific plasma membrane receptors; (c) rapid pH dependent release from the endosome; (d) efficient dissociation of the DNA from complex into the cytoplasm for transport of the DNA to the nucleus; (e) controlled duration and magnitude of expression. In this study, we wish to address perhaps two of the most compelling issues in designing gene delivery vectors: (i) biocompatibility, and (ii) cell targeting. They are discussed separately in the following sections.

2.1 Improving the Biocompatibility of 25kDa PEI

The need for safe and efficient methods for gene delivery remains a critical stumbling block to the routine clinical implementation of human gene therapy (Verma and Somia, 1997). While branched, 25 kDa PEI has become a gold standard amongst the various non-viral vectors due to its relatively high gene delivery efficiency and ready availability, however several groups have reported that PEI is cytotoxic in many cell lines. At PEI concentrations used in typical transfection protocols, cell metabolic activity may be reduced by 40-90% (Lim et al., 2002; Fischer et al., 1999).

To generate biocompatible gene delivery using PEI, many groups have tried shielding the excessively high cationic surface charge on PEI-DNA complexes using various hydrophilic polymers. Although the modified PEI exhibits less toxicity to cells in culture, the trade off is however a less efficient gene expression than PEI25kD
(Pichon et al., 2002; Gosselin et al., 2001). Moreover, little is known about how the polymer structure, including the size, cross-linking density, branching, etc., affects the gene livery efficiency.

In this study, we sought to improve the cytotoxicity of PEI25k using simple filtration method via dialysis in 10,000 Da molecular weight cutoff dialysis tube. After which the filtered PEI25k (hereinafter referred to as PEI25k(F)) will be lyophilized for 4 days. We expect a slight decrease in transfection efficiency, which will be compensated by addition of another low molecular weight PEI600D that serves to tighten the packing of PEI25kD(F)-DNA complexes. We hypothesized that this is due to the branched form nature and large molecular weight of PEI25kD. From geometric point of view, it is quite impossible for many of such large molecules to pack so closely and densely around the DNA that a continuous cation coat is formed around the DNA. There would be ‘gaps’ within this cation coat and we sought to fill up these ‘gaps’ using smaller molecules such as PEI600D so as to condense the DNA further with increased packing of amines around the DNA.

2.2 Cell Targeting Using Nerve Growth Factor

For many envisioned applications in gene delivery, a gene delivery vehicle should provide efficient uptake and expression of the transgene to a selected type of cell and no others, without stimulating a significant immune, inflammatory, or cytotoxic response. One of the major approaches toward this goal is to target vehicle binding to a cell-specific receptor or surface marker and subsequent uptake into cells through receptor-mediated endocytosis. This concept has been pursued for more than a decade (Wu and Wu, 1987, 1988; Berkner, 1988; Cotton et al., 1990, 1993, Fajac et al., 2002). Delivery via receptor-mediated endocytosis begins with the
specific binding of vehicle-conjugated ligands to intrinsic cell-surface receptors. This is followed by internalization of the ligand/receptor complex. The vector should then release from the intracellular compartments into the cytosol, avoiding lysosomal degradation, such that subsequent localization to the nucleus, the locale of ultimate expression, can occur.

Many functional molecules in the nervous system are capable of inducing receptor-mediated endocytosis, for instance, the neurotrophins. Nerve growth factor (NGF), discovered almost half a century ago (Levi-Montalcini, 1987), is the founding and best-characterized member of the neurotrophin family (Chao, 1992). NGF itself is a 27 kDa protein that is held together by three disulfide bridges uniquely arranged in a characteristic cysteine knot motif. It plays a significant role in growth, differentiation, and survival of various types of neurons, such as basal forebrain cholinergic neurons and sympathetic sensory neurons (Thome and Frey, 2001). NGF can also induce the differentiation of PC12 pheochromocytoma cells into neuron-like cells (Greece and Tischler, 1976). These effects are thought to be mediated primarily through binding of NGF to the high affinity NGF receptor TrkA and subsequent signal generation by TrkA (Kaplan et al., 1991; Omichi et al., 1992). A second receptor, p75, a non-selective neurotrophin receptor of the tumor necrosis factor (TNF) superfamily, also binds NGF (and other neurotrophic factors) and may be involved in some aspects of NGF signaling (Chao et al., 1994). Ligand engagement stimulates dimerization and internalization of TrkA receptors through clathrin-coated pits and by macropinocytosis in cell surface ruffles (Jullien et al., 2002). After internalization, NGF is localized with TrkA receptors in endosomes and this is followed by activation of intermediates signaling pathways (Huang and Reichardt, 2003).

Extensive studies such as deletion, point mutation, chimeric recombinant experiments and crystal structure analysis have been carried out on NGF in order to
determine important regions governing its specificity to TrkA receptor. These experiments indicate that the residues in NGF loop 1 region is responsible for p75 receptor binding, while those in loop 2 and loop 4 regions govern the specificity to TrkA receptor. Based on these studies, several groups have successfully developed small molecule mimetics corresponding to TrkA-interacting domain that have NGF agonist activity (Beglova et al., 2000; Maliartchouk et al., 2000; Xie et al., 2000, Ma et al., 2004).

Recently, our group has successfully showed that the chimeric peptide-containing loop 4 of NGF and ten lysine residues (hereinafter called NL4-10K) can bind to and condense DNA as well as direct gene transfer specifically to the cells expressing TrkA receptors. This previous work involves the use of chloroquine, which is thought to protect internalized material from degradation by preventing pH decrease (Luthman and Magnusson, 1983; Erbacher et al., 1996) or acting as an endosomolytic agent (Cirftci and Levy, 2001; Pouton et al., 1998). Although it helps to increase the probability of gene expression, it is also cytotoxic at doses required to augment gene expression, thus preventing in vivo application. In this study, we sought to replace the function of chloroquine with PEI of molecular weight 600 Da which is also known to promote endosomal escape through its ‘proton sponge’ property (Boissif, 1995) (see section 1.5.3 on ‘proton sponge’ property). We hypothesize that the DNA vector consisting of NL4 and PEI600 is also capable of directing gene delivery specifically to TrkA-positive cell only.
Chapter 3

Materials and Methods
3 Materials and Methods

3.1 Peptide Design and Synthesis

A 29-amino acid peptide derived from NGF loop 4-containing region (aa80-108) (Wiesmann et. al., 1999) was selected for NGF receptor targeting and designated as NL4. A DNA binding sequence of 10-lysine residue was added to the C terminus of NL4 to form peptide NL4-10K. NL4 and NL4-10K were prepared using conventional solid-phase, chemical synthesis method and cyclized a disulfide bond formed between C80 and C108 by Cambridge Research Biochemicals (Cleveland, UK). A 10-lysine peptide (designated as 10K) was synthesized by Bio-Synthesis (Lewisville, Texas, USA) as a control. All peptides were provided in dry powder and stored in -80°C.

3.2 Plasmids

The reporter plasmid encoding firefly luciferase used was pCAGluc (kindly donated by Yoshiharu Matsuura, National Institute of Infectious Diseases, Tokyo, Japan) with a composite promoter CAG consisting of the CMV IE enhancer, chicken β-actin promoter and rabbit β-globin polyadenylation signal. Plasmid was amplified in E.coli and purified with HiSpeed Plasmid Kit (Qiagen, Hilden, Germany). The quantity and quality of the purified plasmid DNA was assessed by optical density at 260 and 280 nm and by electrophoresis in 1% agarose gel. The purified plasmid DNA was dissolved in TE buffer and kept in aliquots at a concentration of 1mg/ml.
3.3 Polymers

Branched PEI25kDa and PEI600Da were obtained from Sigma-Aldrich (St. Louis, MO, USA). The stock solutions of PEI25kDa and PEI600Da were prepared in 5% glucose at a concentration of 0.1 M.

3.4 Preparation of Gene Vector Complexes

Gene vector complexes for polymer/DNA or peptide/DNA were formulated as follows. For transfection assay, 0.5 µg of DNA and the corresponding amount of PEI600Da, NL4-10K or 10K was diluted in 5% glucose to 5 µl. Polymer or peptide solution was added to the DNA solution at a dropwise manner and mixed vigorously by vortexing. The complexes were incubated for 30 min at ambient temperature before use. Ternary complexes were generated in the same manner, but polymer solution was first added to DNA solution, incubate for 30 min before peptide solution was added to the polymer/DNA mixtures, making a total volume of 15 µl. The mixtures were mixed vigorously, and again incubated at ambient temperature for 30 min. In some assay, peptide solution was first pipetted to the DNA solution, and the polymer solution was then added. For DNA retardation assay, the gene vector complexes were prepared in the same manner with 0.2 µg of plasmid DNA per complex or triplex.

3.5 Agarose Gel Electrophoresis

Plasmid DNA (0.2 µg) was mixed with Polymer/DNA, peptide/DNA complexes or polymer/DNA/peptide triplexes mixed with a 6X loading buffer were loaded onto an ethidium bromide containing 0.7% agarose gel. Gel electrophoresis was run at room
temperature in TBE buffer at 80V for 50 min. DNA bands were visualized by a UV (254 nm) illuminator.

3.6 Ethidium Bromide Displacement Assay

Ethidium bromide (153 µl of a 0.01% solution) was added to 96 µg of pCAGluc. This DNA-ethidium bromide solution was diluted to a final volume of 6 ml. Different concentrations of the polymers or copolymers were added to each well of a 96-well microplate, to which 50 µl of the DNA-ethidium bromide solution was added to obtain a final volume of 100 µl. After 5 min, the polyplexes were treated with 100 µl of water and gently shaken. The resulting fluorescence at 485 nm excitation and 595 nm emission was measured sing a SPECTRAFluor Plus (Tecan) microplate fluorometer.

3.7 Cell Cultures

Two TrkA-expressing NIH3T3 cells lines, E25 and TRK1, and parental NIH3T3 cells were grown in DMEM (National University of Singapore, Singapore) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). E25 was developed by Dr. Stuart Decker (Parke-Davis Pharmaceuticals) (Decker et. al., 1995), and TRK1 was developed by Dr. Mariano Barbacid (National Center for Oncological Investigation, Madrid, Spain) (Cordon-Cardo et al., 1991), both of which were kindly provided by Dr. Alonzo H. Ross (University of Massachusetts Medical School). To maintain the expression of TrkA in NIH3T3.E25 and NIH3T3.TRK1, 0.5 mg/ml G418 (Sigma-Aldrich, St. Louis, MO, USA) or 50 U/ml hygromycin B (Calbiochem, La Jolla, USA) were used respectively. The cells were maintained in a humidified incubator with 5% CO₂ at 37°C.
3.8 Zeta Potential and Size of the Complexes

Appropriate amount of peptide at desired ratios in 250 µl of 5% glucose was added in a dropwise manner to 20 µg of DNA in 250 µl of 5% glucose while vortexing. After 30 min incubation, the samples were diluted to about 1.5 ml with 1mM KCl buffer to ensure that measurements were made under conditions of low ionic strength where the surface charge of the particles can be accurately measured. The zeta potentials of the complexes were then analyzed by phase analysis light scattering using a Brookhaven ZetaPALS zeta potential analyzer (Brookhaven Instruments Corporation, USA). Default settings on the ZetaPALS were used, i.e. dielectric constant, refractive index and viscosity were assumed to be the same as for water, and the Smoluchowski approximation was used. Determinations were carried out at 22°C and all buffer solutions used were filtered through a 0.22 mM filter before use. For size measurement, the complexes were prepared in the same manner as for zeta potential measurement and then diluted to 3 ml with 1mM KCl buffer. Determinations were carried out at 22°C at a fixed angle of 90° by dynamic light scattering using a Brookhaven ZetaPALS submicron particle size analyzer (Brookhaven Instruments Corporation, USA).

3.9 In Vitro Gene Transfer

For the transfection of NIH3T3.E25, NIH3T3.TRK1 and NIH3T3, the cells were split one day prior to transfection and plated in 48-well plates at a cell density of 2.5 x 10⁴ per well with 250µl indicated medium. After overnight incubation at 37°C, 5% CO₂ and 95% relative humidity, the cell culture medium were replaced with OPTI-MEM (Invitrogen, Carlsbad, CA, USA) before transfection. DNA complexes containing 0.5
µg of plasmid DNA were added and incubated with cells for 4 h. After which the medium were changed to normal culture medium and incubated for another 24 h. Cells were then washed and permeabilized with the reporter cell lysis buffer (Promega, WI, USA). The luciferase activity in cell extracts was measured using a luciferase assay kit (Promega, WI, USA). Each measurement was carried out for 10 seconds in a single-well luminometer (Berthold Lumat LB 9507, Germany). The relative light units (RLU) were normalized by the total protein concentration of the cell extracts, measured with a protein assay kit (Bio-Rad, Hercules, CA, USA).

3.10 In Vivo Gene Transfer

For in vivo study, adult male Wistar rats (8 weeks of age, 180-200g) were used. The spinal cord injection procedure was performed under anesthesia by peritoneal injection of sodium pentobarbital (60 mg/kg). After the skin around L4-L5 was exposed, the intralumbar injection was accomplished via 1 ml syringe connected with 26-gauge needle. A slight movement of the tail indicated the proper injection into the subarachnoid space. Three µg DNA/60 µl of triple complexes were injected into each rat. The injection was carried out over 2-5 min and the syringe was left in place for a further 5 min to limit diffusion of the complexes from the injection site due to the backflow pressure. The skin was closed with surgical clips after the injection. The animals were kept warm until recovered.

One day after the injection, animals were perfused transcardially with 0.1M phosphate-buffered saline (PBS, 200 ml/rat) under anesthesia. Spinal cord was exposed after perfusion. Lumbar spinal cord and dorsal root ganglion were collected and homogenized with 400 µl and 100 µl of PBS using an ultrasonicator (Sonics, CT,
USA) at 100 W, 20 Hz for 5 seconds. Ten µl of the samples was used for the luciferase activity assay at room temperature using a luciferase assay kit (Promega, WI, USA). Each measurement was carried out for 10 seconds in a single-well luminometer (Berthold Lumat LB 9507, Germany). The relative light units were normalized by the total protein concentration of the cell extracts.

3.11 Cell Viability Assay

NIH3T3.E25 cells were cultured in DMEM supplemented with 10 % FBS at 37 °C, 10 % CO₂ and 95% relative humidity. For cell viability assay, the cells (10,000 cells/well) were seeded into 96-well microtiter plates (Nunc, Wiesbaden, Germany). After 24 h, culture media were replaced with serum supplemented tissue culture media containing serial dilutions of polymer or polymer/DNA complexes solutions and the cells were incubated for 24 h. In some experiments involving NL4-10K peptide, the cells were treated with PEI600/DNA/NL4-10K at various charge ratios and incubated for 48 h. After which 20 µl of sterile filtered MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (5 mg/ml) stock solution in phosphate buffered saline (PBS) was added to each well reaching a final concentration of 0.5 mg MTT/ml. After 4 h, un-reacted dye was removed by aspiration. The formazan crystals were dissolved in 100µl/well DMSO (BDH laboratory Supplies, England) and measured spectrophotometrically in an ELISA plate reader (Model 550, Bio-Rad) at a wavelength of 595 nm. The spectrophotometer was calibrated to 0 absorbance using culture medium without cells. Quintuplicate determinations for each treatment were preformed. The relative cell growth (%) related to control cells cultured in media without polymer, polymer/DNA or PEI600/DNA/NL4-10K was calculated by \[ \frac{[A]_{test}}{[A]_{control}} \times 100 \].
Chapter 4

Improving the Biocompatibility of 25kDa PEI
4 Improving the Biocompatibility of 25kDa PEI

4.1 Results

4.1.1 Cytotoxicity of Filtered PEI25k

PEI of molecular weight 25 kDa, although has high transfection efficiency, but also has been reported to be toxic to cells, rendering it unsuitable for in vivo application. The toxicity was thought to be a result of excess free PEI monomers and some leftover chemicals from the polymer synthesis, which would interact with and damage cells. Therefore, we filtered and lyophilized PEI25k to get rid of PEIs which are of molecular weight 10 kDa and below. The cytotoxicity of filtered PEI25kD, referred to as PEI25k(F), was then accessed in NIH3T3.E25 cells using MTT assay. In this assay, free polymers, instead of polymer/DNA polyplexes were used to measure the cytotoxicity. This was done to simulate a worst case scenario and to obtain larger sensitivity results, since, in general, toxicity is reduced when polyplexes with DNA are formed (Godbey et al., 1999). Cells treated with original PEI25k (hereinafter referred to as PEI25k(O)) induced cell death rapidly over increasing polymer concentration with 100% cell death at 1000 µM (Fig. 4-1). Although PEI25k(F) also induced 100% cell death at the same concentration, however, at lower polymer concentration, PEI25k(F) was less toxic than PEI25k(O) in the E25 cell lines.

4.1.2 Improving the DNA-Condensation Capability of PEI25k(F)

In order to access the DNA-condensation capability of PEI25k(F), various amount of polymer were complexed with 0.2 µg of plasmid DNA and electrophorized in 0.7% agarose gel. Fig. 4-2B shows that the mobility of plasmid DNA was fully
Chapter 4: Improving the Biocompatibility of 25kDa PEI

retarded by PEI25k(F) at N/P ratio (PEI amines to DNA phosphates ratio) of 7. In comparison with PEI25k(O) Fig. 4-2A), the DNA retardation occurred at N/P ratio of 5. We also quantified the ability of PEI25k(F)/(O) to condense DNA by measuring the fluorescence emitted upon addition of ethidium bromide to the polyplexes. Fig. 4-3 shows that the N/P ratios at which PEI25k(F) and PEI25k(O) completely suppressed the fluorescence are consistent with those obtained from agarose gel shift assays. Besides, at lower N/P ratios, PEI25k(O) is able to condense plasmid DNA better than PEI25k(F).

These results suggest that the reduced DNA condensation of PEI25k(F), when compared with PEI25k(O) at low N/P ratios, seems to be a consequence of lost of lower molecular weight PEIs (≤10 kDa). Therefore, to tighten the complexation of PEI25k(F) with plasmid DNA, we introduced PEI of molecular weight 600 Da (PEI600) at 1%, 3% and 4% into PEI25k(O) (PEI of molecular weight 25000 Da). The resulting copolymers, containing 99%, 97% and 96% of PEI25k(F) are named PEI25k(F) 99, PEI25k(F) 97 and PEI25k(F) 96, respectively. The various copolymers were complexed with 0.2 µg of plasmid DNA and subject to agarose gel retardation assays. With the introduction of PEI600, total charge neutralization improved from N/P ratio of 7 to 5, 4 and 5 respectively for PEI25k(F) 99, PEI25k(F) 97 and PEI25k(F) 96 (Fig. 4-2C, D and E). This is supported by ethidium bromide displacement assays, where the fluorescence dropped sharply at N/P ratios between 4 and 5 (Fig. 4-3). Furthermore, the copolymers were able to condense DNA as well as PEI25k(O) at low N/P ratios.
4.1.3 Transfection Efficiency of PEI25k and Various Copolymers In Vitro

We investigated the transfection efficacy of PEI25k(F) in NIH3T3.E25 cell lines, where PEI25k(O) was employed as a positive control. The cells were transfected in vitro with 1 µg of plasmid DNA complexed with the two polymers at N/P ratios ranging from 5 to 15. Transfections were made in the presence of 10% serum, and luciferase activities were measured after 24 h. As shown in Fig. 4-4, PEI25k(O) at charge ratio 10 mediated highest transfection efficiency, with a drop in transgene expression at N/P ratio 15, probably due to higher toxicity. In comparison, PEI25k(F) also attained the highest reporter gene expression at N/P ratio 10, but the efficacy was 2 to 4-fold lower than that of PEI25k(O) at all tested N/P ratios.

Next we investigated the effect of using PEI600 as small inserts to PEI25k(F) have on the transfection efficiency. Only PEI25k(F) with inserts at 1% and 3% were tested since PEI25k(F) 96 did not offer further improvement in the packing of DNA. NIH3T3.E25 cells were treated with 1 µg of DNA complexed with various copolymers. At N/P ratio of 5, PEI25k(F) 99 showed a marked increase in transgene expression as compared to PEI25k(O) at similar N/P ratio (where ***P<0.001) (Fig. 4-4). The luciferase activities were at 10-fold and 30-fold higher than PEI25k(O) and PEI25k(F) at N/P ratio 5, respectively. However, transfection efficacy at higher N/P ratios was inferior to that of PEI25k(O). Moreover, no improvement on the transfection efficiency of PEI25k(F) was observed from using 1% PEI600 inserts at high N/P ratios. Similar trend was observed for PEI25k(F) 97 where there was a sharp drop in transgene expression after N/P ratio 5.
4.1.4 Biophysical Characterization of PEI25kD and Various Copolymers

To know actual sizes of the particles, we measured the size of the complex formed by light scattering. Table 4-1A compared the sizes of DNA particles formed by various kinds of polymer and copolymer. For PEI25k(O), we were not able to measure the particle size on our machine at all tested N/P ratios due to excessive aggregation. In contrast, filtered PEI25k had an average size of ~300 nm for ratios 2/1 to 20/1, and was below 200 nm when the ratio was increased to 30/1. Increasing the PEI600 inserts to PEI25k(F) resulted in a slightly reduction in size to ~250 nm for ratios 2/1 to 20/1 and below 200 nm for 30/1. The reduction in size was more apparent for PEI25k(F) 97 than PEI25k(F) 99. Based on the above results, the use of a lower molecular weight PEI with PEI25k resulted in a relatively smaller and more stable polyplexes than using PEI25k alone. Also, filtered PEI25k caused less aggregation amongst the nanoparticles.

To investigate the surface charge of the filtered 25 kDa PEI, as well as to find out the correlation between the amounts of PEI600 inserts used and surface charge, we measured the \( \zeta \) potentials of various polymers at varying N/P ratios. Table 4-1B summarizes the results. For PEI25k(F), except at N/P ratio 2, where the \( \zeta \) potential assumed a negative value of -37.1 mV, the gene vectors at higher N/P ratios were in the positive range. As expected, the \( \zeta \) potential increased with increasing N/P ratios due to an excess of cationic polymer. With the addition of PEI600 at 1%, there was a significant increase in zeta potential at N/P ratio 5, from +38.9 mV to 42.6 mV, while the surface charges at higher charge ratios remain approximately the same. When the PEI600 inserts were increased to 3%, PEI25k(F) 97 had relatively higher surface charges than PEI25k(F) at all tested ratios. In particular, the increase was most prominent at N/P ratio 5.
4.1.5 Cytotoxicity of PEI25k/PEI600 Copolymers

One of the goals in using PEI600 as inserts was to increase the transfection efficiency of PEI25k(F). At the same time, we hoped that the low molecular weight PEI would not increase the toxicity of the copolymers. However, cytotoxicity assessment using MTT assay showed that the copolymer PEI25k(F) 99 and 97 induced cell death rapidly, with total cell death at 500 µM of copolymer used. In addition, the percentage of cell survival of both copolymers was approximately 20% lower than PEI25k(O) at all concentrations tested (Fig. 4-1). This probably explains the higher transfection efficiency seen at low N/P ratio, as well as low transgene expression at higher N/P ratios due to higher toxicity.
4.2 Discussion

The primary requirements for a clinically effective vehicle for human gene therapy are efficient gene transfer and safety. Non-viral vectors, including liposomes and polymers, are typically safer but much less efficient than recombinant viruses. In addition, many polymers still exhibit significant cytotoxicity in important cell lines. As a result, less than 15% of clinical trials have employed non-viral vectors, and the majority of these used lipofection rather than polymers (119).

Cationic polymers such as PEI25kDa has been reported to be one of the most efficient non-viral gene vectors, however, the main obstacle for its use in gene therapy is the cytotoxicity associated with it. One of the reasons is thought to be a result of membrane damaging effects (Chosakulnimitr et al., 1995). Studies had demonstrated that the cytotoxicity of polyplexes was closely related to the cytotoxicity of the free polymers, especially at high N/P ratios (Fechner, 2001). We hypothesized that one of the main contributing factors is the high polydispersity of PEI25kDa. In a solution of PEI25kDa, besides having PEIs of molecular weight 25kDa, there are also a wide range of PEI molecules in the initial PEI25kDa solution. These wide arrays of PEIs when not bound to DNA, may exhibit its toxic effects on the cell membranes through membrane permeabilization. To address this possibility, we attempted to filter off PEIs of molecular weight 10 kDa or lower. Cytotoxicity assay using MTT showed that PEI25k(F) could achieve better cell viability at polymer concentrations below 1000 µM. However, the trade off is lower transfection efficiency as compared to unfiltered PEI25kDa. This can be explained by the fact that the removal of PEIs caused inefficient packing of DNA especially at low N/P ratios, as evident from agarose gel retardation assay (Fig. 4-2B) and ethidium bromide displacement assay (Fig. 4-3), hence resulted in relatively large polyplexes of size 284-398 nm (Table 4-
1A), making the PEI25k(F)-DNA complexes unfavorable for the purpose of cell uptake. Another possible reason is that the interaction of cationic molecules with plasma membrane decreases considerably after filtration, thus the smaller degree of attachment to cell membranes contributes to lower transfection efficiency.

PEI molecules pack around the DNA in a simple self-assembly manner, where the interaction between the cationic polymers and the negatively charged DNA is mediated by electrostatic interactions (Vijayanathan et al., 2002). In packing of PEI around DNA, the latter undergoes localized bending and distortion into colloidal particles of rods, toroids (Dunlap et al., 1997; Golan et al., 1999) or spheroids (Liu et al., 2001). In any case, it is quite impossible for PEI to pack so densely together that it forms a continuous sheath about the condensed DNA. This is due to the convex nature of the polyplexes as well as electrostatic repulsion between individual PEI molecules. Therefore, there would be ‘gaps’ in the outer shell of the polyplexes formed by PEI molecules. As such, we sought to fill in these gaps with smaller molecular weight PEIs to improve the packing as well as the transfection efficiency of PEI25k(F). PEI of molecular weight 600 Da was chosen due to its low cytotoxicity, as shown in (Fig. 4-1). We found that at low N/P ratio, there was a marked improvement in the transgene expression mediated by PEI25k(F) and PEI600 copolymers, as compared to PEI25k(O) and PEI25k(F) alone (Fig. 4-4). Upon particle size measurement, the copolymers-DNA complexes were found to be relatively smaller size than PEI25k(F)-DNA complexes, which suggest better cell uptake. The increase in transfection efficiency could also be explained by surface charge analysis. At N/P ratio of 5, the zeta potential of PEI25k(F)-DNA complexes increases by 4 to 5 mV when PEI600 inserts of 1% and 3% were used, respectively. This means that more PEI molecules were packed into the PEI-DNA complexes, thus there will be more amines per PEI-DNA complex. With more amines in the endosomes upon cellular
uptake, the concentration of protons increases which in turn facilitates better endosomal escape of the polyplexes. Furthermore, the polyplexes will be protected from degradative enzymes due to better buffering capacity.

Zeta potential is also an indication of stability. The higher the zeta potential, the more stable the colloid is. As the PEI-DNA complexes gain more positive charges, the increase in repulsive force between individual complexes keep them from aggregating. The increase in stability is thought to be advantageous. A few groups had reported detecting intact PEI-DNA complexes at the perinuclear as well as in the nucleus (Godbey et al., 1999, 2000; Bieber et al., 2002). This suggests that with the increase in stability of the polyplexes, the likelihood of the gene vectors being transported to either the perinuclear or nucleus and eventually transgene expression is higher too.

Although there was a marked increase in transgene expression at low N/P ratio, the use of PEI600 inserts at high N/P ratios however did not improve the transfection efficacy. In fact, at N/P ratio 15, the reporter gene expression was lower than that of using PEI25k(F) alone (Fig. 4-4). Although at higher N/P ratios (≥10), the particle size of copolymers-DNA complexes decreased as compared to PEI25k(F) (Table 4-1A), the zeta potential stayed relatively the same at each respective ratios (Table 4-1B). It could be that at such high N/P ratio, some PEIs did not incorporate into the polyplexes. Instead, they existed as free polymers in the solutions, contributing to more positive charge being detected, and hence higher zeta potential. Therefore, no improvement in transfection efficiency was observed at high charge ratios. Moreover, as mentioned before, these free polymers will interact with and damage cell membrane, which eventually leads to cell death.

Cytotoxicity assays on E25 cells using the PEI25k(F) and PEI600 copolymers revealed higher toxicity as compared to PEI25k(O) and PEI25k(F) (Fig. 4-1). It is
quite puzzling that the addition of PEI600 at 1% and 3% to PEI25k(F) could caused such a drastic drop in cell viability, since PEI600 is known to be of quite low toxicity (Fig. 4-1). The most probable explanation could be that with the increase in number of PEI molecules packed around the plasmid DNA, the number of PEIs that are loosely associated with the complex surfaces increases also. Therefore, these loosely bound PEIs can also act as a free polymer on cell surfaces, permeabilizing and damaging the cell membranes.

In conclusion, the above data show the existence of gaps between PEI molecules on the outer layer of PEI-DNA complexes. It is possible to improve the transfection efficiencies through better packing of the plasmid DNA by inserting smaller PEI molecules of the right size and charge. This will not only enhance cell-surface binding, but can also create PEI-DNA complexes of higher buffering capability. However, the free PEI must also be removed from the solution in order to achieve a better transfection system.
Fig. 4-1: Cytotoxicity of PEI25k and PEI25k/PEI600 copolymers (PEI25k(F) 99 contains 99% PEI25k(F) with 1% PEI600D. Likewise, PEI25k(F) 97 contains 97% PEI25k(F) with 3% PEI600D) in NIH3T3.E25 cell lines. The cells were treated with various concentrations of polymers for 24 h in a serum-containing medium. Cell viability was determined by a MTT assay expressed as percentage of control, i.e. the untreated sister cultures. Each point represents the mean of four cultures.
A) PEI25k(O)

N/P Ratio: | DNA | 1/1 | 2/1 | 3/1 | 4/1 | 5/1 | 6/1 | 7/1
---|---|---|---|---|---|---|---|---

![Image of gel electrophoresis for PEI25k(O)]

B). PEI25k(F)

N/P Ratio: | DNA | 3/1 | 4/1 | 5/1 | 6/1 | 7/1 | 8/1 | 9/1
---|---|---|---|---|---|---|---|---

![Image of gel electrophoresis for PEI25k(F)]
C) PEI25k(F) 99

D) PEI25k(F) 97

D) PEI25k(F) 96

Fig. 4-2: Electrophoretic mobility of plasmid DNA through a 0.7% agarose gel was reduced by (A) PEI25kD(O), (B) PEI25kD(F), (C) PEI25kD(F) 99, (D) PEI25kD(F) 97 and PEI25kD(F) 96. Various amounts of polymers and copolymers corresponding to specific N/P ratios were mixed with 0.2 µg of DNA in a volume 10 µl for 30 min before electrophoresis.
Fig. 4-3: Ethidium bromide displacement assay of PEI25k(O), PEI25k(F), PEI25k(F) 99, and PEI25k(F) 97. Various amounts of polymers or copolymers with respect to the indicated N/P ratios were added to 96 µg DNA premixed with 0.01% of ethidium bromide. The polyplexes were then treated with water. The resulting fluorescence was measured at 485 nm-excitation and 595 nm-emission. Each data point reflects the mean value from three samples. The value of 1 denotes the fluorescence intensity without polymer.
Fig. 4-4: Effect of transfection efficiencies mediated by PEI25k(F) and PEI25k(F)/PEI600 copolymers in NIH3T3.E25 cells. Cells were treated with DNA complexes by PEI25k(O/F) or PEI25k(F)/PEI600 copolymers in serum-containing medium for 24 h in 24 well plates. 1 µg of pCAGluc plasmid was used per well. ***P<0.001 compared to PEI25k(O) at corresponding N.P ratio (n = 4).
## Table 4-1: Biophysical parameters of polymer / copolymer DNA complexes.

Table 4-1: Biophysical parameters of polymer / copolymer DNA complexes. 50 µg of DNA was complexed with the respective polymers / copolymers in 5% glucose at the indicated charge ratios. (A) Particle size (nm) determination was carried out at fixed angle of 90° by dynamic light scattering. (B) Zeta potential (mV) was analyzed by phase analysis light scattering and the *Smoluchowski* approximation was used. All measurements were determined under a temperature of 22°C and all buffer solutions used were filtered through a 0.22 mM filter before use.
Chapter 5

Cell Targeting Using Nerve Growth Factor
5 Cell Targeting Using Nerve Growth Factor

5.1 Results

5.1.1 PEI as an Endosome-Disrupting Agent

From previous work, it was affirmed that complexes made from a luciferase-encoding reporter plasmid, pCAGluc, and NL4-10K alone had relatively weak transfection efficiency (unpublished data from lab). An endosome-disrupting agent, chloroquine, was therefore employed to augment gene expression. Due to toxicity problems with chloroquine in vivo, we chose PEI600 as the endosomolytic agent. This low molecular weight polycationic polymer is capable of condensing DNA, yet with relatively low background transfection efficiencies and low toxicity as compared with higher molecular weight PEI such as PEI25k (Fig. 4-1). The excess positive charge on PEI/DNA complexes surface can cause unspecific binding, thereby reducing the specificity of a target complex (Schaffer and Lauffenburger, 1998). Since in our formulation order NL4-10K is added to preformed PEI600-DNA, it therefore follows that the neutrally charged PEI600-DNA polyplexes offer best compromise in relation not only to specific binding of targeted complexes but also to the stimulation of host immune response (including complement activation) and systemic clearance mechanism (e.g. liver, macrophages). In order to determine the N/P ratio that corresponds to the electroneutrality point, we measured the zeta potential of PEI600-DNA complexes over various ratios. Table 5-1A shows that at N/P ratio of 5, the surface charge of PEI600-DNA complexes is at -1.75 mV, which is closed to neutrality, suggesting that the negative charges on DNA are almost fully shielded by PEI600. Therefore, the N/P ratio 5 was chosen as a pilot test ratio to prepare triplex that consists of plasmid DNA, PEI600 and NL4-10K.
5.1.2 Biophysical Properties of the NL4-10K, PEI600/NL4-10K, 10K and PEI600/10K Gene Vectors

The condensation of DNA into particulate structures is a prerequisite for gene delivery. For this reason, we first examined the capability of NL4-10K in condensing DNA and to form particulate complexes. Various amount of NL4-10K were complexed with 0.1 µg of pCAGluc and and the mobility was accessed through an agarose gel under electrophoresis. DNA retardation by NL4-10K alone showed that the mobility of plasmid DNA was reduced with increasing peptide concentrations (Fig. 5-1A). At N/P ratio of 2/1, the complex became immobile, indicating complete charge neutralization of DNA. Next we examined the effect of DNA condensation with the addition of PEI600 with N/P ratio fixed at 5/1. The triplexes were prepared as described in materials and methods with the formulation order of PEI600 first added to DNA followed by NL4-10K. As shown in Fig. 5-1C, the triplexes were completely retarded at the peptide/DNA ratio of 1, indicating that the DNA was more tightly packed in the presence of PEI600. This was supported by size measurement of the gene vectors by laser light scattering which showed that NL4-10K gene vectors had a size of 232.7 ± 5.0 nm at peptide/DNA ratio 5, which decreased to 158.4 ± 5.5 nm with the addition of PEI600 at PEI/DNA/peptide ratio of 5/1/5 (Table 5-1B). The other peptide 10K, which was used as a negative control peptide, totally retarded the DNA at peptide/DNA ratio 3, and this ratio was shifted to 1 with the addition of PEI600 (Fig. 5-1B and D).

The electric potential at the surface of polymer/DNA/peptide complex particles will influence their colloidal stability and their interaction with blood components and the negatively charged surface of cells. To investigate the surface charge properties, we measured the ζ potential of various complexes prepared in 1mM KCl buffer.
Before the addition of NL4-10K, PEI600/DNA at the fixed N/P ratio 5 was at neutral charge \((\text{Table 5-1A})\). After NL4-10K was added to the preformed polyplexes at peptide/DNA ratio 5, the surface charges increased to \(\sim 23\) mV. Interestingly, complexes prepared by using only NL4-10K and pCAGluc alone at ratio 5 also assumed the same \(\zeta\) potential. Increasing the amount of peptide in the PEU600/DNA/Nl4-10K triplexes resulted in a negligible increase in \(\zeta\) potential (\(\text{Table 5-1B}\)). For 10K gene vectors, 10K/DNA had a \(\zeta\) potential of \(\sim +11\) mV and increased to \(\sim +23\) mV for PEI600/DNA/10K at charge ratio 5/1/5 (\(\text{Table 5-1B}\)).

### 5.1.3 NL4-10K-containing Triplexes Mediates In Vitro Gene Transfer in a Dose-dependent Manner

To demonstrates that NL4-10K-containing triplexes mediates gene transfer in vitro, transfection assays were performed in parental NIH3T3 cells and two TrkA-expressing cell lines, namely, NIH3T3.TRK1 and NIH3T3.E25. The triplexes were prepared with PEI600 first complexed with 0.5 \(\mu\)g of pCAGluc at fixed N/P ratio of 5, NL4-10K of varying amounts were added after 30 min incubation. \(\text{Fig. 5-2B}\) shows that in NIH3T3.E25 cells, the complexes formed by using NL4-10K/pCAGluc ratio of 0.5 resulted in modest gene delivery, which was comparable to that mediated by the other two negative controls, PEI600-DNA at N/P ratio 5 and PEI600-DNA-10K at ratio 5/1/5, as well as the control of transfection done in parental NIH3T3 cells (\(\text{Fig. 5-1A}\)). There was a tremendous increase in transgene expression when the amount of peptide was between the peptide-DNA ratios of 1 to 10. However, the transfection efficiency did not increase linearly with increasing peptide used. After peptide-DNA ratio of 1, there was a slight drop in transgene expression. This transfection assay was repeated in NIH3T3.TRK1 cells, with the inclusion of peptide-DNA ratio 2.5.
Similar trend was observed in the gene transfer efficiency of NIH3T3.TRK1 (Fig. 5-2C). Highest transgene expression was observed at peptide-DNA ratio 2.5, after which the transfection efficacy reduced with increasing NL4-10K used.

### 5.1.4 Transfection Efficiency Mediated by NL4-10K-containing Triplexes is Dependent on Formulation Order

To investigate the effect of formulation order of vector complexes on the transfection efficiency, the ternary gene vectors consisting of PEI600, NL4-10K and DNA were formulated in two different ways. Either PEI600 was added to preformed gene vectors consisting of DNA and NL4-10K, or complexes were generated vice versa through the addition of NL4-10K to preformed PEI600/DNA complexes. The ternary gene vectors were then transfected in TrkA-expressing NIH3T3.E25 cells. When PEI600 at a polymer/DNA ratio 5 was added to NL4-10K/DNA complexes at various ratios ranging from 5 to 10, the transgene expression remained approximately at the same level as that of the control PEI600/DNA and PEI600/DNA/10K (Fig. 5-3), indicating that triplexes prepared in this way does not mediate gene delivery and it is not dose-dependent. In contrast, when the ternary gene vectors were generated vice versa, i.e. PEI600 was first complexed with DNA at N/P ratio 5, with varying amount of NL4-10K added later, the transgene expression increased / decreased in a dose-dependent manner as aforementioned (Fig. 5-3).

### 5.1.5 Specificity of NL4-10K-Mediated Gene Delivery

The reason for introducing PEI600, a low-molecular-weight cationic polymer with low toxicity into NL4-10K-containing gene vectors was to make use of its
endosome-disrupting ability to help in the escape of NL4-10K gene vectors from the endosomes upon cellular uptake. Although PEI600/DNA at N/P ratio of 5 was chosen due to its electroneutrality, there is a possibility of using a higher amount of PEI600 to attain higher transfection efficiency without sacrificing specificity. Therefore, in this experiment, PC12 cells were transfected with ternary complexes consisting of either 10K, NL4-10K or no peptide at all with increasing amount of PEI600 complexed with 1µg of plasmid DNA. The results are shown in Fig. 5-4A. At charge ratio 5/1, PEI600 alone in PC12 cells had very low transfection efficiency and the addition of 10K peptide into the PEI600/DNA complexes did not improve the efficiency. When NL4-10K was introduced into the PEI600/DNA complexes, transgene expression increased in a dose-response manner, reaching a RLU/mg protein of 2 x 10^5. When the N/P ratio of PEI600/DNA was increased to 10 and 20, PEI600 alone could mediate gene delivery comparable to that of using NL4-10K. Also, the addition of NL4-10K failed to offer further improvement in transfection efficiency. Although the 10K peptide did not mediate gene delivery at N/P ratios 5 and 10, there was however significant non-specific transgene expression at N/P ratio 20. Taken together, these data suggest that using PEI600-DNA at N/P ratios higher than 5/1 will result in non-specific cell uptake and hence disrupt the intended cell-targeting effect of NL4-10K.

To examine the specificity of the NL4-10K peptide in mediating gene delivery, we first compared the transfection efficiencies of the peptide in two Trk1-expressing cell lines NIH3T3.TRK1 and NIH3T3E25 cell with the parental NIH3T3 cells which are TrkA negative. Triplexes were formed at PEI600/DNA/NL4-10K ratio of 5/1/5 with 0.5 µg of pCAGluc per triplex. In NIH3T3 cells, NL4-10K-mediated gene delivery showed modest transfection efficiency, with less than 10^5 luciferase activity per mg protein (Fig. 5-4B). In contrast, NL4-10K-mediated reporter gene expression in
NIH3T3.TRK1 and NIH3T3.E25 was 51-fold and 440-fold higher, respectively, than that in the parental NIH3T3 cells (Fig. 5-4B).

To further demonstrate the targeting property of NL4-10K, the above mentioned cell lines were transfected with triplexes containing 10K short peptide instead of NL4-10K. As shown in Fig. 5-4B, the luciferase gene expression in NIH3T3.TRK1 and NIH3T3.E25 cells were comparable to that of NIH3T3 cells, suggesting a targeting effect of NL4-10K. These findings provided evidence that NL4-10K may mediate gene delivery to TrkA-expressing cells only through the activity of NL4 loop in NL4-10K.

5.1.6 Optimizing the Charge Ratios between PEI600, NL4-10K and DNA

From Table 5-1B, we observed that the $\zeta$ potential of NL4-10K/DNA complex at ratio 5 was $\sim+23$ mV using 20 $\mu$g of pCAGluc. Similarly, the $\zeta$ potential for PEI600/DNA/NL4-10K at ratio 5/1/5 was also at $+23$ mV. Increasing the N/P ratio of NL4-10K to DNA did not significantly increase the $\zeta$ potential. Both observations led to the hypothesis that the $\zeta$ potential offered by the triplexes saturated at about $+23$ mV, any further increase in the amount of NL4-10K peptides added remained unbound to the DNA and they could be homogeneously distributed in the solution. These unbound NL4-10K peptides may compete with the triplexes and thus cause the decrease in luciferase gene expression as observed in Fig. 5-2A and B. One possibility of increasing the amount of NL4-10K bound to DNA is to reduce the N/P ratio of PEI600/DNA, thus freeing more ‘empty gaps’ for NL4-10K to interact with the negatively charged DNA. However, the N/P ratio of PEI600/DNA should be high enough to provide buffering capacity for endosomal escape.
To investigate this hypothesis, NL4-10K at N/P ratio 5 with DNA was complexed with preformed PEI600/DNA polyplexes at various charge ratios ranging from 0.5 to 5, the resulting triplexes were then transfected in NIH3T3.TRK1 cells. As shown in Fig. 5-5A, PEI600/DNA at N/P ratio of 1 and 2.5 mediated reporter gene expression about 9-fold and 4-fold, respectively, higher than that of PEI600/DNA at ratio 5. Fixing the PEI600 ratio at 1, next we investigated the NL4-10K-mediated gene delivery with increasing peptide. Fig. 5-5B shows that a significant increase in transgene expression was observed at PEI600/DNA/NL4-10K ratio of 1/1/5 and 1/1/10, at 6-fold and 10-fold, respectively, as compared to ratio of 5/1/5.

5.1.7 Biocompatibility of NL4-10K-containing Triplexes

As mentioned before, the use of cationic polymer PEI may be toxic to cell since it is known to permeabilize cell membranes. Furthermore, the highly charged 10K domain in NL4-10K peptide may also induce cytotoxicity. To access the possible in vitro cytotoxicities of NL4-10K-containing triplexes, we performed the MTT assay in NIH3T3.E25 cells. Triplexes were prepared with PEI600/DNA N/P ratio fixed at 1/1 while varying the N/P ratio of NL4-10K/DNA. At all tested charge ratios, the triplexes did not induce significant cell death and were able to maintain E25 viability at about 90% (Fig. 5-6). As a positive control, PEI25kD was observed to cause a dose-response cell death trend over the range of 0-50 charge ratios.
5.2 Discussion

Macromolecular uptake by cells is made possible by the presence of ligand-specific receptors on the cell surface. The basic principle of receptor-mediated gene delivery is to subvert this efficient transport system in such a way that ligands complexed with DNA are recognized by the receptor and are carried across the cell membrane. Here we present a non-viral gene transfer vector for targeted gene delivery into TrkA-positive cells. This gene transfer vehicle consists of the hairpin motif of NGF loop 4 linked to 10 lysine residues for targeting and nucleic acid binding purposes, respectively, and a low molecular weight polyethylenimine, PEI600.

In our previous work (Zeng et al., 2004), we had successfully demonstrated that NL4-10K-containing complexes, with the help of chloroquine, is capable of directing \textit{in vitro} gene delivery to TrkA receptors. In this study, instead of chloroquine, low molecular weight, branched PEI600 was employed as an endosomal disrupting agents. The polymer was chosen mainly due to its low immune stimulating effect and low cytotoxicity. Additionally, although it binds to and condenses DNA, the polymer itself mediates very low transgene expression at low polymer/DNA ratios, as compared to high molecular weight PEI. The latter property is favourable for our application since there would not be unspecific gene expression besides those mediated by NL4-10K Therefore, the properties of PEI600 render it suitable to be incorporated into NL4-10K/DNA complex for \textit{in vivo} application. In order to choose a suitable PEI600/DNA N/P ratio in the triplex, we conducted a series of zeta potential measurements over various N/P ratios ranging from 2 to 30. At ratio 5/1, the surface charge of the PEI600-DNA polyplexes becomes neutral, i.e., they are neither positively nor negatively-charged, and it increases progressively with increasing N/P ratios (Table 5-1A). The net positive surface charge, although contributes to efficient
condensation and tight compaction of DNA, may however leads to non-specific cell-uptake, probably due to the excess positive charge on the particle surface and the domination of electrostatic interaction between the particles and cell surface. In view of this, we investigated using increasing PEI600/DNA ratio in NL4-10K-containing triplexes and found that N/P ratios higher than 5 led to non-specific cell-uptake (Fig. 5-4A). Therefore, we fixed the preliminary PEI600-DNA complexes at N/P ratio of 5.

The formulation order of vector complexes was found to have an enormous effect on the gene delivery efficiency. A 200-fold increase of transgene expression was observed when DNA was first complexed with PEI600 and NL4-10K was added afterward. When the complexes were generated vice versa, transgene expression remained on the same level as that of control PEI600/DNA at ratio 5/1 and PEI600/DNA/10K at charge ratio 5/1/5. These differences could be explained by analysis of the structure of the resulting gene vectors. When DNA was first complexed with PEI600 at a charge ratio of 5, the resulting polyplexes had a \( \zeta \) potential of \(-1.75 \pm 0.53 \) mV. Further addition of NL4-10K to the preformed polyplexes resulted in a \( \zeta \) potential of \( 23.2 \pm 1.1 \) mV and \( 25.3 \pm 2.1 \) mV for N/P ratio of peptide/DNA 5/1 and 10/1, respectively (Table 5-1B). Whereas when DNA was complexed with the peptide first resulted in a \( \zeta \) potential of \( 23.3 \pm 1.3 \) mV, this value remained about the same with the addition of PEI600 at charge ratio 5. Therefore, in the first scenario, although the negatively charge DNA had been completely neutralized by PEI600 at N/P ratio 5, there were still 'gaps' on the polyplexes to allow the binding of NL4-10K via the 10K DNA-binding domain. It is possible that this formulation order resulted in a ternary structure with PEI600 and 10K domain bound to the DNA in the core of the complex, while the NL4 TrkA targeting domain was flanked on the periphery of the complex due to the \( \beta \)-strands in the chimeric peptide that act as spacers between NL4 and the 10K/DNA complex. According to this model,
the complexes are apparently taken up into the TrkA-positive cells via receptor-mediated endocytosis, and the ‘proton sponge’ effect of PEI600 eventually induces the release of the complexes into the cytosol. In the second formulation order where peptide was added first, the addition of PEI600 did not cause a significant change in the ζ potential, more specifically, PEI600 could not increase the ζ potential of the complexes further. It could be that the surface potential of the triplexes had already saturated, thus binding of PEI600 to the DNA should not be possible due to strong electrostatic repulsion. Since PEI600 was not incorporated into the gene vector complexes, thus the reporter genes could not escape from the endosomal compartment. This explains the low transfection efficiencies observed with triplexes prepared in this manner (Fig. 5-3).

One of the main goals in this study is to establish that TrkA cell-specific gene transfer is obtained by the targeting domain of NL4 in the NL4-10K/DNA-containing triplex, even with the addition of PEI. In our previous publication (Zeng et al., 2004), we found that the uptake of NL4-10K/DNA complexes is mediated by TrkA, but not p75, TrkB or TrkC. Having fixed the PEI600/DNA ratio at 5, the cell-type specificity of the triplexes was investigated in two NIH3T3 fibroblast lines expressing exogenous TrkA receptors, and also in parental NIH3T3 cells, which are TrkA-negative. Both NIH3T3.TRK1 and NIH3T3.E25 cells, where the percentage of TrkA-positive cells was approximately 67% and 75% (Zeng et al., 2004), respectively, achieved significantly higher reporter gene expression than in NIH3T3 cells.

To further demonstrate the TrkA selectivity of the NL4-10K peptide, TrkA-expressing cells were transfected with triplexes containing 10K peptide, which lacks the NL4 targeting domain. Transfections performed in NIH3T3.TRK1 and NIH3T3.E25 cells led to similar but minimal transgene expression, suggesting the involvement TrkA receptors in NL4-10K-mediated gene delivery. However, it is
conceivable that the differences between the two peptides is due to difference in the surface charge of resulting triplexes, since there are about 5 more basic amino acids in NL4-10K than that in 10K and this could influence the degree of triplexes adhering to negatively charged cell surfaces. Upon measuring the $\zeta$ potential of triplexes containing either NL4-10K or 10K, we found that the surface charge was similar for both triplexes, at about 23 mV (Table 5-1B). Moreover, DNA was fully retarded by NL4-10K and 10K at peptide/DNA ratios 0.5-1 (Fig. 5-1C and D), therefore the differences in transfection efficiency among the peptides can be attributed primarily to the involvement of NL4 domain and hence TrkA receptor rather than to different biophysical gene vector parameters.

At fixed PEI600/DNA ratio of 5, we consistently observed that increasing the amount of NL4-10K used in the triplex did not cause a corresponding increase in transfection efficiency in both NIH3T3.TRK1 and NIH3T3.E25 cells (Fig. 5-2B, C). In fact, after certain NL4-10K/DNA ratio, transgene expression decreased with increasing peptide used. When the N/P ratio of PEI600/DNA was adjusted from 5 to 1, the transgene expression increased significantly, even at higher peptide/DNA ratio. We proposed that this is attributed to the limited binding site available on each plasmid DNA molecule. According to our formulation order where PEI600 is added before NL4-10K, the number of NL4-10K molecules that is bound to the DNA is dependent on how many binding sites is left available for NL4-10K after PEI600 interacts with the negatively charged DNA. Therefore, less NL4-10K will be incorporated into the triplex if more PEI600 was used. Moreover, the electrostatic repulsion between PEI600/DNA polyplexes and NL4-10K molecules also affects the number of NL4-10K molecule bound to DNA. This phenomenon is also evident from $\zeta$ potential analysis. We observed that the surface charge of NL4-10K/DNA complexes at ratio 5 saturates at about +23 mV (Table 5-1B). Triplexes formed by adding NL4-
10K to preformed PEI600/DNA polyplexes at N/P ratio 5 also assumed the same surface charge, i.e. at +23 mV, and \( \zeta \) potential seems to saturate at this value even with additional peptide used (Table 5-1B). As such, excess NL4-10K that remains unbound is distributed in the triplex solution, and will compete with triplexes containing the reporter plasmid for TrkA receptors. Although the free NL4-10K peptides are capable of inducing receptor-mediated endocytosis once bound to TrkA, no reporter gene expression will be observed since they are empty vectors. Therefore, there is a need to reduce the amount of PEI600 used in order to incorporate more NL4-10K peptide into the triplexes and we found the optimized ratio of PEI600/DNA/NL4-10K at 1/1/10 \textit{in vitro}.

The gene vector system using NL4-10K as targeting ligand reported here could potentially be useful in addressing neurodegenerative disorders involving neurons expressing TrkA receptors. Several neuronal cell types that are implicated in important disease states express TrkA and therefore respond to NGF, including sensory, sympathetic, and cholinergic neurons. For example, it has been suggested that NGF therapy may delay the onset of Alzheimer’s disease (Barinaga, 1994; Lindsay, 1996). The neurons that are responsible for Alzheimer’s disease are the basal forebrain cholinergic neurons that express TrkA. Therefore, this peptide reported here could potentially be used to direct therapeutic genes or agents into targeted cells only.
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(B)

<table>
<thead>
<tr>
<th></th>
<th>ζ potential (mV)</th>
<th>particle size (nm)</th>
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<tbody>
<tr>
<td>DNA/10K (1/5)</td>
<td>11.5 ± 1.7</td>
<td>175.0 ± 5.1</td>
</tr>
<tr>
<td>PEI600/DNA/10K (5/1/5)</td>
<td>23.4 ± 0.8</td>
<td>843.0 ± 341</td>
</tr>
<tr>
<td>DNA/NL4-10K (1/5)</td>
<td>23.3 ± 1.3</td>
<td>232.7 ± 5.0</td>
</tr>
<tr>
<td>PEI600/DNA/NL4-10K (5/1/2.5)</td>
<td>22.0 ± 1.0</td>
<td>193.4 ± 5.7</td>
</tr>
<tr>
<td>PEI600/DNA/NL4-10K (5/1/5)</td>
<td>23.2 ± 1.1</td>
<td>158.4 ± 5.5</td>
</tr>
<tr>
<td>PEI600/DNA/NL4-10K (5/1/10)</td>
<td>25.3 ± 2.1</td>
<td>232.8 ± 5.9</td>
</tr>
<tr>
<td>PEI600/DNA/NL4-10K (1/1/5)</td>
<td>24.3 ± 2.5</td>
<td>159.9 ± 2.4</td>
</tr>
<tr>
<td>PEI600/DNA/NL4-10K (1/1/10)</td>
<td>24.6 ± 2.4</td>
<td>168.0 ± 1.1</td>
</tr>
<tr>
<td>NL4-10K/DNA/PEI600 (5/1/5)</td>
<td>21.7 ± 1.8</td>
<td>226.3 ± 1.4</td>
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</table>
Table 5-1: Biophysical parameters of polymer-DNA, peptide-DNA and polymer-DNA-peptide complexes. (A) Zeta potential of PE600/DNA polyplexes at various N/P ratios. (B) Zeta potential and particle size of peptide-DNA and polymer-DNA-peptide complexes in 1mM KCl. (C) Stability of complexes indicated by measurement of the particle size over a period of more than 2 hours. Particle size determination was carried out at fixed angle of 90° by dynamic light scattering. Zeta potential (mV) was analyzed by phase analysis light scattering and the Smoluchowski approximation was used. All measurements were determined under a temperature of 22°C and all buffer solutions used were filtered through a 0.22 mM filter before use.
A) NL4-10K/DNA

N/P ratio

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<th>0</th>
<th>0.5</th>
<th>1</th>
<th>1.5</th>
<th>2</th>
<th>3</th>
<th>4</th>
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</table>

B) 10K/DNA

N/P Ratio:

<table>
<thead>
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<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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</table>
Fig. 5-1: DNA retardation by (A) NL4-10K, (B) PEI600 and NL4-10K, (C) 10K and (D) PEI600 and 10K in agarose gel under electrophoresis. 0.1µg of plasmid DNA was complexed with different amount of peptides at the indicated ratios. The complexes were electrophoresed in 0.7% agarose gel at 80 V for 45 min. For (C) and (D), triplexes were formed by mixing PEI600 with DNA at N/P ratio of 5 and peptides were added to the preformed PEI600/DNA polyplexes after 30 min incubation.
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(A)
Fig. 5-2: Dose-dependent response of transgene expression mediated by NL4-10K. NL4-10K-mediated gene delivery in (A) NIH3T3 cells, (B) NIH3T3.E25 cells and (C) NIH3T3.TRK1 cells. To prepare PEI600/DNA/NL4-10K, pCAGluc was first complexed with PEI600 at N/P ratio of 5 and peptides were added afterward at indicated N/P ratios. All cells were treated with 0.5 µg of pCAGluc/well in a 48-well.
Fig. 5-3: Effects of the formulation order of vector triplexes on the gene delivery efficiency. Either DNA was first complexed with NL4-10K at N/P ratio of 5 to 15, PEI600 (N/P = 5) was added afterward or vice versa. TrkA-expressing NIH3T3.E25 cells were treated with triplexes prepared with both formulation orders in serum containing medium for 24 h in 48-well plates. 0.5µg pCAGluc plasmid was used per well.
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(A)

- No peptide
- 10K/DNA (2.5/1)
- NL4-10K/DNA (2.5/1)
- 10K/DNA (5/1)
- NL4-10K/DNA (5/1)
Fig. 5-4: Specificity of NL4-10K mediated gene delivery. (A) NL4-10K-mediated gene delivery without chloroquine in PC12 cells. To prepare PEI600/DNA/NL4-10K, pCAGluc was first complexed with PEI600 at the indicated N/P ratio and peptides were added 30 minutes later at N/P ratios of 2.5 and 5. PC12 cells were transfected with triplexes containing 1 µg of pCAGluc/well in a 24 well plate. After 4 h, an equal volume of normal culture medium was added and the cells were incubated for 24 h before luciferase expression assay. *P<0.05 or **P<0.01 compared to the controls without peptides. (B) Two TrkA-expressing cell lines, NIH3T3.TRK1, NIH3T3.E25 and parental NIH3T3 cells were transfected with either PEI600/DNA/NL4-10K or PEI600/DNA/10K triplexes. Each triplex consisted of 0.5 µg of pCAGluc complexed with PEI600 first at N/P ratio of 5 and NL4-10K/10K was added later at charge ratio 5. **P<0.01 compared to transfection in NIH3T3 cells (n = 4).
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(A)
Fig. 5-5: Optimizing the transfection efficiency mediated by NL4-10K and PEI600-containing triplexes. (A) Triplexes were prepared with PEI600 first complexes with 0.5 µg of plasmid DNA/well at indicated charge ratios. NL4-10K was added after 30 min incubation at fixed N/P ratio of 5. (B) Triplexes were prepared with PEI600/DNA ratio of 1/1, and NL4-10K were added afterward at various N/P ratios. *P<0.05 compared to control PEI600/DNA/NL4-10K at 5/1/5 (n = 4).
Fig. 5-6: Cytotoxicity of NL4-10K-containing triplexes in NIH3T3.E25 cells. PEI600 was mixed with DNA at N/P ratio 1, and NL4-10K was then added to form PEI600/DNA/NL4-10K triplexes at the indicated total N/P ratio. PEI25kD/DNA complexes formed by mixing PEI25kD with plasmid DNA at the indicated ratios were used as a positive control. Triplexes were added at 0.1 µg DNA/well of a 96-well microplate. Cell survival was estimated using a MTT assay and expressed as a percentage of control, the untreated sister cultures.
References


