CONSTRUCTION OF NERVE GROWTH FACTOR LOOP 4-
CONTAINING POLYPEPTIDES FOR FACILITATED GENE
TRANSFER TO NEURONS

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2004
Construction of Nerve Growth Factor Loop 4-Containing Polypeptides for Facilitated Gene Transfer to Neurons

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A THESIS SUBMITTED FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF MEDICINE
NATIONAL UNIVERSITY OF SINGAPORE
&
INSTITUTE OF BIOENGINEERING AND NANOTECHNOLOGY

March 2004
ACKNOWLEDGEMENTS

First and foremost, I wish to express my appreciation to my supervisor, A/P Shu Wang for his totally supporting on this study and for truly understanding what this research is all about. And to A/P Heng-Phon Too and A/P Hanry Yu, my co-supervisors for the in-depth discussions and useful suggestions.

I would also like to acknowledge our exceptional research group at Institute of Bioengineering and Nanotechnology for providing such a fabulous environment for the study. Especially thank Mr. Shujun Gao for the assistance in animal studies, and thank Dr. Xu Wang for the technical support in immunostaining study and confocal microscopy. My thanks also to Ms. Yuexia Ma for preparing the primary culture. My gratitude also to Dr. Alonzo H. Ross from the University of Massachusetts Medical School for kindly providing two TrkA-expressing NIH3T3 cell lines.

Finally, I would like to express my gratitude to my family for their generosity, faith, and superb guidance during the lengthy PhD study. To my father, Yaoying Zeng - immunologist and researcher - for rendering inspiring ideas. To my mother, Xiaochang Cai - dermatologist and nurturer - for the continuous encouragement. And my wife, Ruijuan Du who herself has been pursuing a PhD in molecular microbiology during the same period for believing in me from the start and lightening my life.
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<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
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<td>Alzheimer's disease</td>
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<td>ADA</td>
<td>adenosine deaminase</td>
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<td>BBB</td>
<td>blood-brain barrier</td>
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<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CR</td>
<td>cysteine-rich cluster</td>
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<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
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<td>DRG</td>
<td>dorsal root ganglia</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>ERK</td>
<td>extracellular receptor-activated kinase</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>HA</td>
<td>hemagglutinin</td>
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<td>HBS</td>
<td>HEPES-buffered saline</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>HSPGs</td>
<td>heparin sulfate proteoglycans</td>
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<td>HSV</td>
<td>herpes simplex virus</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>LLR</td>
<td>leucine-rich region</td>
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<td>MND</td>
<td>motor neuron disease</td>
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<td>NGF</td>
<td>nerve growth factor</td>
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<td>NLS</td>
<td>nuclear localization sequence</td>
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<td>nuclear pore complex</td>
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<td>NT-3</td>
<td>neurotrophin-3</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>OTC</td>
<td>ornithine transcarbamylase</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PD</td>
<td>Parkinson's disease</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<tr>
<td>PEI</td>
<td>polyethyleneimine</td>
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<tr>
<td>PI-3K</td>
<td>phosphotidylinositol-3-OH kinase</td>
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<tr>
<td>PLC-γ1</td>
<td>phospholipase Cγ1</td>
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<tr>
<td>PNA</td>
<td>peptide nucleic acid</td>
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<tr>
<td>RLU</td>
<td>relative light unit</td>
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<tr>
<td>SC</td>
<td>spinal cord</td>
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<tr>
<td>SCID</td>
<td>severe combined immune deficiency</td>
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<tr>
<td>SCID-X1</td>
<td>X-linked severe combined immunodeficiency</td>
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<tr>
<td>SH2</td>
<td>src homology domain 2</td>
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<tr>
<td>TIL</td>
<td>tumor-infiltrating lymphocyte</td>
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LIST OF PUBLICATIONS AND PATENT

Publications:


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Patent:

SUMMARY

Gene delivery vectors that restrict the expression of therapeutic genes to a particular type of cells are critical to gene therapy in a complex structure, such as the central nervous system. Therefore, the development of targeted gene delivery to diseased subtypes of neurons will benefit the success of gene therapy for neurological disorders. In this study, chimeric polypeptides were constructed for targeted gene transfer to cells expressing nerve growth factor (NGF) receptor TrkA.

Firstly, a recombinant polypeptide composed of a targeting moiety derived from loop 4-containing hairpin motif of NGF and a DNA-binding moiety of 10-lysine sequence was expressed in *E. coli*. The recombinant cationic polypeptide facilitated gene delivery to PC12 cells that express the NGF receptors. It activated NGF receptor, TrkA and its downstream signaling pathway in PC12 and promoted the survival of neuronally differentiated PC12 cells deprived of serum. The polypeptide could also bind plasmid DNA and enhance polycation-mediated gene delivery in NGF receptor-expressing PC12 cells, but not in COS7 cells lacking NGF receptors. The enhancement of gene transfer in PC12 was inhibited by pretreatment of free, unbound polypeptides, suggesting a NGF-receptor-specific effect of the polypeptide. These pilot observations demonstrated the concept of using receptor-mediated mechanism for targeted gene delivery to neurons.

To eliminate the effect of the bulky fusion protein in the recombinant polypeptide, a chemically synthesized peptide simply composed of the...
targeting moiety derived from NGF loop 4 and the DNA binding moiety of 10 lysine residues was employed for more systemic study. This peptide activated signal transduction pathways of the NGF receptor TrkA in PC12 cells and supported the survival of the cells after serum deprivation with better efficiency. After forming complexes with plasmid DNA, the peptide dose-dependently increased reporter gene expression in PC12 cells, which could be inhibited by excess NGF. The peptide-mediated gene expression was not affected in PC12 cells by co-incubation with a blocking antibody against the low affinity NGF receptor p75\textsubscript{NTR} and was significantly enhanced in NIH3T3 cells stably transfected with TrkA cDNA, suggesting the involvement of the high affinity NGF receptor TrkA without the participation of p75\textsubscript{NTR}. The peptide-mediated gene expression in rat primary cortical neurons was localized mainly in those expressing TrkA and hardly seen in the cells stained positively with anti-TrkB or TrkC antibodies. Moreover, the peptide did not assist gene transfer in TrkA-poor, but TrkB- and/or TrkC-positive primary cerebellar granule neurons and primary cortex glial cells. The present study demonstrated as well that the peptide enhanced polyethylenimine (PEI)-mediated \textit{in vivo} gene transduction in rat dorsal root ganglia, a site with TrkA-expressing neurons.

In summary, the chimeric polypeptides reported would be useful in gene delivery to and gene therapy of the nervous system and other tissues/organs with cells expressing TrkA.
CHAPTER 1
INTRODUCTION
1.1 Gene Therapy

1.1.1 Background of Gene Therapy

As early as 1963, J. Lederberg addressed the concept of gene therapy in an article "Biological Future of Man" (Wolff and Lederberg, 1994):

“We might anticipate the in vitro culture of germ cells and such manipulations as the interchange of chromosomes and segments. The ultimate application of molecular biology would be the direct control of nucleotide sequences in human chromosomes, coupled with recognition, selection and integration of the desired genes…”

This represents the earliest statement of manipulation of the human genome. Considering this thought was raised far beyond the recombinant DNA era, the idea was quite avant-garde. The concept of gene therapy lies in the postulation that genetic diseases could be treated by direct correction of the genetic defect itself via replacing or supplementing the mutant gene with normal and functional genes. The material support for the gene therapy concept came from the knowledge of cell transformation by tumor virus. These tumor viruses have been so evolved that they could stably introduce new genetic information into the mammalian cells, which propose that they may also be used to introduce normal genes to correct the genetic defect and cure diseases if deprived of their own harmful functions (Friedmann and Roblin, 1972; Jackson et al., 1972; Friedmann, 1976; Anderson, 1984; Cline,
In fact, some of the viruses have been so efficient in transfection that they become widely used vectors in gene delivery research.

However, in the first attempt at human gene therapy, gene delivery was performed with a nonviral method. For many years, \( \beta \)-thalassemia had been considered as the initial disease target for gene therapy. In 1980, University of California at Los Angeles (UCLA) researcher, Dr. Martin Cline performed a recombinant DNA transfer into cells of the bone marrow of two patients with \( \beta \)-thalassemia without the approval of UCLA Institutional Review Board (IRB). In this unsuccessful attempt, cloned human \( \beta \)-globin gene was delivered with the use of calcium phosphate-mediated DNA transfer (Mercola et al., 1980; Cline et al., 1980). However, it was proved later that the regulation of hemoglobin synthesis was so complicated, which made \( \beta \)-thalassemia difficult to tackle by gene therapy still in its early days.

In light of Dr. Cline's controversial experiment, the discussions of gene therapy focused on the argument of its place in medicine and its ethical acceptability rather than its technical issues like efficiency, targeting and disease models. It was not until 1989 that the first approved clinical gene transfer took place, in which NeoR gene-marked tumor-infiltrating lymphocytes (TIL) were transferred into patients with advanced cancer (Rosenberg et al., 1990). This first federally approved human genetic engineering experiment demonstrated that an exogenous gene, although only a marker gene, could be safely transferred into a patient and the gene could be detected in cells taken back out of the patient. In the following year,
Michael Blaese and French Anderson from the U.S. National Institutes of Health performed the first approved gene therapy procedure on four-year-old Ashanti DeSilva with a rare genetic disease called severe combined immune deficiency (SCID) (Blaese et al., 1995). The genetic defect of this illness lies in the adenosine deaminase (ADA) gene, which leaves the patient extremely vulnerable to infection due to the lack of a healthy immune system. In Ashanti’s gene therapy protocol, gene-corrected autologous T cells were infused intravenously. Laboratory tests have shown that the therapy strengthened Ashanti’s immune system and she continued to lead a normal life. Although this procedure was not a cure, ADA-corrected T cells only work for a few months, and the process must be repeated every few months, this was the first proof of therapeutic benefit of gene therapy.

With the revolutionary advance in cellular and molecular biology and human genetics in the past two decades, gene therapy has progressed from theory to practice within a short period of time. The number of clinical trials in gene therapy continues to grow, with as much as 636 clinical trials identified and 3500 patients already involved. The diseases addressed include cancers, monogenic diseases, infectious diseases, vascular diseases and other diseases. The genes transferred include suicide genes and genes encoding cytokines, antigens, tumor suppressors, markers and receptors etc. For updated information on gene therapy trials worldwide, refer to the website http://www.wiley.co.uk/genmed/clinical/. Up to now, gene therapy (somatic) has became a widely accepted therapeutic option for serious diseases.
However, the same gene transfer technology, with which therapeutic gene is delivered to the patient’s cells may also be used for the purpose of functional enhancement, which is to “improve” the non-disease traits. In fact, the side effects from the present gene therapy protocols seem so minimal that genetic engineering for non-disease conditions is about to appear. A well-known example was that a US biotechnology company has developed the technology for gene transfer into the hair follicle cells and tried to apply it to deliver genes to promote hair growth for the treatment of chemotherapy-induced hair loss in cancer patients (Hoffman, 2000). And of course, the application of the technology could be easily adapted to treat the healthy balding men. Even though there is no big deal about treating baldness with gene therapy itself, the risk-benefit analysis should be taken into account while using gene therapy for a broad range of enhancement purposes. Since gene therapy is still in its infantile stage, the long-term side effects to patients after altering the genetic information are not fully understood, the unpredictable dangers caused by gene therapy still exist. Only when enough knowledge of the long-term effects are obtained from somatic cell gene therapy in the treatment of disease that the technology should be applied for the non-disease conditions. Similar but more complicated considerable concern exists in germline gene therapy, in which both medical and ethical issues are deeply involved and in which sufficient knowledge from both somatic gene therapy in human and germline genetic engineering in animals is necessary before moving to human germline gene therapy.
Gene therapy holds great promise for treating diseases. But beyond all those hypes and discussions, one has to realize that gene therapy, more precisely ‘gene therapy research’ has not made noticeable impact on the medical practice in the past decades and will not until the full development of sophisticated gene delivery systems. As we can imagine, an ideal gene delivery vector will be injectable, will target the diseased cells, will transfer therapeutic genes efficiently to most of these cells safe and sound, will direct the insertion of therapeutic genes into proper region of the genome or just stay as stable episomes, will be regulated by either administered agents or by normal physiological signals and will be cost-effective to produce. Most importantly, it will cure disease. Despite numerous clinical trials have been carried out, no notable clinical successes have been shown. Yet, with the invaluable knowledge provided by these clinical trials, progresses are being made and gene therapy will almost certainly revolutionize the practice of medicine in the future and provide an ultimate treatment for a vast range of diseases that are plaguing mankind today.

1.1.2 Gene Delivery with Nonviral Vectors

1.1.2.1 The importance of gene delivery vectors

With the completion of the human genome project and the development of functional genomics, a greater understanding of the molecular basis of genetic disease will render enormous therapeutic genes for the purpose of gene therapy. By then, the only hurdle to the application of these information in gene therapy is the development of safe and efficient DNA delivery
systems. Recently, naked DNA has been used for gene transfection into cells with the help of physical methods such as electroporation, gene gun, ultrasound and hydrodynamic pressure (Niidome and Huang, 2002). Since no carrier is involved, this simple method avoids carrier-related issues like complex formation and safety. But the drawbacks are also obvious, which include no protection of DNA from serum nucleases attack, rapid clearance of DNA by mononuclear phagocytes, limited expression level, and no cell specificity. Although naked DNA combined with physical methods could be used for gene delivery, however, to obtain specificity, targeted vectors are necessary. The vectors with cell specificity are the ideal means by which DNA molecules are delivered to the target cells.

1.1.2.2 The viral vectors

Generally, there are two classes of vectors for gene delivery. The first class is known as viral vector (Somia and Verma, 2000). This group of vectors is derived from common human viral pathogens. With the use of genetic manipulation, these viruses have their genomes modified or “gutted” to prevent viral replication, inflammation, cytotoxicity, immunogenicity, and permit loading of therapeutic genes. The most commonly used viral vectors are derived from retrovirus, lentivirus, adenovirus and adeno-associated virus. These viral vectors can deliver gene efficiently into a broad range of cell types and are widely used in both basic research and therapeutic application.

However, there are some inherited limitations that prevent viral vectors to become a prevailing option in the gene therapy research field (Somia and
Verma, 2000; Williams and Baum, 2003; Dobbelstein, 2003). Although most of the deleterious genes have been eliminated from the genomes of these viral vectors, unexpected and unpredictable side effects may still exist and the safety issue remains a big concern. One major obstacle facing the viral vectors is the immune response of the host. The host immune system recognizes the viral proteins and eliminates the virus-infected cells by cellular immunity, whereas, the strong secondary immune response evoked by memory cells rules out the possibility of repeat administration of viral vectors (Dai et al., 1995; Kafri et al., 1998). To be “stealthy” in the host, most of the viral vectors are designed to have their own proteins synthesis silenced after transduction. Meanwhile, to be efficient in transfection, the “gutless” viral vectors still need the full complement of viral structure proteins, which may elicit the host immune response. In fact, for adenoviral vectors, the immune response of the host could become so serious that it might be fatal. The 17-year-old Jesse Gelsinger was the first patient to die in a Phase I gene therapy clinical trial, in whom the death could be directly relevant to the vector—an adenoviral vector (Marshall, 1999; Lehrman, 1999; Smaglik, 1999). Gelsinger suffered from ornithine transcarbamylase (OTC) deficiency, an inherited liver disease that causes life-threatening levels of ammonia to build up in the blood. In an attempt to correct this deficiency, a crippled form of adenovirus (the second generation of adenoviral vector deleted for the E1 and E4 genes) was used to deliver the OTC gene. But instead of curing the disease, it triggered an “activation of innate immunity”, followed by a “systemic
inflammatory response." Within hours, Gelsinger's temperature shot up to 104.5 degrees Fahrenheit. He went into a coma on the second day and was put on dialysis and then on a ventilator. His lungs filled with fluid. When it became impossible to oxygenate his blood adequately, he died. The tragic death of Gelsinger has shocked the whole research community. Many questions have been raised about the future of human gene therapy, while the safety for patients in gene therapy became the biggest concern.

Another safety concern in gene therapy using viral vectors is the insertional oncogenesis. In a clinical trial, 10 patients with X-linked severe combined immunodeficiency (SCID-X1) were transfused with their own gene-corrected bone marrow-derived progenitors and stem cells. These cells were transduced with retroviral vectors carrying the therapeutic gene, which encoded the common γ chain of the interleukin-2 receptor (γc), a protein that is defective in SCID-X1 patient. Nine of the 10 patients showed significant long-term improvements in the immune function for this fatal disease (Hacein-Bey-Abina et al., 2002). However, the two youngest patients have developed T cell leukemia due to the insertion of the retroviral vector near the promoter of the proto-oncogene LMO2 (Hacein-Bey-Abina et al., 2003). Compared with the risk study in animals, the chance of insertional oncogenesis in the retrovirus-mediated gene therapy (2 out of 10) was surprisingly high. More amazingly, both patients had the retroviral insertion at the same LMO2 gene locus, which was unlikely a untargeted and random event. A possible explanation was that the γc transgene, which encoded a potent anti-apoptotic
product, provided the strong selective advantages to the transducted cells and led to the high frequency of insertional mutagenesis and subsequent clonal dysregulation.

1.1.2.3 The nonviral vectors

The idea of using virus as gene delivery vector is tempting for its simplicity in principle and high efficiency, but the challenges are also apparent. To overcome the problems plaguing the viral vectors, alternative approaches must be explored. In an attempt to create synthetic carriers that have the virtues of viral vectors but without their negative attributes, a second class of gene delivery vectors referred as nonviral vectors are developed (De Smedt et al., 2000; Niidome and Huang, 2002; Davis, 2002; Vijayanathan et al., 2002; Thomas and Klibanov, 2003). These nonviral vectors include mainly cationic lipids and cationic polymers (also known as polycations). With the high positive charge density, they function to interact with the negatively charged plasmid DNA to form lipid-DNA complexes (lipoplexes) or polycation-DNA complexes (polyplexes).

Synthetic nonviral vectors may have some potential advantages over the viral counterparts and safety concern further strengthens the need for the development of nonviral vectors. Nonviral vector could be non-toxic, non-pathogenic and non-immunogenic, which allow large-dosage and/or repeated administration to achieve the same efficacy of viral vectors. The therapeutic gene delivered by nonviral vector could remain episomal and avoid the risk of oncogenesis caused by random integration of viral vector. The transient
expression of the nonviral vectors could be easily overcome with repeated injection. Synthetic vectors may also have large capacity of therapeutic gene, which is required for the delivery of either antisense oligonucleotides or artificial chromosomes. Also, it is much easier to retarget a nonviral vector to a specific cell type than a viral one. The cost and ease of manufacturing has also become a real issue for gene delivery vectors. Viral vectors are biological agents that can only be made in the living cells. To carry out good manufacturing practice (GMP) and quality assurance/quality control (QA/QC) procedures in these biological systems is not an easy thing. On the other hand, synthetic nonviral vector could avoid the using of the tissues or cells as bioreactors, which may simplify the whole manufacturing process. All the above characters suggest that synthetic nonviral vectors should be the main vectors for routine gene therapy in the future.

1.1.2.4 The barriers to nonviral gene delivery

Although nonviral vectors have potential advantages over the viral counterparts, it is still early to say which vector will prevail. The problem of ‘which vectors will prevail’ even exists in the field of nonviral vector itself. For cationic lipids, the formation of lipoplexes depends largely on the interaction among lipid molecules in addition to lipid-DNA interaction. The hydrophobic segments of lipid molecules are the major determinant for the characteristics of the resulting lipoplexes, which results in only limited control over the parameters like particle size, shape, stability or interactions with cell surface, other lipid and DNA (Smisterova et al., 2001;Simberg et al., 2001;Zuhorn et
al., 2002b). In contrast, the formation of polyplexes does not require the interaction among polycation molecules and polycation-DNA interaction is the major driving force. This property leaves greater control over the particle characteristics. In addition, the polycations can be easily modified by chemical methods to achieve higher efficiency and specificity. The polycations also have more flexibility in terms of molecular weight, polymer structure and polymer to DNA ratio. With all the properties, polycations seem potentially superior to cationic lipids in their pharmaceutical prospective (Gebhart and Kabanov, 2001).

For nonviral vectors, the major challenge is to improve the efficiency of gene delivery to a level that surpasses that of viral systems. However, to reach such a goal, nonviral vectors need to overcome a series of barriers before adequate amounts of therapeutic genes are delivered to the nucleus. These barriers may include:

(1) The stability of nonviral systems in the extracellular environment: Both nonviral vectors and the delivered genes are required to remain intact in extracellular space, such as intercellular or intravascular milieu before reaching their target cells. The existence of nuclease results in rapid degradation of the DNA after their intravenous or intramuscular injection. This issue could be partially overcome by the cationic polymers that condense or complex with the negatively-charged DNA and therefore resist the nuclease-related degradation (Li et al., 1999; Adami and Rice, 1999; Yang et al., 2001). The second factor in the biological milieu that might compromise the stability
of the nonviral gene delivery complexes is the increased ionic strength. In a high ionic strength environment, the interactions between polycation and DNA become weak. Aggregation of the complexes may also happen due to the weakened interparticle electrostatic repulsive force. The endogenous negatively-charged molecules may also destabilize the nonviral complexes. These negatively-charged components like serum albumin, glycoprotein may compete with nonviral vectors for DNA binding or facilitate the complex aggregation (Oupicky et al., 1999; Ruponen et al., 1999; Wiethoff et al., 2001). To deal with complex disintegration, cationic polymers with high charge density like polyethyleneimine (PEI) are used (Ruponen et al., 1999). To prevent complex aggregation, polyethylene glycol (PEG) molecules are attached covalently to provide a steric barrier (Kwok et al., 1999; Hwang and Davis, 2001).

(2) The cellular uptake of nonviral gene delivery systems: The plasma membrane forms the first barrier for the transport of gene to the nucleus. Since most biological molecules are unable to diffuse through the phospholipid bilayers, certain pathways are required for the passage of therapeutic gene across the membrane. The attachment of naked DNA to the cell surface is the very beginning step in the process of intracellular gene transfer. However, it is not a spontaneous one due to the high negative charge density of both DNA and cell surface. The positive charges of nonviral vector can neutralize the negative charge of DNA and thereby increase the attachment of DNA to the cell surface. The heparin sulfate proteoglycans
(HSPGs) on the cell surface are thought to be one of the molecules that mediate the binding and the subsequent internalization of the nonviral gene delivery systems (Mislick and Baldeschwieler, 1996). HSPGs are omnipresent on all cell surfaces, which not only function in various cellular processes, but also mediate the entry of several viruses (Bernfield et al., 1999). Experiments showed that the presence of HSPGs significantly improved the gene delivery by nonviral vectors. Although HSPGs may enhance nonviral gene delivery, receptors are the means by which the specificity of gene transfer are fulfilled. Another strategy to increase the DNA attachment is the conjugation of targeting ligands to nonviral vectors or directly to DNA. Through the interaction of cell surface receptors and ligands, the ligand-containing nonviral systems may be directed to particular cell types.

After surface attachment of DNA particles, cells are able to take them up by a process known as endocytosis. In this process, the particles are surrounded by an area of plasma membrane, which buds off inside the cells to internalize the ingested materials. Depending on the targeted cell type and receptors as well as the properties of nonviral vector/DNA particles, various endocytosis pathways may involve. The most common process is receptor-mediated endocytosis, in which clathrin-coated pits take part in the internalization of nonviral vector/DNA complexes (Friend et al., 1996; Zuhorn et al., 2002a). Studies indicate that some clathrin-independent pathway may also involve. One of these mechanisms involves the uptake of DNA particle in small invaginations of the plasma membrane called caveolae (Gottschalk et
al., 1994; Hofland et al., 2002). Other clathrin-independent processes like phagocytosis and macropinocytosis that are common in “professional phagocytes”, but rather rare in other cell types, have also been detected in nonviral gene delivery to several mammalian cell lines (Francis et al., 1993; Labat-Moleur et al., 1996; Matsui et al., 1997; Harbottle et al., 1998). It seems that all these mechanisms participate with different extent, but for efficient nonviral gene delivery, it is really necessary to understand which are most important in certain individual cell types.

(3) The escape of nonviral systems from endosomes: Following the internalization, the endocytic vector-containing vesicles fuse with early endosomes, which locate in periphery of cytoplasm with an acidic internal pH of ~6. The early endosomes function as a sorting compartment, from where the internalized materials are redistributed. There are two possible outcomes for the internalized materials (Clague and Urbe, 2001). In recycling endosomes, the internalized materials are thought to be returned to the cell surface. One evidence demonstrating this possibility is that adenovirus with deficient endosome-escaping ability has been found to be rapidly internalized and at least partially recycled back to the cell surface (Greber et al., 1996). Another possible fate for the materials taken up by endocytosis is their transportation via late endosomes to lysosomes, in which the endocytosed materials are degraded by the action of acid hydrolases (Luzio et al., 2001). It is conceivable that similar fates may happen to those nonviral gene delivery systems without endosome-escaping ability once internalized. Although there
is no information available on which outcome is more likely for nonviral systems, both will obviously reduce their intracellular trafficking to the nucleus. Therefore, efficient endosomal escape ability is one of the key factors that should be considered for designing efficient nonviral vectors.

Some of the current existing nonviral vectors may have shown intrinsic endosomolytic ability. For cationic lipid vectors, lipid mixing between the endosomes and vectors is thought to be the mechanism involved (Xu and Szoka, Jr., 1996). It was postulated that the negatively-charged phosphatidylserine in endosomal membrane interacts with the cationic lipids, which leads to liposome fusion and transgene release. In the case of polycation vectors, the exact mechanism involved in endosomal escape is still being defined and some hypotheses have been proposed. One of these is known as the ‘proton sponge’ hypothesis, which is used to explain the endosomolytic ability of polycations with ionizable amine groups (Boussif et al., 1995). In early endosomes, the slightly acidic environment is maintained by the action of membrane H⁺ pumps that transport the proton against the concentration gradient across the endosomal membrane (Grabe and Oster, 2001). Polyethylenimine (PEI) is a well-known cationic polymer for its high gene transfer efficiency. The unique feature of PEI is its high positive charge density with one protonable amino nitrogen in every 3 atoms. Branched PEI contains 25, 50 and 25% of primary, secondary and tertiary amines respectively and has high buffer capacity over a broad pH range. The hypothesis assumes that under neutral pH, PEI is only partially protonated
and under acidic pH in endosomes and lysosomes, the highly branched PEI absorbs a large amount of proton ions like sponge. This buffering effect leads to the increased influx of $\text{H}^+$ into endosomes followed by the influx of $\text{Cl}^-$ and $\text{H}_2\text{O}$, which causes osmotic swelling and rapture of endosome and thus allows the release of transgene into the cytosol. The ‘proton sponge’ hypothesis is supported by the fact that ionophore, which reduces the transmembrane pH gradient, also reduces the release of PEI/DNA complexes and thus inhibits the transgene expression (Kichler et al., 2001). However, the ‘proton sponge’ ability of PEI and other protonable polymer under physiological ionic strength and in the complexation with DNA may change significantly, which suggest the necessity of further re-evaluation of the hypothesis (Godbey et al., 2000).

While not all the present nonviral vehicles are effective in endosome-escape, viruses, however, have developed successful strategy to overcome the endosomal membrane. Learning from virus is beneficial to the development of the nonviral vectors. The infection process by enveloped influenza virus has provided one of the best-known mechanisms for endosome-disrupting. The influenza viruses enter the host cells by receptor-mediated endocytosis. In acidic endosomes, the viral membrane fuses with endosomal membranes, followed by the release of genetic materials into the cytosol and the initiation of virus replication. The crucial molecule in this fusion process is a glycoprotein called hemagglutinin (HA) (Carr et al., 1997). HA is a trimer composed of three HA1 and HA2 subunits, which forms a spike
and protrudes from the virus surface. In a natural conformation, each HA1 subunit forms a globular domain at the tip of spike, which may bind to the host cell membrane and initiate viral entry. Each HA2 subunit is composed of 4 domains, which are a N-terminus fusion peptide, a short $\alpha$-helix, a nonhelical loop and a long $\alpha$-helix. At neutral pH, three long $\alpha$-helixes from HA2 subunits form a three-stranded coiled coil. The whole HA2 subunits are buried within the HA1 subunits with each HA2 subunit linked to one HA1 subunit by a disulfide bond at the base of the molecule. At low pH in endosomes, the fusion proteins are exposed after a series of molecular events. First, the three globular HA1 domains separate from each other; second, the nonhelical loop region of each HA2 changes into an $\alpha$-helix and forms a long $\alpha$-helix together with the existing short and long $\alpha$-helix. These three 88-aa $\alpha$-helixes form a 13.5-nm-long three-stranded coiled coil, which protrudes from the viral membrane with the fusion peptide exposed at the tip. The insertion of these fusion peptides into the endosomal membrane triggers membrane fusion process.

To mimic the endosome-escaping mechanism evolved by viruses, the small peptide domains from virus that have crucial function in the fusion process are used to equip the nonviral gene delivery systems. One of these is the above-mentioned N-terminal fusion peptide from influenza virus HA2 subunit. This peptide is an amphiphilic anionic peptide, which undergoes conformational change in response to the variation in pH (Lear and DeGrado, 1987). At neutral pH, this peptide adopts a non-helical conformation due to
the repulsion of the negatively-charged glutamic acids and aspartic acids. At low pH, it transforms into a helical amphipathic structure with hydrophobic residues arranged on one side that may interact and destabilize the lipid bilayers. Several synthetic amphiphilic peptides have been developed to mimic the pH-induced membranes fusion by viral peptide. The synthetic peptide GALA containing repeat sequence of glutamic acid-alanine-leucine-alanine transforms from a random coil at pH 7.5 to an amphipathic α-helix at pH 5.0 (Subbarao et al., 1987). With the use of GALA, an increase of transfection efficiency of nonviral vectors has been observed (Haensler and Szoka, Jr., 1993; Simoes et al., 1998; Simoes et al., 1999). A cationic version of GALA, known as KALA was designed for both DNA-compacting and endosome-disrupting (Wyman et al., 1997). In transfection in vitro, pCMVLuc/KALA complexes produced luciferase activity 100-fold greater than that found in the optimal poly-L-lysine/DNA complexes.

Some pharmacological agents have also been used to enhance DNA release form endosomes. Chloroquine, a weak base that accumulates in acidic compartments like late endosomes and lysosomes, is commonly used to increase the transfection efficiency of nonviral gene delivery systems. Possible functions of chloroquine in this process are (i) increasing the intralysosomal pH and reducing the degradation by decreasing the hydrolytic enzyme activity (Wibo and Poole, 1974; Poole and Ohkuma, 1981; Maxfield, 1982); (ii) inhibition of endosome/lysosome fusion (Hedin and Thyberg, 1985; Stenseth and Thyberg, 1989); (iii) destabilization of endosomal
membrane (Zhou and Huang, 1994). All above mechanisms would increase the possibility of endosomal escape of transgene.

(4) The cytosolic transportation: Once escape from endosomes, transgenes have to trespass cytosol to reach their final destination - nucleus. However, there is no known mechanism for active transport of DNA in cytosol. Moreover, the high viscosity of cytosol makes diffusion of transgenes in cytosol even difficult. An observation suggests that nucleic acid fragments larger than 2000bp are almost immobile in cytoplasm, whereas fragments up to 500bp can diffuse freely (Lukacs et al., 2000). The presence of cytosolic nucleases may also result in significant degradation of DNA (Lechardeur et al., 1999; Pollard et al., 2001). Observations have suggested that in lipoplex-mediated gene delivery, DNA molecules are set free into the cytosol after endosomal escape (Xu and Szoka, Jr., 1996; Cornelis et al., 2002); in polyplex systems, DNA molecules are still at least partially complexed with polycations (Pollard et al., 1998). Supporting evidence came from the experiments with microinjection of both lipoplex and polyplex directly into cytosol. On injection of lipoplex, transgene expression is much less than the injection of DNA alone (Zabner et al., 1995); in contrast, the microinjection of polyplex results in significant transgene expression (Pollard et al., 1998). There is still no clear explanation for the enhanced expression of transgenes that are still complexed with polycations like PEI or poly-L-lysine. Possible mechanisms may lie in the facilitated diffusion due to small size of polyplexes and the protection of DNA from nucleases in complexed form.
(5) The nuclear localization: For transgenes to be expressed, they must enter the nucleus, in which transcription may take place. Like the cell membrane, the nuclear membranes are also lipid bilayers that serve as a barrier between the cytoplasm and the nucleus. Transgenes and other molecules are unable to diffuse through the nuclear membrane. Unlike the cell membrane, no evidence suggests a similar process like endocytosis that occurs on the cell membrane can help the transport of materials into the nucleus. However, there are still three possible pathways for the nuclear localization of the transgenes. DNA can pass through the nuclear pore complex (NPC), the only channel for the trafficking of macromolecules between the cytoplasm and the nucleus; DNA can enter the nucleus during the breakdown and reform of nuclear envelope in mitotic cells; or DNA may traverse the nuclear envelope. Of these three possible routes, the second one is perhaps quite widespread but with limited application in gene delivery to the postmitotic cells; the third seems least likely and has no experimental support.

The NPC is a large multiprotein structure that spans across the nuclear envelop and extends into both cytoplasm and nucleoplasm. In close state, the NPC allows passive diffusion of molecules with diameter up to 9 nm (or protein up to 50 kDa); during active transport, the NPC permits the passage of larger molecules with diameter up to 25 nm (or protein up to 1000 kDa) (Mattaj and Englmeier, 1998; Ryan and Wente, 2000). The nuclear import is an energy-consuming and carrier-dependent process that may transport proteins (like transcription factors) or RNAs. In karyophilic proteins, one or
more special sequences that function as nuclear targeting signal may be found (Kalderon et al., 1984; Lanford and Butel, 1984; Robbins et al., 1991; Siomi and Dreyfuss, 1995). These sequences are designated as nuclear localization sequence (NLS). During nuclear transport, free cytoplasmic transport factors known as karyopherins associate with the NLS of karyophilic protein to form a pore-targeting complex. The complex then docks on the cytoplasmic side of NPC followed by translocation through the pore in an energy-dependent process that is still not clearly understood.

Despite the mechanism of trafficking through NPC has not been fully understood, researchers have attempted to apply this knowledge in gene delivery. One possible way for targeting the DNA to the nucleus is to include the binding sites for karyophilic proteins in the transgene sequence. With the binding of karyophilic proteins, nuclear transport of transgene may be facilitated. SV40 enhancer sequence that can bind to a variety of transcription factors has been known to help the DNA nuclear transport (Dean, 1997). For more specific nuclear transport, the integration of tissue specific promoter sequence that interacts with specific transcription factors in cytoplasm was also examined. It has been demonstrated that the incorporation of promoter for smooth muscle gamma actin facilitates the plasmid transport to the nucleus of smooth muscle (Vacik et al., 1999). Therefore, this strategy may not only improve the nuclear transportation of transgene, but also may act in a tissue-specific manner. Using this strategy, the design of nonviral vectors may become less difficult. With a well-designed transgene that includes
binding sequence of transcription factors and utilizes the cell machinery evolved for nuclear transport of karyophilic proteins, it is not necessary to include a nuclear transport mechanism in the vectors themselves. Alternative way to facilitate DNA nuclear transport is to conjugate the NLS peptide to the transgene. NLS peptide covalently associated with DNA has been proved to help transgene nuclear localization (Zanta et al., 1999). The concept has also been demonstrated with DNA noncovalently associated with NLS via charge interaction or specific peptide nucleic acid (PNA) sequence (Branden et al., 1999). Despite all these experiments, still no solid evidence proves that the conjugated NLS peptides to DNA actually function as nuclear localization signal.

Within all these possible hurdles to nonviral gene delivery, it is hard to say which step poses the most difficult barrier. It seems that the relative contribution of each step to the overall gene delivery may vary in accordance with the targeting cell types. Therefore, to design a versatile vector that is suitable for all cell types may not be practical. It will be more likely that the nonviral vector should be tailored accordingly for efficient gene delivery to a specific tissue. Moreover, to achieve high efficiency, a successful nonviral vector should be capable of tackling multiple barriers. To put all these barrier-tackling modules in one nonviral vector without compromising each other is still a serious challenge to nonviral delivery system.
1.1.2.5 The improvement of nonviral vectors

To achieve effective therapeutic transgene expression, researchers have designed nonviral vectors that surmount different obstacles encountered at both systemic and cellular levels. Based on the tackling issues, these nonviral gene vehicles have been categorized into several groups: (i) the vectors that condense and protect DNA and increase complex stability; (ii) the vectors that target delivery of DNA to specific cell types; (iii) the vectors for intracellular targeting to cytosol or nucleus; (iv) the vectors that can dissociate from DNA in cytosol; (v) the vectors that can control DNA release in tissues for continuous and controlled expression. Although these vectors are capable to overcome certain specific barrier, an ideal nonviral vector that addresses multiple barriers still needs to be developed. In general, an ideal nonviral vector should have the following basic properties: Condense the DNA into a small package; Target specific tissue via cell surface receptors; Avoid nonspecific uptake; Escape the endosome; Cross the nuclear membrane.

Actually, researchers of both viral and nonviral vectors are trying to achieve those same objectives from different starts. Viral vectors researchers use a ‘top-down’ approach, in which they remove those immunogenic or toxic or other noxious components from the viral vectors so that they become safe for clinical use. Nonviral vector builders, meanwhile, use a ‘bottom-up’ approach, in which components that may improve gene transfer are added piece by piece into nonviral gene delivery systems so that they gain the required efficiency for clinical application. At present, viral vectors
predominate in current clinical gene therapy trials because they are efficient in foreign genes delivery. These viral vectors have evolved such delicate mechanisms to overcome cellular barriers that for them intranuclear delivery of foreign gene seems one of the most natural things to do. On the other hand, to build a totally synthetic nonviral vector that mimics all those sophisticate mechanisms in viral vectors may be difficult. Although we have learned a lot from the viruses, the development of safe and efficient gene delivery system requires the rational incorporation of these viral strategies into a single nonviral vector system, which is still being developed.

1.1.3 Targeted Gene Therapy

1.1.3.1 Targeted gene therapy

The basic criteria of gene therapy were defined long before the appearance of any practical applications. It required the therapeutic genes to be efficiently delivered to the relevant cells and expressed at appropriate level, which implied the necessity of targeted gene therapy. Despite this early recognition, the early generations of vectors were designed to provide only the basic gene transfer capability without addressing the issue of specificity. In viral gene delivery, the cellular uptake was restricted by the native tropism of the original virus, whereas in nonviral gene delivery, the uptake was largely a nonspecific process. In these researches, the efficiency was listed as the primary object for vector development.
In the early beginning, the original idea of retargeting vectors for selective gene transfer has been studied only for the purpose of improving the vector efficiency itself. The results of early in vivo gene therapy trials have been disappointing, which were mainly due to the extremely low rate of cell transduction. To improve the gene therapy outcomes, retargeting of vectors has been used as one of the strategies to improve vector efficiency. The conjugation of ligands to the vectors may increase the attachment of the gene delivery complexes to the cell surface. To have high efficiency, ligands to those most abundantly expressed receptors were intensively studied.

In the following studies, it becomes apparent that the lack of targeting ability has limited the application of gene therapy to many disease candidates. One obvious example comes from cancer gene therapy, in which the delivery of toxic genes is one of the most commonly used anti-tumor strategies. In this case, the expression of toxic genes should be restricted to the tumor cells only, while expression in any other non-tumor cells will be of serious consequence. In the context of lack of available vectors with targeting ability, to confine both the therapeutic effect and the therapy-related side effects, the initial in vivo cancer gene therapy aimed at those localized tumors within certain natural body compartments such as glioma, pleural mesothelioma and peritoneal carcinomatosis. But even in these space-confined tumors, ectopic gene delivery still occurred. In this regard, vectors without tumor cell targeting ability are apparently not applicable to tumors with metastasis, where the target cells spread widely throughout the body.
Similarly, to implement gene therapy to a wide range of disease candidates, it is highly desirable that the therapeutic genes are only expressed in specific cell types, but not in other cell types. The purpose of targeted gene therapy is to restrict the therapeutic effects in only the target cells and thus avoid the possible side effects of gene products in the non-target cells. This is especially important while the target cells are widely distributed like cells in hematopoietic system or if the target cells are dispersed among highly heterogeneous populations like those in central nervous system.

Nowadays, it is a common recognition that besides safety and efficiency, specificity is another very important issue to be considered in developing gene delivery systems for gene therapy. The development of cell or tissue-targeted vectors presents one of the great challenges for gene therapy research.

1.1.3.2 Approaches to targeted gene therapy

There are three major approaches toward targeted gene therapy. *Ex vivo* therapy is one initial approach used in clinical gene therapy trials. In this method, target cells were selected by isolation from the body and modified by vector-mediated gene transduction *in vitro*. The gene-modified cells were then transferred back to the body to obtain therapeutic benefit. This procedure was often used in treating hematological diseases and diseases of the immune system, where the cells could be easily removed and returned. Since cell transduction *in vitro* may be obtained at relative higher level, there is no
requirement for the targeting ability of vectors, which makes it easier for clinical application. In fact, in those human trials with positive findings, *ex vivo* method has been used to treat diseases like X-linked severe combined immunodeficiency and hemophilia (Nathwani et al., 2003; Gaspar et al., 2003). Unfortunately, only limited diseases are amenable to this *ex vivo* intervention, other strategies for targeted gene therapy are required.

In clinical application, it is most common that the therapeutic complexes are administered to a mixed cell population hoping for the delivery of transgene to certain cells. In this scenario, ligand-directed targeting of gene is one major approach for targeted gene therapy. Since ligands are able to recognize the receptors that are specific for a certain cell type, the addition of ligands may bestow the gene delivery vector the ability to distinguish between target cells and non-target cells. At present, numerous studies are attempting to modify the cell specificity of both viral and nonviral vectors for transductional targeting. The third approach for targeted gene therapy is based on the modification of therapeutic gene, which is known as transcriptional targeting. In this strategy, therapeutic gene is placed under the control of cell-specific transcriptional regulatory sequences like promoter, enhancer and/or silencer. Since the transcription of therapeutic gene is activated only in target cells, the therapeutic effect of gene product is limited to the target cells. By combining both transductional and transcriptional targeting, it is possible to increase the selectivity at two different levels for the same target.
1.1.3.3 Targeting of nonviral vectors

As there is no panacea, there is no versatile vector for gene therapy to all kind of diseases. Gene delivery vector has to be customized to fit the requirement of various diseases. To target certain diseased cells, a vector needs to be modified by integrating ligands that recognize the cell-specific receptors. One crucial issue for retargeting the gene delivery systems is to reduce the nonspecific attachment to cell surfaces. For current viral systems, the biodistribution of vectors is largely determined by the native tropism of the parental virus. For viral vector retargeting, it is necessary to reduce the binding to native receptors and add a new targeting ligand while keeping the efficiency of post-attachment steps in the viral vectors. Similarly, for nonviral vector retargeting, although there is no native receptor binding that needs to be taken away, it is required to minimize the nonspecific attachment arising from the highly positive charge of the cationic vectors. Conceivably, such nonspecific interactions may affect or even cover the ligand-directed targeting and thus deviate transgene complexes from the target site. Current strategy to address this issue has been to shield the vectors with either hydrophilic polymers like PEG or with ligand directly (Xu et al., 1999; Kircheis et al., 2001a; Kircheis et al., 2001b). By masking the surface charge, the intrinsic targeting effects could be avoided. This method has been applied to retarget both lipid and polymer vectors (Fenske et al., 2001; Kircheis et al., 2001c).

It should be recognized that the realization of ligand-directed targeted gene therapy for a wide spectrum of diseases is based on the introduction of
novel targeting ligands into the vectors to direct therapeutic gene to new cell surface receptors. Depending on the targeted receptors, various ligands have been used to conjugate nonviral vectors. Targeted gene delivery has been described for a number of cells such as tumors, hepatocytes, epithelium, macrophages, lymphocytes and hematopoietic stem cells etc (Anwer et al., 2003). Ligands tested include galatose, transferrin, folate, mannose, lectins, antibodies and receptor-binding peptides (Ziady AG and Davis PB, 2002). These ligands include not only endogenous ligands for receptors, but also exogenous ligands like antibodies and phage-derived peptides, developed de novo. However, there is still a clear appeal to introduce more useful cell-specific ligands that can be added to the vectors. For many cells of interest to gene therapy, suitable cell-specific ligands have yet to be discovered. Obviously, to develop efficient targeted gene therapy, it requires not only the engineering of functional vectors, but also the introduction of new ligands to direct the vectors to the cells of interest.

1.2 Gene Therapy in the Nervous System

1.2.1 Gene Therapy in the Nervous System

1.2.1.1 The appeal to gene therapy in the nervous system

The neurological disorders have been generally regarded as tragic diseases due to their chronic and suffering pathological processes and usually poor prognosis. The impairment of the nervous system always provokes special dread in people. Diseases like Parkinson’s disease (PD) or
Amyotrophic lateral sclerosis (ALS) may progressively rob control of the body, while spinal cord injury may do the same damage in just an instant. Alzheimer’s disease (AD) may take away the very essence of one’s personality and even life by affecting the cognitive area of the brain and eventually the whole brain. These declines in functional abilities impose a particularly heavy burden not only to the patients themselves, but also to their caregivers and family members as well.

The disorders of the nervous system have been classified into many categories with each of which containing a long list of diseases depending on the etiologies, the affected anatomic structures and/or the functional losses. They include not only the disorders of higher function, motor control, the special senses, spine and spinal cord, body function, but also headache and pain, neuromuscular disorders, epilepsy, cerebrovascular disorders, neoplastic disorders, autoimmune disorders, disorders of myelin, infections, trauma and toxic disorders and degenerative disorders. The detailed classification of the nervous system disorders derives from the tradition of neurology, which has been focusing on diagnosis with accurate neuroanatomical localization of the lesion or pathophysiological definition of the disease process. Armed by the powerful neuroimagery technologies like X-ray computerized tomography and magnetic resonance imaging, it becomes easier for neuroanatomical diagnosis in those structurally based conditions; meanwhile, the techniques like pathology, molecular biology, clinical neurophysiology and functional brain imaging help the elucidation of
the pathophysiological basis of the disorders. However, in contrast to the expertise on diagnosis, therapeutic approaches to neurological diseases have been very limited.

The neurological diseases are more common than we might think. For example, AD is the most common form of neurodegenerative disease in people over 65 years. It is estimated that more than 15 million people are affected by the disease worldwide and almost half of those over 85 years show signs of the disease (Palmer, 2002). In the United States alone, over four million people suffer from AD and another million have PD, the second most common neurodegenerative disorder after AD. Since age is the single most important risk factor for these degenerative diseases, the number of individuals who are developing AD and PD is becoming a considerable concern with the increased lifespan of people. Statistics postulate that more than 10 million people will suffer from AD in the US by the year 2025, the number will approach 20 million by the year 2050. Currently, the estimated cost for the care of these AD patients and the lost of productivity in both the patients and their caretakers is $200 billion annually in the US (Kawas CH and Katzman R, 1999). The number will increase to a more staggering amount unless effective means for preventing and/or treating these disorders are developed.

Unfortunately, medical researchers have made only limited progress in the battle against the neurological diseases mainly because of the inability of the injured nerve tissue in self-repairing, which is commonly observed in other
tissues, and the vulnerability of the nervous system to insulting conditions. The current neurological treatments are incompetent in preventing the relentless loss of neurons, needless to say the perspective of complete functional recovery. In the past two decades, significant advance has been made in understanding the molecular biology of the nervous system in both health and disease (Shilling and Kelsoe, 2002). These advances help to uncover the implicating molecules and genes in the nervous system disorders and provide the possibility of various new approaches for treating neurological diseases such as growth factor therapy, stem cell therapy and immune therapy. Particularly, with the substantial advances in understanding the pathogenesis of these diseases, new molecular based-therapy like gene therapy that delivers the therapeutic gene to the affected nervous system offers the possibility to prevent neuron loss and degeneration in the brain, to induce new growth and/or even to restore function.

1.2.1.2 The applicability for gene therapy in the nervous system

One of the difficulties in treating neurological diseases is presented by the anatomical structures such as the cranium, the meninges and the blood-brain barrier (BBB) that are protecting the nervous system. The application of classical pharmacological treatment is restricted by these structures specific to the nervous system, particularly by the BBB. The BBB prevents access to the brain of numerous potentially therapeutic molecules, since 98% of all small-molecule drugs and 100% of all large molecule drugs do not cross the BBB (Foubister, 2003). The possible routes of administration for such
molecules are intracerebral or intracerebroventricular injection or infusion using osmotic mini-pump if long-term treatments are required. Even after delivery via these routes, the molecules can only penetrate a short distance into the brain that limits the therapeutic effect. With intracerebroventricular administration, however, the molecules may also diffuse to the sites other than the target region via cerebrospinal fluid circulation and cause unexpected side effects. Gene therapy provides one possible strategy to overcome the limitations of classical pharmacotherapy. This approach uses therapeutic gene directly to affect the local and/or focal expression of a therapeutic protein to achieve a desired therapeutic effect, therefore offers a simple and effective way to delivery therapeutic agents to the brain.

From the viewpoint of therapeutic strategy, gene therapy is also applicable to the neurological diseases. A common pathological event observed in the neurological disorders is neuronal death. Although the etiologies may be various, the administration of neurotrophic or anti-apoptotic factors presents a major strategy for treating such disorders. To maintain the regional specialized function of the nervous system, this kind of therapeutic intervention should be supplied locally, which may be achieved by therapeutic transgene expression. Moreover, the enhanced neurotransmitters synthesis is another strategy for treating some neurodegenerative diseases. For example, the dopaminergic function needs to be strengthened in PD patients, whereas enhanced cholinergic function in AD patients will be beneficial. Obviously, apart from the affected pathways in these disorders, many other pathways in
the nervous system may share the same neurotransmitter and receptor system for signal transduction. In these cases, to avoid the side effects that arise from the activation of other pathways sharing the same neurotransmitters, it is favorable to achieve the local production of neurotransmitters by therapeutic gene expression rather than by using a systemically administered drug.

Therefore, beyond the common disease targets like cancers, monogenic diseases, vascular diseases and infectious diseases, the neurological disorders are becoming new candidate diseases for gene therapy, researchers are now developing different strategies to combat a wide range of neurological disorders like neurodegenerative diseases, chronic pain, spinal cord injuries or even strokes.

1.2.2 Targeted Gene Delivery to the Nervous System

1.2.2.1 The challenges and requirements for gene therapy in the nervous system

There are some characteristics of the nervous system that are challenging the development of gene therapy. The nervous system is the most complex system in the body. It is estimated that there are about 100 billion neurons in human brain that can be functionally classified into thousands of different types (McKay, 1989). The synaptic connections among these neurons are also complicate. The estimated number of synapse in these neurons is on the astonishing order of $10^{15}$. These neurons present a wide variety of
morphologies and functions; they are delicately organized into distinct anatomic structures. These highly organized structures determine not only the basic sensory and motor functions but also the higher functions like the memory and learning capacities as well as the personality of a person. It is conceivable that any changes in the connectivity or activity of neurons may cause serious consequences to the patients. Furthermore, the nervous system presents the system where most genes are expressed. The neurons, the basic units of the nervous system, are post-mitotic and non-dividing cells. They have different functions according to their individual patterns of gene expression, their anatomical locations and their synaptic connections. To make thing more complicate, there are non-neuronal cells in the nervous system, which are astrocytes, oligodendrocytes and microglia, with each of them performing different functions. Therefore, in such a highly heterologous system, it is important to apply therapeutic genes only to target cells during gene therapy.

The neurological diseases tend to affect selective, defined groups of neurons with specific biochemical and functional characteristics to produce the disease phenotype. For example, the degeneration of dopaminergic neurons in the substantia nigra results in the triad of bradykinesia, rigidity and tremor in Parkinson’s disease; the degeneration of lower motor neurons in the spinal cord and upper motor neurons in the brain results in weakness without affecting the sensory or higher cortical functions in motor neuron disease.
(MND). Thus, these affected neurons are the primary targeted regions for gene therapy in these neurological diseases.

As previously mentioned, successful gene therapy depends both on safe and efficient gene delivery vectors. However, the targeting ability is also considered as another important property for a successful vector, which is especially crucial for gene therapy in the nervous system.

**1.2.2.2 Targeting of nonviral vectors to the nervous system**

To allow localized delivery of the therapeutic gene and to reduce the opportunities of unexpected side effects, cell type-specific gene delivery to the neuronal cells is required for the success of *in vivo* gene therapy of the neurological disorders. Among the viral vectors that are commonly used for gene delivery, those derived from adeno-associated virus (AAV) infect neurons preferentially through the interaction of capsid proteins with HSPG moieties on the cell surface (Bartlett et al., 1998); those derived from herpes simplex viruses (HSV) show highly efficient retrograde and anterograde transport within the nervous system (Chiocca et al., 1990). Obviously, this neurotropic property is directed by the native tropism and the viruses can infect a broad range of cell types. To generate viral vectors that target one specific type of neurons, the envelope proteins that mediate the binding of virus to specific receptor need to be genetically modified and this is not an easy task. Technically, it would be easier to develop nonviral system for targeting a specific group of neurons using novel ligands that bind to the specific receptors on the neuron surface. And the development of targeted
nonviral vectors is also necessary to overcome the possible risk associated with viral vectors.

In general, targeted nonviral vector system to neurons is still a field under development, which provides only countable publications. In a recent study, Peluffo et al used an intergrin-targeting multifunctional protein for gene delivery to the central nervous system (Peluffo et al., 2003). This nonviral system shows no obvious *in vivo* toxicity and targets different cell populations like neurons, astrocytes, microglia and endothelial cells. In other study using bifunctional synthetic peptide, Collins et al synthesized a polylysine-molossin peptide that binds integrin for gene delivery into primary cultures of rat cerebral cortex neurons (Collins et al., 2003). Apparently, the above systems using ligands that bind intergrin, a receptor widely expressed in many cells is not suitable for targeting a specific group of neurons. In a better neuronal targeting system, one research group used Hc fragment of tetanus toxin as targeting ligand to conjugate with polylysine (Knight et al., 1999) or to integrate in a multi-domain system for gene delivery to neuronal cell *in vitro* (Box et al., 2003). The research on developing nonviral targeted system for gene delivery to a subtype of neurons is also scarce. One group used neurotensin as ligand to target the neurotensin receptor expressing in neurons like those of the nigrostriatal and mesolimbic dopaminergic systems in conjugation with polylysine (Martinez-Fong et al., 1999), the system was tested for gene transfer to rat substantia nigra (Alvarez-Maya et al., 2001). Another study used a monoclonal antibody to target RET, a receptor tyrosine
kinase expressed in both neuroblastoma cells and cells in substantia nigra (Yano et al., 2000). Obviously, the above-mentioned vectors may only be applied in certain disease conditions. To realize gene therapy in the nervous system, various targeted vector systems are still required for specific delivery of therapeutic gene to various affected regions in different neurological disorders. For targeted gene therapy in the nervous system, nerve growth factor (NGF) of neurotrophin family may be a promising targeting ligand in nonviral gene delivery system.

1.2.3 NGF and NGF Peptidomimetics

1.2.3.1 NGF and its receptors

Neurotrophins are a family of structurally and functionally related homodimeric neurotrophic factors that include NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and neurotrophin-6 (NT-6) (Barde et al., 1982; Ernfors et al., 1990; Hallbook et al., 1991; Gotz et al., 1994). These highly homologous dimeric proteins play important roles in the development and maintenance of the peripheral and central nervous system (Snider, 1994). Their biological functions are mediated through two classes of cell surface receptors: the low affinity $\text{p}75^{\text{NTR}}$ receptor and the high affinity Trk receptors (Chao and Hempstead, 1995; Lee et al., 2001). The $\text{p}75^{\text{NTR}}$ receptor belongs to the tumor necrosis factor receptor family and all neurotrophins bind with similar affinities to the $\text{p}75^{\text{NTR}}$ receptor ($K_d \sim 10^{-9}$ M). The roles of the $\text{p}75^{\text{NTR}}$ receptor include promotion of apoptosis, survival and regulation of other Trk activities (Carter et al.,
The Trk receptors are members of receptor tyrosine kinase superfamily, to which the neurotrophins bind selectively with high affinities ($K_d \sim 10^{-11}$M) and act as trophic factors (Cordon-Cardo et al., 1991; Kaplan et al., 1991; Soppet et al., 1991; Berkemeier et al., 1991; Klein et al., 1991a; Klein et al., 1991b; Ip et al., 1992). Three different Trk receptors are known in vertebrates. TrkA is the receptor for NGF; TrkB binds to BDNF and NT-4/5, whereas TrkC is the preferred receptor for NT-3. The relationship of neurotrophins and their Trk receptors appears somewhat promiscuous. This is suggested by the fact that different Trk receptors may bind to the same neurotrophin (e.g. NT-3 can also bind to TrkA and TrkB with lower affinity than for TrkC.) and that different neurotrophins may bind to the same Trk receptor (e.g. BDNF and NT-4/5 may bind to TrkB.).

NGF, the best-characterized member of the neurotrophin family, was discovered half century ago, which promotes the survival of sensory neurons in the dorsal root ganglia (DRG) and sympathetic neurons (Levi-Montalcini, 1987). X-ray crystallography of dimeric NGF revealed a novel tertiary protein fold that results in a rather asymmetric molecule (McDonald et al., 1991). Each NGF monomer shows a flat and elongated shape with the central part of the molecule composed of two pairs of twisted, anti-parallel $\beta$-strands. These $\beta$-strands are connected at one end of the monomer by three hairpin loops, while the three disulfide bridges at the other end are clustered to form a
cysteine-knot motif that further stabilizes the fold and locks the molecule in its conformation. In biologically active form, two NGF monomers are arranged in parallel orientation that allows the two pairs of β-strands from each monomer to pack against each other and generates a closed-packed homodimer with an overall dumbbell-like shape. The two central β-strands (strands A and B and strands C and D) of the two monomers assemble to form the ‘handle’ of the dumbbell. These four β-strands provide the major interactions that stabilize the dimer. On one end of the dumbbell are the cysteine-knot motif, the N- and C-termini, and the loop L3 connecting strands B and C, which are pointing away from the membrane. On the other end, there are three hairpin loops L1, L2 and L4, as well as four short β-strands arranged in two anti-parallel β sheets. The structures of other neurotrophin family members such as BDNF, NT-4/5 and NT-3 have also been revealed, which have shown to be structural homologues to NGF (Holland et al., 1994; Robinson et al., 1995; Butte et al., 1998; Robinson et al., 1999). Sequence alignment analysis shows about 50% of sequence identity among the neurotrophins, which maps onto strands A, B and D, and loop 3 and many of these conserved residues participate in dimer formation (Wiesmann et al., 1999; Wiesmann and de Vos, 2001).

The Trk receptors are highly sequence homologous with their extracellular portion being classified into five distinct subdomains (Schneider and Schweiger, 1991; Kaplan and Stephens, 1994; Patapoutian and Reichardt, 2001). These subdomains are a cysteine-rich cluster (CR1, domain 1), a
leucine-rich region (LRR, domain 2), a second cysteine-rich cluster (CR2, domain 3), and two immunoglobulin (Ig)-like domains (Ig 1 and Ig 2, domain 4 and 5). The intracellular tyrosine kinase domain is linked by a putative helical membrane-spanning region to the extracellular part. The overall 3-D structures of these Trk receptors are still unknown. Functional studies show that they may share the common signaling mechanisms. The binding of ligand to receptor activates intrinsic tyrosine kinase domains, which catalyzes phosphorylation of several tyrosine residues. Following the binding of src homology domain 2 (SH2)-containing proteins to these phosphotyrosines, three major signaling pathways are switched on (Sofroniew et al., 2001). Among these pathways, the Ras/extracellular receptor-activated kinase (ERK) and phospholipase Cγ1 (PLC-γ1) signaling are thought to be involved in neuronal differentiation and growth by activation of gene expression, whereas phosphotidylinositol-3-OH kinase (PI-3K) signaling may promote neuronal survival by inhibition of apoptosis.

In addition to Trk receptors, neurotrophins may also signal through the p75NTR receptor. This receptor is a transmembrane glycoprotein. The intracellular domain of p75NTR receptor is a death domain that may regulate apoptosis and cell death (Bamji et al., 1998), but not a tyrosine kinase domain as observed in Trk receptors. The extracellular portion of p75NTR contains four cysteine repeat motifs (Smith et al., 1994). The exact role of p75NTR is not known. Its biological activity depends on the binding neurotrophins as well as the expression of other Trk receptors (Frade et al., 1996; Chao et al.,
1998; Meldolesi et al., 2000). For instance, in the absence of TrkA expression, the binding of NGF to p75NTR will result in apoptosis in TrkA-negative cells; in the presence of TrkA, p75NTR enhances the binding of NGF to TrkA and promotes cell survival (Bamji et al., 1998).

1.2.3.2 NGF peptidomimetics

NGF and other members of neurotrophin family have great potential in promoting neuronal survival and neurite outgrowth. These polypeptide growth factors also have biological functions in several other cell types like lymphoid, epithelial, oligoglia and mast cells. Disregulation of neurotrophins or their receptors is evident in many human disorders such as Alzheimer’s disease, stroke, pain, neuropathy and cancers, in which both hyperactivity and hypoactivity of these ligand-receptor systems may be the etiological factors (Hefti, 1997; Saragovi and Gehring, 2000). Therefore, neurotrophins and their receptors are important therapeutic targets in these diseases (Saragovi et al., 1999). However, direct applications of neurotrophins are limited, because these neurotrophins which have poor stability in vivo may induce unfavorable side effects due to their wide array of local and systemic biological activities and are expensive to produce (Barinaga, 1994). One approach to address these limitations is to develop synthetic, small molecule mimetics of neurotrophins with optimal pharmacological profiles (Saragovi and Gehring, 2000; Massa et al., 2002). The development of agents that act directly at neurotrophin receptors as agonists, partial agonists or antagonists is one of the neurotrophin mimetic strategies. These agonists or antagonists would
mimic or disrupt the interaction of the neurotrophins with the extracellular portion of Trk (or p75^{NTR}) receptors and be expected to contain structural determinants of neurotrophin actions that interact with the receptors.

Rational design of small mimetics that bind neurotrophin receptors requires the understanding of structural basis of neurotrophin-receptor interactions. Several strategies have been used to identify the domains in neurotrophin that interact with the receptors. In loss-of-function approach, individual residues or small domains are deleted or replaced by alanine (alanine scanning mutagenesis) or substituted with homologous segments from other members of the same family (homologous scanning mutagenesis), whereas in gain-of-function studies, chimeric molecules produced by exchanging residues or small domains between different members of neurotrophin family are prepared. Both the lose-of-function and the gain-of-function effects in these mutants or chimeric proteins are examined by receptor binding as well as biological assays. Moreover, the crystallographic studies of the neurotrophins, the fragments of the receptors and the complex of neurotrophins and receptor fragments provide further evidence of the molecular contacts.

Various techniques have been applied to discover the domains of NGF that interact with its receptors. In a peptide mapping approach that used synthetic peptides with sequences corresponding to specific NGF regions to inhibit NGF activity, residues 29-35 were identified as a key active site (Longo et al., 1990). Subsequent crystallography and molecular modeling studies of
NGF revealed that NGF contained three surface hydrophilic β-hairpin loops (loop 1, 2 and 4) that were likely to be the candidate sites for ligand-receptor interaction (McDonald et al., 1991; Holland et al., 1994). The previously identified residues 29-35 were mapped to loop 1 region of NGF. Studies further confirmed that synthetic peptide consisted of residues 25-39 inhibits NGF activity and NGF-p75NTR receptor binding (LeSauteur et al., 1995; Van der Zee et al., 1996). Recombinant substitution study suggests the importance of Lys32 and Lys34 in binding to p75NTR receptors (Ibanez et al., 1992). Molecular modeling further supports loop1 as critical site for binding to p75NTR (Shamovsky et al., 1999). The NGF domains that interact with TrkA have also been deduced via different approaches, such as chemical modification, recombinant protein and NGF-TrkA-d5 co-crystallization (McDonald and Chao, 1995; Woo and Neet, 1996; Kullander et al., 1997; Woo et al., 1998; Wiesmann et al., 1999). The above studies indicated that the TrkA binding sites primarily consist of residues in NGF loop 2 (residue 40-49), loop 4 (residues 91-97) and the N-terminus (residue 1-8).

In traditional approaches, random screening of chemical compound libraries has been used to discover small molecules with receptor binding activity. However, these methods are relatively unproductive. An alternative approach to identify small molecules that bind neurotrophin receptors is via structure-based design (Longo and Mobley, 1996; Cunningham and Wells, 1997; Kieber-Emmons et al., 1997; McInnes and Sykes, 1997). The sequence diversity observed in the loop regions suggests the importance of these turns.
in defining the specificity of neurotrophins to the receptors. Many synthetic peptides mimicking these specific domains of native neurotrophins (peptidomimetics) have been shown to function via the neurotrophin receptors. These peptidomimetics are often cyclized by intramolecular disulfide bond for applying conformational constrain and inducing structure more closely matching those corresponding loops in native neurotrophins. In an early study, cyclized monomeric peptidomimetic C(92-96) corresponding to NGF loop 4 was found to inhibit NGF bioactivity and $[^{125}\text{I}]$NGF binding to TrkA-receptor expressing E25 cells (LeSauteur et al., 1995). Subsequent NMR studies demonstrated striking similarity between the calculated structure of peptidomimetic C(92-96) and the structure of corresponding loop 4 region derived from NGF X-ray studies (Beglova et al., 1998). In vivo study showed that C(92-96) delivered to rat cortex led to an inhibition of NGF-induced upregulation of choline acetyltransferase (ChAT) activity and a decrease in the size of cholinergic boutons, suggesting the blocking effect of this peptidomimetic to NGF in vivo activity (Debeir et al., 1999). In another design of peptidomimetic based on the same NGF loop 4 region, a cyclized dimeric rather than monomeric form termed ‘P92’ was synthesized (Xie et al., 2000). P92 was found to promote survival and neurite outgrowth of cultured DRG sensory neurons with activity well under that of native NGF, suggesting a partial agonist profiles. This research also provides substantial evidence supporting the hypothesis that P92 acts via TrkA receptors.
1.2.3.3 Targeting NGF receptor-expressing neurons

Obviously, the development of NGF peptidomimetics that bind directly to NGF receptors may not only be useful in modulating the biological effects of NGF-receptor interaction, but also provide novel targeting ligands for targeted gene delivery systems. Using NGF peptidomimetics as targeting ligand may overcome those limitations produced by the use of native NGF protein. Also, there is apparent necessity for specific gene delivery to those sites with NGF receptor, especially to those with TrkA receptor expression. One of the TrkA-expressing sites locates in basal forebrain, which contains cholinergic neurons that are affected in Alzheimer’s disease (AD) (Winkler et al., 1998). Widespread neuronal degeneration occurs in AD, which appears to be related to the accumulation of a toxic protein, amyloid in the extracellular space in the brain. This results in preferential degeneration of cholinergic neurons in the basal forebrain as well as structure in the hippocampus and parahippocampal gyrus. Targeted delivery of therapeutic genes to these sites has particular potential in treating AD. Another site with TrkA-expressing neurons locates in dorsal root ganglion (DRG) that contains peripheral sensory neurons (Mu et al., 1993; McMahon et al., 1994). Small diameter neurons in DRG are the major population with TrkA expression. These neurons are affected in sensory polyneuropathy characterized by degeneration of peripheral sensory nerve fiber, a common neurological condition for which no current therapies exist. Selective delivery of therapeutic gene to these DRG neurons may be used to combat this disorder.
Outside the nervous system, NGF and NGF receptor expressions have been also found in cells of the immune and inflammatory systems (Sofroniew et al., 2001; Vega et al., 2003). Several types of leucocytes are known to express TrkA, which include mast cells, CD4+ T lymphocytes, B lymphocytes, monocytes, and macrophages. Many of these cells also have the capability to express NGF. In addition, TrkA receptor expression has been observed in various cancer cells (Saragovi and Gehring, 2000). Increased level of TrkA expression has been associated with abnormal growth in prostate endothelium. Some breast cancer cells have been observed to overexpress TrkA. Therefore, NGF peptidomimetic that binds to TrkA may also be used for targeted gene delivery to these cells and tissues.

1.3 Aim of the Study

The realization of targeted gene therapy for various diseases depends on the introduction of different novel targeting ligands into the vectors accordingly. These novel ligands would direct therapeutic genes to the targeted cell surface receptors. For targeted gene delivery to the nervous system, NGF could be a promising ligand, which may target TrkA, a receptor expressed in basal forebrain cholinergic neurons that are affected in Alzheimer's diseases. However, to overcome the limitation of using native NGF as targeting ligand, smaller and more stable NGF mimetics are desirable.

In this study, a NGF loop 4-containing hairpin motif will be employed as a novel ligand for targeted gene delivery. Both bacterially produced
recombinant proteins and chemically synthesized peptides will be used to prove the concept. The binding of this hairpin motif to TrkA receptor will be examined by biochemical and biological assays. The transfection efficiency as well as specificity of these hairpin motif-containing gene delivery vectors will be tested by \textit{in vitro} gene transfer. Toxicity of this novel gene delivery vector will also be examined in both \textit{in vitro} and \textit{in vivo} studies. This targeted system will also be assayed for \textit{in vivo} gene delivery to TrkA-expressing neurons.

Hopefully, this study will provide an example for using ligand mimetic for targeted gene delivery to a subtype of neurons, and also present a platform for designing more efficient TrkA-targeted vector by including other functional domains.
CHAPTER 2
MATERIALS AND METHODS
2.1 Studies Using Bacterially Produced Polypeptides

2.1.1 Plasmid Construction

A 29-amino acid sequence derived from NGF loop 4-containing region (#80-108) was selected for NGF receptor targeting and designated as NL4. The numbering of the amino acid refers to mature NGF (Wiesmann et al., 1999; Wiesmann and de Vos, 2001). A DNA binding sequence of 10-lysine residues was added to the C-terminus of NL4 to form a chimeric peptide NL4-10K. The DNA sequences encoding NL4 or NL4-10K were PCR-amplified from a pcDNA3.1/GS plasmid containing the NGF gene (humFL ORF: H-X52599M; Invitrogen, Carlsbad, CA, USA), using a common 5' primer (5'-TGTACCACGACTCACACC-3') and 3' primers (5'-GCAAGCTTTCAACAGGCCGTATCTATCCG-3') for NL4 or (5'-GCAAGCTTTCAACAGGCCGTATCTATCCG-3') for NL4-10K respectively. These NL4 or NL4-10K encoding sequences were inserted in-frame into pET-40b(+) expression plasmid (Novagen, Madison, WI, USA) between the Scal and HindIII sites, downstream of the coding sequences for DsbC and a His6 tag.

2.1.2 Polypeptide Expression, Purification and Detection

The plasmids were transformed into the E. coli expression host strain BL21(DE3)pLysS from Novagen (Madison, WI, USA). A single colony of freshly transformed bacteria was suspended in 10ml of LB containing 30 µg/ml kanamycin and 34 µg/ml chloramphenicol and incubated for 4hr till an
OD 600 of ~0.6. The cultures were then used to inoculate 400 ml of LB medium containing 30 μg/ml of kanamycin and 34 μg/ml chloramphenicol. After rapid shaking at 37 °C for 2hr, cells were induced with IPTG and grown for another 2hr. The bacteria were harvested and lysed in 20 mM Na₂HPO₄, 0.5 M NaCl, 10 mM imidazole, pH 7.4. After sonication and centrifugation, the supernatant was purified by nickel-chelating affinity chromatography on an ÄKTAexplorer FPLC (Amersham Biosciences, Buckinghamshire, UK). The extract was purified on a 1 ml HisTrap column (Amersham Biosciences). Elution was carried out with increasing imidazole concentration. The fractions containing purified polypeptide were pooled and dialyzed against HEPES-buffered saline (HBS, 150mM NaCl, 20mM HEPES, pH 7.4) at 4 °C overnight. The polypeptides were electrophoresed on 12.5% SDS-PAGE gel and transferred to nitrocellulose membranes. An anti-His horseradish peroxidase (HRP) conjugates (Qiagen, Hilden, Germany) was used for detection.

2.1.3 Cell Lines and Reporter Plasmid

A PC12 (rat pheochromocytoma) cell line was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in RPMI-1640 medium (National University of Singapore, Singapore) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA) and 5% horse serum (Sigma-Aldrich, St. Louis, MO, USA). PC12 cells are known to express TrkA and p75NTR receptors and extensively used to study functions of the receptors. A COS7 (African green monkey kidney fibroblast) cell line was obtained from ATCC and cultured in Dulbecco's
modified Eagle’s medium (DMEM) (National University of Singapore, Singapore) supplemented with 10% FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin. The cells were maintained in a 37 °C, 5% CO₂, humidified incubator.

To determine the transfection efficiency of this nucleic acid delivery system, a reporter plasmid, pCAGluc (a gift from Yoshiharu Matsuura, National Institute of Infectious Diseases, Tokyo, Japan), encoding firefly luciferase under the control of the composite promoter, CAG was employed. CAG comprises the chicken β-actin promoter and the cytomegalovirus immediate early enhancer.

2.1.4 Detection of TrkA, Erk and Akt Activation

Immunoblotting experiments were conducted to test whether the chimeric polypeptides could, like NGF, induce autophosphorylation of TrkA and activate its signal transduction pathways. PC12 cells were seeded in 6-well plates at a density of 2x10⁶ cells/well and cultured in RPMI 1640 medium containing 0.5% FBS and 0.25% horse serum for 2 days. The low serum concentration was necessary to reduce the basal level of phosphorylation. The medium was refreshed 2 hr before treatment. The cells were incubated with the polypeptides or NGF in serum-free medium for 15-20 min. The cells were then washed in phosphate-buffered saline (PBS), lysed, and sonicated. The cell lysates were resolved by SDS-PAGE and transferred to a nitrocellulose membrane for immunoblotting. The primary antibodies used include Phospho-TrkA (Tyr490) polyclonal antibody, Phospho-p44/p42 MAPK
(Thr202/Tyr204) monoclonal antibody, and Phospho-Akt (Ser473) monoclonal antibody, all obtained from Cell Signaling Technology (Beverly, MA, USA). Colorimetric detection was performed according to the Amplified Opti-4CN Substrate Kit (Bio-Rad, Hercules, CA, USA) after using HRP-conjugated secondary antibodies.

2.1.5 Cell Survival Assay

For PC12 survival assay, the cells were seeded in collagen-coated 96-well plates in serum-free RPMI 1640 containing 10 ng/ml NGF and cultured for 6 days. The medium was then changed to serum-free RPMI 1640 containing polypeptides at the indicated concentrations. Ten ng/ml of NGF, an optimal concentration for PC12 survival, was used as positive control. After incubation for 4 days, cell viability was estimated by MTT assay (Promega, Madison, WI, USA). Cell survival effects of the polypeptides were expressed as percentage of NGF-induced survival.

2.1.6 DNA Retardation Assay

This assay qualitatively assesses the ability of polypeptides to bind to DNA and thus to retard its migration (by decreasing its charge/mass ratio) through an agarose gel under electrophoresis. Plasmid DNA (0.1 μg) was mixed by vortexing with polypeptides at various ratios in HBS in a total volume of 20 μl. After 30 min incubation at room temperature, the mixtures were loaded on a 0.8% agarose gel and electrophoresed at 80 v for 30 min. Gels were visualized under UV after electrophoresis.
2.1.7 Gene Delivery Assay

For gene transfection experiments, plasmid DNA/polypeptide/polymer ternary complexes were used. To form the complexes, polypeptides and DNA were dissolved in OPTI-MEM medium. Equal volume of polypeptide was added dropwise to equal volume of DNA while vortexing at desired ratios. After incubation for 30 min at room temperature, polyethylenimine (MW ~600 dal, PEI600) from Sigma-Aldrich (St. Louis, MO, USA) or protamine (Sigma-Aldrich, St. Louis, MO, USA) diluted in OPTI-MEM was added to the complexes to form triple complexes. The required amount of PEI600 was calculated by taking into account that 1 µg DNA contains 3 nmol of phosphate and that 1 µl of 10 mM PEI600 holds 10 nmol of amine nitrogen. This nitrogen/phosphate ratio is defined as N/P ratio and used as a measure of the charge balance in a polycation/DNA complex. For the purposes of calculating the N/P ratio for the polypeptide used in the study, the number of basic amino acid residues in the DNA-binding domain was used as the number of “N” moieties per molecule.

The gene delivery complexes were tested for transfection efficiency in vitro in PC12 (TrkA and p75NTR positive) cells and COS7 (NGF receptor negative) cells. The cells were grown to 50-70% confluence in microplate wells (96- or 24-well plates). DNA complexes were added and incubated with cells for 4 hr. The cells were then cultured in normal culture medium for 24 hr before luciferase activity assay. After the incubation, cells were 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 sec 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 using a protein assay kit (Bio-Rad, Hercules, CA, USA).

2.2 Studies Using Chemically Synthesized Peptides

2.2.1 Peptide Design and Synthesis

The amino acid sequences of NL4 and NL4-10K were previously described. Briefly, a 29-amino acid sequence derived from NGF loop 4-containing region (#80-108) 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. Both NL4 and NL4-10K were prepared using conventional solid phase, chemical synthesis method and cyclized by a disulfide bond formed between C80 and C108 by Cambridge Research Biochemicals (Cleveland, UK). A 10-lysine peptide (designated as 10K) was synthesized by BioSynthesis (Lewisville, Texas, USA) as a non-targeted control. All peptides were provided in dry powder and stored in –80°C.

2.2.2 Cell Cultures

Rat PC12 pheochromocytoma cells that express TrkA and p75NTR receptors were also employed in the study using synthetic peptides and cultured using the above-mentioned condition. Two TrkA-expressing NIH3T3
cell lines, E25 and TRK1, and parental NIH3T3 cells were grown in DMEM supplemented with 10% FBS. E25 was developed by Dr. Stuart Decker (Parke-Davis Pharmaceuticals) (Decker, 1995), and TRK1 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, CA, USA) was used respectively. The cells were maintained in a humidified incubator with 5% CO₂ at 37°C.

Primary cultures of cortical neurons and glial cells were established from the cortex of embryonic Wistar rats at gestational day 20. Meninges-free cortices were dissected and individual cells were dispersed mechanically by trituration of minced tissue in 3 ml DMEM medium supplemented with 2% FBS. The suspension was allowed to settle in a centrifuge tube. The cells in the supernatant were collected by centrifugation at 1000 rpm for 5 min and re-suspended gently in DMEM with 10% FBS. Viability of the cells was assessed prior to plating using trypan blue. To obtain neurons, cells were plated onto microplate pre-coated with poly-L-lysine/laminin at a density of 7.5×10⁵ viable cells/cm². After two-hour incubation to allow neurons to attach, the medium and unattached cells were removed and serum-free DMEM/F12 medium with 1% N2 supplement (Invitrogen, Carlsbad, CA, USA) was added into the plate. The neurons were then incubated at 37°C in 5% CO₂ in a humidified incubator.
incubator for 2-5 days before transfection. To obtain glial cells, the cells collected from the cortexes were plated at a density of $4 \times 10^5$ viable cells/cm$^2$ on poly-L-lysine/laminin coated dishes and grown to confluence for 7 days in DMEM/F12 medium supplemented with 10% FBS. The cells were detached and plated into coated plates one day before transfection.

Primary cultures of rat cerebellar granule neurons were established from the cerebellum of 8-day-old Wistar rats. Meninges-free cerebella were dissected and individual cells were isolated mechanically by trituration of minced tissue in 3 ml DMEM medium supplemented with 10% FBS. Cells were diluted with 10 ml DMEM with 10% FBS and let settled in a centrifuge tube. The supernatant was collected and centrifuged at 900 rpm for 5 min. The pellet was re-suspended gently in DMEM with 10% FBS. Viability of the cells was assessed prior to plating using trypan blue. Cells were plated into wells pre-coated twice with poly-L-lysine (50 mg/ml) at a density of $7.5 \times 10^5$ viable cells/cm$^2$. After 2 hr of attachment, the medium and unattached cells were removed and serum-free DMEM/F12 medium with 1% N2 supplements added afterwards. The cells were then incubated at 37°C in 5% CO$_2$ in a humidified atmosphere for 5 days before transfection.

2.2.3 Biochemical and Biological Assays

For biochemical assays of TrkA, Erk and Akt activation, PC12 cells were seeded in a 6-well plate with the cell density of $2 \times 10^6$/well and cultured in RPMI-1640 containing 0.5% fetal calf serum and 0.25% horse serum for 2 days. The low serum concentration was necessary to reduce the basal level
of phosphorylation. After cultured in fresh serum-reduced medium for 2 hr, the cells were stimulated for 15 min with 5 μM of NL4, NL4-10K or NL4-10K/DNA complexes, and 10 ng/ml of NGF (Invitrogen, Carlsbad, CA, USA) was used as positive control. The cell lysates were then prepared and electrophoresed on 12.5% SDS gels and transferred to nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). Membranes were incubated with Phospho-TrkA (Tyr490) polyclonal antibody, Phospho-p44/p42 MAPK (Thr202/Tyr204) monoclonal antibody or Phospho-Akt (Ser473) monoclonal antibody from Cell Signaling Technology (Beverly, MA, USA). Colorimetric detection was performed with Amplified Opti-4CN Substrate Kit from Bio-Rad (Hercules, CA, USA). To inhibit the tyrosine kinase activity of TrkA, PC12 cells were pre-incubated with 100 nM K252a (Calbiochem, Ja Jolla, CA, USA) and 10 μM AG879 (Calbiochem, Ja Jolla, CA, USA) before addition of peptides or NGF.

For PC12 survival assay, the cells were seeded in collagen-coated 96-well plates in serum-free RPMI 1640 containing 10 ng/ml NGF and cultured for 6 days. The medium was then changed to serum-free RPMI 1640 containing NL4 or NL4-10K at the indicated concentrations. 10 ng/ml of NGF, an optimal concentration for PC12 survival, was used as positive control. After incubation for 4 days, cell viability was estimated by MTT assay (Promega, Madison, WI, USA). Cell survival effects of NL4 and NL4-10K were expressed as percentage of NGF-induced survival.
2.2.4 Reporter Plasmid, DNA Binding Assay and Preparation of DNA Complexes

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 DNA 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 1 mg/ml.

For DNA binding assay of the synthetic peptides, plasmid DNA (0.1 μg) was mixed by vortexing with NL4-10K or NL4 at various ratios in HBS in a total volume of 20 μl. After 30 min incubation at room temperature, the mixtures were loaded on 0.8% agarose gel and electrophoresed at 80 v for 30 min. Gels were visualized under UV after electrophoresis.

For gene delivery experiments, either plasmid DNA/peptide complexes or plasmid DNA/peptide/polymer ternary complexes were prepared with various nitrogen/phosphate ratios, which was defined as N/P ratio and used as a measure of the charge balance in a polycation or peptide/DNA complex. For the purposes of calculating N/P ratio for the peptides used in the study, the number of basic amino acid residues in the DNA-binding domain was taken as the number of “N” moieties per peptide molecule. The required amount of
DNA was calculated by taking into account that 1 µg DNA contains 3 nmol of phosphate. To form DNA/peptide complexes, peptides and DNA were dissolved in OPTI-MEM medium (Invitrogen, Carlsbad, CA, USA). Equal volume of peptide was added dropwise to equal volume of DNA while vortexing at desired ratios. Complexes were incubated for 30 min at room temperature before transfection. To form ternary complexes, polyethylenimine (MW ~600 dal, PEI600) from Sigma-Aldrich (St. Louis, MO, USA) was diluted in OPTI-MEM to contain 10 nmol of amine nitrogen per µl. Equal volume of PEI600 was mixed with equal volume of DNA at the indicated ratios while vortexing. After 30 min incubation, peptides under testing were added to the complexes at ratios indicated to form triple complexes. PEI/DNA complexes using a high molecular weight PEI (MW ~25 kDa, PEI25kDa) (Sigma-Aldrich), were prepared and used as a positive control in toxicity assays.

2.2.5 Zeta Potential and Size of the Complexes

Appropriate amount of a 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 1 mM 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 μm 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 1 mM 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).

### 2.2.6 Gene Transfer

For *in vitro* gene transfer, cell culture medium was replaced with OPTI-MEM before transfection. DNA complexes were added and incubated with cells for 4 hr in the presence or absence of 100 μM chloroquine. After the incubation in normal culture medium for another 24 hr, cells were 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 sec in a single-well luminometer (Berthold Lumat LB 9507, Germany). The RLU were normalized by the total protein concentration of the cell extracts, measured with a protein assay kit (Bio-Rad, Hercules, CA, USA).

For *in vivo* study, adult male Wistar rats (8 weeks of age, 180-200 g) 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.

Three days after the injection, animals were perfused transcardially with 0.1 M PBS (200 ml/rat) under anesthesia. Spinal cord was exposed after perfusion. Lumbar spinal cord and dorsal root ganglia were collected and homogenized with 400 µl and 100 µl of lysis buffer (Promega, WI, USA), respectively. After three freeze-thaw cycles, the lysates were centrifuged at 14,000 g for 5 min at 4°C. Twenty µl of the supernatants 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 sec in a single-well luminometer (Berthold Lumat LB 9507, Germany). The RLU were normalized by the total protein concentration of the cell extracts.

2.2.6 Flow Cytometry, Immunocytochemistry and Immunohistochemistry

For flow cytometric analysis of neurotrophin receptor expression in TrkA-expressing cell lines NIH3T3.E25 and NIH3T3.TRK1 or primary cortical or cerebellar neurons, cells were dislodged from 60 mm culture dishes with 1 mM EDTA in Hank’s buffered saline, fixed by 4% paraformaldehyde in PBS, permeabilized by 0.2% Tween 20 in PBS, blocked with 1% bovine serum
albumin (BSA) in PBS, and stained with rabbit anti-TrkA or anti-TrkB or anti-TrkC antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hr. After washing with PBS, cells were then stained by sheep anti-rabbit IgG-FITC (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. Cells were washed and re-suspended with PBS. Flow cytometry was processed with Coulter Flow Cytometer at the Clinical Research Center, National University of Singapore.

For immunostaining of primary cortical neurons, cells were grown on coverslips and transfected as described above. Cells were briefly washed with PBS and fixed with ice-cold methanol at -20°C for 20 min. The air-dried samples were washed with PBS and blocked with 1% BSA in PBS, followed by incubation with rabbit anti-TrkA or anti-TrkB or anti-TrkC antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-luciferase antibody (Promega, WI, USA) for 1 hr. After washing with PBS, cells were incubated with donkey anti-rabbit IgG-Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and donkey anti-goat IgG-FITC (Research Diagnostics, Flanders, NJ, USA) for 30 min. Coverslips were mounted in FluoSave Reagent (Calbiochem, La Jolla, CA, USA) and examined under a Zeiss confocal microscope at the Clinical Research Center, National University of Singapore.

For immunohistochemistry, all rats under anaesthesia were perfused with 0.1 M PBS followed by 2% paraformaldehyde in 0.1 M PBS. Spinal cords were removed and post-fixed in the same fixative for 24 hr. Then the samples were transferred into 0.1 M PBS containing 15% sucrose and kept overnight.
at 4°C. Frozen horizontal sections of the spinal cord were cut at 30 µm thickness and mounted on slides. Sections were washed for 20 min in 0.1 M PBS and then incubated overnight with anti-OX42 monoclonal antibody (Harlan Sera Lab, Leicestershire, UK; dilution 1:500). After incubation, sections were rinsed in PBS for 15 min and reacted with Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA) against mouse IgG for 1 hr. They were then treated with 3,3’-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO, USA) as a peroxidase substrate. The sections were counterstained with 1% methyl green, dehydrated and mounted in Permount.
CHAPTER 3
EXPERIMENTAL RESULTS
3.1 Studies Using Bacterially Produced Polypeptides

3.1.1 Expression of Recombinant Cationic Polypeptides

Recombinant DNA method was used to produce a recombinant polypeptide comprising a hairpin motif of NGF as a targeting ligand and a 10-lysine sequence as a DNA binding moiety. The targeting ligand was derived from amino acid residues 80-108 of NGF that contains the four residues of loop 4 and part of the C and D β strands (designated as NL4). The loop 4-flanking regions of NGF were included in order to stabilize the native conformation of the loop 4 through the formation of hydrogen bonds between two β strands. The three-dimensional structure was further stabilized by the disulfide bond formed between C80 and C108 of NGF. The gene encoding NL4 together with a 10-lysine sequence at its C-terminus or NL4 alone was introduced into pET40b(+) expression vector by molecular cloning (Figure 3.1). These sequences were expressed in E.coli system together with DsbC, an E. coli disulfide bond isomerase that should enhance polypeptide stability, solubility and folding. The product was designated as DsbC-NL4-10K and the control, without the 10-lysine sequence, was designated as DsbC-NL4 (Figure 3.2A). Western blotting demonstrated the expression of DsbC-NL4-10K and DsbC-NL4 in E.coli using a His-tag antibody (Figure 3.2B). Purification of proteins was performed by His-tag affinity chromatography with FPLC.
Figure 3.1 Schematic of expression plasmids. Genes encoding NL4 or NL4-10K sequence were generated by PCR and inserted into the expression vector pET-40b(+) using restriction sites Scal and HindIII.
Figure 3.2 Structure and production of recombinant polypeptides. (A) NGF loop 4-containing sequence (#80-108) was expressed in *E. coli* as a fusion polypeptide, designated as DsbC-NL4. The other fusion polypeptide with the appendage of a DNA-binding sequence of 10-lysine at the C-terminus to DsbC-NL4 sequence was also produced in *E. coli* to form a NGF receptor-targeted vector, designated as DsbC-NL4-10K. (B) Western blotting with antibody against His$_6$ tag confirmed the expression of DsbC-NL4-10K and DsbC-NL4 in *E. coli*. 
3.1.2 Activation of TrkA, Erk and Akt by DsbC-NL4-10K

Activation of TrkA receptor, an NGF receptor, by DsbC-NL4-10K was tested in TrkA-positive PC12 cells. NGF is known to induce the autophosphorylation of TrkA on Tyr490 (Obermeier et al., 1993) and activate the pathway of Ras-MAP kinase, Erk1/Erk2 (p44 and p42 MAP kinases) (Payne et al., 1991). Activation of TrkA receptor by NGF is also known to trigger Akt signaling cascade, which plays a critical role in cell survival (Franke et al., 1997). Similarly, DsbC-NL4-10K induced TrkA phosphorylation on Tyr490 and activated Erk1/Erk2 (Figure 3.3). Furthermore, DsbC-NL4-10K activated Akt pathway (Figure 3.3). At the same concentration used, DsbC-NL4 also activated Erk and Akt, although to a lesser extent. The result suggests that DsbC-NL4-10K binds to TrkA receptor and the appendage of 10-lysine to the C-terminal of DsbC-NL4 does not affect the receptor binding and bioactivity of the polypeptide.
**Figure 3.3 Activation of TrkA, Erk and Akt by DsbC-NL4-10K.** PC12 cells were incubated in RPMI-1640 medium containing 0.5% FBS and 0.25% horse serum as stated in Materials and Methods and treated for 15 min with 10 ng/ml NGF, 5 µM DsbC-NL4, 5 µM DsbC-NL4-10K or serum free RPMI-1640 without additives. The cell lysates were collected for Western blotting using antibodies against Phospho-TrkA, Phospho-p44/p42 MAPK or Phospho-Akt.

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3.1.3 Promotion of PC12 Cell Survival by DsbC-NL4-10K

To assay the neuroprotective effect of DsbC-NL4 and DsbC-NL4-10K polypeptides, differentiated PC12 cells deprived of serum and NGF were used as an evaluation model (Franke et al., 1997; Dago et al., 2002). Withdrawal of serum and NGF from medium induces loss of differentiated PC12 cell viability; recombinant polypeptides were added to medium to prevent cell death. Figure 3.4 showed that addition of DsbC-NL4 prevented PC12 cells death with 71-92% of the maximum survival promoted by 10 ng/ml NGF. For DsbC-NL4-10K, 12-67% of the maximum survival promoted by NGF was observed. This observation also indicates that DsbC-NL4-10K binds to TrkA receptor.
Figure 3.4 Promotion of neuronally differentiated PC12 cells survival in serum-free medium by DsbC-NL4-10K. Differentiated PC12 cells were deprived of serum and NGF for 4 days. DsbC-NL4 or DsbC-NL4-10K was added at the time of serum and NGF withdrawal and 10 ng/ml NGF was used as a positive control. Cell survival was estimated using a MTT assay. Cell survival mediated by the recombinant polypeptides was expressed as percentage of maximal NGF promoted survival (n=4).
3.1.4 Binding of DsbC-NL4-10K to Plasmid DNA

The binding ability of DsbC-NL4-10K to plasmid DNA was monitored by DNA retardation assay. While DsbC-NL4-10K complexed with plasmid DNA, the positively charged amino groups (N) in 10-lysine tail will neutralize the negatively charged phosphate groups (P) of DNA and thus result in the loss of electrophoretic mobility of DNA. At proper N/P ratio, the binding oligolysine will neutralize all the negative charge of DNA and plasmid will be immobilized on gel. Figure 3.5 showed that plasmid DNA gradually lost its mobility with the increase of DsbC-NL4-10K concentration. However, plasmid DNA was not totally retarded even at a theoretic N/P ratio of 10. This is understandable for that the high molecular mass of DsbC-NL4-10K polypeptide on one hand can provide shielding effect for protecting DNA, on the other hand can hinder more condensed complex formation. Particles formed at N/P ratio of 10 had their sizes lied between 200-300 nm while measured by Light Scattering Method. This indicated that DsbC-NL4-10K could still condense plasmid DNA to certain extent. While DsbC-NL4 and bovine serum albumin (BSA) were used as control, no change in mobility of DNA was observed at all testing w/w ratios. This suggests that it is the 10-lysine tail in DsbC-NL4-10K that binds DNA.
Figure 3.5 DNA retardation by DsbC-NL4-10K. DsbC-NL4-10K, but not DsbC-NL4 or bovine serum albumin (BSA), binds to plasmid DNA and retards its migration in agarose gel under electrophoresis.
3.1.5 Enhanced Polycation-mediated Gene Delivery to PC12 by DsbC-NL4-10K

The ability of DsbC-NL4-10K to improve polycation-mediated gene transfer was tested in PC12 cells, which express NGF receptors. For this purpose, the ternary complexes were prepared. DsbC-NL4-10K was mixed with DNA first; PEI600 was then added at a fixed N/P ratio of 20. Figure 3.6 showed that PEI600 alone was not an efficient vector. With the help of DsbC-NL4-10K, 6 times higher gene transfer was observed. As controls, the addition of either DsbC-NL4, the polypeptide without 10-lysine sequence or 10K, a synthetic 10-lysine peptide as non-targeted control, didn't enhance the gene delivery. The results suggest that the NL4 domain, but not the 10-lysine sequence plays major role in the enhancement of gene transfer to PC12.

To further verify the contribution of DsbC-NL4-10K, the expression patterns of reporter gene after ternary complexes-mediated gene transfer were compared in NGF receptor-expressing PC12 and NGF receptor-negative COS7 cells. As shown in figure 3.7A, DsbC-NL4-10K enhanced PEI600-mediated gene delivery in PC12 cells; in COS7 cells, DsbC-NL-10K had little contribution to gene delivery. Besides PEI600, the capability of DsbC-NL4-10K to enhance gene delivery by protamine, a small arginine-rich cationic molecule was also tested. The protamine-containing complexes were formed in a similar way as PEI600-containing complexes with a protamine/DNA (w/w) ratio of 2. Figure 3.7B showed that DsbC-NL4-10K improved protamine-mediated gene delivery with a dose-response manner in...
PC12 at low dosage and at higher dosage, the efficiency gradually decreased. In COS7, however, DsbC-NL4-10K showed little benefit. The observation that the enhancement by DsbC-NL4-10K to both protamine- and PEI600-mediated gene transfer was observed in PC12, but not in COS7, suggests a NGF-receptor-specific effect of the recombinant cationic polypeptide.

To obtain further evidence of the specific nature of gene delivery mediated by DsbC-NL4-10K, PC12 cells grown in 24-well plate were incubated with free DsbC-NL4 or DsbC-NL4-10K 30 min before transfection. This pre-treatment greatly reduced the transfection efficiency of DsbC-NL4-10K/DNA/PEI600 complexes, as compared to cells pre-treated with BSA (Figure 3.8). In contrast, pre-treatment with DsbC-NL4, DsbC-NL4-10K or BSA resulted in the similar transgene expression when PEI600/DNA complexes are used. This implies that DsbC-NL4-10K-mediated gene delivery occurs through receptor binding, because pre-treatment with free DsbC-NL4 or DsbC-NL4-10K saturates the receptors.
Figure 3.6 Enhanced PEI600-mediated gene transfer by DsbC-NL4-10K in PC12. DsbC-NL4-10K/DNA/PEI600 ternary complexes were used to transfect PC12 cells. To form the complexes, DsbC-NL4-10K polypeptide and pCAGluc plasmid (0.1 µg/well in 96-well plates) were incubated at various ratios at room temperature for 30 min in 20 µl OPTI-MEM. After that PEI600 was added with a fixed N/P ratio of 20, the complexes were incubated for another 30 min. DsbC-NL4 or 10K (a synthetic 10 lysine sequence) was also used as non-targeted control for DsbC-NL4-10K. Results are expressed in relative light units (RLU)/mg protein ± SE (n=6). *p<0.05, compared to the respective controls using 10K.
Figure 3.7 Comparison of DsbC-NL4-10K-mediated gene delivery in PC12 and COS7 cells. (A) DsbC-NL4-10K enhanced PEI600-mediated gene transfer in PC12, but not in COS7. DsbC-NL4-10K/DNA/PEI600 complexes formed as previously described were used to transfect PC12 and COS7 cells (0.1 µg DNA/well in 96-well plate). (B) DsbC-NL4-10K
enhanced protamine-mediated gene transfer in PC12, but not in COS7. To form DsbC-NL4-10K/DNA/protamine complexes, DsbC-NL4-10K polypeptide and pCAGluc plasmid (0.1 µg/well in 96-well plates) were incubated at various ratios at room temperature for 30 min in 20 µl Opti-MEM. After adding protamine (2 µg per µg DNA), the complexes were incubated for another 30 min. Results are expressed in RLU/mg protein ± SE (n=6).

Figure 3.8 Competitive inhibition of DsbC-NL4-10K-mediated gene delivery to PC12 cells by DsbC-NL4-10K pre-treatment. PC12 cells were pre-treated with DsbC-NL4, DsbC-NL4-10K or BSA before transfection. DsbC-NL4-10K/DNA/PEI600 complexes were formed as previously described. PC12 cells were exposed to 0.5 µg DNA per well in a 24-well plate. Results are expressed in RLU/mg protein ± SE (n=6). *p<0.05, compared with control pre-treated with BSA.
3.2 Studies Using Chemically Synthesized Peptides

3.2.1 Biochemical and Biological Effects of NL4-10K Peptide

NL4-10K comprises the four residues of NGF loop 4 and the flanking β-strand sequences (amino acids #80-108, NL4) linked to a C-terminal nucleic acid binding domain of ten consecutive lysine residues (10K). The first and last amino acid residues of NL4 are cysteine residues, which were oxidized to form an intramolecular disulfide bond to stabilize the native structure of loop 4. The amino acid sequences of NL4 and NL4-10K are listed in Figure 3.9.

Activation of TrkA receptors by NL4-10K was tested in TrkA-positive PC12 cells. NGF is known to induce the autophosphorylation of TrkA on Tyr490 (Obermeier et al., 1993), which in turn elicits signaling cascades that give rise to the bioactivity of NGF. Amongst the involved proteins are Erk1 and Erk2, part of the Ras-MAP kinase pathway (Payne et al., 1991), and Akt (Franke et al., 1997). Similarly, both NL4 and NL4-10K induced TrkA phosphorylation on Tyr490 and activated both Erk1/Erk2 and Akt (Figure 3.10A), indicating that both NL4 and NL4-10K may bind to TrkA receptors and the appendage of 10 lysines to the C-terminal of NL4 did not affect the receptor binding and bioactivity of NL4-10K. Furthermore, after complexed with plasmid DNA, NL4-10K maintained its effects to activate TrkA, Erk and Akt (Figure 3.10A), suggesting that there were enough accessible NL4 domains on the surface of the complexes for TrkA-binding. To further assess the hypothesis that NL4 (with or without the 10K addition) acts via TrkA, two inhibitors, K-252a and
AG879, were used to block TrkA protein tyrosine kinase activity (Xie et al., 2000). As expected, pre-treatment with these inhibitors greatly reduced the extent of NL4- and NL4-10K-induced phosphorylation of Erk1 and Erk2 (Figure 3.10B). These biochemical assays demonstrate that NL4 (with or without the 10K domain) may act through TrkA to activate some of the same signal transduction pathways that NGF activates.

One manifestation of survival-promoting bioactivity of NGF is its ability to promote survival of PC12 cells grown in serum-free medium, which otherwise results in a loss of cell viability (Franke et al., 1997; Dago et al., 2002). Differentiated PC12 cells were grown in serum-free medium containing NL4 or NL4-10K. The cell survival rate, measured by the MTT assay, was compared to the survival rate promoted by NGF (10 ng/ml, optimal concentration). Over a range of concentrations from 0.125 to 2.5 µM, the increasing NL4 concentration resulted in the increasing cell survival rates from 68-109% of the optimal NGF-promoted survival rates (Figure 3.11). A similar dose response was observed for NL4-10K between 0.125 to 5 µM.
Figure 3.9 Structures of chimeric NL4-10K and its control NL4.
Figure 3.10 Effects of NL4-10K on TrkA receptor. (A) Activation of TrkA, Erk and Akt by NL4-10K. PC12 cells were incubated in RPMI 1640 medium containing 0.5% FBS and 0.25% horse serum and treated for 15 min with 10 ng/ml NGF, 5 μM NL4, 5 μM NL4-10K, NL4-10K/DNA complexes (N/P ratio of 5) or serum free RPMI 1640 without additives. The cell lysates were collected for Western blotting using antibodies against Phospho-TrkA, Phospho-p44/p42 MAPK or Phospho-Akt. (B) Blocking of NL4-10K-induced Erk activation by TrkA inhibitors. PC12 cells were pre-incubated with or without 100 nM K-252a or 10 μM AG879 for 10 min and then treated as described in (A). The cell lysates were collected for Western blotting using antibody against Phospho-p44/p42 MAPK.
Figure 3.11 Promotion of neuronally differentiated PC12 cell survival in serum-free medium by NL4-10K. Differentiated PC12 cells were deprived of serum and NGF for 4 days. NL4 and NL4-10K were added at the time of serum and NGF withdrawal and 10 ng/ml NGF was used as a positive control. Cell survival was estimated using a MTT assay. Cell survival mediated by the peptides was expressed as a percentage of maximal NGF promoted survival (mean ± SE, n=6).
3.2.2 NL4-10K Binds to DNA and Mediates *In Vitro* Gene Transfer

To examine the possibility of using NL4-10K as a gene delivery vector, we first tested whether the peptide could bind to plasmid DNA and neutralize its negative charges in a DNA retardation assay. Figure 3.12 showed that the mobility of plasmid DNA was reduced with increasing peptide concentrations. Between the N/P ratios of 1 and 5, the complex became immobile, indicating complete charge neutralization of DNA. In contrast, NL4, which does not contain a DNA-binding domain, did not retard DNA migration.

The physical properties of the complexes were also measured. At N/P ratio of 5, the particle size of 10K/DNA polyplexes was 175.0 ± 5.12 nm (n=3), while the size for NL4-10K/DNA complexes only slightly increased to 232.7 ± 4.97 nm (n=3), as expected due to the addition of NL4 domain. Both particles were stable over the 160 min measurement time. Polydispersity of the particles was typically in the range 0.1 - 0.3, indicating a rather narrow size distribution and hence suggesting minimal aggregation in both samples. The zeta potentials of both 10K/DNA and NL4-10K/DNA complexes were in the same positive range around 20 mV.

The complexes formed at different peptide/DNA ratios by self-assembly of NL4-10K and reporter plasmid pCAGluc were then tested in PC12 cells for gene expression (Figure 3.13). Pilot tests confirmed the necessity of using the endosome-disrupting agent chloroquine in NL4-10K-mediated gene expression. In the presence of chloroquine, complexes formed by 0.3 nmol of
NL4-10K with 1 µg of pCAGluc (N/P ratio of 1) resulted in modest gene delivery, but the transfection efficiency increased markedly with an increasing amount of NL4-10K. Neither NL4 (targeting domain only) nor 10K (DNA-binding domain only) nor a mixture of the two exhibited this dose-responsive trend. With 1.5 nmol of NL4-10K per µg of DNA (N/P ratio of 5), transfection was 110 times more efficient than that achieved with a mixture of NL4 and 10K peptides (Figure 3.13).
Figure 3.12 DNA retardation by NL4-10K in agarose gel under electrophoresis. 0.1 µg of plasmid DNA was complexed with different amount of peptide at the indicated ratios. The complexes were electrophoresed in 0.8% agarose gel at 80 v for 30 min.
Figure 3.13 Efficiency of NL4-10K-mediated gene delivery in vitro. NL4-10K was used to deliver pCAGluc plasmid DNA to PC12 cells. Short peptides, NL4, 10K, and a mixture of NL4 and 10K peptides (NL4 & 10K) were used as controls. Cells were transfected with complexes containing 1 \( \mu \)g of pCAGluc/well in 24-well plates in the presence of 100 \( \mu \)M chloroquine. Luciferase expression was assayed 24 hr after the transfection. Results are expressed as the mean of RLU/mg protein ± SE (n=4). **P<0.01 compared to controls.
3.2.3 NL4-10K Mediates Gene Delivery through TrkA

To clarify whether that NL4-10K-mediated gene delivery was targeted to TrkA, several types of cells with different expression profiles of NGF receptors were used. These cells were first assayed for their neurotrophin receptor expression by flow cytometric analysis, using the antibodies from Santa Cruz that has been shown to be able to stain Trk receptors in the rat cortex (Miller and Pitts, 2000). In NIH3T3 cells, cortex glial cells and primary cerebellar granule neurons that are known to be TrkA-negative or TrkA-poor, the percentage of TrkA-positive cells was very low or close to zero. In TrkA-transfected NIH3T3.E25 and NIH3T3.TRK1 cells, approximately 75% and 67% of the cells were TrkA-expressing respectively (Figure 3.14A), similar to the finding from the previous analysis (Bulseco et al., 2001). Interestingly, about 87% of PC12 cells and 48% of primary cortical neurons were TrkA-positive (Figure 3.14A). Figure 3.14B showed TrkB- and TrkC-expressing cortical neurons were approximately 24% and 16% respectively, and the corresponding cerebellar granule neurons were around 30% and 24% respectively.
Figure 3.14 Flow cytometric analysis of Trk receptors in various cell lines and primary cultured cells. (A) Histogram summarizing the percentage of cells expressing TrkA receptor (mean ± SE, n=3). Cells were incubated with rabbit anti-TrkA, followed by staining with sheep anti-rabbit IgG-FITC. Typical flow cytometry results are shown under the histogram. (B) Histogram summarizing the percentage of primary cortical neurons and cerebellar granule neurons expressing TrkA, TrkB or TrkC (mean ± SE, n=3). Cells were incubated with rabbit anti-TrkA or anti-TrkB or anti-TrkC, followed by staining with sheep anti-rabbit IgG-FITC. Figures under the histogram are typical results from flow cytometric analysis.
The specificity of NL4-10K-mediated gene delivery through NGF receptors was assessed via a competitive inhibition assay using excess NGF, NL4, or NL4-10K to compete with NL4-10K/DNA complexes for access to NGF receptors. Treatment by NGF would also produce a rapid clearing of TrkA, up to 70%, from the PC12 cell surface (Jullien et al., 2002). As shown in Figure 3.15A, the presence of NGF, NL4 or NL4-10K during transfection reduced the efficiency of gene delivery by 74-90%. The presence of excess 10K peptide, however, did not have a significant effect. These findings indicate that the NL4-10K-mediated gene delivery into PC12 cells was dependent on those sites that can be recognized and bound by NGF.

Since PC12 cells also express p75NTR receptors that could bind NGF, another competitive assay was carried out using p75NTR-blocking antibody (Advanced Targeting Systems, San Diego, CA, USA) (Curtis et al., 1995) during NL4-10K-mediated transfection to investigate the possible involvement of p75NTR. This antibody, as well as its control IgG, did not affect NL4-10K-mediated gene expression significantly (Figure 3.15A), suggesting that the gene delivery through p75NTR was less likely. This is further supported by gene transfer experiments in two TrkA-expressing, p75NTR-negative NIH3T3 cell lines, E25 and TRK1 (Cordon-Cardo et al., 1991; Klein et al., 1991a; Decker, 1995). NL4-10K-mediated reporter gene expression in E25 and TRK1 was 25-fold and 133-fold higher, respectively, than that in the parental NIH3T3 cells (Figure 3.15B). The finding provided solid evidence that
NL4-10K could mediate gene delivery through TrkA without involvement of p75\textsuperscript{NTR}.

To investigate the potential ability of NL4-10K to target gene delivery to specific subpopulations of neurons expressing TrkA, but not those expressing TrkB or TrkC, primary cortical neurons, cerebellar granule neurons and glial cells were used. The expression of TrkA and other Trk receptors in primary cultures of cortical neurons was documented in previous studies (Miranda et al., 1996; Seabold et al., 1998; Miller and Pitts, 2000; Dubus et al., 2000; Lee et al., 2000) and confirmed by flow cytometric analysis (Figure 3.14), which further provided the proportion of three Trk receptors in primary cortical neurons. In glial cells, the presence of TrkA is still a matter of contention and, if present at all, TrkA is expressed in a small percentage of glial cells and at low levels (Sofroniew et al., 2001). Primary cerebellar granule neurons are known to express significant levels of TrkB and TrkC, but not TrkA (Nonomura et al., 1996), which was also shown in the present flow cytometric analysis (Figure 3.14B). Complexes formed at the peptide/DNA (nmol/µg) ratio of 0.75 (N/P ratio of 2.5) by self-assembly of NL4-10K, or its control peptide 10K, and reporter plasmid pCAGluc were used together with 100 µM chloroquine in transfection. Figure 3.15C shows that NL4-10K/pCAGluc complexes were 22-fold more efficient than the non-targeted control using 10K in gene transfer to primary cortical neurons. In cerebellar granule neurons, however, the difference between the transfection efficiencies of NL4-10K and 10K was not significant (Figure 3.15C). In primary glial cells,
both NL4-10K and 10K were inefficient in gene delivery (Figure 3.15C). Immunostaining of transfected primary cortical neurons was also performed using antibodies against TrkA, TrkB or TrkC together with an antibody against luciferase to further examine the TrkA-specificity. The double staining disclosed a high degree of co-localization of luciferase with TrkA-positive cells, but poor co-localization for the reporter gene product with TrkB or TrkC (Figure 3.16).
Figure 3.15 Specificity of NL4-10K-mediated gene delivery. (A) Competitive inhibition of NL4-10K-mediated gene delivery into PC12 by NGF. PC12 cells were transfected, in the presence of 100 µM chloroquine, with NL4-10K/pCAGluc complexes prepared with a peptide/DNA (nmol/µg) of 1.5 (N/P ratio of 5), with or without co-incubation with free NGF, NL4, 10K, NL4-10K or anti-p75<sup>NTR</sup> antibody. Luciferase activity is expressed as mean of RLU/mg protein ± SE (n=4). *P<0.05 and **P<0.01 compared to transfection without additives. (B) NL4-10K-mediated gene delivery in TrkA-expressing NIH3T3 cell lines and parental NIH3T3 cells. Two TrkA-expressing cell lines, NIH3T3.E25, NIH3T3.TRK1 and parental NIH3T3 cells were transfected as described in (A). Luciferase expressions were compared (n=4). **P<0.01 compared to transfection in parental cell line NIH3T3. (C) NL4-10K mediates gene delivery into primary cortical neurons, primary cerebellar granule neurons and glial cells. Primary cortical neurons and glial cells from the rat cortex and primary cerebellar granule neurons were transfected with complexes containing 1 µg of pCAGluc/well in a 24-well plate in the presence of 100 µM chloroquine. After 4 hr, an equal volume of normal culture medium was added and incubated for 24 hr before luciferase expression assay. **P<0.01 compared to the 10K controls (n=4).
Figure 3.16 Co-localization of Trk receptors and luciferase immunoreactivity in primary cortical neurons after transfection with NL4-10K/pCAGluc complexes. Cells were grown on coverslips. Rabbit anti-TrkA or anti-TrkB or anti-TrkC together with goat anti-luciferase were used for staining after transfection, followed by staining with donkey anti-goat IgG-FITC and donkey anti-rabbit IgG-Cy3. The mounted coverslips were observed and photographed under confocal microscope. Luciferase immunoreactivity was shown in green, Trk receptor in red, and double stained cells in yellow.
3.2.4 NL4-10K-containing Complexes Mediate Gene Delivery to DRG

In Vivo

As chloroquine was not suitable for in vivo application, this study introduced PEI600, a cationic polymer with endosome-disrupting ability and low toxicity, into NL4-10K/DNA complexes and tested gene transfer of the ternary complexes in the absence of chloroquine. PEI600 was mixed with DNA and NL4-10K was then added to form PEI600/DNA/NL4-10K ternary complexes. Figure 3.17A showed that the gene transfer efficiency by PEI600 alone in PC12 cells was low at all tested N/P ratios and the addition of 10K peptide into the PEI600/DNA complexes did not improve the efficiency. However, while NL4-10K was introduced into the PEI600/DNA complexes formed at N/P ratios of 5 and 10, gene expression increased with a dose-response manner, with the highest reading at ~2 $\times 10^5$. When the N/P ratio of PEI600/DNA reached 20, the addition of NL4-10K peptide failed to offer further improvement.

A large portion of dorsal root ganglion (DRG) cells expressed TrkA receptors (Ruit et al., 1990) and intrathecally applied neurotrophins may penetrate into the DRG (Jones et al., 1999). To test the NL4-10K-mediated gene delivery in vivo, rats were given PEI600/pCAGluc/ NL4-10K complexes via intralumbar injection. In the spinal cord near the lumbar injection site, transfection mediated by NL4-10K was lower relative to that offered by the 10K control. In contrast, luciferase gene expression in the DRG resulting from PEI600/pCAGluc/NL4-10K was twice as high as that mediated by
PEI600/pCAGluc/10K (Figure 3.17B), suggesting a targeting effect of NL4-10K in the region with TrkA.
Figure 3.17 Gene delivery mediated by NL4-10K-containing complexes. (A) NL4-10K-mediated gene delivery without chloroquine in PC12 cells. To prepare PEI600/DNA/NL4-10K, pCAGluc was first complexed with PEI600 at a N/P ratio indicated and peptides were added afterward at N/P ratios of...
2.5 or 5. PC12 cells were transfected with complexes containing 1 µg of pCAGluc/well in a 24-well plate. After 4 hr, an equal volume of normal culture medium was added and the cells were incubated for 24 hr before luciferase expression assay. *P<0.05 or **P<0.01 compared to the controls without peptides (n=6). (B) NL4-10K-mediated in vivo gene delivery into dorsal root ganglia (DRG). PEI600/DNA/NL4-10K complexes were formed as described above, at a peptide/DNA (nmol/µg) ratio of 1.5 (N/P ratio of 5). Complexes were administered intrathecally to rats under anesthesia. Lumbar spinal cord (SC) and DRG were collected 3 days after injection. Results are expressed in RLU/mg protein ± SE (n=4). *P<0.05 compared to the 10K control.
3.2.5 Biocompatibility of PEI600/DNA/NL4-10K Ternary Complexes

Because of the introduction of cationic polymer PEI600 that disrupts endosomal membranes into the complexes and also because of the high positively charged 10K domain, \textit{in vitro} cytotoxicity of PEI600/DNA/NL4-10K complexes was tested by MTT assay in primary cortical neurons. PEI600 was mixed with DNA at N/P ratio of 10, NL4-10K was then added to form PEI600/DNA/NL4-10K complexes at the indicated total N/P ratio. Results showed the lack of toxicity of the complexes at the tested N/P ratios (Figure 3.18A). As a positive control, PEI25kDa/DNA complexes showed apparent toxicity to primary cortical neurons (Figure 3.18A). Tissue compatibility of PEI600/DNA/NL4-10K complexes was assayed by using microglial activation marker OX42 to detect inflammatory responses that could be triggered directly by a gene delivery vector or indirectly by cell/tissue injury (Popovich et al., 1997). The complexes showed no toxic effects in the spinal cord region close to the injection site, while the positive control PEI25kDa/DNA complexes triggered a clear inflammatory response, as assessed by proliferation of OX42-stained microglial cells (Figure 3.18B).
Figure 3.18 Biocompatibility of NL4-10K-containing complexes. (A) Cytotoxicity of NL4-10K-containing complexes in primary cortical neurons. PEI600 was mixed with DNA at N/P ratio of 10, and NL4-10K was then added to form PEI600/DNA/NL4-10K complexes at the indicated total N/P ratio. PEI25kDa/DNA complexes formed by mixing PEI25kDa with plasmid DNA at the indicated ratios were used as a positive control. Complexes were added at 0.1 µg DNA/well of 96-well plate. Cell survival was estimated using a MTT assay and expressed as a percentage of control, the untreated sister cultures. (B) Immunoreactivity of OX42 in the spinal cord after intrathecal injection of NL4-10K-containing complexes. PEI600 was mixed with DNA at N/P ratio of 10, and NL4-10K was then added at N/P ratio of 5 to form PEI600/DNA/NL4-10K complexes. PEI25kDa/DNA complexes formed with N/P ratio of 15 was used as a positive control. Three µg DNA/60 µl of complexes were injected intrathecally. Samples were collected 3 days after injection and the tissue sections were stained with anti-OX42, followed by immunoperoxidase histochemistry.
CHAPTER 4
DISCUSSION
Cell-specificity is one of the main objects for the development of gene delivery system. This property is highly desired for the effective application of gene therapy, especially in the nervous system. In this study, two NGF loop 4-containing chimeric polypeptides produced by either biological or chemical methods were used to prove the concept of targeted gene transfer to cells expressing NGF receptor TrkA.

4.1 Studies Using Bacterially Produced Polypeptides

In the first part of this study, to prove the concept of using NGF mimetic as targeting ligand for gene delivery, a recombinant polypeptide comprising a NGF loop 4-containing hairpin motif and a decalysine sequence was expressed in *E. coli* together with a DsbC fusion portion. This polypeptide DsbC-NL4-10K was able to direct gene transfer to NGF-receptor expressing cells. The binding of the polypeptide to NGF receptor was demonstrated by its ability to activate TrkA receptor and its downstream signaling pathways and to promote the survival of differentiated PC12. Specificity of the polypeptide-mediated gene transfer was evident by its efficiency in NGF receptor expressing cells, but not in receptor-negative cells and the inhibition of transgene expression by pre-treatment of free polypeptides.

4.1.1 The design and production of polypeptides

The design of the polypeptide was based on the current knowledge of NGF structure and its interaction with NGF receptors. Various approaches, from site-directed mutagenesis, deletion, chimeric molecule construction to
crystal structure analysis, have been used to study structural determinants of NGF effects. These studies have confirmed the amino acid residues that are important for receptor binding are localized in N-terminal region (#1-8), variable region II (#40-49) and variable region V (#96-97) (The variable region V corresponds to loop 4 in (Wiesmann et al., 1999; Wiesmann and de Vos, 2001)) (Ibanez, 1995). These studies raise the possibility of designing small peptide units that biochemically and biologically mimic the large and complex native NGF. It has been reported that small peptide mimetics of NGF activate TrkA-related signal transduction and promote NGF-like neurotrophic effects (LeSauteur et al., 1995; Beglova et al., 1998; Xie et al., 2000; Beglova et al., 2000; Maliartchouk et al., 2000). These peptide mimetics need to be cyclic in order to be biologically active; they must not only be sequence analogs but also structural analogs of NGF loops.

In the first part of the study, a 29-amino acid long hairpin motif from NGF was employed as targeting ligand. This loop 4-containing motif includes sequence from C80 to C108 [The numbering of amino acids refers to (Wiesmann et al., 1999; Wiesmann and de Vos, 2001)]. In addition to the four amino acid residues of loop 4, this structure includes as well parts of the C and D β strands of NGF that may stabilize the natural conformation of the loop through the formation of hydrogen bonds between two β strands. The three-dimensional structure is further stabilized by the disulfide bond formed between C80 and C108 after oxidation. Different from a full-length native NGF with six residues of cysteine to form three disulfide bonds, the segment of
NGF used in the present study contains only two residues of cysteine. Thus, mismatched disulfide bond linkage, a problem often seen in the production of recombinant native polypeptide, is avoided and unfolding/refolding of recombinant polypeptide to form correct disulfide bonds would be unnecessary, making this polypeptide preparation relatively easier. By using a hairpin motif instead of a simple loop artificially cyclized at two ends, it is expected that the loop conformation and the flexibility required for induced-fit ligand recognition by NGF receptors will be better conserved. Previous studies have suggested that a short positively charged lysine tail was enough for DNA binding (Gottschalk et al., 1996). Here, a 10-lysine sequence was introduced at the C-terminus of the hairpin motif to form the chimeric polypeptide-based nonviral vector. The sequence was expressed together with a DsbC protein, an *E. coli* disulfide bond isomerase to enhance the protein stability, solubility and folding. While used as a DNA vector, this DsbC fusion protein on one hand could potentially protect DNA from enzyme attack owing to the shielding effect provided by the bulky domain; on the other hand, it might also hinder the binding and condensation of DNA. A short synthetic peptide might avoid the problem caused by a bulky structure, however, a recombinant polypeptide approach such as the one used in this study offers opportunities to produce a single polypeptide chain based-vectors by incorporating multiple functional domains to overcome the barriers at different stages of gene transfer, from cellular internalization, endosomal escape, cytoplasmic transport to nuclear translocation.
4.1.2 The polypeptide-containing complexes

To study the targeting ability of the recombinant polypeptide for \textit{in vitro} gene transfer, protamine, a small arginine-rich peptide, as well as low MW PEI600 were used to form DsbC-NL4-10K-containing complexes. Similar systems have been used in studies using protamine (Medina-Kauwe et al., 2001a; Medina-Kauwe et al., 2001b). Here PEI600 was introduced due to its low toxicity and moderate endosome-disrupting and DNA-binding ability (Ahn et al., 2002). With these complexes, other endosome-disrupting agent like chloroquine, which might be unsuitable for \textit{in vivo} study was obviated. As observed in this study with these ternary complex systems, a low polypeptide/DNA ratio was required to enhance polycation-mediated gene delivery in PC12; at higher ratio, excess and/or unbound polypeptides might give rise to competitive inhibition by saturating the binding sites on the cell surface. Therefore, the intrinsic competition between the polypeptide and the polycation for DNA binding in these ternary systems requires the optimization of polypeptide/polycation/DNA ratio. With high polycation/DNA ratio, the targeting polypeptide could be expelled and vise versa. One possible solution to this issue is to include a functional domain with endosome-escaping mechanism in the same recombinant polypeptide molecule. This endosome-disrupting sequence could be N-terminal amphiphilic peptide of influenza virus HA2 or synthetic amphipathic peptides like GALA or KALA (Cho et al., 2003).
In brief, this preliminary research demonstrated the concept of using a loop 4-containing hairpin motif from NGF for targeted gene delivery with bacterially produced recombinant polypeptide. This motif has shown its biochemical and biological functions by activating the TrkA-related signal transduction pathways and preventing the cell death of differentiated PC12. While incorporating a DNA-binding sequence of 10 lysines, the motif may also direct the gene delivery to NGF receptor-expressing cell. Since this polypeptide was expressed as a DsbC fusion protein, the bulky fusion domain may affect both the binding of NGF loop 4-containing hairpin motif to its receptor and the binding of the 10-lysine sequence to the DNA. To overcome these issues, a chemically synthesized peptide simply composed of the targeting moiety derived from NGF loop 4 and the DNA-binding moiety of 10 lysine residues was employed for more systemic study.

4.2 Studies Using Chemically Synthesized Peptides

The second part of this study further showed that a chemically synthesized peptide containing the hairpin motif of NGF loop 4 linked to a nucleic acid binding domain was capable of directing gene delivery to TrkA receptors. Several lines of evidence support this conclusion: (1) The peptide, even after binding to DNA, possessed NGF-like bioactivities in TrkA-positive PC12 cells; (2) The peptide dose-dependently directed gene delivery to PC12 cells; (3) Gene transfer to PC12 cells was inhibited by co-incubation of excess amounts of NGF or free NGF loop 4 containing peptides, but not by a p75NTR receptor blocking antibody; (4) The peptide significantly enhanced gene
expression in p75NTR-negative NIH3T3 cells heterologously transfected with TrkA cDNA; (5) The peptide did not mediated gene expression in primary cerebellar granule neurons, as well as cortex glial cells, that are TrkB- and/or TrkC-positive, but TrkA-negative; (6) The peptide would have to contain both TrkA-targeting and DNA-binding domains in order to be functional in gene delivery; (7) The peptide-mediated gene expression required the use of endosome-disrupting agents, being consistent with the understanding that NGF-TrkA binding triggers endocytosis. Thus, this may be the first report using NGF loop fragments and TrkA receptors in targeted gene delivery to neurons.

4.2.1 The design of targeting ligand

The design of the peptide vector was inspired by the successful development of small NGF mimetics (LeSauteur et al., 1995; Longo et al., 1997; Beglova et al., 1998; Xie et al., 2000; Beglova et al., 2000; Maliartchouk et al., 2000) and based on the molecular basis of NGF-receptor interaction. NGF loop 4 is a hydrophilic hairpin loop exposed on NGF surface, serving as one of the two primary NGF loop domains interacting with TrkA receptors (Drinkwater et al., 1993; Kullander et al., 1997). Using the sequence of this region, small molecule ligands of TrkA with less than 10 residues of amino acids had been successfully generated (LeSauteur et al., 1995; Beglova et al., 1998; Xie et al., 2000; Beglova et al., 2000). These small NGF mimetics have cyclized structures, being both sequence and structure analogs to the NGF loop 4. In the present study, a 29-amino acid peptide (NL4) derived from
amino acid #80-108 of NGF was synthesized and cyclized with a disulfide bond formed between C80 and C108 for TrkA targeting. In addition to loop 4 sequence, the peptide includes as well parts of the C and D β-strands of NGF, in an attempt to use natural β-strand structures to stabilize the native conformation of the loop 4. Such a hairpin motif structure may also provide flexibility required for induced-fit ligand recognition of receptor. To construct a DNA vector, a 10-lysine sequence was introduced to the C-terminus of NL4 to form NL4-10K. While the 10K part binds DNA, β-strands in the chimeric peptide may function as spacers between NGF loop 4 and the 10K/DNA complex, allowing independent action of the targeting domain and the DNA-binding domain. Furthermore, using a fragment of NGF polypeptide containing a hairpin motif, instead of the full-length polypeptide chain, susceptibility to proteolytic degradation and immunogenicity of the polypeptide during in vivo application would be reduced.

4.2.2 The binding of ligand to TrkA

Consistent with the notion that the NGF loop 4 is one of the key sites interacting with TrkA (LeSauteur et al., 1995; Beglova et al., 1998; Xie et al., 2000; Beglova et al., 2000), the peptides NL4 and NL4-10K developed in the study activated TrkA and its downstream signaling components, Erk and Akt. The inhibition of NL4-mediated Erk activation by TrkA kinase inhibitors K252a and AG879 indicated that the action was most likely through TrkA receptor itself. NL4 and NL4-10K peptides also promoted the survival of differentiated PC12 deprived of serum and NGF, providing another evidence that the
peptides bind to and act via TrkA. Moreover, NL4-10K showed similar activity as NL4 peptide even when it was complexed with DNA, a property critical to its use as a gene delivery vector. It is noticeable that without DsbC fusion portion that may produce spatial hindrance and/or affect loop 4 conformation, the chemically synthesized peptides show higher efficacy in TrkA-binding as evidenced by their biochemical and biological functions, comparing to their recombinant counterparts.

4.2.3 The selectivity in peptide-mediated gene delivery

A challenging issue of this study is to establish the TrkA selectivity of NL4-10K peptide in gene delivery process. Preliminary data collected support the notion that the uptake of NL4-10K/DNA complexes is mediated by TrkA, but not p75NTR, TrkB or TrkC. Several previous studies in PC12 cells have confirmed that p75NTR does not take part in the actual physical internalization of NGF (Eveleth and Bradshaw, 1992; Kahle et al., 1994; Gargano et al., 1997; Jullien et al., 2002). It has been confirmed as well in Chinese hamster ovary fibroblasts over-expressing TrkA that TrkA, in the absence of p75NTR, is fully capable of mediating NGF endocytosis (Zapf-Colby and Olefsky, 1998). Consistently, in the present experiments a p75NTR receptor blocking antibody failed to inhibit NL4-10K-mediated gene transfer in PC12 cells that express both low affinity p75NTR and high affinity TrkA receptors and the gene transfer took place efficiently in two stably transfected NIH3T3 cell lines expressing only TrkA. Structurally, several residues in NGF loop 4 are also of importance in binding to p75NTR, as demonstrated by site-directed mutagenesis studies.
These residues however need to function together with NGF loop 1 (residues 29-35) for binding to p75NTR (Longo et al., 1990; LeSauteur et al., 1995; Shamovsky et al., 1999). As for TrkB and TrkC, the low efficiency of NL4-10K peptide in the transfection experiments using primary glial cells and cerebellar granule neurons, which mainly express TrkB and/or TrkC, but not TrkA (Condorelli et al., 1995; Nonomura et al., 1996), does not support the possibility that these two receptors had participated in NL4-10K-mediated gene transfer.

Ligand-induced dimerization of cell surface receptors has been regarded as a general mechanism for the initiation of downstream processes, including endocytosis of ligand/receptor complexes (Sofroniew et al., 2001), although extensive evidence has emerged to challenge this dogma (Mischel et al., 2002; Pattarawarapan and Burgess, 2003). Dimeric native NGF may induce TrkA dimerization, initiating NGF signal transduction. Monomeric and monovalent ligands of TrkA have been shown to function as partial agonists, acting allosterically through the receptor rather by inducing receptor dimerization (Maliartchouk et al., 2000). In general, peptides used in this study are a monomeric ligand and their bioactivities may be the result of conformational changes of TrkA receptors. With the introduction of 10-lysine sequence into the peptide, these positively charged basic amino acid residues could bind to the weakly negatively charged cell surface, creating locally concentrated peptides that offer the opportunity for receptor dimerization. After forming complexes with DNA, more than one peptide
would be available on the surface of each complex. Theoretically, in NL4-10K/pCAGluc complexes formed at N/P ratio of 1, there will be ~1400 NL4-10K molecules available for binding to each plasmid DNA molecule; at higher N/P ratio, the number becomes even larger. It is possible that neighboring pairs of tethered peptides act as dimers to facilitate receptor dimerization and subsequent receptor-mediated endocytosis of the complexes. Further structural analysis of receptor-ligand complexes is required to decipher the mechanisms involved.

4.2.4 The peptide-containing complexes

A typical vector for receptor-mediated gene transfer is composed of a cationic polymer of synthetic origin chemically conjugated with a targeting ligand. This approach requires chemical reactions that could be harsh enough to inactivate sensitive targeting ligands. In the case of using proteins that are difficult to be purified or synthesized as targeting ligands, the relative large amounts of material required for chemical conjugation could be prohibitively expensive, limiting the development and testing of such gene delivery systems and the scale-up necessary for gene therapy. The peptide developed in this study contains a lysine-based DNA-binding domain, necessitating the use of an endosomolytic agent, such as chloroquine. For *in vivo* gene delivery, the peptide could be used together with those cationic polymers that may promote endosomal escape, e.g. PEI, to form DNA complexes through charge interaction between the macromolecules. By preparing a gene delivery system with this simple self-assembly method, one would avoid the
use of harsh and reagent-wasteful chemical reactions and expect to see better-conserved biological activities of peptide-based targeting ligands. The including of cationic polymers, like PEI, in such a ternary system also reduces the amount of peptide needed to condense DNA.

In this study, low MW, branched PEI600 was used to form NL4-10K-containing complex. PEI600 has repeated basic units with a backbone of two carbons followed by a nitrogen atom and contains primary, secondary and tertiary amino groups, each of which has the potential to be protonated, therefore disrupting endosome membrane (Ahn et al., 2002). This polymer was picked up in this study mainly due to its low immune stimulating effect and low cytotoxicity. Although it binds to and condenses DNA, the polymer itself mediates very low level of gene transfection at low polymer/DNA ratios, thus acting as one component of the ternary complex without pronounced effects on the specificity of gene transfer. With the increase of PEI600 in ternary complexes, however, the gene delivery may become nonspecific, probably due to the excess positive charge on the particle surface and the domination of electrostatic interaction.

4.2.5 The use of oligolysine as DNA carrier

In this research, to prove the specificity of this newly developed targeting ligand in gene delivery is the primary objective. Therefore, a simple design consisting of a targeting ligand and a simple DNA-binding sequence of 10 lysines was chosen. This bi-functional peptide was chemically synthesized, which may avoid the chemical conjugation process and thus obviate the
random conjugation and polydispersity of polymer. The use of oligolysine may also avoid the possible nonspecific electrostatic interaction due to the high-density positive charge of cationic polymers, which could mask the targeting effect via ligand-receptor interaction. However, for efficient intracellular trafficking, endosomolytic agents like chloroquine are still required to increase the efficiency of this peptide. To improve this targeted gene delivery system, the incorporation of other DNA carriers could be one possible direction in the future. Gene transfer agents like PEI that have intrinsic endosomolytic property may also eliminate the use of chloroquine and make the system more suitable for \textit{in vivo} application.

\textbf{4.2.6 The possible intrinsic gene delivery at high +/- charge ratio}

An important concern for the research of targeted gene delivery systems is the possible intrinsic gene delivery for cationic DNA carrier used at high +/- charge ratio. To prove the targeting effect of a novel ligand in a gene delivery system, it will be ideal to conjugate the ligand to a DNA carrier that would not produce any nonspecific attachment to the target cell surface. However, the current existing DNA carriers, most of which depend on the electrostatic action of cationic polymer and anionic DNA, have potential intrinsic binding ability to some cell surface molecules like glycosaminoglycans, especially while the complexes are produced at high +/- charge ratio (Mislick and Baldeschwieler, 1996). To minimize the intrinsic effects of the DNA carrier, hydrophilic polymers like polyethylene glycol (PEG) have been employed to mask the DNA carrier complexes in hoping for a charge shielding effect.
However, this strategy may also impair the targeting ability of the conjugated ligand (Kunath et al., 2003). At present, to differentiate between the intrinsic targeting effect provided by the DNA carrier itself and the extrinsic targeting effect provided by the targeting ligand remain a serious challenge in this field.

In the previous research of targeted gene delivery, oligolysine (such as a sequence of 16 lysine residues [K]_{16}) has been used as DNA-binding domain together with integrin-targeting domain (a RGD-containing sequence with 11 amino acids in length) in the synthetic peptides known as RGD[K]_{16} and [K]_{16}RGD (Hart et al., 1995; Harbottle et al., 1998). In these studies, the optimized +/- charge ratio for in vitro transfection was ~4.28. At this high +/- charge ratio, the control peptide [K]_{16} produced no significant transgene expression. Combined with the evidence from competitive inhibition assay, the authors claimed the specificity of gene delivery by their integrin-targeting peptides. In one related research (Hart et al., 1997), the optimized +/- charge ratio was even as high as 6:1.

The role of electrostatic binding in complexes with high +/- charge ratio did raise the concern. In one study (Collins et al., 2000) using a synthetic 31 amino acid DNA vector (polylysine-molossin) comprising a 15-amino acid integrin-targeting sequence and [K]_{16}, the authors have shown that at +/- charge ratio of 4/1, although the [K]_{16}/DNA and polylysine-molossin/DNA complexes both may bind to target cell surface, however, only polylysine-molossin/DNA complexes resulted in efficient transgene expression, whereas [K]_{16}/DNA complexes were completely ineffective. Therefore, for targeted
vector systems using oligolysine as DNA binding domain, it is possible that while used at high +/- charge ratio, the attachment of vector/DNA complexes to the cell surface may involve both ligand-receptor interaction and electrostatic interaction. The nonspecific electrostatic interaction may not be essential, especially while the affinity of ligand to receptor and the density of the targeted cell surface receptor are high. On the other hand, the inefficient transgene expression mediated by [K]16/DNA alone suggests that cell surface attachment is not enough for gene delivery, while the internalization of complexes via binding to the targeted receptor may be important for transgene expression. With this receptor-dependent internalization process, the targeted vectors may also confer transfection specificity.

In this study, a chemically synthesized 39-amino acid peptide (NL4-10K) was used for targeted gene transfer. There are some potential advantages in this design for the purpose of reducing possible of electrostatic effect in peptide/DNA complexes. Previous studies have shown that 8-10 lysines is adequate for DNA binding (Gottschalk et al., 1996; Medina-Kauwe et al., 2001a; Medina-Kauwe et al., 2001b). A DNA-binding domain of 10 lysine sequence (10K) instead of [K]16 was used, hoping that with less lysines per molecule, the nonspecific electrostatic binding to the cell surface would reduce. A 29-amino acid sequence (NL4) was used for targeting. With a larger targeting domain and a smaller DNA binding sequence in the same molecules (29 amino acids vs. 10 amino acids), the targeting domain may
provide better spatial hindrance effect to shield the positive charge of lysines, thus, reduce the electrostatic binding of the complexes.

In this gene delivery study, optimized complexes of NL4-10K/DNA have been formed at +/- charge ratio of 5/1, which is similar to that used in the studies using $[\text{K}]_{16}$ considering the less positive charge density in NL4-10K. At this ratio, the 10 lysine sequence alone gave no transgene expression, whereas NL4-10K was two orders of magnitude more efficient, which at least suggests the specificity of NL4-10K-mediated tranfection. In the co-incubation study using same molar concentration of free NL4, NL4-10K and 10K (the control sequence of 10 lysines), the presence of 10K, which was supposed to compete with NL4-10K/DNA complexes for the negatively-charged binding sites on the cell surface, didn’t affect the NL4-10K/DNA-mediated transgene expression; however, the presence of NL4, which was supposed to compete with the complexes for ligand-binding sites or NL4-10K, which was supposed to compete with the complexes for both ligand-binding sites and the negatively-charged binding sites, significantly reduce the transgene expression. These results suggest that in the case of NL4-10K/DNA complexes, the charge interaction may be less important for the attachment of NL4-10K/DNA complexes to the cell surface, while the ligand-receptor interaction may be more important.

4.2.7 The competitive inhibition assay

Competitive inhibition with free ligands is one of frequently used methods for determining the specificity of targeted gene delivery. As mentioned above
in the targeted gene delivery study using RGD[K]₁₆ (Hart et al., 1995), in the presence of excess free RGD peptide, transgene expression was reduced by 70%. In another study using epidermal growth factor (EGF) as targeting ligand and poly-L-lysine as DNA-binding agent (Frederiksen et al., 2000), the presence of excess free EGF only reduced the transfection by ~34-64%. In the study that coupled the transferrin to PEI (Kircheis et al., 1997), the excess free transferrin reduced the transfection by ~80-86%. From these observations, it is likely that depending on the targeting ligands and the DNA-binding agents and also the targeted cells used, a targeted gene delivery system could have various extent of background transgene expression even in the presence of excess free ligands.

There are some possible explanations for the background transgene expression in the competitive inhibition assay. Theoretically, at high +/- charge ratio, there would be thousands of targeted vector bind to each plasmid DNA. This multivalent structure likely have a greater avidity for binding through ligand-receptor interaction than the free ligands, which make this kind of competitive inhibition assay a little bit different from the traditional one. At this high +/- charge ratio, portion of the complexes could also bind to the cell surface via electrostatic interaction, and then enter the cell via the subsequent receptor-mediated internalization. However, there is still no substantial evidence to support this theory. According to inhibition assay in this study, free NL4 peptide and NGF reduced the transfection by 74% and 82% respectively, whereas free NL4-10K peptide reduced the transfection
by 90%. Therefore, this only leaves 10-24% that are not inhibited by free ligands co-incubation, which is an acceptable result according to other similar research.

4.2.8 The possibility of receptor-mediated gene delivery using a targeted oligolysine-based system at high +/- charge ratio

To develop a targeted gene delivery system, the introduction of novel ligands is one important aspect of the research. To fulfill ligand-directed distribution of therapeutic gene to a target site, a DNA carrier system should be free from inherent properties that would bias bio-distribution. Unfortunately, the currently used cationic DNA carriers may produce non-specific interaction that would affect the contribution of the targeting ligands. Therefore, to study the targeting effect of a novel ligand, a DNA carrier system that has less intrinsic properties should be chosen. From this viewpoint, oligolysine is a better choice than other polymers like PEI that also have endosomolytic property.

To avoid the polydispersity of polyllysine, a short synthetic lysine sequence (10K) was used in this study simply for its function of DNA binding or condensing. This 10K sequence itself was poor in gene delivery to TrkA-expressing cells, while only the 10K sequence together with targeting ligand (NL4-10K) was efficient. Since both NL4-10K/DNA and 10K/DNA complexes have showed similarity in particle size and zeta potential, thus the difference in their gene delivery ability was unlikely to be explained by the difference in their physical properties. Also, masking the negative charge on cell surface by
free 10K did not reduce the NL4-10K-mediated gene delivery, while masking the TrkA receptor by free NL4-10K did, indicating the targeting effect of the NL4 domain at this high +/- charge ratio. Therefore, in this NL4-10K-mediated gene delivery, although there could be small portion of nonspecific interaction, receptor-specificity gene delivery has been predominant.

4.2.9 The application

High-level and cell-specific gene expression are required to effectively apply gene therapy approaches to treat many neurological disorders, including stroke, ischemia, epilepsy, head and spinal cord trauma, Parkinson’s diseases, Huntington’s disease, Alzheimer’s disease, amyotrophic lateral sclerosis, and neurogenetic disorders. The chimeric peptide reported here could potentially be useful in gene therapy of the disorders, in which neurons expressing TrkA are affected. In Alzheimer’s disease, the neurons with most prominent pathological changes are basal forebrain cholinergic neurons that express TrkA (Winkler et al., 1998). A gene delivery system that targets TrkA may help to transfer therapeutic genes into the neurons to augment cholinergic functions. Outside the nervous system, NGF and TrkA expression are detected in B lymphocytes, T lymphocytes, mast cells, monocytes and macrophages, suggesting a role for NGF in immune and inflammatory functions (Sofroniew et al., 2001). In addition, human tumor tissues up-regulate neurotrophin receptors and are responsive to NGF (Saragovi and Gehring, 2000). The peptide reported in this study is expected to offer potential benefits for gene delivery to these cells and
tissues. As the peptide alone activates TrkA receptor-related signal transduction and exerts NGF-like biological activities, it can also be used directly as therapeutic agents in the treatment of the aforementioned disorders.

In summary, the present study has demonstrated that an NGF loop 4-containing hairpin motif, both chemically synthesized and bacterially produced recombinant one, can function as a novel ligand for targeted gene delivery. The motif can bind to TrkA, mediate in vitro gene transfer via TrkA efficiently, and has shown potential targeting ability for in vivo gene delivery to neurons without toxicity. The present study provides, for the first time, an example for targeted gene delivery to a subtype of neurons and presents a platform to build more sophisticated TrkA-targeted vectors by incorporating other functional domains.
CHAPTER 5
REFERENCES


reovirus type 3 receptor is secondary to inactivation of p21ras and mitogen-activated protein kinase. DNA Cell Biol 18: 763-770.


APPENDIX A: AMINO ACID SEQUENCES

1. **Name**: human NGF

Sequence:
SSSHPIFHRG EFSVCDSVSV WVGDKTATAD IKGKEVMVLG EVNINNSVFK 50
QYFFETKCRD PNPVDSGCRG IDSKHWSNSY TTTHTFVKAL TMDGKQAAWR 100
FIRIDTACVC VLSRKAVRR

2. **Name**: DsbC-NL4

Sequence:
MKKGFMLFTL LAAFSGFAQA DDAAIQQTLa KMGIKSSDIQ PAPVAGKTV 50
LTNSGVLYIT DDGKHIIQGP MYDVSSTAPV NVTNKLMLKQ LNALEKEMIV 100
YKAQPEKHVI TVFTDIITCY CHKLHEQMAD YNALGITVRY LAFPRQGLDS 150
DAEKEKMAIWI CAKDRNKAFD DVMAGKSVP ASCDVLAH ALGQVLGVS 200
GTPA VLSNG TLVPQYQQPK EMKEF IDEHQQ KMTSGKSTS GSHHHHHHHS 250
AGLVPRGSC TTHTFVKALT MDGKQAAWRF IRIDTAC

Features:
- aa 1-236: DsbC
- aa 244-249: His_{6} tag
- aa 259-287: NL4

3. **Name**: DsbC-NL4-10K

Sequence:
MKKGFMLFTL LAAFSGFAQA DDAAIQQTLa KMGIKSSDIQ PAPVAGKTV 50
LTNSGVLYIT DDGKHIIQGP MYDVSSTAPV NVTNKLMLKQ LNALEKEMIV 100
YKAQPEKHVI TVFTDIITCY CHKLHEQMAD YNALGITVRY LAFPRQGLDS 150
DAEKEKMAIWI CAKDRNKAFD DVMAGKSVP ASCDVLAH ALGQVLGVS 200
GTPA VLSNG TLVPQYQQPK EMKEF IDEHQQ KMTSGKSTS GSHHHHHHHS 250
AGLVPRGSC TTHTFVKALT MDGKQAAWRF IRIDTACKKK KKKKK

Features:
- aa 1-236: DsbC
- aa 244-249: His_{6} tag
- aa 259-297: NL4-10K

4. **Name**: NL4

Sequence:
CTTTHTFVKA LTMDGKQAAW RFIRIDTAC

Features:
- aa 1-29 correspond to aa 80-108 of nerve growth factor (NGF) and include loop 4, a known receptor binding region
- C1 and C29 form a disulfide bridge

5. **Name**: NL4-10K

Sequence:
CTTTHTFVKA LTMDGKQAAW RFIRIDTACK KKKKKKKKK

Features:
- aa 1-29 correspond to aa 80-108 of nerve growth factor (NGF) and include loop 4, a known receptor binding region
- C1 and C29 form a disulfide bridge
- aa 30-39 form a nucleic acid binding domain

6. Name: 10K

Sequence:
KKKKKKKKKKK
APPENDIX B: NUCLEIC ACID SEQUENCES

1. NA SEQ 1
Description: Forward primer for NL4 and NL4-10K
Sequence:
5’-TGTACCACGA CTCACACC-3’

2. NA SEQ 2
Description: Reverse primer for NL4
Sequence:
5’-GCAAGCTTTTC AACAGCCGT ATCTATCCG-3’

3. NA SEQ 3
Description: Reverse primer for NL4-10K
Sequence:
5’-GCAAGCTTTTC ATTTTTTTTT TTTTTTTTT TTTTTTTTT TACAGCCGT ATCTATCCG-3’

4. NA SEQ 4
Description: Coding sequence for DsbC-NL4
Sequence:
```
atgaagaaag gttttatgtt gtttactttg ttacgcccgt tttcaggctt 50
tgctcaggct gatgacgcgg caattcaaca acacgttagcc aaatggcga 100
tcaaaagcag cgatattcag cccccggcctg tagctggcgc gcagacatgtt 150
tgctcaggct gatgacgcgg caattcaaca acacgttagcc aaatggcga 200
tcaggggccca atgtagcag ctggccggcc aagctgggct aatgggcga 250
ctatagctgt tttaagcag ttaaatgcgc gatgacgcgg caattccagcc 300
tttaaaagccg aacagcagcc aaccgtgtgta ttgcagcagc 350
tgctcagcag ccaggtaaac gcagacatgtt ttgcagcagc 400
tgagagcagc aagatacagc ccagacctac gcagacatgtt ttgcagcagc 450
tgctcaggct gatgacgcgg caattcaaca acacgttagcc aaatggcga 500
tcaggggccca atgtagcag ctggccggcc aagctgggct aatgggcga 550
tcaggggccca atgtagcag ctggccggcc aagctgggct aatgggcga 600
tgctcaggct gatgacgcgg caattcaaca acacgttagcc aaatggcga 650
tgcacgacag ccaggtaaac gcagacatgtt ttgcagcagc 700
tcaggggccca atgtagcag ctggccggcc aagctgggct aatgggcga 750
tgcacgacag ccaggtaaac gcagacatgtt ttgcagcagc 800
tgctcaggct gatgacgcgg caattcaaca acacgttagcc aaatggcga 850
```

5. NA SEQ 5
Description: Coding sequence for DsbC-NL4-10K
Sequence:
```
atgaagaaag gttttatgtt gtttactttg ttacgcccgt tttcaggctt 50
tgctcaggct gatgacgcgg caattcaaca acacgttagcc aaatggcga 100
tcaaaagcag cgatattcag cccccggcctg tagctggcgc gcagacatgtt 150
tgctcaggct gatgacgcgg caattcaaca acacgttagcc aaatggcga 200
tcaggggccca atgtagcag ctggccggcc aagctgggct aatgggcga 250
tcaggggccca atgtagcag ctggccggcc aagctgggct aatgggcga 300
tcaggggccca atgtagcag ctggccggcc aagctgggct aatgggcga 350
tcaggggccca atgtagcag ctggccggcc aagctgggct aatgggcga 400`
tgccgatcac cgtgcgttat cttgctttcc cgccgccaggg gctggacagc 450
gatgcagaga aagaaatgaa agctatctgg tgtgcgaaag ataaaaacaa 500
agcgttttgat gatgtgatgg caggttaaaag cgctgcacca gcctgcttgcg 550
acgtggatat tgccgaccat tacgcaacttg gcgtccagct tggcgttagc 600
gtacctccgg cagtttgctg gagcaatggc acactttttc cgggctaccc 650
gccgcccagaa gagatgaaag aatTTTctca gcaacatcca aaaatgacca 700
gcggtaaagg atcaactagt ggttctggtc atcaccatca ccactacctc 750
gccgggtctgg tgccacgcgg tagttgtacc acgactcaca ctttcttcaaa 800
ggccttgacc atggatggca agcaggtgct cttgcgtgtt atccggatatg 850
atatgcggcttg taaaaaaaaa aaaaaaaaaa aaaaaaaaaa