DNA CONденсATION AND PACKAGING FOR POTENTIAL APPLICATION IN GENE THERAPY

by

PAUL KABIRU

(Under the Direction of George Majetich)

ABSTRACT

The phenomenon of DNA condensation has been studied for decades, often as a model of DNA packaging within viruses and chromosomes, due to its medical importance as a key step in gene therapy. The approach of using oligopeptides and low molecular weight natural polyamines as DNA packaging systems has received a lot of interest due to the biochemical knowledge that in vivo, DNA is usually associated with peptides composed of 5-20 amino acids in viruses and polyamines such as spermine and spermidine in chromosomes that facilitate DNA condensation and packaging.

Amphiphilic polymers such as Poly(L-lysine)-b-Poly(ethylene glycol) and poly(ethylenimine)-alt-Poly(ethylene glycol) have also shown a lot of potential as DNA condensation and packaging systems.

In this study, utilization of natural polyamines, oligopeptides, and amphiphilic polymers in DNA condensation, packaging and their potential application in gene therapy is explored.

INDEX WORDS: Gene therapy, DNA condensation, DNA condensate morphologies, mechanism of DNA condensation, viral vectors, synthetic vectors, low molecular weight natural polyamines, oligopeptides, stabilization of oligopeptides/DNA condensates, amphiphilic polymers
DNA CONDENSATION AND PACKAGING FOR POTENTIAL APPLICATION IN GENE THERAPY

by

PAUL KABIRU
B.S., Kennesaw State University, 2003
M.S., Georgia Institute of Technology, 2006

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA
2009
ACKNOWLEDGEMENTS

I would like to express my gratitude and appreciation to Dr. George Majetich and Dr. John Stickney for their support, advice and guidance. I would also like to thank my committee members, Dr. Robert Phillips, Dr. Jeffrey Urbauer and Dr. Vladimir Popik for their advice, assistance and for serving on my Committee.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF SYMBOLS AND ABBREVIATIONS</td>
<td>ix</td>
</tr>
<tr>
<td><strong>CHAPTER</strong></td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Gene Therapy</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Brief History of Gene Therapy</td>
<td>4</td>
</tr>
<tr>
<td>1.3 DNA Condensation</td>
<td>5</td>
</tr>
<tr>
<td>1.4 DNA Condensate Morphologies</td>
<td>6</td>
</tr>
<tr>
<td>1.5 Mechanism of DNA Condensation</td>
<td>7</td>
</tr>
<tr>
<td>1.6 Viral Vectors</td>
<td>11</td>
</tr>
<tr>
<td>1.7 Synthetic Vectors</td>
<td>12</td>
</tr>
<tr>
<td>2 STRUCTURAL INFLUENCE OF LOW MOLECULAR WEIGHT NATURAL POLYAMINES AND OLIPOPEPTIDES ON DNA CONDENSATION</td>
<td>14</td>
</tr>
<tr>
<td>2.1 Driving Forces of DNA Condensation</td>
<td>14</td>
</tr>
<tr>
<td>2.2 Utilization of Low Molecular weight Natural Polyamines (Spermine and Spermidine) and Oligopeptides as DNA Condensing Agents</td>
<td>15</td>
</tr>
</tbody>
</table>
2.3 Effect of the Nature of the DNA Condensing Agents and Condensation Environment on DNA Condensation Capability and Size, Stability and Morphology of the DNA condensates

2.4 Stabilization of Oligopeptide/DNA Condensates

3 DNA CONDENSATION BY AMPHIPHILIC POLYMERS

3.1 Covalent Conjugation of Poly(ethylene glycol) onto Poly(L-lysine) (PLL) and Poly(ethylenimine) (PEI)

4 FUTURE STUDIES

REFERENCES
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Enzyme-prodrug therapy</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>Model for the overall process of DNA condensation in which both rods and toroids are formed</td>
<td>8</td>
</tr>
<tr>
<td>1.3</td>
<td>Transmission electron micrographs of DNA and hexamine cobalt chloride condensates at different times</td>
<td>9</td>
</tr>
<tr>
<td>1.4</td>
<td>Model for the condensation of DNA pre-associated with divalent cations</td>
<td>10</td>
</tr>
<tr>
<td>1.5</td>
<td>A model of the effects of architectural protens e.g IHF or HU on DNA condensation process</td>
<td>11</td>
</tr>
<tr>
<td>2.1</td>
<td>Schematic representation of DNA condensation by multivalent cations</td>
<td>15</td>
</tr>
<tr>
<td>2.2</td>
<td>Schematic representation of solid phase peptide synthesis (SPPS) of H₂N-Lys-Trp-Lys-Lys-Ac</td>
<td>17</td>
</tr>
<tr>
<td>2.3</td>
<td>Low molecular weight natural polyamines and amino acids</td>
<td>19</td>
</tr>
<tr>
<td>2.4</td>
<td>Potential hydrogen bonding sites in DNA base pairs</td>
<td>20</td>
</tr>
<tr>
<td>2.5</td>
<td>Transmission electron microscopy (TEM) images of spermidine- and spermine-induced DNA condensates produced in the presence and absence of IHF</td>
<td>23</td>
</tr>
<tr>
<td>2.6</td>
<td>Typical plots of the relative intensity of scattered light against concentrations of polylysine, Lysine₅ and Lysine₄</td>
<td>24</td>
</tr>
<tr>
<td>2.7</td>
<td>Formation of stable DNA/oligopeptide condensates</td>
<td>26</td>
</tr>
<tr>
<td>2.8</td>
<td>Model for interaction of amphiphilic peptides with DNA</td>
<td>27</td>
</tr>
<tr>
<td>Symbol</td>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>IHF</td>
<td>Integration host factor</td>
<td></td>
</tr>
<tr>
<td>HU</td>
<td>Histone-like protein from E.coli strain U93</td>
<td></td>
</tr>
<tr>
<td>Ala (A)</td>
<td>Alanine</td>
<td></td>
</tr>
<tr>
<td>Val (V)</td>
<td>Valine</td>
<td></td>
</tr>
<tr>
<td>Ile (I)</td>
<td>Isoleucine</td>
<td></td>
</tr>
<tr>
<td>Leu (L)</td>
<td>Leucine</td>
<td></td>
</tr>
<tr>
<td>Phe (F)</td>
<td>Phenylalanine</td>
<td></td>
</tr>
<tr>
<td>Pro (P)</td>
<td>Proline</td>
<td></td>
</tr>
<tr>
<td>Trp (W)</td>
<td>Tryptophan</td>
<td></td>
</tr>
<tr>
<td>Tyr (Y)</td>
<td>Tyrosine</td>
<td></td>
</tr>
<tr>
<td>Arg (R)</td>
<td>Arginine</td>
<td></td>
</tr>
<tr>
<td>Asn (N)</td>
<td>Asparagine</td>
<td></td>
</tr>
<tr>
<td>Gln (Q)</td>
<td>Glutamine</td>
<td></td>
</tr>
<tr>
<td>Lys (K)</td>
<td>Lysine</td>
<td></td>
</tr>
<tr>
<td>Ser (S)</td>
<td>Serine</td>
<td></td>
</tr>
<tr>
<td>Thr (T)</td>
<td>Threonine</td>
<td></td>
</tr>
<tr>
<td>His (H)</td>
<td>Histidine</td>
<td></td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------</td>
<td></td>
</tr>
<tr>
<td>Alk</td>
<td>Acetamide</td>
<td></td>
</tr>
<tr>
<td>PEG</td>
<td>Poly (ethylene glycol)</td>
<td></td>
</tr>
<tr>
<td>PLL</td>
<td>Poly(L-lysine)</td>
<td></td>
</tr>
<tr>
<td>PEI</td>
<td>Poly(ethylenimine)</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 GENE THERAPY

The delivery of foreign genes or oligonucleotides with therapeutic activity in cells (gene therapy) has tremendous potential to provide effective management, prevention and treatments for many detrimental human diseases such as chronic diseases (for example, cardiovascular disorders) genetic disorders and cancer.\textsuperscript{1-3} A growing understanding of the genes involved in these diseases has raised optimism about our capability to cure these diseases. For example, this knowledge can be utilized in various anticancer strategies such as:

**Replacement of a Deficient Tumor Suppressor Gene**

Tumor suppressors act as transcription factors and are involved in re-establishment of the balance between growth and apoptosis. Malfunction of these genes leads to cell immortalization, multidrug resistance, and uncontrolled cell division.\textsuperscript{4} Introduction of functional tumor suppressor genes into human carcinoma cells has been reported to facilitate induction of apoptosis of cancer cells and remission of drug resistance caused by the defective genes.\textsuperscript{5,6}
Inhibition of a Dominant Oncogene

Inactivation or weakening the expression of oncogenes can lead to inhibition of uncontrolled cell proliferation, angiogenesis and metastasis formation. This can be achieved by introduction of a gene coding for an antisense RNA which hybridizes with the target mRNA of the desired oncogene thereby facilitating degradation of the duplexed mRNA. Therefore, by utilizing this methodology, specific inhibition of targeted oncogenes can be achieved. Moreover, genes coding for ribozyme molecules that act specifically on mRNA expressed by a defective gene can also be utilized to “knock out” the effects of the cancerous gene.7

Suicide Gene Therapy

Introduction of prodrug-converting enzyme genes, which perform conversion of non-toxic prodrugs into therapeutically active toxins that causes cell death, into target cells can be utilized in order to limit non-specific toxicity (Figure 1.1). The genes of these enzymes can be either under control of tumor specific promoters, thus limiting cytotoxicity to tumorous tissues or cell-specific targeting ligands can be introduced into the vectors used to deliver the genes which can facilitate delivery of the genes only to the cells of interest, thus limiting non-specific toxic effects.8
Figure 1.1  Enzyme-prodrug therapy.\textsuperscript{8}

Amplification of Tumor Cells Immunogenicity

Malignant cells display antigenic differences to normal cells; in some cases, however, their immunogenicity remains undiscovered by the host’s immune system. Therefore, one of the strategies that can be utilized to eliminate the malignant cells is to enhance their immunogenicity so that they can be recognized by the host’s immune system. This strategy can be utilized by cell-specific delivery of vectors containing antigen genes into target cells, which can lead to an immune response against the target cells after the expression of the presented antigens.\textsuperscript{9,10}
Practical utilization of this knowledge is nonetheless limited by our ability to manipulate and replace the defective genes or insert others encoding the correct protein in a safe and effective manner. Thus, it is rather paradoxical that the weak link in gene therapy is the vehicle rather than the “drug” itself. Viral or non-viral vectors are used to condense and delivery therapeutic genes into the cells of interest since naked DNA is unable to efficiently cross cellular barriers by passive diffusion because of its large size, strong negative charge, hydrophilicity and susceptibility to nuclease degradation.\textsuperscript{11}

\section*{1.2 BRIEF HISTORY OF GENE THERAPY}

The first approved gene therapy procedure was performed on September, 1990, on a patient suffering from Severe Combined Immunodeficiency Syndrome (SCID), a rare genetic disease caused by adenosine deaminase deficiency. A retroviral vector was used to transduce T lymphocytes with a normal copy of the gene encoding the enzyme adenosine deaminase. Considerable improvement in the patient’s health was noticed after the procedure.\textsuperscript{12}

On September, 1999 however, Gene therapy suffered a major setback with the death of 18-year-old Jesse Gelsinger. Jesse was participating in a gene therapy trial for ornithine transcarbamylase deficiency (OTCD), a rare metabolic disorder that is marked by dangerous levels of ammonia in the bloodstream. He died after suffering a massive immune response triggered by the adenoviral vectors used as the gene’s transport vehicle.\textsuperscript{12}

On January, 2003, Food and drug administration (FDA) placed a temporary halt on all gene therapy trials using retroviral vectors after two children participating in a gene therapy trial for
SCID in a French gene therapy trial developed leukemia. However, on April, 2003, FDA eased the ban on gene therapy trials using retroviral vectors for treatment of life-threatening diseases. On May, 2006, Researchers at the National Cancer Institute (NCI), part of the National Institutes of Health, successfully reengineered lymphocytes, to target and attack cancer cells in patients with advanced metastatic melanoma. This was the first time that gene therapy had been used to successfully treat cancer in humans.

On April, 2008, a team of British doctors from Moorfields Eye Hospital and University College in London reported the results from the world’s first clinical trial to test a revolutionary gene therapy treatment for inherited blindness using viral vectors. They reported that the experimental treatment was safe and could improve sight in patients suffering from inherited childhood blindness (Leber's congenital amaurosis), caused by a single gene abnormality.

On March, 2009, University of California researchers reported a dramatic reduction in HIV viral loads in patients treated with gene therapy using viral vectors.

1.3 DNA CONDENSATION

In vitro condensation of DNA to nanoparticles by multivalent cations has been extensively studied for decades, in order to understand the factors involved in the control of DNA compaction in living cells and viruses, which can facilitate development of novel gene delivery vehicles. As a highly negatively charged polyelectrolyte, the double-stranded DNA adopts an extended random coil conformation in dilute solutions which is driven by repulsion between neighboring segments and entropic gain of flexibility. However, in the presence of multivalent and polyvalent cations, e.g., hexammine cobalt chloride, spermidine, spermine, cationic oligopeptides, cationic lipids, polylysines and polyethylenimine, compaction of DNA into
various compact nanoparticle structures, e.g., spheroids, toroids and rods or intermolecular DNA aggregation is induced. The ability of cationic molecules to condense DNA indicates that charge neutralization by electrostatic interaction between the negatively charged phosphate groups of DNA and the positively charged multivalent cations is a major factor governing DNA condensation.

Compaction occurs when > 89% of the DNA phosphate charges are neutralized by the counterions. According to the counterion condensation theory, 76%, 88%, 92%, and 94% of DNA phosphate charges are neutralized by mono-, di-, tri-, and tetravalent cations respectively. Therefore in aqueous solutions, DNA condensation requires cations of charge +3 or greater. Monomolecular DNA collapse occurs at micromolar DNA concentrations or below, while aggregation is induced at higher concentrations. The understanding of such compaction and aggregation processes is important in development of effective non-viral DNA compaction agents.

1.4 DNA CONDENSATE MORPHOLOGIES

The size, shape and morphology of DNA condensates influence their physiological properties. Thus, understanding the factors that govern their structure is vital for improving the biological application of the condensates. The principal morphologies of DNA condensates are toroids, rods and spheroids, with the toroids and rods being the most common morphologies and toroids the dominant morphology. Hud et al. noted that the observed distribution of toroids and rods depends on both thermodynamics and kinetics of DNA condensate formation, with rods representing a metastable state, which converts to the more thermodynamically stable toroids by internal conversion or by complete decondensation followed by recondensation into toroids.
They observed a relative equilibrium population of 97% toroids and 3% rods for a sample of 3kbDNA condensed by hexamine cobalt chloride.\textsuperscript{24,27} However, a high proportion of rods relative to the toroids is observed when moderately nonpolar condensing agents or solvents are used. This possibly occurs due to lowering of the free energy of exposed heterocyclic bases within the DNA modulated by the nonpolar environment which favors sharp local kinking (a prerequisite of rod formation) over gradual DNA bending which occurs in toroids.\textsuperscript{32-34} On the other hand, spherical globules becomes the predominant condensate morphology when large excess of the condensing agent is used to condense the DNA.\textsuperscript{35}

1.5 MECHANISM OF DNA CONDENSATION

Hud et al postulated that, the process of DNA condensation occurs in stages on different time scales. The initial stage, which is usually complete within the millisecond time frame, involves spontaneous formation of nucleation loops, which is thought to be a spontaneous event facilitated by thermal fluctuations in DNA conformation. The nucleation loops are stabilized by the condensing agents. This stage also involves intramolecular condensation that promotes formation of proto-toroids and proto-rods (Figure 1.2). Addition of previously uncondensed DNA which results to initial particle growth also occurs during this stage. The next stage involves a shift of condensate morphology to relatively more thermodynamically stable toroids and fewer rods, in addition to continued particle growth. An equilibrium between toroids and rods can be reached within the minute time scale during this stage, with the relative population shifting from \(~79\%\) toroids and \(~21\%\) rods at two minutes after initiation of condensation to \(~97\%\) toroids and \(~3\%\) rods at 41 minutes after initiation of condensation. Within hours to days, the final stage which involves aggregation of individual condensates can be observed (Figure 1.2 and 1.3).\textsuperscript{26,27,36}
Figure 1.2  Model for the overall process of DNA condensation in which both rods and toroids are formed.27
Figure 1.3 Transmission electron micrographs of DNA and hexamine cobalt chloride condensates at different times. (A) 2 min after the initiation of condensation; (B) 16 min after the initiation of condensation; (C) 41 min after initiation of condensation; (D) 71 min after initiation of condensation. The scale bar in A is 100 nm.

However, it has been postulated that the presence of divalent cations, e.g. Mg(II), in DNA solution prior to addition of condensing agents leads to stabilization of intra- and intermolecular helix-helix contacts, but not to a threshold sufficient for DNA condensation, which nucleate DNA condensation upon addition of the multivalent condensing agent. This facilitates formation of intertwined aggregates of toroids (Figure 1.4).
Moreover, a substantial change in relative condensate morphologies is observed when DNA condensation is conducted in the presence of multivalent cations (e.g. spermine and spermidine) and architectural prokaryotic proteins such as integration host factor (IHF) and histone-like protein from Escherichia coli strain U93 (HU). As indicated above, when the DNA condensation is conducted only in the presence of multivalent cations, the relative toroid to rod population is about 97% to 3% respectively. However, in the presence of the multivalent cations and the architectural proteins (e.g. IHF or HU), the relative population of rods increases to more than 90%. This change in relative toroids to rods population is postulated to be promoted by intercalation of conserved proline residues from the architectural proteins into DNA base pairs which induces pronounced kinks in the DNA double helix. Intercalation of the proline residues into the DNA nucleation loops during the initial stage of DNA condensation increases the probability for the loops to collapse upon themselves forming nucleating structures that favor formation of rods (Figure 1.5).
Figure 1.5 A model of the effects of architectural proteins, e.g., IHF or HU, on DNA condensation process. Bold arrows indicate the steps that become more favorable in the presence of IHF or HU and the black ellipsoids represent IHF or HU.39

1.6 VIRAL VECTORS

Viral vectors are very effective in facilitating delivery, integration and long-term expression of foreign genes in the cells of interest since viruses have been tailored by evolution for transferring their genes from one cell to another, since they can only replicate their genome inside the host cells. However, they suffer from severe drawbacks, for example, limited insert size of virally packaged therapeutic gene, possibility of generating replication-competent virus through recombination with the host genome, generation of toxic inflammatory responses, and possibility
of random integration into host chromosome which can lead to activation of oncogenes or inactivation of tumor suppressor genes.\textsuperscript{40-43} For example, in 2002, two patients suffering from severe combined immune deficiency (SCID) were successfully treated by gene therapy using retroviral vectors at the Necker Hospital in Paris, but they subsequently developed leukemia.\textsuperscript{44} Therefore, non-viral vectors, such as liposomes, peptides and cationic polymers, have received a lot of attention as alternatives to viral vectors. Although they display relatively low transfection efficiency when compared to viral vectors, they are non-pathogenetic and they do not elicit immune response.\textsuperscript{45-48} The low transfection efficiency is caused by, poor stability of the DNA/condensing agents complexes, poor cellular uptake of the complexes, inadequate release of the complexes from the endosomes into the cytoplasm, and inefficient transport across the cytoplasm to the nucleus.\textsuperscript{49, 50}

1.7 SYNTHETIC VECTORS

In the past decade, cationic polymers have emerged as important synthetic alternatives to viral vectors. They possess many advantages, for example, absence of specific immune response, large DNA loading capacity, simplicity of preparation, ease of large scale production, ability to readily and accurately determine their molecular structure and purity of the compounds and capability to synthesize versatile multifunctional compounds which can be systematically modified to optimize their efficacy in response to in vitro and in vivo experimental results. These systems, however, display suboptimal transfection efficiency. The low transfection efficiency is postulated to be due to several obstacles that hamper gene delivery into a cell. They include: extra- and intracellular degradation of therapeutic DNA by nucleases, poor cellular penetration, ineffective intracellular trafficking, and inadequate nuclear localization.\textsuperscript{51-55} Therefore, the basic
obstacle that synthetic vectors have to overcome is the inability to condense DNA into stable well defined nanoparticles that can resist extra- and intracellular degradation by nucleases. Viral vectors efficiently overcome these obstacles by protecting and stabilizing their genetic cargo while simultaneously facilitating cellular penetration and appropriate trafficking. Thus, one of the potential approaches towards optimization of synthetic vectors is to develop ‘artificial viruses’, i.e. synthetic vectors that incorporate functional elements mimicking viruses. The design of such virus-like modular artificial systems depends heavily on in-depth biochemical knowledge of the mechanisms underlying the various strategies used by viruses to efficiently infect their host cells.\textsuperscript{53-55} Some simple synthetic systems for DNA delivery evolved from the biochemical knowledge that the active sites of enzymes, receptor ligands and antibodies are composed of about 5 to 20 amino acids. This insight allows formulation of small synthetic peptides that emulate the active sites of viral proteins, therefore enabling design and synthesis of versatile DNA vectors that have the potential to be as efficient as the viruses, but without the limitations displayed by the viruses.\textsuperscript{27,59}

This thesis explores the utilization of low molecular weight natural polyamines (spermine and spermidine), oligopeptides, and amphiphilic polymers in DNA condensation, packaging and their potential application in gene therapy.
CHAPTER 2

STRUCTURAL INFLUENCE OF LOW MOLECULAR WEIGHT NATURAL POLYAMINES AND OLIGOPEPTIDES ON DNA CONDENSATION

2.1 DRIVING FORCES OF DNA CONDENSATION

Recent advances in gene therapy have greatly contributed to the high interest in DNA condensation studies. Previous thermodynamic studies of multivalent cations-DNA binding have demonstrated that the major driving forces of DNA condensation is the electrostatic interaction and entropic gains facilitated by thermodynamic release of counter-ions (e.g. Na\(^+\), K\(^+\) and Mg\(^{2+}\)) previously bound to DNA. Multivalent and polyvalent cations, e.g. cationic lipids, polyethylenimine, spermidine, spermine, polylysines, cationic oligopeptides and cationic amphiphilic graft copolymers (composed of peptides grafted onto hydrophobic backbone) interact with DNA and facilitate DNA condensation into compact, well-defined nanometer-scale particles. This phenomenon of DNA condensation has been studied for decades as a model of high-density DNA packaging in living systems, particularly in sperm cells and viruses. Association of multivalent or polyvalent cations around the DNA phosphate groups facilitates DNA condensation via a reversible, linear polymer to globule transition. DNA undergoes localized bending or distortion at a certain critical ratio of multivalent cation to DNA, which facilitates compaction of DNA into toroids, rods and spheroids (Figure 2.1).
2.2. UTILIZATION OF LOW MOLECULAR WEIGHT NATURAL POLYAMINES (SPERMINE AND SPERMIDINE) AND OLIGOPEPTIDES AS DNA CONDENSING AGENTS

The size and homogeneity of DNA condensing agents play a critical role in the continued development of non-viral DNA delivery systems, since they impact cellular toxicity, immunogenicity, and the ability to systematically optimize the DNA delivery systems for in vivo applications. The high molecular weight and heterogeneity displayed by cationic polymers often leads to cationic polymer/DNA condensates of non-uniform physico-chemical characteristics, relative cellular toxicity and difficulties to selectively modify the polymers in order to optimize their utility as DNA delivery systems. On the other hand, cationic lipids tend to change size and lipid composition in the presence of blood components; therefore, they are not ideal candidates for in vivo application. These factors have led to emergence of oligopeptides and low molecular weight natural polyamines, such as spermine and spermidine, as attractive alternatives to cationic polymers and lipids since they can easily be synthesized and functionalized, and in

Figure 2.1 Schematic representation of DNA condensation by multivalent cations.
addition they are less cytotoxic and immunogenic as compared to the other non-viral DNA vectors. Small synthetic peptides, also, can easily be synthesized by the standard solid phase peptide synthesis (Figure 2.2).
Figure 2.2 Schematic representation of solid phase peptide synthesis (SPPS) of H$_2$N-Lys-Trp-Lys-Lys-Ac. 69

The approach of using low molecular weight polyamines and peptides as DNA packaging systems evolved from the biochemical knowledge that, in vivo, DNA is usually associated with cationic polyamines and proteins such as spermine and spermidine in chromosomes, histones in chromatin, and certain viral proteins in viruses that facilitate DNA condensation. 70, 71 Small synthetic peptides (5-20 amino acids) that emulate the active sites of viral proteins have shown great potential as DNA vectors since they have the capability to be as effective as viral vectors but without the safety limitations associated with the viral vectors.

The low molecular weight polyamines and oligopeptides can interact with DNA through various interactions at neutral pH (Figure 2.3), such as: Electrostatic interaction between positively charged spermine, spermidine and amino acid side chains (Arg and Lys) and the phosphodiester
backbone of DNA, intercalation of aromatic or cyclic residues (Trp, Phe, Tyr and Pro) into DNA base pairs and hydrogen bonding interactions between hydrophilic side chains (Asn, Ser, Tyr, Thr, and His) and DNA base pairs (Figure 2.4). In addition, aliphatic side chains of amino acids (Val, Ile, Leu, Ala, Pro) and hydrophobic regions of amphiphilic polymers can interact with the hydrophobic regions of DNA.\textsuperscript{72-75}
Amino acids that can potentially interact with DNA via hydrophobic interactions or intercalation into the DNA base pairs

![Structural formulas of amino acids](image1)

Amino acids that can potentially interact with DNA via electrostatic interactions or hydrogen bonding

![Structural formulas of amino acids](image2)

**Figure 2.3** Low molecular weight natural polyamines and amino acids.
2.3 EFFECT OF THE NATURE OF THE DNA CONDENSING AGENTS AND CONDENSATION ENVIRONMENT ON DNA CONDENSATION CAPABILITY AND SIZE, STABILITY AND MORPHOLOGY OF THE DNA CONDENSATES

DNA condensation capability and the size, stability and morphology of peptide-DNA complexes are strongly influenced by the number and composition of amino acid residues in the peptide. For example, Navvelt et al. reported that, unlike a trilysine, a tetralysine provided sufficient threshold
of positive charges and charge density to expedite DNA condensation. The inability of trilysine to expedite DNA condensation was attributed to low charge density resulting from partially protonated lysine residues and chain separation between positive charges on the lysine side chains. It has also been reported that DNA binding affinity of arginine residues is significantly higher than the DNA binding affinity of lysine residues which is facilitated by the relatively high arginine pKₐ (12.48) compared to lysine pKₐ (10.54). In addition, the arginine guanidinium group is capable of forming bifurcated hydrogen bonds with the DNA base pairs which can potentially stabilize the peptide-DNA complex. DNA condensation has also been reported to improve when hydrophobic amino acids are added to cationic peptides due to hydrophobic interactions between the hydrophobic amino acids side chains and hydrophobic regions within the DNA backbone. In addition, hydrophobic amino acids, which can potentially intercalate into DNA base pairs such as tryptophan, proline and phenylalanine, can also improve DNA condensation capability of cationic peptides. Moreover, proline residues also substantially enhance the population of rods in the peptide-DNA complexes. This is postulated to occur as a result of formation of pronounced kinks on DNA modulated by intercalation of the proline residues between DNA base pairs.

Low molecular weight cationic natural proteins and oligopeptides (possessing cations of charge +3 or greater) have previously been reported to facilitate DNA condensation into well defined nanoparticles. In addition, the stability and morphology of the condensates can easily be manipulated by changing the condensation environment and amino acid composition. For example, low molecular weight proteins, spermidine [H₂N(CH₂)₃NH(CH₂)₄NH₂] and spermine [H₂N(CH₂)₃NH(CH₂)₄NH(CH₂)₃NH₂] can condense DNA into toroids and rods with relative populations of approximately 97% toroids and 3% rods through electrostatic interaction between...
the positively charged spermine and spermidine and the negatively charged phosphodiester backbone of DNA. However, when the DNA condensation is performed in the presence of architectural proteins such as integration host factor (IHF), the relative population of rods increases to more than 90%. This change in relative toroids to rods population is postulated to be promoted by intercalation of conserved proline residues present in IHF into DNA base pairs which induces formation of pronounced kinks in the DNA double helix. These kinks facilitate formation of rods (Figure 2.5).84, 85
Figure 2.5  Transmission electron microscopy (TEM) images of spermidine- and spermine-induced DNA condensates produced in the presence and absence of IHF. (A) DNA condensate produced by the addition of spermidine to 3.4 kb DNA. (B) DNA condensate produced by the addition of spermidine to 3.4 kb DNA in the presence of 75 nM IHF. (C) DNA condensates produced by the addition of spermine to 3.4 kb DNA. (D) DNA condensates produced by the addition of spermine to 3.4 kb DNA in the presence of 125 nM IHF. The scale bar is 100 nm.  

Moreover, the number of cationic charges also plays a vital role in DNA condensation. The DNA condensates formed in the presence of spermine (+4 charge) are considerably smaller than those formed in the presence of spermidine (+3 charge), which demonstrates that the spermine is more efficient in condensing and packaging DNA into stable nanoparticles than spermidine (Figure 2.5)
On the other hand, polylysine (+390 charge) is more efficient in condensing DNA than pentalysine oligopeptide (+5 charge), and pentalysine is more efficient than tetralysine (+4 charge). This can be deduced from the low concentration of polylysine required to fully condense DNA as compared to the amount of pentalysine and tetralysine required (Figure 2.6). The trend of the decreasing concentration, required to fully condense DNA, with increasing lysine chain length can be explained in terms of binding affinity. A higher cationic charge leads to a higher binding affinity than that of a lower cationic charge, thereby causing DNA to collapse at lower counterion concentration. Therefore, polylysine’s ability to condense DNA at significantly lower concentration than pentalysine and tetralysine can be attributed to its higher DNA binding affinity.\(^76\)

**Figure 2.6** Typical plots of the relative intensity of scattered light against concentrations of polylysine, Lysine\(_5\) and Lysine\(_4\).\(^76\)
2.4 STABILIZATION OF OLIGOPEPTIDE/DNA CONDENSATES

The main drawback of the condensates formed by interactions between DNA and cationic oligopeptides is their relative instability during in vivo gene delivery which can lead to premature DNA release and subsequent enzymatic degradation. To overcome this dilemma, several strategies have been formulated. For example, the presence of cysteine residues in the DNA condensing cationic oligopeptides leads to interpeptide disulfide bond formation after binding of the oligopeptide to DNA, which leads to stabilization of the condensates. On the other hand, the disulfide bonds can easily be broken in the intracellular reducing environment (Figure 2.7), which facilitates release of the DNA cargo into the cellular matrix. In addition, the degree of stability of the condensates can be modulated by changing the number of thiol groups in the DNA condensing peptides.\textsuperscript{11,86}
Other strategies utilized to increase the efficacy of cationic oligopeptide include substitution of cationic lysine residues with cationic arginine residues, since arginine side chains forms more stable electrostatic interactions with DNA phosphate groups than lysine side chains. In addition, incorporation of amino acid residues that can interact through hydrogen bonding and hydrophobic interaction with the DNA can further optimize DNA condensation capability of the oligolysines. Furthermore, hydrophobic amino acid residues that can potentially intercalate into
DNA base pairs (e.g. tryptophan and phenylalanine) have been shown to facilitate improved DNA condensation potential of oligopeptides. This is postulated to occur due to increased hydrophobicity or provision of a more ordered interaction of the DNA and oligopeptide facilitated by intercalation of the hydrophobic amino acid side chain into the DNA base pairs (Figure 2.8). Wadwa et. al. reported a 40-fold reduction in particle size and a 1000-fold amplification in transfection efficiency of AlkCWK$_{18}$ DNA condensates relative to polylysine$_{19}$, which was proposed to be facilitated by the ability of tryptophan to increase the peptide’s efficiency to condense DNA into small particles. AlkCWK$_{18}$/DNA condensates were relatively uniform particles with diameters of approximately 50-100 nm, whereas polylysine$_{19}$-induced condensates were large aggregated particles of approximately 3100 nm in diameter (Figure 2.9).

![Figure 2.8](image)  
**Figure 2.8** Model for interaction of amphiphilic peptides with DNA.
One of the most promising strategies for improving the DNA condensation and delivery efficiency of oligopeptides is grafting the oligopeptides onto a hydrophobic backbone. Emrick et al. synthesized polyolefin-graft-pentalysine polyelectrolytes (Figure 2.10 and 2.11) which showed the capability of condensing plasmid DNA at N/P of ≥ 2 and forming well defined nanoparticles of ~ 80 nm diameter, with relatively low size distribution (Figure 2.12), which depicted that the DNA was fully packaged within the nanoparticles. The spatial clustering arrangement of the pentalysines on the polyolefin backbone enhanced the DNA condensation and transfection capability of the oligopeptides. Evaluation of the polyolefin-graft-pentalysine polyelectrolytes efficacy showed overall better transfection efficiency and cell viability than commercial transfection agents in human adenocarcinoma epithelial cervical cells, HeLa (Figure 2.9 Electron microscopy of DNA condensates. DNA condensates prepared at 0.5 nmol of peptide/µg of DNA for AlkCWK₁₈ (A) and at 0.8 nmol of peptide/µg of DNA for polylysine₁₉ (B). The calibration bar shown is 50 nm in length.⁸²
2.13 and 2.14). To further improve the potential efficacy of these amphiphilic graft copolymers in vivo, studies are being conducted to evaluate the optimum strategy of incorporating poly(ethylene glycol) into the copolymers without affecting their efficiency, since graft copolymers with poly(ethylene glycol) grafts (Figure 2.11, polymer 2) were shown to be inferior to those without poly(ethylene glycol) grafts. This was postulated to occur due to steric hindrance and reduced charge density modulated by the presence of the poly(ethylene glycol) grafts. As it was previously noted, the presence of poly(ethylene glycol) moieties in gene delivery vectors during in vivo application is very important since poly(ethylene glycol) facilitates biocompatibility, relatively high colloidal stability in biological medium and prolonged circulation in the blood stream.

Figure 2.10  Schematic representation of polyolefin-graft-oligopeptide synthesis.\textsuperscript{88}
Figure 2.11 Structures of graft copolymers, polymer 1e and polymer 2.
**Figure 2.12** DNA Condensates characterization: (a) gel electrophoresis and (b) dynamic light scattering of polymer 1e-DNA condensates at N/P ratio of 3.89

**Figure 2.13** Fluorescence microscope images of human adenocarcinoma epithelial cervical cells (HeLa cells) transfected with complexes of GFP-expressing plasmid DNA and various transfection reagents: polymer 1e (N/P 3), and commercial transfection reagents; jetPEI, Seperfect, and lipofectamine 2000.89
Figure 2.14 Relative fluorescence (left) and cell viability of HeLa cells transfected with complexes of GFP-expressing plasmid DNA and various transfection reagents: polymer 1e (N/P 3), and commercial transfection reagents: jetPEI, Seperfect, and lipofectamine 2000.\textsuperscript{89}
CHAPTER 3

DNA CONDENSATION BY AMPHIPHILIC POLYMERS

3.1 COVALENT CONJUGATION OF POLY(ETHYLENE GLYCOL) ONTO POLY(L-LYSINE) (PLL) AND POLY(ETHYLENIMINE) (PEI)

Poly(L-lysine) (PLL) and poly(ethylenimine) (PEI) are more efficient non-viral gene delivery vehicles than their low molecular weight analogues, oligolysines and natural polyamines (spermine and spermidine). However, their high cationic charge densities adversely affect cell viability, due to detrimental interaction with negatively charged cellular components. To circumvent this dilemma, Poly(ethylene glycol) (PEG) is conjugated onto PLL and PEI (Figure 3.1 and 3.2), to limit the detrimental cellular interactions.94, 95

Covalent conjugation of poly(ethylene glycol) to cationic polymers is a well-known approach for efficient stabilization of the cationic polymers/DNA complexes under physiological conditions by reducing protein adsorption due to the hydrophilic shielding effect of cationic charges and overcoming the solubility limitations of the complexes facilitated by poly(ethylene glycol). In the absence of poly(ethylene glycol), the cationic polymers/DNA complexes can interact with plasma proteins such as albumin (which is responsible for rapid clearance of complexes from the bloodstream), leading to formation of large aggregates which are rapidly removed from the bloodstream. In addition, the complexes can also interact with erythrocytes leading to aggregate formation. These aggregates can obstruct blood vessels which can lead to severe consequences such as stroke.96-99 Therefore, incorporation of poly(ethylene glycol) in cationic polymers is vital.
for ensuring, safety, stability, solubility, prolonged circulation in the blood stream and biocompatibility of the cationic polymers/DNA complexes during in vivo application.  

\[
\begin{align*}
3HC\left[\begin{array}{c}
\text{O} \\
m
\end{array}\right]NH_2 \quad + \quad n\begin{array}{c}
\text{R}_1 \\
\text{HNOCOO}
\end{array} \quad \rightarrow \quad H_3C\left[\begin{array}{c}
\text{O} \\
m
\end{array}\right]NH\begin{array}{c}
\text{C} \\
\text{R}_1
\end{array}N\begin{array}{c}
\text{H} \\
\text{H}
\end{array}n
\end{align*}
\]

\[
\begin{align*}
\text{HBr/AcOH} \quad \rightarrow \\
H_3C\left[\begin{array}{c}
\text{O} \\
m
\end{array}\right]NH\begin{array}{c}
\text{C} \\
\text{R}_2
\end{array}N\begin{array}{c}
\text{H} \\
\text{H}
\end{array}n
\end{align*}
\]

\[
\begin{align*}
\text{R}_1 = (\text{CH}_2)_n\text{NHCOCH}_2\text{Ph} \\
\text{R}_2 = (\text{CH}_2)_n\text{NH}_2
\end{align*}
\]

**Figure 3.1** Schematic representation of synthesis of PEG-b-PLL.  

34
Shielding of the cationic charges by PEG, on the other hand, decreases DNA condensation efficiency of these polymers. For example, PEI-alt-PEG copolymers (PEI $M_n = 423$, PEG $M_n = 258, 575$, and 700) showed relatively low DNA condensation efficiency since DNA condensation occurred at a relatively high N/P ratio of about 11.7, (Figure 3.3). However, PEI-alt-PEG(258) showed better transfection efficiency than PEI, which was postulated to occur as a result of PEI cytotoxicity (Figure 3.4).\textsuperscript{95}
Figure 3.3 Agarose gel electrophoresis of PEI-alt-PEG/DNA complexes at various N/P. (top panel) PEI-alt-PEG(258)/DNA, (middle panel) PEI-alt-PEG(575)/DNA, (bottom panel) PEI-alt-PEG(700)/DNA.
On the other hand, PLL-b-PEG (PLL $M_n=56,200$, PEG Degree of Polymerization = 32) block copolymers showed more efficient stabilization of the copolymer/DNA complexes than PLL/DNA complexes in the presence of serum and better transfection efficiency (Figure 3.5). This occurs due to a reduction of adsorption of negatively charged proteins onto copolymer/DNA complexes facilitated by shielding effect of cationic charges by PEG chains, while the negatively charged serum proteins can readily be adsorbed onto the positively charged PLL/DNA complexes which leads to aggregates formation and retardation of gene delivery.\textsuperscript{94}
Figure 3.5 Serum effect on the transfection efficiency of PLL and PLL-b-PEG (Run No. 2) on 297T transformed immortalized cells.⁹⁴
CHAPTER 4

FUTURE STUDIES

Even though DNA condensation and packaging into stable, well defined nanoparticle condensates is vital for DNA transfection, the other hurdles that hamper the efficiency of non-viral gene vectors such as lack of target specificity, poor endosomal escape, and poor delivery of the DNA cargo to the nucleus, need to be addressed (Figure 4.1). These hurdles not only reduce the efficiency of the non-viral vectors, but also they can potentially elicit some detrimental health effects during in vivo application. For example, lack of target specificity leads to indiscriminate transport of the DNA condensates throughout the body. Therefore, only a small fraction of the therapeutic DNA will potentially reach the target site. In addition, healthy organs and tissues can be detrimentally affected by the biological action/s of the therapeutic DNA. Therefore, lack of target selectivity leads to unacceptable side effects and inadequate therapeutic results. As a result of inadequate therapy, repeated administration of the therapeutic DNA agents is required which might lead to exacerbation of potential side effects. Due to the limitations mentioned above, controlled and localized gene therapy is of utmost importance.
Figure 4.1 Major barriers to peptide-guided gene delivery: (A) condensation of DNA into compact and stable nanoparticles; (B) targeting of the condensates to specific cell-surface receptors; (C) induction of endosomal escape; and (D) nuclear localization and transport across nuclear membrane.\textsuperscript{79}
Molecular conjugates can be coupled to non-viral gene delivery vectors, in order to facilitate deliver of therapeutic genes to target cell. This can be achieved by utilizing ligands that have receptors expressed on the target cells. For example, conjugating epidermal growth factor antibody or antibody-fragments to the gene delivery vectors can facilitate delivery of the vectors to human carcinomas such as glioblastoma, cancers of the lung, breast, head, neck and bladder, since over expression of the epidermal growth factor receptors has been observed in a high percentage in these carcinoma cells. On the other hand, synthetic ligands such as galactose, which mediates liver specific DNA delivery, can also be utilized for cell specific targeting. The cell-specific targeting ligands facilitate internalization of the DNA condensates through receptor-mediated endocytosis. However, the DNA condensates must be able to escape from the endosome into the cytosol, before endosome-lysosome fusion, which modulates degradation of the endosome contents by lysosomal enzymes. Some of the strategies that can be used to modulate the release of DNA condensates from the endosome include use of endosomolytic reagents such as chloroquine or incorporation of endosomolytic peptides such as H5WYG into the DNA condensing graft copolymer. On the other hand, addition of histidine residues into the DNA condensing oligopeptides can facilitate edosomal escape. Chloroquine raises endosomal pH, decreases degradation of endocytosed material by inhibiting lysosomal hydrolytic enzymes and also induces osmotic swelling of the endosomes, which results in release of the internalized DNA condensates into the cytosol due to destabilization of the endosome. On the other hand, histidine amine groups (pKa ~6.0) promote endosomal escape through proton sponge activity. The histidine amine groups are easily protonated in the acidic environment of the endosome, which facilitates pumping in of protons into the endosome. This is accompanied by an influx of chloride ions to maintain charge neutralization, which
results in osmotic swelling of the endosome, and subsequently its rupture, and release of the DNA condensates into the cytosol occurs.\textsuperscript{107,108}

After the release of the DNA condensates from the endosome into the cytosol, the condensates must be efficiently trafficked to the nucleus and transported across the nuclear membrane through the nuclear pore complex (NPC) before DNA transcription can be initiated. However, the pore size of the NPC (~9 nm) limits free diffusion of the condensates into the nucleus.\textsuperscript{106}

Therefore, incorporation of nuclear localization signals (NLS) into vectors which can facilitate trafficking of the DNA condensates to the nucleus and active transport of the condensates into the nucleus is also a vital strategy.

Incorporation of the moieties mentioned above into the vectors can potentially broaden their capabilities as a potential gene delivery vehicles by substantially enhancing their potential to overcome various hurdles that limit efficient gene transfection.
REFERENCES

(1) McNeish, I.; Seckl, M. *Gene Therapy: The Use of DNA as a Drug* 2002, 9, 87-134.


(6) Riva, C. M. *Anticancer Research* 2000, 20, 4463-4471.


(18) Hud, N. V.; Downing, K. H. Proceedings of the National Academy of Sciences 2001, 98, 14925-14930


(22) Bloomfield, V. A. Biopolymers 1997, 44, 269-282.


(38) Swinger, K.; Rice, P. Current Opinion in Structural Biology 2004, 14, 28-35.


(60) Smith, A. E. Annual Reviews in Microbiology 1995, 49, 807-838.


Martin, M.; Rice, K. *The AAPS Journal* 2007, 9, 18-29.


