Release studies of naked DNA, polyplexes and lipoplexes from polymeric films

Ramgopal Yamini

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Release Studies of Naked DNA, Polyplexes and Lipoplexes from Polymeric Films

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A thesis submitted to the Nanyang Technological University
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ABSTRACT

This report details the study done on the stability, release and transfection efficiencies of naked DNA, polyplexes and lipoplexes from different polymeric films in which they were entrapped. The polymeric system studied includes PLGA (53/47), PCL and PEVAC. The release profile was determined by quantifying the amount of DNA released at specific time points over a period of 1 month, utilizing the fluorescence of ethidium bromide. Heparin sulphate and oleic acid were used to decomplex the polyplexes and lipoplexes. A sustained release was obtained from all the 3 polymeric films. In all the cases studied, a rapid initial in vitro release was obtained (pDNA), while the release from the complexed pDNA was sustained for a month. This is attributed to the anionic, hydrophilic nature and solubility of the drug in polymeric matrices. Among the 3 polymers, a fastest release is noted from a semi-crystalline polymer PCL. The release of polyplexes from PCL and PEVAC polymeric systems is dominated by the hydrogen bonding between PEI and the polymers. In case of PLGA, the delayed release is dominated by the charge interaction between PLGA and polyplexes. Lipoplexes were released earlier from films when compared to polyplexes. Also the use of porogens like PEG/TEC and gelatin were explored. The presence of porogen had a significant increase in the release of lipoplexes from PCL and PEVAC films and polyplexes from PLGA films. The bioactivity of the released complexes were determined by in vitro cell transfection using COS 7 cells. Transfection was observed from lipoplexes samples from both PCL and PEVAC samples on day 9. The results also demonstrated that bioactivity of the encapsulated systems were superior to the naked DNA.

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CHAPTER I

INTRODUCTION

1.1 Background:

Remarkable advances in recombinant DNA technology and cell biology have fueled significant progress in the field of gene therapy^{1,2}. A critical factor for the therapeutic application of these advances is an effective methodology for gene delivery.

Gene therapy will benefit a range of diseases from single-gene defects, to chronic diseases such as cancer, to vaccination. The success of gene therapy relies on the ability of gene delivery systems to selectively deliver therapeutic genes to a sufficient number of target cells yielding expression levels that impact the diseased state. Initially gene therapy used viral vectors, but due to safety concerns, attention is now diverted towards non-viral systems as well. The major types of DNA non-viral delivery vehicles, which are currently significant, include liposomes, cationic lipids, cationic polymers and polymeric vesicles. The first two options have been more popular approaches to gene therapy, and have been thoroughly reviewed. More recently, using cationic polymers as the basis of gene delivery systems has begun to show increasing promise. While these systems are not as efficient as viruses, they are associated with fewer initial safety concerns.

The DNA delivery particle, once taken up by the cell, must cross a number of barriers before arriving at the site of activity, the cell nucleus (Figure 1.1). While some strategies have been identified to overcome these barriers, a systematic study of these variables is needed in order for improved carriers to be designed and tested.

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Figure 1.1 Barriers in gene delivery

Chapter 1

All the carrier molecules identified so far are nitrogen rich and act by electrostatically binding to DNA and transferring DNA into the cell presumably via endocytosis. These delivery agents are able to transfer genes to cells both in culture and in the whole organism but there are still problems associated with the inability to target these systems as transfection occurs principally in the first capillary bed encountered –the lung/liver/spleen endothelium. It is thus possible that the first generation of licensed gene therapeutics will have to be administrated locally. Targeted systems are still the ultimate goal especially when considering treatments such as suicidal gene therapy. At the cellular level, the bottleneck to efficient gene delivery is that undeniably the transfer from the endosome to the nucleus as cellular uptake appears to be accomplished relatively easily. Studies are thus needed to clarify the variables associated with this transport step if efficient delivery systems are to evolve from the current crop.

The use of viral vectors^{3,4} has been extensively studied. However there remains questions of safety and immune response of viral proteins, particularly when high viral loads and repeated administrations are needed. In addition, viral vectors are limited by the size of the foreign gene that can be inserted into the viral genome. So a considerable effort is focused on investigating efficient non-viral methods of gene transfer to enhance gene transfection. This includes cationic liposomes⁵, ligand –poly-L-lysine conjugates⁶, particle bombardment⁷, and direct intramuscular injection of Plasmid DNA⁸. However, the important limiting factor with the non-viral methods of gene delivery is their comparative inefficiency to facilitate cellular uptake of DNA and subsequent gene expression⁹. Although not as efficient as the viral vectors and achieving only transient transgene expression, non-viral vectors offer the potential advantages of ease of synthesis, low immune response and unrestricted plasmid size.

Over the past few decades, there has been considerable interest in developing biodegradable polymers as effective drug delivery devices. Various polymers have been used in drug delivery research as they can effectively deliver the drug to a target site¹⁰ and thus increase the therapeutic benefit, while minimizing side effects.

Chapter 1

One of the major shortcomings of viral and non-viral vectors is the so-called transience of the therapy. This is a major issue with treatment of a chronic condition such as cystic fibrosis or SCID. The injected corrective gene finds its way to the nucleus, but does not integrate itself into the cell's DNA. Therefore the benefit from the corrective gene is short-lived, and proportional to the amount of DNA that reaches the nucleus. This necessitates repeated injections, sometimes as often as once every week for the therapy to be effective. Only adeno-associated and retro viral vectors can overcome this deficiency, but these vectors have other problems, such as potential immunogenicity and limited capacity for the size of the gene.

Therefore, extending the duration of action of injected or implanted sources of corrective genes is of benefit to the patient. This may be accomplished by sustaining the release of the gene vector over several days or months. *Sustained release polymeric drug delivery systems have been investigated thus for the delivery of a variety of drug molecules including macromolecules¹¹⁻¹⁴ such as proteins, peptides, oligonucleotides, and DNA*

These delivery systems include microspheres, nanospheres, and polymer matrices. The DNA is either entrapped or dispersed into the polymer matrix or adsorbed on to the surface. Sustained – release DNA delivery systems, in addition to providing a controlled release of DNA could also protect^{15, 16} the entrapped DNA from degradation due to nucleases. Prolonged availability of the DNA released slowly could increase the efficiency of cellular DNA uptake by the cells. This prolonged availability¹⁷ also lead to a prolonged duration of gene expression.

Biodegradable (polyesters, polyanhydrides) and non-biodegradable polymers (ethylene vinyl acetate polymers), either synthetic or natural (collagen, gelatin), have all been investigated for sustained release delivery systems. However, many issues have surfaced with respect to DNA dispersion in polymeric systems.

Because of the extremely large size and polar nature, the encapsulation of plasmid DNA molecules with in a hydrophobic matrix appears to be a challenge. The reported

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encapsulation processes result in some denaturation of the pDNA. The encapsulated DNA is not only degraded during the encapsulation process, but also in the course of polymer degradation. However, these systems deliver DNA as a bolus, without long term sustained release. To date, there is no reported sustained-release dosage form for release of naked or complexed DNA that has good bioactivity and duration of action of the released DNA. Because interstitial administration of a single dose of free DNA can lead to a transient gene expression, it is reasonable to speculate that prolonged, continuous DNA delivery to tissues will enhance gene expression; in addition, sustained release systems provide the patient with the possibility of increased compliance, fewer physician visits, and overall reduction in the cost of disease management.

Chapter 2

CHAPTER II

LITERATURE REVIEW

2.1 Historical Perspective:

Humans have always attempted to improve their health by ingesting or administering drugs. Examples appear throughout written history, from every continent and culture. Noah produced alcohol and Christ was offered a sedative to ease the pain of crucifixion¹⁸. The use of opium was described by Theophrastus in the third century BC, the stimulating power of Nethylxanthines was exploited by ancient Arabian shepherds and priors, and the paralyzing properties of curare were recognized by native South Americans centuries before the arrival of Sir Walter Raleigh. The chemotherapy of cancer, which many consider as a modern development has existed in some form for over 500 years. Vaccination, the intentional exposure to pathogens, was used in China and India to prevent smallpox and other infections centuries before the birth of either Jenner or Pasteur¹⁸.

Even during the 20th century, drug discovery frequently resulted from empiricism and happenstance. The anticancer effects of nitrogen mustard were realized during the development of chemical – warfare agents, and penicillin was discovered after the inadvertent contamination of bacteriological plate. As technology advanced, particularly after 1970, methods of drug and vaccine production become more sophisticated and rational. In parallel with the rise of modern pharmaceutical technology and the explosive ascent of biotechnology, the cellular and molecular basis for the action of many drugs has been uncovered.

Today, the drug designer benefits from an accumulated base of scientific knowledge concerning, for example, the interactions between neurotransmitters and their receptors, the regulation of hormone secretion, and the sensitivity of tumor cells to specific kinds of chemicals.

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New technology and biological insight have led to new classes of therapeutic and prophylactic agents. More complex agents such as chimeric antibodies, gene-based drugs, antisense oligonucleotides, and virus like particles, are emerging as clinically viable entities. New clinical approaches involve cells as well as molecules; the introduction of genetically modified cells into humans has blurred the distinction between conventional pharmacology and transplantation.

This wealth of new technology and the resulting new armaments in the war against disease will incite new strategies for drug and vaccine administration. Most current methods for drug administration are direct descendants of ancient practices, and have changed little over the last few centuries.

Pills and injections have enabled significant medical advances to be made; these methods are inadequate for the delivery of recombinant proteins, which frequently have short half-life, poor permeability in membranes, and serious toxicity when delivered systemically in large doses. Similarly, general methods for the delivery of gene-therapy vectors are still unknown since the uptake, biodistribution, expression, and toxicity of oligonucleotides have yet to be systematically studied. Like the new rational approaches to drug and vaccine design, new delivery technologies must exploit findings from basic design.

Biocompatible polymers have become a critical component in the design of delivery systems. Polymers have long been used in experimental drug delivery systems to provide controlled release of small organic molecules or macromolecules. Now, many decades after their first description, some polymer-based controlled drug delivery systems are approved for use in humans. The promise of polymeric controlled release systems is still largely unrealized, because marriages between the new technology and appropriate clinical problems have been difficult to manage.

Chapter 2

2.2 Gene Therapy – An Introduction:

In the past few years, “gene” has become a household word. We know that we inherit genes from our parents, that genes are made up of DNA (Deoxyribonucleic Acid), and that changes in DNA can cause disease. The popular media has gone a long way in educating the public about the connection between abnormal genes and disease. Unfortunately, for many genetic diseases, current medical treatments are inadequate.

Gene therapy, as first proposed 1972 by Friedman and Roblin¹⁹, aims at the delivery of nucleic acids (DNA or RNA) into target cells in order to cure patients suffering from different diseases. The transferred nucleic acids are mostly used to turn on or restore a gene function (‘gain of function’). A relatively new field in gene therapy applies nucleic acids to suppress specific gene functions (‘loss of function’) by turning off genes with antisense oligonucleotides or double-stranded small interfering RNA (siRNA).

The use of genes in the treatment of diseases provides a whole new type of medical therapeutics. Gene therapy could alleviate or even cure a currently untreatable disease. Alternatively, it could provide a more effective treatment where some therapeutic options already exist. Gene therapy is unique in that it has the potential for removing the cause of the disease rather than treating only the symptoms. With the promise of gene therapy on the horizon, the seriously ill have renewed hope that they might live to profit from the new technology.

The term “human gene therapy” describes the medical procedures that use DNA in the therapeutic treatment of human diseases²⁰. The two basic types of human gene therapy are:

- **Human Germ Line Gene Therapy (HGLGT)**, where transferred genes will eventually reside in most cells of the body, including the germ cells (eggs and sperm); or

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- **Somatic Gene Therapy (SGT)**, where genes are introduced into somatic (not germ line) cells and are not destined to pass on genetic information to future human generations.

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2.3 Diseases and therapeutic strategies:

Gene Therapy offers therapeutic benefit to patients in a number of ways (Table 1). This includes replacing a mutated, non-functional gene as for example in Cystic fibrosis, stimulating an immune response as in Aids's patients or by taking a number of genetic factors in the treatment of cancer.

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Table 1.Candidate diseases for gene therapy²¹			
GENETIC			
Disease	Defect	Incidence	Targets
SCID	ADA in 25% of SCID (SCID/ADA) patients	Rare	Bone marrow cells or T lymphocytes
Hemophilia	A Factor VIII deficiency	1:10,000 males	Liver ,muscle, fibroblasts
	B Factor IX deficiency	1; 30,000 males	Or bone marrow cells
Familial hyper-cholesterolaemia	Deficiency of LDL Receptor	1:1 million	Liver
Cystic fibrosis	Faulty transport of salt in lung epithelium, Loss of CFTR gene	1:3000 Caucasians	Airways in the lungs
Haemoglobinopathies Thalassaemias/sickle cell anemia	Structural defects in alpha&beta globulin gene	1,600 in certain ethnic groups	Bone Marrow cells, giving rise to red blood cells
Gaucher's disease	Defect in the enzyme Glucocerebrosidase	1:450 in Ashkenazi Jews	Bone marrow cells, macrophages
ACQUIRED			
Cancer	Many causes, including Genetic and environmental	1 million /year in USA	Variety of cancer-cell types, liver, brain, pancreas,

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			breast, kidney
Neurological diseases	Parkinson's, Alzheimer's, Spinal cord injury	1million Parkinson's and 4 million Alzheimer's patients in USA	Direct injection in the brain,neurons, glial cells,schwann cells
Cardiovascular	Restenosis Atherosclerosis	13 million in USA	Arteries, vascular endothelial cells
Infectious diseases	AIDS, hepatitis B	Increasing	T cells, liver, macrophages

2.4 Strategies for Gene Therapy:

2.4.1: Ex vivo & In vivo Approaches:

In the *ex vivo* approach cells would be extracted from the patient, transfected with the new gene, expanded in the culture, and reimplanted in the patient²².

In the case of *in vivo* delivery, the gene would be treated as a pharmaceutical agent and administered directly to the patient. In this option, the delivery system would have to avoid the body's defense mechanism, reach the cell to be treated, and transfect the cell insitu²³.

2.5 Technical Aspects of Gene Therapy:

The most fundamental requirement for gene therapy to be successful is that a therapeutic gene can be effectively delivered to a target cell. Once delivered that gene must enter the nucleus of cell wall, where it will act as template for production of protein molecule. The protein then exerts the primary therapeutic effect. This may be, for example, cell killing in the case of tumour therapy or cell preservation in the case of neurogenerative disease.

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2.6 Gene Delivery Systems:

Although gene therapy has not yet been established as standard treatment, it was already applied in various clinical studies, e.g. in the field of cancer therapy (most clinical trials), monogenic diseases (Hemophilia A and B, cystic fibrosis, severe combined immunodeficiency syndrome (SCID)), infectious diseases, vascular diseases, or DNA vaccination ²⁴. Gene therapy may hold the potential to revolutionize modern molecular medicine, provided that appropriate nucleic acid delivery systems ('vector systems') are available.

Currently used delivery systems can be divided into viral and non viral vectors. A variety of viral and non-viral systems are being used experimentally each with distinct advantages and disadvantages (Table 2).

Table 2 Viral and Non-Viral gene delivery systems²¹				
Gene delivery system	Size limitation of genes	High level of sustained gene expression	Scale up problems	Safety problems
Cationic Liposomes	No	No	No	No
Cationic Polymers	No	No	No	No
Naked DNA	No	No	No	No
Retroviruses	Yes	Yes	Yes	Possible mutagenesis
Lenti viruses	Yes	Yes	Unknown	Possible autogenesis

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Adenoviruses	Yes	No	No	Yes
Adeno-associated viruses	Yes	Yes	Yes	Yes

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2.6.1 Viral vectors:

As viruses are natural transfection systems, early exploration of gene therapy mainly focused on this vector. This system consists of viruses that infect cells but are genetically engineered to prevent replication. The replication – incompetent virus contains the coding sequence for a therapeutic gene or set of genes within the viral genome. Upon infection, viruses can be subdivided into integrating and non-integrating. Integrating viruses insert the viral genome into the host DNA, whereas the viral genome from non-integrating virus exists as an episome in the nucleus of infected cells. Examples of non-integrating virus are adenovirus and while retrovirus and lentivirus²⁵ are integrating virus.

Generally, viral transfection systems show high transfection efficiency. However, they show serious drawbacks, for example:

- Repeated administration (transient transfection) is hindered by their immunogenicity
- They show limited transfection specificity
- Limited capacity for the gene
- Activation of proto-oncogenes upon “at random” insertion into the genome is possible, and
- The vectors are difficult to handle pharmaceutically, including problematic scale-up and lack of control over reproducibility.

For the above reasons, there is an increasing move toward developing non-viral systems for DNA delivery.

2.6.2 Non viral vectors:

Non viral delivery systems can circumvent some of the problems associated with the viral vectors and are emerging as favorable alternatives to viral vectors. Advantages of non viral vectors include their low immunogenicity, low acute toxicity, simplicity, unrestricted plasmid size and feasibility to be produced in large scale. There are however, some drawbacks with these non viral vectors, including their lower gene expression than

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viral vectors in gene transfer and their transient gene expression. The commonly used systems include the following,

(1) Direct DNA delivery — the simplest non-viral gene delivery system uses naked DNA expression vector. This method was useful in delivery of DNA into skeletal muscle ²⁶, liver ²⁷, air ways ²⁸, and tumors ²⁹. However, naked DNA goes through rapid degradation upon systemic administration. Although direct injection of plasmid DNA has been shown to lead to gene expression, the overall level of expression is much lower ²⁸ than viral or liposomal vectors.

Other delivery modes include bioballistic bombardment or gene gun. The gene gun uses gold particles coated with DNA, which are transferred to a Mylar carrier sheet. An electric arc generated by a high voltage discharge accelerates the DNA-coated gold particles to high velocity, enabling the effective penetration of target organs in vivo or single cell layers invitro.³⁰.The major draw back in this system is that the target tissues must be surgically exposed, although the DNA transfer is relatively efficient. Direct DNA delivery has also led to the development of the DNA tumor vaccines ³¹

(2) Encapsulated DNA — Encapsulation of DNA into neutral and anionic liposomes has also been proposed as non viral delivery system. An anionic liposome cannot externally bind negatively charged DNA, which must be encapsulated, allowing cell-specific targeting. Therefore the DNA size to be encapsulated is limited ³²

(3) Artificial chromosomes — the most recently described non viral vector is the mammalian artificial chromosome. After diagnosis of a genetic defect, introduction of the mammalian artificial chromosome into a subset of blastomeres by microinjection would allow a sufficient population of cells to express the transgene to eliminate the genetic deficiency ³³

(4) Self assembled complexes — the self-assembled complex is the most commonly employed non viral strategy. This strategy includes primarily the use of complexes consisting of DNA and cationic lipids or cationic polymers.

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Self-assembled non viral vectors

Cationic polymers and cationic lipids are capable of spontaneously forming complexes with DNA after removal of small counter ions from both cationic carriers and DNA (a thermodynamically favored step, which drives and stabilizes complex formation)³⁴⁻³⁵

Cationic polymers

Cationic polymers are commonly used in gene delivery because they can easily complex with the anionic DNA molecules.³⁶ Polymer-DNA complexes, also known as polyplexes, can be used to deliver DNA into cells. The general mechanism of action of polyplexes is based on the generation of a net positively charged complex owing to electrostatic interaction of these cationic polymers with anionic DNA.³⁷ The cationic polyplexes can then interact with the negatively charged cell surface to improve DNA uptake. Versatility of physicochemical properties and easy manipulation are some of the most important advantages of polymeric gene carriers.³⁸ Polymeric matrices with varying properties can be designed by choosing an appropriate distribution of different molecular weights and degree of cross-linking of the polymer, and/or by the incorporation of targeting ligands.³⁹ Agents such as folates, transferrin, antibodies, or sugars such as galactose and mannose can be incorporated for tissue targeting. Industrial-scale manufacturing is feasible at low cost. Commonly used polymers include PEI⁴⁰, PLL,⁴¹ chitosans,⁴² and dendrimers.³⁹ Polymeric transfection systems have advantages, but they are not ideal. In addition to low transfection capabilities, polymeric delivery systems suffer from problems in the control of molecular weight distributions, dispersities of the polyplexes, and other quality control issues.³⁹ Some polymers have inherent potent pharmacological properties (such as hypocholesterolemia induced by chitosans) that make them extremely unfavorable for human use.⁴³

Poly (L-lysine) (PLL)

PLL polymers are one of the first cationic polymers employed for gene transfer⁴⁴. PLL is synthesized by polymerization of the N-carboxy-anhydride of lysine, fractionated and

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characterized in terms of the average degree of polymerization and average molecular weight. They are linear polypeptides with the amino acid lysine as the repeat unit, they are biodegradable in nature. This property is very useful for in vivo applications. However, successful transfection requires co-application of chloroquine, a lysoosmotropic agent, which reduces the lysosomal degradation of lipoplexes⁴⁵ PLL usually serves as a means of condensing DNA into a manageable unit for cellular uptake.

Cationic PLL interacts with anionic DNA molecules and forms a positively charged complex that can interact with the negatively charged cell surface and undergo rapid internalization.³⁸ However, PLL has a low level of transfection efficiency, primarily owing to lack of rapid release of PLL-DNA complexes from endosomes. It was established⁴⁶ that PLL-DNA complexes undergo biodistribution into acidic lysosomes that favor DNA degradation upon cellular internalization. PLL suffers from immunogenicity and toxicity caused by its amino acid backbone.⁴⁷ PLL-DNA complexes also undergo nonspecific binding to cell membranes, thereby limiting their use in cell targeting.⁴⁸ Consequently, PLL has been used in gene delivery as an adjunct to graft copolymers and other endosomolytic agents to improve their uptake and targeting. For example, it was demonstrated that PLL poly (lactic-co-glycolic) acid (PLGA) grafted micelles had high transfection efficiencies and low cytotoxicities compared with PLL alone.⁴⁹ PLL transfection capacity was significantly improved by pretreating the PLL-DNA complex with peptides and an endosomolytic agent such as chloroquine.⁵⁰ Targeting of PLL to liver was significantly improved by its conjugation to a liver tissue-specific ligand, asialoorosomucoid.⁵¹ From a formulation perspective, PLL-DNA complexes demonstrated a high degree of polydispersity, leading to unreliable cellular DNA delivery.³⁷

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Chitosan

Chitosan consists of glucosamine units ((1, 4) - linked poly (2-amino-2 deoxy-B-D-glucose or poly (D-glucosamine))). Mumper⁵² was the first to propose to deliver gene into cell using chitosan as a vector.

Chitosan is a natural biodegradable polymer that is a possible alternative to PEI owing to its low toxicity.^{53,54} Like PLL, chitosan generates a positively charged complex upon interaction with DNA that favors its cellular interaction and subsequent uptake. The transfection efficiency of chitosan is; however, lower than that of other polymers such as PEI, as observed by a comparison study consisting of intratracheal administration of chitosan-DNA polyplexes to mice *in vivo*.⁵³ Chitosan has been used in nonconventional gene delivery. For example, chitosan was recently used⁵⁵ to augment the *in vitro* infectivity of an adenoviral vector in Chinese hamster ovary cells. Topical application of chitosan microparticles⁵⁶ containing the luciferase gene has been demonstrated to express the reporter gene in mouse skin, suggesting a potential use of chitosan for genetic immunization. Chitosan-DNA nanoparticles, upon oral administration, were capable of conferring immunological protection to mice against a peanut antigen, indicating a possible use of Chitosan in delivering genetic vaccines to control food allergens.⁵⁷ However, chitosan at high doses can cause hypocholesterolemia in humans⁴³, thereby limiting its applications.

Dendrimers

Dendrimers have an attractive architecture for gene transfer because their well-defined structure and robust chemistry enables the synthesis of many generations of protonatable amines. These molecules can be prepared by divergent (starting from a central core molecule) or convergent (starting with what will become the periphery of the molecule, building inwards) synthesis strategies. Polyamidoamine (PANAM) dendrimers represent a novel class of polycationic synthetic polymers that can be used for gene transfer.⁵⁸⁻⁶⁰ The 3-dimensional spherical structure of dendrimers offers synthesis control of the molecule in terms of degree and generation of branching. This

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control can produce polymer particles with a very low degree of polydispersity, which is a significant advantage over other polymers such as PLL that generate highly polydisperse particles. Low polydispersity can lead to reproducible gene delivery and a clinically reliable formulation. The cationic amino acid residues in the polymeric structure of PANAM dendrimers can help in DNA condensation and endosome release.³⁹ Superfect and Polyfect (Qiagen, Valencia, CA) are two commercially available sixth-generation branched activated dendrimer formulations for in vitro gene delivery.³⁹ Some reports⁵⁸ suggest that lower generations also can be effective in gene delivery. Dendrimers that protect oligonucleotides from serum nucleases⁶¹ have been used to enhance oligonucleotide delivery.⁶²

Polyethylenimine (PEI)

PEIs were first introduced by Behr in 1995⁶³, and have become one of the gold standards of nonviral gene delivery. PEI is an organic macromolecule with the highest density of protonable amine functions and is therefore ideal to condense nucleic acids into particles of nanometer range⁶⁴. The amino groups allow easy chemical modification, and different biologically active moieties such as cell targeting ligands or compounds promoting intracellular delivery have been coupled to PEI⁶⁵

The basic unit of PEI has a backbone of two carbons followed by one nitrogen atom. It is available in both linear and branched forms. The branched form of PEI contains, primary, secondary and tertiary amines, each with the potential to be protonated. This helps PEI to serve as an effective buffer through a wide range of pH. Since these compounds mimic viral delivery functions, such complex vector systems carrying multiple functional domains have been termed ‘artificial viruses’.⁶⁵

The most striking difference between cationic lipids and cationic polymers is the ability of the latter to more efficiently condense DNA.⁶⁶⁻⁶⁷ The condensation process of DNA with the polycation PEI has been studied extensively. The particle size of DNA/PEI complexes depends on the molar ratio of PEI nitrogen to DNA phosphate (N/P ratio) and

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on the salt concentration. Small individual particles are formed at low salt concentration (< 50 mM NaCl) and/or N/P ratios above 5, whereas formation of large aggregated particles is observed in the presence of salt (> 50 mM NaCl) at lower N/P ratios⁶⁸⁻⁶⁹ The net positive surface charge and an excess of free PEI during complex formation can prevent aggregation by repulsion of positive charges, whereas an increase in salt concentration reduces the hydration layer around the particles and promotes particle aggregation. High molecular-weight polyethylenimine (PEI, 25 kDa) condensed plasmid DNA to a similar extent compared to this low-molecular-weight counterpart (5 kDa) at the same N/P ratio⁷⁰. Using atomic force microscopy, Dunlap and colleagues estimated that the size of polyplexes formed between branched 25-kDa PEI or linear 22-kDa PEI and DNA ranged from 20-40 nm when the charge N/P ratio was 1.6⁶⁴. At this ratio, DNA was completely condensed. Using photon correlation spectroscopy, Kunath and colleagues found that polyplexes between 25-kDa PEI and DNA at a ratio N/P of 6.7 had a size approximately 150 nm⁷⁰ The difference in size can be due to the different ratios used in the study.

Highly branched PEI [e.g., 25-kDa (Aldrich) and 800-kDa (Fluka)] and linear PEI are most frequently used⁷¹ and are found to be capable of transfecting cells efficiently *in vitro* as well as *in vivo*. The PEI polyplexes is also capable of transfecting nondifferentiated COS- 1 cells; however, it can also be extremely cytotoxic due to induction of apoptosis.⁷⁵

PEIs offer a significantly more efficient transfection and protection against nuclease degradation than other polycations, e.g., PLL, possibly due to their higher charge density and more efficient complexation. The high amount of positive charges, however, results in a rather high toxicity of PEI polymers. The toxicity and the fact that these polymers are not biodegradable are limiting factors, especially for its *in vivo* use.^{72,76}

The high density of primary, secondary, and tertiary amino groups exhibiting protonation only on every third or fourth nitrogen at pH 7, confers significant buffering capacity to the polymers over a wide pH range. This property, known as the "proton

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sponge effect"⁷⁷ is probably one of the most important factors explaining the high transfection efficiencies obtained with these polymers. Behr and colleagues proposed the proton sponge effect. The hypothesis postulates that at physiological pH only 1 to 6 nitrogen atoms are protonated. Upon lowering the pH, e.g., in endosomes, the proportion of protonated nitrogen increases and generates a charge gradient, which induces a Cl⁻ influx. The increase in Cl⁻ concentration induces a water influx and ultimately endosomal swelling and rupture. Indirect evidence that supported the hypothesis included a remarkable decrease in the transfection efficiency of PEI-containing polyplexes when the cells were treated with drugs that prevent endosome acidification, such as chloroquine.⁷⁸⁻⁸⁰ The cytotoxicity and transfection efficiency of PEI are directly proportional to its molecular weight.⁸¹ Efforts to reduce the toxicity by synthesis of PEI with graft copolymers such as linear poly (ethylene glycol),⁸² incorporation of low molecular weight PEI, and PEI glycosylation⁸³ are under way.

Not only the molecular weight of the PEIs, but also the degree of branching plays an important role for biological properties of complexes with nucleic acids. Linear PEIs⁸⁴ have been synthesized and investigated⁸⁵, and it was demonstrated that linear PEI of 22 kDa, e.g., ExGen™ 500 (Euromedex, France), displays excellent transfection efficiency⁸⁵⁻⁸⁸. Linear PEI has recently been reported to mediate a cell-cycle-independent nuclear entry of plasmid DNA.⁸⁹ This finding is of particular importance in the therapy of slowly-dividing tissues.

A synergistic effect on the transfection efficiency by the combined use of polycations and cationic liposomes was found by Lee et al.⁹⁰ The highest expression level was achieved by using the combination of PEI 25 kDa (0.65 Ag/Ag of DNA, N/P ratio =4.5) with 10 nmol of DOTAP-Chol. This formulation dramatically increased the luciferase or GFP expression 10- to 100-fold, which was much higher than those of other polycations alone, cationic liposomes alone or the combination.⁹¹

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Figure 2.1 Structures of cationic polymers commonly used for gene delivery (Adapted from [92])

Cationic liposomes

Liposomes have emerged as one of the most versatile tools for the delivery of DNA therapeutics⁹³⁻⁹⁶. Liposomes are vesicles that consist of an aqueous compartment enclosed in a phospholipids bilayer. If multiple bilayers of lipids are formed around the primary core in a concentric fashion, the complex assemblies that are generated are known as multilamellar vesicles (MLV). MLV can be formed by reconstituting the lipid films in buffer. Small unilamellar vesicles (SUV) of specific size (100-500 nm) can be produced by extruding MLV through polycarbonate membranes. SUV (50-90 nm) can be produced by sonication of MLV or larger SUV. Both hydrophilic and hydrophobic

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drugs can be trapped in the liposomes. Liposomes and drug/lipid complexes have been used for the delivery of the anticancer drugs doxorubicin and daunorubicin.⁹⁷

Liposomes can be used as DNA drug delivery systems either by entrapping the DNA-based therapeutics inside the aqueous core or complexing them to the phospholipids lamellae. Anionic lipids are not considered suitable for use in gene transfer, and are not researched as popularly as their cationic counterparts. However, many experiments dealing with the usage of anionic lipids for gene delivery are conducted and some progress has been made.⁹⁸⁻¹⁰¹

Since their introduction approximately 15 years ago, cationic lipids have attracted significant interest because of their promise in gene delivery as non viral carriers of genetic material into living cells.^{102,103}

Cationic liposome formulations generally consist of mixtures of cationic and zwitterionic lipids^{96,104,105} a typical cationic amphiphile generally comprises three important elements: cationic head group, hydrophobic anchor, and linker. The positively charged head group is necessary for binding and complexation of nucleic acid phosphate groups. The lipid anchor is typically either a fatty chain (e.g. derived from oleic or myristic acid) or a cholesterol group, which determines the physical properties of the lipid bilayer, such as flexibility and the rate of lipid exchange. Structural variations in the hydrophobic domain of cationic lipids include the length and the degree of nonsaturation of the alkyl chains¹⁰⁶ Being a bilayer lipid, it also helps in assembling the lipids into a polycationic scaffold as well as in facilitating absorptive endocytosis and/or fusion with cell membranes. The linker group is an important determinant of the chemical stability, biodegradability, and transfection efficiency of the cationic lipid. The linker group provides sites for the introduction of novel side chains to enhance targeting, uptake and trafficking.

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Figure 2-2 Structure of common lipids used as materials for gene therapy. Cationic lipids: DOTMA: [2,3-bis(oleoyl)propyl]-trimethyl ammonium chloride; DMRIE: 1,2-dimyristyloxypopyl-3-dimethyl-hydroxyethyl ammonium bromide; DODAB: dioctadecyldimethyl ammonium bromide; DOTAP: 1,2-diacyl-3-trimethylammonium propane; DC-Chol: 3[N-(N0,N0-dimethylaminoethane)-carbamoyl] cholesterol. Neutral Lipids: DOPE: dioleoylphosphatidyl ethanolamine; DOPC: dioleoylphosphatidyl choline; Chol: cholesterol.

(Adapted from 110)

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All the cationic lipids are classified according to number of positive charges, nature of linker bond, and nature of hydrophobic anchor. A direct correlation between the nature of the linker group of the cationic lipids and their potential toxicity was also demonstrated. Lipids with stable ether linkages (DOTMA, DMRIE) are more toxic than those containing labile ester linkages (e.g. DOTAP).¹⁰⁶⁻¹⁰⁸

A good number of liposomal formulations such as Lipofectamine (Invitrogen, Carlsbad, CA), Effectene (Qiagen, Valencia, CA), and Transfectum (Promega, Madison, WI) are available commercially¹⁰⁹ but most of the kits are useful only for in vitro experimentation.

Mixing of DNA with cationic lipid results in the collapse of DNA to form a condensed structure –termed lipoplex –in which nucleic acids are buried within the lipid. Electrostatic interactions between the positive charges of the cationic head groups and the phosphate DNA backbones are the main driving force for the lipoplex formation. The colloidal properties (size, stability) of the lipoplexes are principally determined by the cationic lipid/DNA charge ratio and not the composition of the lipid or the helper lipid¹¹⁰. The charge ratio (+/-) is defined as the number of amines on the cationic lipid relative to the number of phosphate groups on the DNA. A neutral charge ratio is to be avoided as it results in larger particle size (> 1 μ m).¹¹⁰ It has been proposed that lipoplex with a net positive charged surface¹¹¹ interacts more efficiently with the negatively charged cell surface. Another reason could be related to the size of the lipoplexes prepared at different ratios. At high positive or negative charge ratios, homogenous, relatively small aggregates are formed. At charge ratios close to neutrality, or with slight excess of positive charges, larger aggregates and sometimes precipitation of the lipoplexes have been reported.¹¹²

The order in which the lipid and the DNA is mixed is critical^{110,113} With addition of DNA to lipid, a gradual size increase is noted. With addition of lipid to DNA, the particle size remains roughly constant until the amount of lipid positive charge exceeds the nucleic acid negative charge, whereupon the particles grow rapidly in size.¹¹³

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Generally the *in vitro* transfection efficiency of polyplexes or lipoplexes depends on a large variety of factors including complex preparation (e.g., concentration of the carrier and DNA solutions, order of mixing, ionic strength of the solutions, dose, charge ratio, and speed of mixing), the cells (e.g. cell type, confluency of cells, cell physiological state, degree of cellular metabolism, rate of division and cell cycle) and the composition of the incubation medium, and the time of incubation.

Cationic lipids form micellar structures with DNA. The structures fuse with the cell membrane, sometimes after interactions with surface proteoglycans. The complexes are internalized by endocytosis, resulting in the formation of double-layer inverted micellar vesicle. During maturation of the endosome into a lysosome, the endosomal wall might rupture, releasing the contained DNA into the cytoplasm and potentially towards the nucleus. DNA imported into the nucleus might result in gene expression. Alternatively DNA might be degraded within the lysosomes.

The transfection efficiencies of non viral cationic liposomal vectors are significantly lower than those of viral vectors. Low transfection efficiencies have been attributed to the heterogeneity and instability of cationic lipoplexes.¹¹⁴ Another drawback of the cationic lipids is their rapid inactivation in the presence of serum. Lipofectin mediated transfection was inactivated by serum.^{105,115} In addition, most of the *in vitro* studies that demonstrate the transfection efficiency of cationic lipid based systems were conducted in standardized media formulations such as Opti-MEM (Invitrogen) that do not contain serum. Therefore, *in vitro/in vivo* correlation is misleading and unreliable. Some *in vivo* studies have revealed that the gene transduction responses were short-lived and transient.^{116,117}

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Also, despite the appreciable success of cationic lipids in gene transfer, toxicity is of great concern. Cytotoxicity of cationic lipids has been established in numerous *in vitro*^{118, 119} and *in vivo*^{121, 122} studies. Using a battery of cell proliferation assays (such as [3H] thymidine incorporation) and cytotoxicity tests it was demonstrated that cationic liposomes composed of dimethyldioctadecyl ammonium bromide (DDAB)-DOPE and the commercial transfection-reagent DOTAP were cytotoxic to CaSki cells, a human cervical cancer cell line.¹³⁴ Cationic liposomes were also confirmed to cause dose-dependent pulmonary toxicity in mice upon intratracheal instillation.¹²¹ The study demonstrated that cationic liposomes stimulated the production of reactive oxygen intermediates that have been previously implicated in pulmonary toxicity.¹²¹ Cationic lipids have also been demonstrated to be cytotoxic to phagocytic macrophages.¹³⁷ Reduction in toxicity was observed upon the replacement of DOPE by 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), possibly suggesting the role of the helper lipid DOPE in the toxicity of cationic liposomes.¹²² In other studies, cytokine-mediated pulmonary toxicity and TNF- α induction by cationic lipids in lung tissue have been identified.¹³⁸ Routine transfer of genetic drugs using cationic lipids is impossible without the elimination of these obstacles. Novel synthetic strategies and formulation approaches may yield progress in this direction.

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Figure 2.3 Lipoplex mediated transfection and endocytosis (Adapted from 110)

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2.7 Intracellular Gene Delivery:

The introduction of genetic material into eukaryotic cells has been a major technique to investigate the gene function and the regulation of gene expression^{124,125}. A variety of methods have been developed to accomplish gene transfer in eukaryotic cells.

Various cellular transport systems exist for uptake of macromolecules into the cell. These include pinocytosis¹²⁶ and several receptor mediated mechanisms.¹²⁷ During pinocytosis, a portion of the membrane invaginates and pinches off a vesicle inside. Materials taken up are directed to the lysosomal compartment. This is degradative that is acidic [pH 4.5-5.0], and contains a comprehensive collection of concentrated enzymes at high concentrations involved in breakdown of most biological materials.

The principal receptor –mediated mechanism comprises attachment of ligand to receptor that is associated with, or that becomes associated, with a coated pit.¹²⁷ The coated pit is a specialized area of membrane, containing a particular protein clathrin, which is triggered to form an endosome when a ligand attaches to its receptor. Most coated pits are believed to form endosomes of 100 nm diameter, but they are reported to be larger in some tissues. Other ligand mediated uptake routes¹²⁸ not involving clathrin are also thought to exist. For all these routes of uptake, the size of particles for uptake is likely to be critical. Even when taken up by these endocytic routes, DNA is still separated from the cytoplasm by nuclear cell membrane. An early exit from the endosomal or lysosomal compartment is necessary, both to avoid enzymatic degradation and to reach its target. A mechanism that enables DNA to cross a membrane is therefore essential.

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Figure 2.4 Receptor mediated endocytosis (Adapted from 126)

However, once uptake is achieved, transfer from the endosomes to the nucleus is the rate-limiting step.¹²⁹ Efficient destabilization of the endosomes¹³⁰ and the use of endoosmolytic agents such as chloroquine, certain peptides and adenovirus particles¹³¹ enhance protein expression. The effectiveness of nitrogen –rich polymers such as Polyethylenimine has been ascribed to the ability of this polymer to **reduce the pH** of the endosomal compartment¹³² as well as to facilitate DNA escape from this area¹³³. Intracellular trafficking and the processing of the associated plasmid DNA across intracellular barriers are relatively inefficient processes. Only a minute fraction of the 10^4 - 10^5 plasmid DNA molecules that are usually delivered to a cell in vitro will eventually enter the nucleus^{134,135} and actually be expressed. Improving the efficiency of

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these processes would have a profound effect on the efficiency of non-viral gene delivery systems.

Translocation of DNA from the cytosol to the nucleus is also difficult for plasmids alone, which are transported through the nuclear pore complex¹³³ in an energy dependent manner. The cut-off of the nuclear pores (9-10 nm diameters) is in the order of 300 base pairs for nucleotides; however the transport of the larger molecules is an active process that can be modulated by the inclusion of nuclear localization signals (NLS). In general, NLS are (basic) peptide and protein sequences that are recognized by the cellular machinery^{134, 135} triggering their energy dependent uptake. NLS such as SV40 large T antigen nuclear localization signal peptide (NLS or NLS/NL)¹³² or EBV oriP sequences^{130,133} can be covalently linked to DNA to promote nuclear accumulation of the conjugated DNA. Indeed Zanta¹³¹ and others¹³¹ report a 10 to 1000-fold transfection enhancement as a result of the signal peptide irrespective of the cationic vector or the cell type used.

Figure 2.5 Schematic drawing of DNA delivery pathways with three major barriers: low uptake across the plasma membrane, inadequate release of DNA molecules with limited stability, and lack of nuclear targeting. (A) DNA–complex formation. (B) Uptake. (C) Endocytosis (endosome). (D) Escape from endosome. (E) Degradation (endosome) (F) Intracellular release. (G) Degradation (cytosol) (H) Nuclear targeting. (I) Nuclear entry and expression. (Adapted from 127)

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2.8 Summary: (Gene Delivery Systems)

Regardless of the type of carrier system used, a gene delivery vector must fulfill certain requirements if the therapeutic biopolymer is to reach and exert an influence on the target site. A DNA delivery vector must:

1. Reach and bind to the target cell surface.
2. Be internalized by the cell (e.g. by endocytosis, phagocytosis)
3. Escape from sub-cellular compartments (endosomes) and be transported to the nucleus
4. Express the gene product in the target cells

In order to accomplish these tasks the vector must also condense DNA into particles small enough to enter the target cell, protect DNA from degradation by serum nucleases, and must be non-toxic to the host. The barriers to gene delivery are shown below.

Non-viral systems must rely on cellular mechanisms (non-specific or receptor mediated) to import DNA into the nucleus. Although uptake, transport into the cell nucleus and transfection are inherently inefficient for DNA associated with non-viral vectors (and much less efficient than viral systems), even low levels of gene expression can be sufficient to produce the therapeutic response. However, synthetic vectors can be tagged with nuclear localization sequences and cell targeting ligands in exactly the same way as has been carried out with the polymer-drug conjugate to improve transfection efficiency.

Since the most important aspects of non viral vectors for gene therapy are transfection efficiency and toxicity, many new non viral vectors were synthesized aiming to improve transfection efficiency and to lower toxicity. Different vectors will show different transfection efficiencies depending on the cell type and the transfection conditions (e.g., in vitro versus in vivo). A given lipoplex or polyplex may show high transfection efficiency in a given cell type under specific conditions under which others are less efficient. Each vector will be considered for specific applications and it is unlikely that a universal vector will emerge.

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2.9 Biodegradable Polymers – An Introduction:

An increasing need for the modulated delivery of both conventionally and biotechnology generated drugs of high molecular weight and short half-life has generated considerable interest in the development of biodegradable polymers^{136, 137} and their formulation into drug delivery systems. The use of biodegradable polymers confers the inherent advantage of alleviating the need for surgical removal of the delivery system at a later rate.

Biologically degradable polymers are defined as a class of polymers that degrade to smaller particles due to the presence of chemicals inside the body. **Biodegradable polymers** and **Bioabsorbable polymers** are the two kinds of degradable polymers that have the ability to breakdown in this manner. “**Biodegradation**” can be described as degradation that is promoted by **enzymes**. “**Bioabsorbable**” polymers are those that are degraded by other chemicals in the body. In the discussion that follows, we will not differentiate between the two modes of degradation, and refer to all degradable polymers as biodegradable polymers.

Biodegradable polymers are normally used controlled release systems. A great number of biodegradable polymers have been developed and evaluated for drug delivery usage, but the most widely investigated biodegradable polymers and systems achieving the most success at this time are lactic / glycolic acid polymers. Lactic / glycolic acid polymers are biodegradable. The products of degradation are non-toxic, non-carcinogenic, and non-teratogenic. In general, they do not cause inflammatory reactions in tissues. These polymers are easily processed, have good mechanical properties, and are sterilizable.

PLGA has been successful in drug delivery formulations because their degradation can range from 3 weeks to over a year depending upon the composition of the polymer¹³⁸⁻¹⁴⁴ as well as the method of preparation and formulation.

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The low molecular weight PLGA can be synthesized from lactic acid and glycolic acid by condensation polymerization, and normally called poly (lactic-co-glycolic acid). High molecular weight PLGA is synthesized from **lactide and glycolide by ring opening polymerization and always called poly (lactide-co-glycolide).**

2.10 Polymeric Gene Delivery:

Polymeric delivery systems for macromolecules and other drugs were developed in response to the limitations of systemic, bolus injections of these compounds. Many large molecules (e.g., growth factors) have short half-lives in vivo, on the order of a few minutes. However these factors must present for prolonged periods (e.g., minutes to days) to elicit a tissue response. One approach to this problem is to provide a stable depot for sustained, continuous release.

The adaption of controlled release technologies for the delivery of non viral and viral vectors has the potential to overcome the extracellular barriers that limit gene therapy. The potential advantages of the controlled release systems include

- (i) Drug levels maintained within a desirable range,
- (ii) Localized delivery to a target cell type to avoid adverse side effects,
- (iii) Decreased dose or number of dosages, and
- (iv) Facilitated delivery of fragile compounds.

Controlled release can maintain high levels of DNA concentrations in the cellular microenvironment, which improves gene delivery.² In addition to this, viral and non viral vectors may have a relatively short half life²⁻⁴, and delivery vehicles can prevent their degradation and /or provide a sustained release.

DNA delivery using polymeric controlled release systems follow the following mechanisms. (i) Polymeric release, in which the DNA is released from the polymer, or

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(ii) substrate mediated delivery, in which DNA is retained at the surface. In polymeric release the DNA from the system occurs through a combination of diffusion and degradation of the polymer matrix. For rapid release of DNA, levels would be expected to rise quickly and decline as the DNA is cleared or degraded. For sustained release the concentration may be maintained within an appropriate range by adjusting the release to replace DNA that is cleared or degraded. In case of solid state delivery the DNA is immobilized to the biomaterial or extracellular matrix, which supports the adhesion and places the DNA directly in the cellular microenvironment. In the above mentioned system the DNA is concentrated at the delivery site. Cells cultured on the substrate can internalize the DNA directly from the surface or by degrading the linkage between the vector and the material.

The interactions between the vector and the biomaterial could be nonspecific, including hydrophobic, electrostatic or van der Waals interactions. These interactions decide whether the DNA is released or is bound to the substrate. These interactions have been well characterized for the protein delivery with biopolymers^{144,145}. Nonspecific binding depends on the molecular composition of the vector (e.g., polymer, protein, and lipid) and the N/P ratio of each. Specific interactions include antigen-antibody or biotin-avidin, on the vector and the polymer.

A variety of natural and synthetic materials have been employed for gene delivery. This includes polyesters and polyanhydrides which are hydrophobic in nature. The hydrophilic polymers include hyaluronic acid (HA), collagen, poly (ethylene glycol) (PEG)). Polymers composed of lactide and glycolide are perhaps the most widely used and recognized biodegradable synthetic polymers because of their accepted use as materials for sutures¹⁴⁶. These polymers are FDA approved and are generally considered as to be compatible. These systems are very attractive for long-term sustained delivery for a number of reasons, including their biocompatibility and controllable degradation into natural metabolites¹⁴⁷. Hydrolytic degradation of the polymers produces lactic acid and glycolic acid, two substances naturally involved in metabolic pathways of the body. The degradation rate can be controlled through the composition of the polymer and

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molecular chain weight of the chains. These polymers exhibit relatively even chain scission through out their bulk after being placed in vivo. These properties have led to the use of these materials for drug delivery and as scaffolds for tissue engineering¹⁴⁸. Drug release from these systems typically occurs by a combination of surface desorption, drug diffusion and polymer degradation.¹⁴⁹

Polyanhydrides polymers were originally developed as vehicles for drug delivery¹⁵⁰. These polymers were synthesized from hydrophobic monomers connected by water – labile anhydride bonds. These polymers exhibit chain scission mainly at the surface of the polymer matrix, and the matrix degrades and loses mass from the exterior to the interior⁷⁹. The material maintains its structural integrity as it degrades making it useful as structural replacements in a load –bearing environment.¹⁵¹

Ethylene vinyl co-acetate (EVAc) is a non degrading polymer. It has been extensively used for release of both low- and high molecular weight drugs, primarily because the diffusion can be fine-tuned by changing the ratio of ethylene to vinyl acetate. The diffusional process for drugs is dependent upon the presence of interconnecting network of channels leading to the external environment. Modifications that affect the network of pores, such as drug loading, polymer molecular weight, and the presence of co-dispersants, can be used to control the release rate of the drug. The presence of pores is a function of drug loading. At low drug loading, complete dissolution of the drug within the polymer prevents rapid release. Higher drug loading or the addition of carrier proteins increases the possibility of an interconnecting network of drug particles with a path to the surroundings. As drug adjacent to the surface dissolves and diffuses out of the matrix, open channels remain that permit drug located within the matrix to be solubilized and released.

Poly (ε-caprolactone) (PCL) is semi crystalline biodegradable polyester with a low melting point and a low glass transition temperature¹⁵². PCL is in a rubbery state at room temperature, which contributes to it being a good candidate for developing a biodegradable reservoir or monolithic device for diffusion-controlled drug delivery,

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especially in the contraceptive field. PCL exhibits certain desirable characteristics for drug-delivery applications, including biodegradability, biocompatibility, commercial availability, etc.^{153,154} Protein release has been studied from PCL films, using PEG as a porogen.¹⁵⁴

Collagens are an interesting class of natural polymer. Among 14 forms of collagen, type I collagen is most widely used as biomaterials for cell growth and drug delivery. Collagen can be lyophilized to form a spongy product or can be formed into a hydrogel by thermal mechanisms or covalent cross linking using aldehydes or carbodiimides.¹⁵⁵⁻¹⁵⁸ Collagen is generally extremely crystalline, and exists mainly as triple-helical structures (tertiary structures) which also form supermolecular ordered states (quarternary structure). When the tertiary and quaternary structures are broken down, by heat or by other means, gelatin is obtained, and it may be considered an amorphous form of collagen.

Figure 2.7 Synthetic Polymers in gene delivery

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Naked pDNA Delivery:

Plasmid DNA being negatively charged interacts weakly with the polymers and hence their release rates are controlled by the polymer properties. Many synthetic and natural polymers are negatively charged and rapid release of pDNA will most likely result from repulsive charge interactions between plasmid and polymer.

Much attention has been focused on biocompatible, biodegradable polymers such as PLGA, for the encapsulation of proteins and peptides¹⁵⁹ and for genes.¹⁶⁰⁻¹⁶⁶ PLGA was selected since it is compatible and biodegradable polymer that is approved for in vivo applications.¹⁵⁹

Controlled release of DNA from suture coating, bone implants, tissue engineering scaffolds and vaccines has been reported.

PLGA polymers can provide release rates ranging from a few days to more than 60 days, with the polymer composition and polymer composition regulating the release¹⁶⁷⁻¹⁷⁰. Also the integrity of the DNA can be affected by degradation of the polymer to lactic acid and glycolic acid.¹⁶⁷ Sustained release and delayed release was achieved with hydrogels composed of PEG and lactic acid segments based on the number of lactic acid units.¹⁷¹ Collagen based materials released plasmid DNA for times ranging hours to days.^{172,173} HA (hyaluronan) based hydrogels also release the DNA, the rate of release can be controlled by the extent of cross linking.^{174,175}

Nanospheres loaded with plasmid DNA represent an alternative to the traditional complexation Polymeric “Nanoparticles” are colloidal particles consisting of macromolecules that vary in size from 10 nm to 1000 nm. They are mainly developed as drug delivery systems as an alternative to liposomal technology in order to overcome the problems related to the stability of these vesicles¹⁷⁶ in biological fluids and during storage.

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In general DNA diffuses from nanoparticles through the pores as opposed to decomplexing from cationic polymer or lipid. Transfection levels achieved in vitro with these nanoparticles are significantly lower than with a bolus delivery of lipoplexes^(177, 178). These nanoparticles provided a sustained release intracellular, showing a substantial increase in expression over a week time period.

Leong and co workers have fabricated nanospheres with natural polymers like collagen and chitosan¹⁷⁹ and a synthetic gene carrier micelle using a biodegradable polyphosphoesters. DNA-gelatin nanoparticles, formed by salt-induced complex coacervation of gelatin and plasmid DNA, have been developed. Transfection from these nanospheres was comparable to that obtained by lipofectamine. TruongLe and coworkers used cross linked gelatin and it provided some protection against nuclease degradation¹⁸⁰. The potential of delivering the CFTR gene to the lung airways was demonstrated by instilling gelatin DNA nanospheres into the lower lobes of adult rabbits. The protein expression is transient. They have also explored the potential of a biodegradable polyphosphoester, namely poly (cholesteryl oxo – carbonylamido ethyl) methyl bis (ethylene) ammonium iodide ethyl phosphate. The particle size is around 100-200 nm and transfection were tried out in HEK 293 and HeLa cells. They were found to be less cytotoxic than the widely used polycationic carrier of poly-L-lysine and lipofectamine.¹⁸⁰

Hsu et al prepared nanospheres containing plasmid DNA using water –in-oil-in-water (W/O/W) double emulsion and solvent evaporation method. PLGA microspheres have been shown to protect DNA from degradation from nucleases.

Labhasetwar and Levy⁷⁹ have investigated sustained release biodegradable nanospheres for the delivery of plasmid DNA-reporter genes for alkaline Phosphatase and Luciferase. Gene transfection was demonstrated in 293-T and COS-7 cells and also in vivo in a non-union femoral fracture (osteotomy) rat model. These nanospheres demonstrated sustained release of the encapsulated DNA under in vitro physiological conditions with an 82% cumulative DNA release over 17 days. The transfection efficiency of the nanospheres in

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tissue culture was two to five orders of magnitude greater than the gene expression with the same amount of plasmid DNA in solution.

Also cationic groups can be attached to the material to promote DNA binding. Collagen was cationized through modification with amino groups or poly L lysine.¹⁸³ Degradation of collagen led to the release of the bound DNA. Denis-Mize¹⁸⁴ et al have used the cationic surfactant (cetyltrimethylammonium bromide) to coat the PLG microspheres and plasmid DNA was adsorbed to it. Transfection was studied in dendritic cells. Also Zheng et al and Sacks et al have used PEI (polyethylenimine) or poly L lysine to bound or blend with the material and some cellular transfection was noted *in vitro*.^{185,186}

Apart from nanospheres DNA was delivered from polymeric scaffolds. Shea et al¹⁸⁷ have used an implantable PLG scaffold delivering 500 ug of DNA. Transfection was noted in cells within and adjacent to the scaffolds. Collagen mini pellets were able to transfect cells intramuscularly for 60 days with 50 ug of DNA¹⁸⁸. Also collagen implanted into a bone defect transfected cells locally with 1 mg of DNA.¹²⁴ Alternatively, plasmid DNA has also been incorporated into inorganic calcium phosphate co-precipitates that are adsorbed onto PLG matrices, and are mostly released within 48 h.¹⁸⁹

Ethylene vinyl acetate discs implanted intravaginally induced gene expression for 28 days.¹⁹⁰ Another study by Jong et al¹⁹¹ using EVAc rods showed that the DNA was released without degradation and it retained the ability to transfect cells *in vitro*. The release of DNA at both low and high loading appears to occur through a complex combination of diffusion and swelling. At higher loadings the EVAc rods were able to release 500 ug of DNA during a month long period with majority of the release occurring in the first one week.

Klugherz et al¹⁹² formulated a plasmid DNA polymeric (PLGA) coronary stent coating that provides localized gene delivery and expression while permitting stent expansion and deployment. They have shown that the DNA transfection (*in vitro*) could be due to the double emulsion method they used. Gene expression was seen in cells which were

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very close to the stent placement region. They hypothesized that the transfection could be due to the DNA present in the microemulsions. When these come into contact with cell membranes will directly transfect cells. The in vivo study showed that the DNA diffused from the polymer coating into the arterial walls.

Liang et al ¹⁹³ devised a novel non-viral gene delivery method using nanofibrous scaffold. A core-shell DNA nanoparticle was developed by solvent-induced condensation of plasmid DNA. This was subsequently encapsulated in a triblock copolymer, polylactide -poly (ethylene glycol)-polylactide in the same solvent environment. A non-woven scaffold was formed from a mixture of encapsulated DNA nanoparticles and biodegradable PLGA. During electro spinning, the polylactide shell provided protection to the encapsulated DNA. The plasmid DNA was released in a controllable fashion and was shown to transfect cells.

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Plasmid DNA complex delivery:

In general it is important to determine whether the stability of the incorporated DNA was maintained during its encapsulation into microspheres and the subsequent scaffold fabrication. However, the plasmid integrity tends toward the open conformation at later times of release. Retention of the supercoiled conformation during encapsulation and release is desired since it can lead to greater gene expression than do the open or linear confirmations¹⁹⁴ Possible methods for minimizing the degradation include co-incorporation of an antacid such as $Mg(OH)_2$ into the process, which has been shown to stabilize proteins by increasing the microclimate pH.¹⁹⁵ Alternatively, the addition of EDTA to the DNA solution of complexation of the DNA with poly (L-lysine) (PLL) or polyethylenimine (PEI) prior to encapsulation can protect the plasmid from enzymatic degradation and maintain the supercoiled structure.^{196,197}

Also to achieve an efficient therapy using a matrix containing non-condensed pDNA, high amounts of the plasmid are required.¹⁹⁸ Condensing with cations needs a low amount of pDNA thus increasing its encapsulation efficiency. They also help to increase the incorporation efficiency.¹⁹⁹ During immobilization; the binding of plasmid DNA to a cationic material may exhibit limited cellular internalization due to the strong interactions between the DNA and the material. To avoid this DNA can be complexed in solution with the condensing agents and immobilized to the polymers. The presence of complexing agents can maintain the stability of DNA complexes.^{200,201}

Dunne et al²⁰² encapsulated protamine sulphate compacted DNA in PLGA micro particles. It was found that DNA complex increased protection against DNA nucleases and decreased shear effects during processing. The released DNA was able to transfect in HEK 293 cells in vitro. Interestingly they found that the DNA release was faster than other investigations.^{203,204} This could be possibly due to high surface loading and /or the presence of basic groups in protamine sulphate which can accelerate degradation/controlled release from PLG/PLGA polymers.^{205,206} Also the encapsulation

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provided greater protection to the DNA during homogenization steps of the solvent evaporation procedure (microsphere synthesis). This could be probably because of the compaction of the DNA most likely reduces the shear forces the DNA molecules is exposed to during the homogenization.

Capan et al.²⁰⁷ produced PLGA microspheres loaded with pDNA /PLL. It was found out that supercoiled pDNA was degraded in the uncomplexed pDNA loaded microspheres. The percentage of supercoiled pDNA in the microspheres was only 16.6% for the uncomplexed pDNA but increased to 76.6% and 85.6% upon 1:0.25(w/w) and 1:0.333 (w/w) complexation with PLL, respectively. Another study by the same group involved the use of polyvinyl alcohol (PVA) as a surfactant to stabilize the particle formation. The particle size decreased from 6.6 to 2.2 μm as the PVA concentration increased from 1-7%. The released pDNA was in the supercoiled form. More controlled and sustained release can be achieved by using a combination of a hydrophobic polymer with high molecular weight and surfactant with low viscosity. But no studies on transfection were reported.

Another study involving PLL complexation in PLGA microspheres²⁰⁸ reported the sustained release of the complexes and the released DNA from the complexes were able to transfect the CHO cells in vitro. The released DNA was then complexed with DOTAP/DOPE transfection reagent, as the PLL complex had broken down upon release. They achieved a similar transfection with that of the fresh control. This suggests that the complexation of free pDNA with PLL was able to maintain the functional activity of pDNA, which seems to be affected during microsphere preparation.

Howard et al.²⁰⁹ studied the formulation and characterization of a PLGA microparticle carrier containing PEI polyplexes for the deposition of intact polyplexes in intestinal lymphoid tissue. PEGylated PEI/DNA complexes were used in this study to avoid aggregation of polyplexes at higher loading (1mg/ml). The polyplexes loaded microspheres showed a high burst effect followed by a slight sustained release. In contrast, almost zero order release was found with the DNA only encapsulated system.

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Significant levels of β -galactisidase expression above background readings were found in the spleen of rats 72 hrs after a single oral dose of the microencapsulated PEGylated polyplexes. Transgene expression in the spleen indicates that the microparticle carrier or the released polyplexes have followed a migratory pathway from the intestine to peripheral sites.

Oster et al.²¹⁰ formulated cationic microparticles by blending poly (lactide-co-glycolide) (PLGA) (50:50), with different cationic agents, either PEI 25 kDa (polyethylenimine) or CTAB (cetyl-trimethyl-ammonium-bromide). DNA adsorbed onto microspheres with 10% or 50% PEI generally had higher transfection efficiencies than CTAB but reached lower expression levels than the PEI/DNA polyplexes alone. This showed the intact releases of at least part of the complexed DNA. The transfection efficiency in non-phagocytic cells was elevated compared to naked DNA.

Cationic PLGA microparticles covalently modified with branched polyethylenimine (PEI) were synthesized by Sudhir et al.²¹¹ Enhanced pDNA loading and enhanced buffering capability were obtained. PEI conjugation did not impart cytotoxicity on the particles and allowed for improved transfection of phagocytic APC's. It was also found that the presence of branched PEI on their surface rendered the particles more hydrophilic leading to accelerated degradation and enhanced release of protein and DNA.

Release of Oligonucleotide/Polyethylenimine complexes from PLGA microspheres were evaluated by De Rosa et al.²¹² It was found that PEI affected the microsphere morphology inducing pore formation yielding an accelerated release. It was also shown that the addition of PEI strongly increased microsphere loading, but also induced a more rapid release profile. Also the encapsulation reduced the cytotoxicity of the complex and an improved intracellular penetration of the released oligonucleotide with the localization in the nucleus, especially when high polyethylenimine concentrations were used.

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Adsorption of PEI/DNA complexes to silica nanoparticles ^(213,214) resulted in transgene expression in vitro comparable to that observed by bolus delivery.

Hyung et al ²¹⁵ studied gene delivery from tissue engineering scaffolds. This type of substrate mediated delivery, an alternative to sustained release, is based on the immobilization of DNA complexes to the polymer surface for subsequent delivery to cells cultured on the substrate. Substrate-mediated delivery from PLGA scaffolds achieved similar or higher levels of transgene expression as bolus delivery with lower quantities of DNA and was able to increase the number of transfected cells relative to bolus. Zain et al ²¹⁶ characterized substrate binding and transfection of DNA polyplexes and lipoplexes that are non-specifically adsorbed to either tissue culture polystyrene or fetal bovine serum coated polystyrene. Serum adsorption was used as a simple approach to modulate the interaction of the complexes with the culture substrate.

Interactions between the complexation agents and the polymer have been adapted to immobilize DNA complexes specifically to a substrate. Poly (L-Lysine) (PLL) and PEI were modified with biotin residues for subsequent complexation with DNA and binding to a neutravidin substrate. ^{217,218} Non specific binding of DNA complexes to substrates has been employed with other systems to mediate delivery. This approach was studied by Bielinska et al ²¹⁹ where the complexes formed with polyamidoamine (PAMAM) dendrimers have been bound onto PLG and collagen membranes coated phosphatidyl glycerol (1-5%)

Polyplexes were entrapped in porous PLGA sponges and showed significant transfection in vitro ²²⁰ ; it was found that at low PEI/DNA loading, the gene transfer is inefficient. At optimal loading the complexes were well dispersed throughout the matrix and transfection is seen in more cells. At higher loading PEI/DNA formed aggregates with in the matrix that sequester the DNA, making it less accessible for gene transfer.

The release of these complexes was also a function of N/P ratio. Low N/P ratios facilitated release from synthetic polymers ²²¹, while high N/P significantly limited

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release^{199,222} At charge ratio of 2:1 or higher, DNA is fully condensed and can no longer bind to PEI, so the excess PEI is retained in PLGA, and may be responsible for the retarded release; on the other hand, there was no toxicity exerted from PEI in these sponges. The PEI would be expected to ionically bind to PLGA, making it less able to crosslink proteins on the cell surface, which leads to membrane disruption.

A novel approach by Segura et al²²³ applied surface tethering of DNA complexes to enhance gene delivery. The cationic polymers helps to condense the DNA for efficient internalization and the surface tethering functions to maintain the elevated concentrations in the cell microenvironment for cells adhered to the substrate. DNA complexes (PLL/DNA) are tethered to surfaces that support cell adhesion thorough the functional groups attached to cationic polymers. Cells cultured on these surfaces were transfected by the surface –associated DNA. The transfected cells are localized to the sites at which the DNA is tethered, potentially providing a mechanism to spatially regulate gene delivery.

Zheng et al²²⁴ blended Poly (ε-CBZ-L-lysine) with poly (D, L-lactic –co-glycolic acid) or poly (L-lactic acid). Films were formed from this. The films were surface modified by the attachment of polyethylenimine (PEI), covalently. Only covalently attached PEI was capable of mediating transfection. Also PEI was attached to commercially available tissue culture polystyrene on which type I collagen had been immobilized. Cells grown over this showed transfection. PEI is known to have a membrane disrupting effect. So the transfection could be due to the concept of entry of DNA into the cells via a membrane disrupting effect rather than the entry of DNA/PEI particles into the cell. However it is also possible that cellular proteases might cleave surface PLL or collagen and thereby release PEI/DNA complexes that enter the cell. Slow release of PEI/DNA from the surface resulting from cleavage of polylysine or collagen by cellular proteases might count for the fact that the transfection rates continued to increase over several days. Plasmid DNA attached to the PEI can transfect cells plated on the surface over several days.

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Collagen-based release may limit vector degradation, and can induce transgene expression for up to 60 days¹⁸⁸ Berry et al²²⁵ showed that GAM (gene activated matrix) retained the DNA within them. DNA complexed with poly-D-lysine (PLL) encapsulated in a collagen sponge was implanted into severed rat optic nerves as a means to promote neuron survival and regeneration. It promoted a sustained expression in optic nerve for 3 months. At present it is not known whether sustained RGC (Retinal ganglion cells) survival reflects sustained expression either from a single transfection event or from repeated transfections from the depot store of DNA in the GAM.

Scherer et al²²⁶ immobilized polyplexes and lipoplexes on collagen sponges and studied their release from the matrix. Substantial transfection was achieved both in vitro and in vivo. PEI/DNA complexes and lipoplexes were slowly released from collagen, and addition of a protective copolymer to the complexes increased release, presumably by decreasing interactions between collagen and the complex. DNA formulated as a copolymer –protected gene vector as opposed to naked DNA allows its use at low dose while retaining the transfecting capacity in the context of carrier mediated gene delivery.⁶

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2.11 Summary:

Gene therapy is considered to be a promising approach to treat life-threatening diseases. The key challenge for plasmid –based gene therapy is to surmount the limiting steps in intracellular movement, including endosomal release, cytoplasmic transport, and nuclear uptake, and to enhance the retention of plasmids in the nucleus.

Gene delivery systems are designed to control the location of the gene within the body by affecting the distribution and access of a gene expression system to the target cell, and/or recognition by a cell-surface receptor followed by intracellular trafficking and nuclear translocation. Gene delivery systems should serve to both protect a gene expression system from premature degradation in the extra cellular milieu and to effect nonspecific or cell specific delivery to a target cell. To achieve this, both viral and non-viral vector systems have been developed over the years. Among the various gene carriers, viral vectors are still the most widely investigated because of their efficiency in transfection. Although viral vectors are highly efficient in introducing DNA into cells, they possess some serious disadvantages, such as the possible induction of the immune responses and problems with pharmaceutical –grade large scale production. Therefore an alternative for viral vectors, attention was focused on the design of non viral vectors. These include cationic polymers such as poly L lysine (PLL), poly (ethylene imine) (PEI), and poly (dimethylamino methacrylate) (pDMAEMA) or cationic lipids such as DOTAP or DOPE. However, the current non viral carriers are less efficient in their transfection activity than viral vectors.

DNA-based tissue engineering is being investigated as a way to grow new tissues and organs. Ideally local delivery of plasmid DNA will allow appropriate levels of transgene expression for a prolonged period. For example, sustained releases of plasmid DNA from PLGA matrices can lead to the transfection of large number of cells at a localized site, leading to the production of therapeutic proteins needed for tissue regeneration.

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While biomaterials based gene delivery offer many advantages over more conventional methods, there are several challenges that relate primarily to the release profile. Since many of the polymers are negatively charged, plasmid DNA (highly negative surface charge density) interacts weakly with these polymers, leading to an early in vitro release.(high burst release) Also for polymers such as PLGA, the degradation rate of the polymer affects the stability of the pDNA, making it to lose its bioactive form, resulting in low transfection efficiencies. To overcome these pDNA is complexed with condensing agents like PEI/PLL/Protamine sulphate or Lipofectamine. These agents help to condense the pDNA, so that less amount of pDNA needed for transfection. Also in some cases the complex protects the pDNA during fabrication steps. They also play a role in protecting the pDNA from degradation by acidic pH (PLGA degradation) and cellular nucleases.

In the studies by Capan et al ²⁰⁷ and Gebrekidan et al ²⁰⁸, it was found that the addition of complexing agents delayed the release from PLGA microspheres. A very low burst effect was noticed. This could be linked to the charge interactions occurring between the polyplexes and PLGA, delaying the release. In contrast to their results, studies by Dunne et al ⁽²⁰²⁾, De Rosa et al ¹⁹⁹, Kasturi et al ²¹¹ and Howard et al ²⁰⁹ showed that PEI/DNA complexes loaded on the microspheres released earlier than pDNA. Various reasons have been forwarded to explain the faster release of complexed pDNA:porosity, in the case of De Rosa et al ¹⁹⁹; for Howard et al ²⁰⁹ the faster release came from PEGylated PEI-DNA, and may be due to faster water uptake. Porogens²²³ that increase the rate of release are reported: both give burst effects with complexed and uncomplexed pDNA..

The role of complexing agents was also studied in collagen. Sacks et al ²²⁷ found similar rates for DNA and for Lipofectamine/DNA complex, whereas Scherer et al ²²⁶ obtained a sustained release with polyplexes loaded with a protective polymer (PROCOP).

The nature of the released DNA is of great relevance to its transfection efficiency. There are very few studies that report on the nature of the released pDNA complexes from polymer films; only Sacks et al ²²⁷ have reported that the pDNA released from the

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pDNA-LIP (from collagen films) was in a condensed form since its transfection capability was higher than that observed with pDNA that was released from pDNA – collagen. Most studies^{202,208,209} simply complexed released pDNA with complexing agents, then showed transfection. Clearly, it is desirable to release the pDNA in its complexed state in order to improve the transfection efficiency. This thesis work is aimed at examining the nature of the released pDNA from various polymers, either from dispersed naked DNA or complexed DNA forms.

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2.12 Rationalization of Objective for Thesis:

From the above discussion, it is clear that sustained release dosage forms for pDNA are not currently available or optimized. Such dosage forms face many challenges to develop.

A vital challenge in these polymer based therapeutics is the potential difficulty in maintaining the stability of the molecule of interest during encapsulation, storage, and release. Double - stranded DNA remains extremely stable in solution (37 deg Celsius) above pH 7 for more than 3 weeks, but is very susceptible to low pH resulting in rapid degradation into single-stranded DNA and / or oligonucleotides. Acidic pH is known to damage DNA by depurination.²²⁹ It has been noted that during the incubation of PLGA microspheres in buffer at 37 deg, there is a gradual transition from supercoiled to nicked to linear isoform of the released DNA.

It is important to retain the stability of the incorporated DNA during its encapsulation into polymers and the subsequent scaffold fabrication. Retention of the supercoiled conformation during encapsulation and release is desired since it can lead to greater gene expression than do the open or linear conformations.¹⁹⁴ Saccharides were added to the aqueous solutions in the microsphere fabrication process to stabilize the DNA during lyophilization.³¹ Possible methods for further minimizing degradation are co-incorporation of an antacid such as $Mg(OH)_2$ into the process, which has been shown to stabilize proteins by increasing the microclimate pH.¹⁹⁵ Alternatively, the addition of EDTA to the DNA solution or complexation of the DNA with poly(L-lysine) (PLL) prior to encapsulation can protect the plasmid from enzymatic degradation and maintain the supercoiled structure.^{196,208}

There is a gap in the literature regarding DNA stability due to the exposure to organic solvents and high shear forces during encapsulation. Another issue is the quantification of the released pDNA, especially when it is in the complexed form. Howard et al⁽²⁰⁹⁾ and Mairead et al²⁰² highlighted the use of decomplexing agents like SDS/heparin/PAA

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to quantify the polyplex release. Such studies do not report how much of the released DNA was complexed; this is an issue we address in this study. The use of decomplexing agent is vital, in a way it helps to quantify the amount of pDNA from polyplexes is free or in complexed form. This information is then to be related to the observed transfection by the released DNA, something else that has not been reported to date.

To sum up, the main objectives of this study are:

- (a) The incorporation of DNA into polymeric matrices, and the associated study of the effect of processing on the stability of the DNA;
- (b) Quantitative study of the release of pDNA (naked) and complexed DNA (lipoplex and polyplex) from various degrading and non-degrading polymer matrices
- (c) Quantification of the nature of the released DNA; how much is complexed?
- (d) The effects of degradation on the released DNA
- (e) What are the critical factors that influence the release of pDNA and complexed DNA? The answers lead to the development of a sustained-release form for complexed DNA
- (f) Transfection of cells by the released DNA

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2.13 Outline of Thesis:

To achieve the above objectives, the research work followed the steps below. This study consists of 5 main parts

- Standardization and characterization of polyplexes and lipoplexes (Chapter 4, Part I)

Includes fluorescent intensity measurement, zeta potential, particle size measurement (DLS), transfection efficiency and toxicity.

- Solvent effect on polyplexes and lipoplexes (Chapter 4, Part II)

Fluorometry and transfection

- Effect of porogens like PEG/TEC on the release profiles of pDNA and polyplexes (Chapter 5, Phase I – Early studies)

Fluorescence intensity measurement with and without heparin sulphate for polyplexes

SEM to study the morphology of films and GPC to study the degradation effect.

- Effect of Gelatin on the release profiles of pDNA and polyplexes (Chapter 6, Phase II – Release studies with gelatin loaded films)

Fluorescence intensity measurement with and without heparin sulphate for polyplexes

Fluorescence intensity measurement with and without oleic acid for lipoplexes

SEM to study the morphology of films and GPC to study the degradation effect.

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- Study the bioactivity of the released complexes in an in vitro cell culture system like COS -7 cell line. (Chapter 7, Phase III)
- Future studies were discussed in this (Chapter 8)

Chapter 3

CHAPTER III

MATERIALS AND METHODS

3.1 Materials:

pDNA: pEGFP-*Luc*: pEGFP *Luc* encodes a fusion of green fluorescent protein (EGFP) and Luciferase from the firefly *Photinus pyralis*. The EGFP sequence contains a red – shifted variant of wild-type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells. The excitation maximum is at 488 nm and emission is at 507 nm. It is a cotransfection marker that allows normalization of transfection efficiencies by fluorescence microscopy of living cells or by a standard luciferase assay.

JM 109 Competent cells: These were used for the transformation of the foreign pDNA. These were obtained from Promega.

Kanamycin Sulphate: This was used for the antibiotic selection for the pDNA. It was used at a concentration of 50 µg/ml. It was supplied by Gibco.

DNA Extraction kit: pDNA was extracted using Qiagen filter giga columns. This was provided by Qiagen.

Polyethylenimine (PEI): The commercially available branched PEI (25 K) was used in the experiments. It was provided by Sigma.

LipofectamineTM reagent: It is suitable for the transfection of DNA into eukaryotic cells and is a 3:1 (w/w) liposome formulation of the polycationic lipid 2, 3-dioleoyloxy-N-[2 (sperminecarboxamido) ethyls-N, N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane filtered water. It was provided by Invitrogen.

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Heparin Sodium: Heparin sodium is the salt of heparinic acid. It was obtained from porcine intestinal mucosa. It's anionic in nature and used for the separation of DNA from PEI. It was used at a 10 times higher concentration as PEI. It was dissolved in water. It was provided by Sigma.

Oleic Acid Sodium Salt (Sodium Oleate): Oleic acid has the following molecular formula $C_{18}H_{33}NaO_2$. It was purchased from TCI chemicals, Japan. This was used for the separation of pDNA from lipoplexes. It was used as 10 times higher as the liposomes. It was dissolved in water.

Ethidium Bromide: It is a fluorescent dye used for DNA quantification. It is a mutagenic compound which intercalates with double stranded DNA and RNA. The fluorescence of ethidium bromide increases by 25 fold on binding double-stranded DNA. It binds with the single stranded DNA and triple stranded DNA although not very strongly.

Quant – iTTM PicoGreen dsDNA Reagent (PGR): This is an ultra sensitive fluorescent nucleic acid stain for quantitating double-stranded DNA (ds DNA) in solution. It was provided by Molecular ProbesTM invitrogen detection technologies.

COS - 7 cells: This is an African green monkey kidney fibroblast-like cell line suitable for transfection by vectors a half enhanced expression in the presence of SV40 T antigen. It is a transformed cancerous cell line. This was provided by ATCC. All the transfection experiments were done using this.

DMEM: Dulbecco's Modified Eagle Medium with 4.5g/L Glucose and L-Glutamine was obtained from GIBCO. This was used in the cell culture experiments.

All the remaining cell culture reagents like antibiotics, trypsin etc were purchased from GIBCO. Foetal bovine serum (FBS) was purchased from Sigma and heat inactivated in the lab. Cell culture plates (24 well, 96 well) were provided by Costar.

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Luciferase Reporter Assay kit: This provides a firefly luciferase substrate formulation (Substrates A and B) and cell lysis buffer. This is meant for measuring luciferase expressed by transfected cells. It was purchased from BD Clontech.

QuantiLim Recombinant Luciferase: It was a 61kDa monomeric protein that catalyzes the oxidation of beetle luciferin with the concomitant production of light. It was used for obtaining the standard curve for the luciferase assay.

Micro BCA TM Protein Assay Reagent Kit: This reagent is a detergent –compatible bicinchoninic acid formulation for the colorimetric detection and quantization of total protein. This kit has been optimized for use with dilute protein samples (0.5-20µg/ml). Used for the quantification of total cell protein. It was purchased from Pierce Biotechnology, Rockford, IL

Poly (lactic–co–glycolic acid) 53/47: This is a biodegradable polymer of molecular weight 90 K (I.V 0.88). This was provided by PURAC, Asia.

Polycaprolactone (PCL) (C₆H₁₀O₂)_n: It is a non-toxic, biodegradable polymer. It has an average M_n ~80,000 by GPC. It was obtained from sigma-aldrich.

Poly (ethylene-co-vinyl acetate): This is a non degradable polymer, with 40wt. % (vinyl acetate).It has the molecular formula (-CH₂CH₂-)_x[-CH₂CH(O₂ CCH₃)-]_y. It was provided by Sigma.

Polyethylene glycol (PEG): It is used as a plasticizer and as a porogen. This has a Mw Av 3000. It is purchased from TCI chemicals, Tokyo.

Triethyl citrate (TEC): This was provided by Aldrich. It also acts as a plastizicer and porogen.

Polyvinyl alcohol (PVA): This is used as an emulsion stabilizer. It is 99 % hydrolyzed and has Mwt 89,000- 98,000. It is provided by Aldrich.

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Gelatin: Type A, medium bloom: 175, (Mn 40,000).It is provided by Sigma. It is used as a porogen.

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3.2 Methods:

Micro plate Fluorescent Reader:

pDNA as such and pDNA released from Lipoplexes and Polyplexes were quantified with Bio-Tek, FL_X 800 - Multi Detection Micro plate Reader .pDNA from polyplexes was separated using heparin salt. In case of lipoplexes oleic acid was used to separate the complexes. Separate standard curves were constructed with and without heparin salt and oleic acid. It was also used for the N/P ratio calculation for the preparation of polyplexes and lipoplexes.

pDNA was tagged with ethidium bromide and quantified using the following wavelengths EX: 485 nm, EM: 590 nm. The working range for ethidium bromide was 0.1 µg- 15µg /ml. All the samples were measured in a 96 well dark bottom plates, meant for the measurement from Costar. These plates were used to minimize the background effect.

For quantifying lower concentration pDNA (< 100 ng/ml) Pico green reagent was used. Quantification was done using the manufacturer's procedures. The following excitation and emission wavelengths were used EX: 485 nm, EM: 520 nm.

UV Spectrophotometer:

DNA concentrations and protein quantification from cell lysates (BCA kit) were measured in this. DNA samples were measured at 260 nm and protein at 280 nm. Generally pure DNA has an A_{260}/A_{280} ratio of 1.8-2.0. All the samples were checked for this ratio. Protein samples were quantified at 560 nm using the UV-1700 Pharmaspec from Shimadzu.

Dynamic Light Scattering (DLS):

The effective diameters of DNA, polyplexes (PEI/DNA) and Lipoplexes (Lipofectamine/DNA) were measured using this. This was measured in DLS,

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Brookhaven Instruments BI-200SM system. The light source is a 35mW Helium Neon Laser with a wavelength at 632.8 nm. The temperature was controlled at 25⁰ C +/- 0.1⁰ C. To minimize the effect of stray light, a 90⁰C angle was chosen for data analysis. The correlation decay functions from DLS were analyzed by the cumulants method to obtain the effective diameter and the polydispersity. The constrained regularized CONTIN method was used to obtain the particle size distributions

Zeta Potential:

Zeta Potential measurements for the polyplexes and lipoplexes were done using a Zeta Sizer Nano system (Malvern Instruments). The instrument was equipped with a 10-miliWatt helium neon laser producing light at a wavelength of 633 nm. Zeta potential determination is based on the electrophoretic mobility of the complexes in an aqueous medium. Measurements of zeta potential in high-ionic-strength culture medium was impossible because of inhibition of the electrophoretic mobility of relatively large polyplexes by an excess of small and fast ions. For this reason the samples were prepared in water and measured using disposable cuvettes at 25⁰ C and a fixed scattering angle of 90⁰ C.

Polymeric Film Casting Knife:

A custom made casting knife made of anodized aluminum was used for casting films. It has a beveled blade applicator body. The film gap can be adjusted in 10 micron steps from 0-6 mm by means of two integrated micrometric screws..

Field Emission Scanning Electron Microscopy (FESEM):

This was used to analyze the surface morphology of the polymeric films. JEOL JSM-6340 Field Emission SEM at 5.0 KV was used for the study. The sample films were dried and mounted on a metal holder using a double sided copper tape. This was coated with gold using a Pulsed Plasma System before observation.

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Gel Permeation Chromatography (GPC):

Degradation studies were conducted on the aging of PLGA, PCL and PEVAC films, using GPC. Polymer samples were immersed in water and removed from it at specific intervals. Films were dried in vacuum oven for 7 days, after which they were dissolved with chloroform overnight. Samples were prepared at a concentration of 1mg/ml. Samples were filtered by 0.22 μ m filters and then filled into 2 ml vials sealed for auto-injection. Average molecular weight was calculated using a series of polystyrene standards that ranged from 640 to 4,843,000 in their molecular weight.

Column: PLgel 5 μ m MIXED –D (Serial No : sm-m0-53-65)

Length/I.D. (mm): 300 $\times$ 7.5

Detector: Refractive Index (254 nm)

Mobile Phase: Chloroform

Flow rate: 1 ml/min

Temperature: 35 °C

Standard: Polystyrene standards that range from 640 to 4,843,000 in their molecular weight.

Differential Scanning Calorimetry: (DSC)

Thermal Properties like crystallinity and melting points of PCL and PEVAC films were studied using DSC (DSC 2920, TA Instrument, USA), equipped with refrigerated cooling system (RCS). Testing was performed under flowing nitrogen (50 ml/min). Samples were prepared in standard aluminium pans at room temperature. Reference cell were loaded with an empty pan.

Fourier Transform Infrared Spectrometer: (FTIR)

The interaction between the ethylene groups between PEI and the polymer films (PCL, PEVAC) were analyzed with the Perkin Elmer system 2000.

Cell Culture:

All the cell culture work is done in the cell culture lab, using the following equipments

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Laminar hood, CO₂ incubator, Liquid nitrogen tank and Fluorescence microscope.

Inverted Fluorescent Microscopy:

This inverted phase contrast microscope (LeicaDMIL) is used for the qualitative analysis of GFP protein expression. It has a blue fluorescence filter with the following Ex: 470/40 nm and Em: 515 nm. This was used to quantify the green fluorescence emitted by GFP positive cells. It was attached a DC 300 F CCD camera (3.3 mega pixel CCD with Array RGB filter for brilliant images). It was provided by Leica Microsystems.

Luminometer:

This was used to quantify the gene expression. The sirus luminometer (Berthold systems) measures standard glow chemistry (Luminescence). Once the transfected cells were lysed, they were diluted with the cell lysis buffer. Then they were treated with treated with the substrates A and B. The samples were transferred to the glass tube provided for the luminescence measurement .The luminescence were measured using the sirus luminometer.

Preparation of Plasmid (pEGFP-*Luc*):

The plasmid was replicated in a high copy JM109 *Escherichia coli* strain grown in selective kanamycin (50 µg/ml) supplemented Luria-Bertani medium, isolated by alkaline lysis followed by anion exchange chromatography using the Giga Qiagen kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Purity of the plasmid was tested using UV spectroscopy (E 260/280 nm ratio). After isolation, the pDNA was dissolved to an end concentration of 1.2 µg/ml TrisHCL buffer (pH 8.0).

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CHAPTER IV

PHASE I PREPARATION OF POLYPLEXES, LIPOPLEXES AND STABILITY ISSUES

4.1 Part I: Preparation and Characterization of DNA Complexes

4.1.1 Optimization of Polyplexes (PEI/DNA Complexes)

4.1.1.1 Preparation of PEI

PEI obtained from sigma was prepared at a high concentration stock solution. About 1.4 gms of PEI is weighed and dissolved in 5ml water. The solution was passed through a 0.2 μ m filter further diluted with filtered water to 1.4 g/25 ml. This corresponds to 56 mg/ml. Further it was diluted with filtered water to the required concentration.

4.1.1.2 Preparation of PEI/DNA Complexes

pDNA was diluted from the stock solution using water. It was diluted to a working range of 0.2 μ g-0.5 μ g /0.5 ml. PEI was diluted using water. Keeping the DNA amount the same, PEI is added to it in different amounts to optimize the ratios. The complexes were analyzed fluorimetrically using ethidium bromide dye. To test their transfection efficiencies, these complexes were loaded to cells at different loadings ranging from 0.2 μ g to 0.5 μ g. These were analyzed using luciferase assay kit and their protein analysis done with micro BCA kit.

4.1.1.3 Analysis of DNA Complexation by Ethidium Exclusion

Complexation of DNA by polycationic polymers was evaluated using a fluorescence micro plate reader. Complexes were prepared as mentioned above, incubated for 30 mins at room temperature. To this ethidium bromide was added at a volume of 63 μ l (80 μ g/ml, 5:1 molar ratio of probe to nucleotides). The degree of

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ethidium exclusion was measured as the decrease in fluorescence relative to that of a control (free pDNA)

4.1.1.4 Measurement of charge of PEI/DNA

The Zeta potentials of polyethylenimine (PEI) – DNA complexes formed with various amounts of PEI were determined using an electrophoretic light scattering technique. Measurements were made in complexes in DMEM. All glass and plastic ware was washed three times with pre-filtered water to avoid particulate contamination. For each formulation, mean particle electrophoretic mobility was measured in a thermostatically controlled micro-electrophoresis cell equilibrated to 25⁰C. Two repeat measurements were made with the samples. All samples were prepared in duplicates. Zeta potential was calculated according to the Smouluchowski equation. All samples were prepared at a concentration of 10 µg/ml.

4.1.1.5 Measurement of size of PEI/DNA

This is done using Dynamic Light Scattering (DLS). DNA was prepared at high concentration, and then filtered using 0.2 µm filter. The concentration of the DNA was checked spectrophotometrically before and after filtration. PEI was prepared in the same way. PEI is added to the DNA and vortexed immediately for a few seconds. The final concentration of the polyplexes used for DLS measurement was 33.5 µg/ml. These complexes were incubated for 20 mins and the particle size was measured using DLS. At each measurement the polydispersity is kept with in the range (0.05-0.2). All samples were prepared in duplicates.

4.1.1.6 Transfection:

Cos – 7 cells were seeded in a 24 well tissue culture grade plates at 2×10^4 cells per well and grown overnight to approximately 80% under normal growth conditions. One hour before the complexes were added to the cells the primary growth medium was replaced with 0.2ml of fresh medium (DMEM) (Dulbecco's Minimum Essential Medium). About 100 µl of prepared polyplexes and lipoplexes were

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topped up with 300 μ l of fresh SFM (DMEM with the absence of foetal bovine serum) .This was added drop wise to each well .The cells were incubated with complexes at 37⁰C in a 5% CO₂ incubator for 5 hrs unless otherwise stated. Media was replaced with 0.5 ml of 2X medium (DMEM with 10% SFM) and left overnight .The medium was changed with 1X medium, the next day. After 48 hrs the cells were lysed with 100 μ l of cell lysis buffer and analyzed using luciferase assay kit, as described below:

4.1.1.7 Analysis of Luciferase Expression:

After removal of medium, cells were washed twice with PBS and harvested from culture dishes by scraping in or with the presence of cell lysis buffer. After the addition of 100 μ l lysis buffer, the plate was incubated at 37⁰ C for 30 mins. The collected samples were centrifuged at 14,000 rpm for 2 mins. About 5- 10 μ l of the lysed cell solution was treated with the substrate A and B. The luminescence intensity was measured using a luminometer. A standard curve was used to convert luminescence reading (RLU - Relative Light units) to fg of the enzyme. This curve is constructed using the recombinant luciferase enzyme. The enzyme activity was normalized using the protein content of cell extracts. Protein concentration in the lysates was assayed by a modified Lowry method (Micro BCA assay- Pierce). The samples were incubated with the BCA reagent and incubated at 60⁰ C for 60 mins. The absorbance was measured at 560 nm, using a UV spectrophotometer. This was converted to absolute amounts using a bovine serum albumin provided with the kit (mg of cell protein). Finally the enzyme activity was expressed as fg of enzyme activity /mg of cell protein.

4.1.1.8 Cellular Toxicity:

In vitro toxicity of the transfected samples were assayed using the Micro BCA assay. The standard curve was constructed using the BSA provided with the kit. After lysis of the cells with the lysis buffer, the lysates were treated with the working bichinoic reagent provided with the kit. The samples were incubated for 60 mins at 60⁰ C .The absorbance was measured at 560 nm. Cells transfected with naked DNA were used as a control.The toxicity is calculated as follows,

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$$\text{Relative Toxicity \%} = \frac{\text{protein amount of control cells} - \text{protein amount of transfected cells}}{\text{protein amount of control cells}} \times 100$$

4.1.2 Optimization of Lipoplexes: (LPF/DNA Complexes)

4.1.2.1 Preparation of Lipoplexes:

LipofectamineTM reagent is obtained from Invitrogen Life technologies. It is obtained as a stock solution 2mg/ml in water.

pDNA was diluted 0.2-0.5 µg DNA in 50 µl of DMEM (SFM) medium/water and mixed gently. LipofectamineTM was gently mixed before use, and then diluted to 0.5-5 µl in 50 µl of DMEM (SFM) with gentle mixing. The diluted DNA was mixed gently with the diluted LipofectamineTM (total volume = 100 µl), and incubated for 30 minutes at room temperature.

By keeping the DNA amount same, LipofectamineTM was added to it in different amounts to optimize the ratios.

For zeta potential measurement, complexes were prepared and diluted in water, whereas it is diluted in DMEM (SFM) for transfection purposes. These complexes were also analyzed using agarose gel electrophoresis as well as fluorimetrically using ethidium bromide dye. Lipoplexes complexes were prepared as per manufactures instruction.

4.1.2.2 Analysis of Lipoplexes Complexation by Ethidium Bromide Exclusion:

Done similarly as mentioned for polyplexes above (4.1.1.3)

4.1.2.3 Measurement of Charge of Lipoplexes:

This is done similarly as it is done for polyplexes (4.1.1.4)

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4.1.2.4 Measurement of Size of Lipoplexes:

In case of lipofectamine the final concentration of lipoplexes was 16.75 µg/ml. A high concentration stock of lipofectamine was used. It was added to the double filtered water.

4.1.2.5 Transfection:

Similar to the above mentioned (4.1.1.6)

4.1.2.6 Analysis of Luciferase Expression:

Similar to the above mentioned (4.1.1.7)

4.1.2.7 Cellular Toxicity:

Similar to the above mentioned (4.1.1.8)

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Results & Discussion:

4.2 Optimization of Polyplexes:

4.2.1 Analysis of DNA Complexation by Ethidium Exclusion:

Figure 4.1 Effect of charge ratio (+/-) on drop in the fluorescence intensity of PEI/DNA complexes

Figure 4.1, shows that ethidium exclusion is a useful indicator of complexation between polycation and DNA plasmid. As polycation was introduced to the DNA the fluorescence emitted due to ethidium/DNA complexes decreased. Condensation was evident when DNA was complexed in water with PEI.

At N/P ratios of 2 or higher, the complexation was “complete”, showing that all DNA was present in the complexed form.

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4.2.2 Measurement of Charge of PEI/DNA Complexes:

Figure 4. 2 Effect of charge ratio (+/-) on the charge of the PEI/DNA complexes

The zeta potential decreases proportionally with lower PEI concentrations (Figure 4. 2). The negative potential at an N/P ratio of 0 is due to the negative charges from unbound DNA. The zeta potential rises to a maximum of between +33 and +38 mV with the N/P ratio ranging between 1 and 10. These data indicated that there was a little free polycation present; not all of the polycation become complexed with DNA, as expected from the excess N/P ratio. The zeta potentials of the complexes were determined in medium (DMEM). Zeta potential of PEI alone is +38 mV.

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4.2.3 Measurement of Particle Size of PEI/DNA Complexes:

Figure 4. 3 Effect of charge ratio (+/-) on effective particle size of PEI/DNA complexes

The size of PEI/DNA with PEI of varying ratios is presented in Figure 4.3. Preparation of particles in water have resulted in particle sizes between 75 -110 nm. This indicates that PEI/DNA complex formation is an efficient, spontaneous process driven by electrostatic interactions. As N/P ratio increases initially, the complex is compacted due to the electrostatic interactions, as expected. However, above the N/P ratio of 8, particle size increase from 75 nm to 100 nm. It is not known at present whether the particle growth represented swelling of individual particles or aggregation. It could also result in expansion due to charge repulsion as an excess of positive charge is added.

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4.2.4 Measurement of transfection activity of PEI/DNA Complexes:

Figure 4.4 Effect of charge ratio (+/-) on transfection activity from COS-7 cells of PEI/DNA complexes

Figure 5.4 shows the transfection efficiency of polyplexes of different N/P ratios in COS-7 cells. In general, the transfection efficiency in COS-7 cells was high (10^6 - 10^7 fg/mg of protein) and showed a typical bell-shaped profile with the optimum around N/P ratio of 4 $\mu\text{g}/\mu\text{g}$. It is interesting to note that at an N/P ratio of 12 and 24 the polyplexes are less effective than at the lower ratios, apparently because of increased toxic effects of PEI.

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4.2.5 Measurement of relative toxicity of PEI/DNA Complexes :

Figure 4.5 Effect of charge ratio (+/-) on cellular toxicity of PEI/DNA complexes

Cellular toxicity of the polyplexes was studied. Results from the figure 4.5 shows that toxicity increase with the increase in the N/P ratios. No toxicity is noted from N/P 0.5 to 1; as the N/P ratio increase from 4 the toxicity % also increases reaching a maximum of 45 % at the N/P 24.

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4.2.6 Transfection Activity Vs Cellular Toxicity of PEI/DNA Complexes:

Figure 4.6 Transfection activity Vs cellular toxicity of PEI/DNA complexes

Figure 4.6 compares transfection efficiency against cell toxicity. The transfection efficiency follows a skewed bell shaped curve and the transfection activity drops gradually after N/P 4, to 10^6 at N/P 24. The toxicity is also very high about 45% at this ratio.

To summarize, it is evident that at low N/P ratios (0.5-1), the particles are bigger, and transfection efficiencies are low, due to its toxicity. As the ratio increases to 4, the zeta potential value increase to +36.57 mV, with a particle size drop to 97 nm and the toxicity value increase to 27%. The transfection activity also increase to 10^7 .

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The particle size increases to 100nm at N/P 12. This shows that the relatively large polyplexes complexes induce cell death probably either via more efficient uptake of polyplexes and also unbound PEI or a more intimate interaction and disruption of the plasma membrane resulting in higher toxicity.

From the above data, N/P = 4 was chosen as a standard for the film loading studies. The transfection activity (10^7 fg/mg of protein) is highest at the N/P ratio of 4, in spite of the toxicity. It is important to choose complexes with the high transfection to load in polymeric films. Once the polyplexes are loaded in the polymeric films, its bioactivity is lost up to 29 % with the solvent chloroform. Polyplexes were released at a very slow rate from polymeric films. The lower amount released from the polymeric films is not toxic to the cells.

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4.3 Optimization of Lipoplexes:

4.3.1 Analysis of Complexation by Ethidium Bromide Dye Exclusion:

Figure 4.7 Effect of charge ratio (+/-) on drop in the fluorescence intensity of LPF/DNA complexes

As in the case of polyplexes, the same trend is observed with the lipoplexes, drop in the fluorescence intensity with the addition of positively charged lipofectamine. Figure 4.7 shows that there was not much drop in the fluorescence intensity is noted as the mass ratio increase from 2 to 12.

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4.3.2 Measurement of Charge of LPF/DNA Complexes:

Figure 4.8 Effect of charge ratio (+/-) on the charge of the LPF/DNA complexes

The zeta potential of lipofectamine/DNA complexes in DMEM were consistent (Figure 4.8). The negative potential at an N/P ratio of 0 is due to the negative charges from unbound DNA. The zeta potential rises to a maximum of between +31.3 and +57.3 mV at the mass ratios between N/P 2 and N/P 12.

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4.3.3 Measurement of Particle Size of LPF/DNA Complexes:

Figure 4.9 Effect of charge ratio (+/-) on effective particle size of LPF/DNA

Figure 4.9, shows the effect of N/P ratio on the size of the lipoplexes. Generally the particle size of pDNA is 105 nm. The particle size drop from there to 59 nm with the N/P ratio of 6. This shows that the pDNA is being condensed with the addition of lipofectamine. But as the N/P ratio increase from 6 to 12, it seems that the particle size starts to increase from 58.6 nm to 62.85 nm. This could be due to the agglomeration of the complexes or expansion due to charge repulsion as an excess of positive charge is added.

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4.3.4: Measurement of Transfection Activity of LPF/DNA Complexes:

Figure 4.10 Effect of charge ratio (+/-) on transfection from COS-7 cells of LPF/DNA complexes

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4.3.5 Measurement of Relative Cellular Toxicity of LPF/DNA Complexes:

Figure 4.11 Effect of charge ratio (+/-) on cellular toxicity of LPF/DNA complexes

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4.3.6 Transfection Activity Vs Cellular Toxicity of LPF/DNA Complexes:

Figure 4.12 Transfection activity Vs cellular toxicity of LPF/DNA complexes

The transfection efficiency of lipoplexes of different N/P ratios is shown in Figure 5.10 in COS-7 cells. From N/P 2-6, as the zeta potential increases from +31.33 to +48.7, particle size drops from 65.7 nm (N/P = 4) to 58.62 nm (N/P = 6). The transfection activity increase from 10^5 fg/mg of protein to 10^7 fg/mg of protein, along with the cellular toxicity increasing from 22.9% to 54.75%. This shows that as the zeta potential increases, particle size drops, transfection increases and toxicity also increases.

The maximum transfection efficiency is noted at the N/P ratio at 8, reaching up to 10^8 fg/mg of protein. The zeta potential increase to +50.132 mV, the particle size slightly becoming

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larger (60.37 nm). The toxicity also increases to 55.7% at this ratio. After this ratio the transfection efficiency drops with the increase in toxicity, particle size and zeta potential.

It could be noted that the transfection activity drops after the N/P ratio 8. This could be due to the lipoplexes becoming more and more positively charged as shown with the drop in fluorescence intensity in Figure 4.7 dropping from 830 at N/P 8 to 498 at N/P 12. It is interesting to note that this is supported by the increase in the zeta potential value from 50 at N/P 8 to 57.32 at N/P 12 (Figure 4.8). The particle size also increases from 60.3 at N/P 8, to 62.85 at N/P 12 (Figure 4.9). The toxicity % increase to 62.85% at N/P 12. This could cause more cell death and the transfection drops at higher N/P ratios.

From the above studies N/P 8 was chosen as a standard for the complex preparation for the film loading part. The transfection activity (10^8 fg/mg of protein) is highest at the N/P ratio of 4, in spite of the toxicity. It is important to choose complexes with the high transfection to load in polymeric films. Once the polyplexes are loaded in the polymeric films, its bioactivity is lost up to 56 % with the solvent chloroform. Lipoplexes were released at a very slow rate from polymeric films. The lower amount released from the polymeric films is not toxic to the cells.

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4.4 Part II: Separation of pDNA from Polyplexes and Lipoplexes:

Naked DNA released from polymeric films can be quantified as such using ethidium bromide. The amount of polyplexes and lipoplexes released from the polymeric films can be quantified only indirectly, after decomplexation using the naked pDNA released from the complexes. For this purpose the DNA needs to be decoupled from the complexes.

In case of polyplexes, heparin salt was used for the decomplexation. For this study the ratio of PEI: Heparin salt (w/w) is 1:2.5. Heparin salt was prepared as a stock solution (10 mg/ml) in water. From this about 4 μ l was added to the complexes and mixed well. The samples were incubated for 1 hr at 37 °C. Later the samples were analyzed by the addition of ethidium bromide 63 μ l (25 mg/ml) in PBS. Separate standard curves were constructed both with and without the presence of heparin salt.

For Lipoplexes, oleic acid was used for decomplexation. Oleic acid was prepared as a 10 mg/ml stock solution. From this about 5 μ l of it was added to the lipoplexes. The ratio between lipofectamine to oleic acid is 1:5. These complexes were incubated about 4 hours at 37 °C. After 4 hours the samples were treated with ethidium bromide and measured using a fluorescence micro plate reader. Separate standard curves were constructed with and without the presence of oleic acid. Oleic acid was added as 5 times (w/w) as Lipoplexes.

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4.5 Part III: Stability of pDNA, Polyplexes and Lipoplexes in Solvents:

In the PhD studies, naked DNA, polyplexes and lipoplexes were loaded at high concentrations in polymeric films. As the incorporation of the DNA into the polymers involved the use of solvents, it was important to determine the effect of solvents on the pDNA activity. This was studied as follows:

All of the solvents were filtered through a 0.22 μ m filter. For the naked DNA, a 7 μ l (Stk DNA 4 mg/ml) solution in 93 μ l of DMEM (Dulbecco's Minimum Essential Medium) was treated with 400 μ l of acetone; vortexed at 2500 rpm for a minute. The solution was allowed to stand at room temperature for 30 mins, followed by its transfer to a wide-mouth bottle for the evaporation of the solvent. Once the solvent was evaporated it was treated with 500 μ l of fresh DMEM. For chloroform, the samples were treated with chloroform for 30 mins. This was followed by the gentle removal of the solvent settling at the bottom, followed by 15 minutes air drying.

In the cases of lipoplexes and polyplexes, the same procedure was followed. The complexes were prepared in the same way as mentioned above. For polyplexes, PEI was added at an N/P ratio of 4 and for lipoplexes the ratio used was 8. Once the evaporation of the solvent was over the samples were analyzed through flurometry for decomplexation. The complexes were also used for transfection at a loading of 3 μ g/ml. Complexes made with fresh DMEM at the same loading were used as a control. From this 3 μ g were loaded to cells. COS -7 cells were used for these studies.

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Results & Discussion:

4.5.1 Effect of Solvents on the Dissociation of Complexes:

This was done to check the complex stability with the use of solvents. For this the samples were prepared as mentioned above. About 50 μ l of the samples (pDNA/SFM) were taken, treated with ethidium bromide (63 μ l), and the fluorescence measured using microplate fluorescence reader. The controls used were fresh pDNA at a loading (28 μ g). Separate study done with polyplexes and lipoplexes at loading (28 μ g in water) does not show any detectable fluorescence.

If the solvent affects the stability of the polyplexes and lipoplexes, free pDNA will be released from them. The released pDNA will be detected by means of fluorometry using EtBr as a dye. The loss in activity of the complexes as determined using a microplate fluorescence reader is summarized below.

Table 3: Effect of solvents on the loss of activity (%) of pDNA, PEI/DNA and LPF/DNA (Flouresence intensity – EtBr study)

Samples	Treatment with Solvents	Loss of activity (%)
pDNA	Acetone	27
pDNA	Chloroform	61
Polyplexes	Acetone	0
Polyplexes	Chloroform	14
Lipoplexes	Acetone	2-3
Lipoplexes	Chloroform	2-3

4.5.2 Effect of Solvents on the Transfection Efficiency:

COS-7 cells were treated with the solvent treated and fresh samples. Samples were incubated with the cells for 5 hours in serum free medium (SFM) (DMEM with the absence of FBS (foetal bovine serum)). After incubation the samples were removed and replaced with 2X SFM overnight. After replacement with 1X medium, cells were grown for 2 days. Gene transfections were analyzed after 48 hrs.

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The below figures (4.13, 4.14, and 4.15) show the effect of solvents acetone and chloroform on the transfection efficiencies of the naked DNA, polyplexes and lipoplexes. A high loading of about 3 μg was added to the cells, instead of the normal loading of 0.5 μg . This is done, since at low loading (0.5 μg) the treated complexes may not be able to show any detectable transfection due to the solvent treatment. To overcome this 3 μg of both treated and untreated samples were used, to get a measurable transfection from solvent treated samples. 3 μg of fresh samples were used so that it can be compared with the treated samples.

Figure 4.13 Effect of solvents on the transfection efficiency of pDNA

The effect of solvents on pDNA (Figure 4.13) shows that chloroform is very detrimental to pDNA. About 57% of activity is lost during the treatment, whereas in the case of acetone, only 31% of the activity is lost. Chloroform has a water solubility of 0.8 g/100 ml. It is possible that it could cause DNA precipitation, but not enough to cause a visible aggregation. So the drop in transfection could be due to precipitation of DNA in the presence of chloroform. Chloroform is a non-solvent for pDNA, partly due to its lower dielectric constant, as explained below.

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Dielectric constant of the solvent:

DNA precipitation is largely dependent on the dielectric constant of the solution, and the greater this parameter, the more difficult it is to aggregate the macromolecule. Chloroform has a dielectric constant of 4.8 compared with water, which has 78.3. So DNA molecules may aggregate, and this may lead to lowered transfection. The extent of aggregation is not enough for these to be visible to the eye.

Figure 4.14 Effect of solvents on the transfection efficiency of polyplexes

The solvent effect on polyplexes was shown in Figure 5.14. It seems that the solvent acetone is detrimental in this case. Only 13% activity is retained when compared to the untreated samples (control- polyplexes/SFM at 28 μ g loading). It indirectly shows that about 87% of the activity is lost during the treatment form. This could be due to the nature of acetone. Acetone is generally used in the precipitation of proteins. So the lower activity with the acetone treated samples could be related to polyplexes aggregation. Aggregation results in larger particle size and their cellular

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uptake is hindered. This could result in less transfection when compared with controls. In the case of chloroform treated samples, about 71% activity is retained.

Figure 4.15 Effect of solvents on the transfection efficiency of polyplexes

Figure 4.15 shows the effect of chloroform and acetone on lipoplexes. In this case also acetone seems to be detrimental, retaining about 4% of the activity, losing the rest. Acetone is used in the extraction of neutral lipids from a mixture of lipids²³⁰. So it is possible that it extracts the neutral lipid (DOPE) in lipofectamine. The absence of DOPE, will result in the drop in transfection²³¹. With chloroform, about 44% activity is retained when compared with the controls.

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Figure 4.16 Comparison of solvent effects between polyplexes and lipoplexes

So naked DNA with chloroform showed a 57% loss in activity, compared to 31% loss with acetone. Whereas when PEI/Lipofectamine was used to condense the pDNA, there seems to be an increase in transfection with the solvent treated samples. (solvent treated pDNA Vs solvent treated lipoplexes/polyplexes) (Figure 4.16)

The activity retained using polyplexes/chloroform is about 71%. This is very high in terms of percentage as only 44% is retained with the lipoplexes after chloroform treatment. But if it is compared in terms of transfection activities between polyplexes and lipoplexes for samples treated with solvents, lipoplexes are higher than polyplexes (7×10^6 Vs 2×10^6). Whereas in untreated samples it is (2×10^7 Vs 1×10^6). (Figure 4.16)

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In general the bioactivity measurement using flourometry of the solvent treated samples is not very accurate. So the bioactivity measurement using transfection efficiencies seems to be more appropriate.

Since the chloroform seems to affect the polyplexes and lipoplexes much less than acetone, it was decided to use chloroform as a solvent for casting films in future studies.

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CHAPTER V

PHASE I PREPARATION OF POLYMERIC FILMS, POLYPLEXES LOADING AND RELEASE STUDIES – EARLY STUDIES

5.1 Preparation of PEI:

PEI obtained from Sigma was prepared at a high concentration stock solution. PEI is weighed and dissolved in water. About 1.4 gms of PEI is weighed and dissolved in 5ml water. The solution was passed through a 0.2 μ m filter and diluted with filtered water to 1.4 g/25 ml. This corresponds to 56 mg/ml. Further it was diluted with Serum Free Medium (SFM) to the required concentration.

5.2 Preparation of PEI/DNA Complexes:

pDNA was diluted from the stock solution using water. It was diluted to a working range of 28 μ g in 100 μ l (280 μ g/ml). Keeping the DNA amount unchanged, PEI is added at a level 4 times higher than pDNA (PEI-112 μ g). An N/P (w/w) ratio of 4 is used in these studies. The N/P ratio was estimated to be the ratio of primary amine in the polycation to phosphates (nucleotides) in the DNA that was placed in solution.²³² The complex was vortexed vigorously. The complexes were incubated at room temperature for 30 mins. A slight agglomeration was noticed during incubation. The complexes were broken down by pipetting and vortexing before it was mixed with the polymer solution.

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5.3 Incorporation of Complexs into the Films :

PLGA films:

A 10% (w/v) of PLGA solution was prepared using chloroform as a solvent. From this about 200 μ l were used for the film preparation. The polyplexes and PLGA solution were mixed together in the volume ratio of 1:2 (100 μ l:200 μ l) and this emulsion is then mixed vigorously at 1800-2000 rpm (The addition order is important: PLGA solution is added to PEI/DNA complex and not the other way round. This helps in forming a better emulsion and the better the emulsion, betters the quality of film formation). The emulsion was then spread out over a glass plate and the film caster (after it has been properly calibrated and set to the desired thickness: $t_w = 0.8$ mm) was used to cast the film inside the laminar hood. The film was allowed to dry for 3 days to remove the solvent in the laminar hood. The film was then plucked out from the plate after it had dried completely (i.e. the solvent had evaporated completely). Blank films with the absence of pDNA and polyplexes were also made.

PCL films:

A 6.6% (w/v) PCL solution was made by dissolving the polymer in chloroform. 312 μ l of this solution was mixed with 125 μ l of PEI/DNA or pDNA in SFM. The ratio between the SFM and polymer is 1:2.4. PCL solution was added to the SFM and vortexed at 2200 rpm and cast as a film. The ratio was slightly changed from the PLGA films, so as to get a good emulsion, before casting the film.

PCL/PVA/PEG films:

PCL was dissolved in chloroform as mentioned above. To this 10% PEG (w/w) was added and dissolved. pDNA and polyplexes were prepared as mentioned above. After their incubation, they were treated with 0.5% of PVA (w/w) of

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polymer. PCL/PEG blend (315 μ l) was added to the polyplexes/pDNA solution. The ratio between the polyplexes and polymer is 1:2.4.

PCL/TEC films:

A 10% (w/w) of TEC was added along with the PCL and dissolved using chloroform. About 315 μ l of this polymer solution was mixed with 100 μ l of SFM (pDNA, polyplexes or SFM alone) and vortexed as mentioned above. The ratio between the polyplexes and polymer is 1:2.4.

PEVAC films:

10 % (w/v) of PEVAC solution was prepared using chloroform. 500 μ l of this solution was mixed with the SFM and vortexed. The ratio between the polyplexes and polymer solution is 1:2.4.

5.4 In vitro Release Study:

The films were placed in a 12 - well cell culture plates. About 700 μ l of PBS (Phosphate buffered saline) at pH 7.4 was used for the in vitro release studies. Plates were incubated at 37 $^{\circ}$ C and at different time points (t=4, 7, 14, 21, 28) days. The buffer was removed and replaced with the fresh buffer. About 1% ampicillin was used as an antibiotic in the PBS to avoid contamination.

5.5 Quantification of the Release:

pDNA released from the films was quantified using EtBr as mentioned in section 3.2.

Polyplexes were quantified using heparin sulphate as mentioned in section 3.2.

5.6 Degradation study:

A separate set of films were made as mentioned above for the degradation study. Films were taken at different time points, dried under vacuum for 4 days. The temperature was maintained at 37 deg celcius. Once the films were dried they were dissolved with chloroform (2 mg/ml) and analyzed using GPC.

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5.7 Dissolution study:

The incorporation efficiency of plasmid DNA into the films was determined using a combination of TE (Tris-EDTA) buffer and chloroform. Films were dissolved in chloroform. TE buffer was added to this. The two phases were separated by centrifugation at 13,000 rpm. Following centrifugation, the aqueous layer was collected. A second aliquot of TE buffer was added to the chloroform dissolved polymer solution and mixed by inversion. This second extraction was performed to improve the yield of extracted plasmid. The concentration of the DNA in the extracted solution was quantified using EtBr in a flurometer. For samples with PEI/DNA, additionally heparin sulphate was added, to decouple the polyplexes.

5.8 SEM Study:

The dried films were used for the SEM. SEM samples were prepared as mentioned in section 3.2.7

5.9 FTIR Study:

PCL and PEVAC films were prepared separately with 5%, 10% and 20% of PEI (w/w) and used for the FTIR study. Control films were prepared with the absence of PEI in them.

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Results & Discussion:

5.9 PLGA Films:

5.9.1 Release of naked DNA and Polyplexes from PLGA (53/47) films:

Figure 5.1 Release of pDNA Vs polyplexes from PLGA (53/47) films

The in vitro release profile of pDNA and polyplexes from polymeric system was studied by immersing the films in water. The samples were taken at different time points quantification of the samples were done using Etbr with the presence and absence of heparin sulphate as mentioned before. (Section 3.2)

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The release of pDNA from the PLGA films shows a triphasic behavior (Figure 6. 1). Till day 4, there is a fairly rapid release of pDNA (the so-called “burst” effect). The first phase of the release is attributed to the pDNA (undissolved in the polymer) on or close to the surface. This contributes about 67% of the pDNA. In the following phase release occurs primarily by diffusion through the polymer matrix, and is very slow. Hence we observe a “plateau” in the release profile. This lasts from day 4 to 14. The third phase of release can be attributed to polymer bulk erosion. In this phase a rapid release of DNA was observed. In general, PLGA polymers degrade by a bulk homogenous hydrolytic process. On exposure to water a rapid decrease in the molecular weight occurs as susceptible bonds in the polymeric chain are cleaved producing smaller fragments. Eventually these fragments become small enough to be soluble in the aqueous media ²⁰⁵. PLGA polymers with a 53/47 ratio of lactic acid to glycolic acid degrade rapidly and the lower the molecular weight of the polymer the more rapidly the polymer degrades.^{206,207}

When PEI was used to condense the DNA, the overall release slows down considerably. No burst effect was seen with the PEI/DNA samples loaded in PLGA. Also a very slow release was noted till Day 14. From day 14 onwards, erosion-controlled release sets in. The condensation of DNA with PEI has prevented the large burst effect as seen in the case of free pDNA. The burst effect observed for pDNA could be ascribed to specific polymer-pDNA interactions. Many synthetic and natural polymers are negatively charged and thus repulse the negatively-charged pDNA. This contributes to the burst effect observed for pDNA release. In the case of polyplexes, PEI being positively charged could interact with the negatively charged PLGA, in the same way that it interacts with the pDNA. At N/P (w/w) ratio of 4, there should be an excess of free PEI which could interact with the PLGA. This charge interaction could delay the release of complexes as compared with naked DNA. In addition, it is also possible that the NH groups on the PEI will interact with the C=O groups on the PLGA, resulting also in complexation. As will be seen below, we believe the dominant interaction is

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hydrogen bonding (between the C=O segments in the polymer and NH in the PEI). It is seen that inspite of the pore (100 μ) (chapter 6, figure 6.3) made on the polymer films, the complexes still cannot be released till the polymer starts to degrade. So the polyplexes could be released only after the polymer matrix starts to degrade. But the release is very slow. This could be due to the dominant charge interaction between PEI and PLGA. PEI has a positive charge of +38 mV and PLGA (53/47) has a negative charge of (-14.7 mV). Additionally the hydrogen bonding between PLGA and PEI, hinders the release.

All the released samples from the PEI/DNA were in the form of complexes as shown by the sample treatment (with and with out heparin sulphate.). During transfection experiments the released pDNA (naked) was able to trasfect the cells. But the released PEI/DNA complex did not show any transfection. The explanation is as follows: as the polymer erodes, the eroding PLGA fragments are still attached to the PEI-DNA complexes. This is due to the dominant hydrophobic interaction and/or the excess positive charge on the PEI interacting strongly with the negatively-charged PLGA fragments. This results in some shielding of the complex, and reduces its transfection efficiency. The shielding of the complexes is verified using zeta potential of the released polyplexes from the PLGA films at different time points. The zeta potential values are summarized below,

*Chapter 5***Table 4: Zeta potential of polyplexes released from PLGA films**

Days	Test Films – PLGA films with Polyplexes
7	-0.54
14	-1.26
21	-0.69

In order to overcome the problems with the release and transfection from the PLGA films it was decided to load the same complexes in slow degrading polymers like PCL and a non degrading polymer like PEVAC.

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Figure 5.2 GPC data for the degradation of PLGA films

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5.10 PCL Films:

Naked DNA and PEI/DNA were loaded in the same way as was done for the PLGA films. Release study was done for the following intervals (days= 2, 4, 8, 14, 21, 28). The released DNA was quantified using the Etbr. pDNA released from the PEI/DNA complexes were quantified in a similar way. The bound DNA was decomplexed from the complexes using heparin sulphate and quantified using ethidium bromide.

5.10.1 Release of pDNA and Polyplexes from PCL films:

Figure 5.3 pDNA Vs polyplexes release from PCL films

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The above graph is plotted as the % release pDNA Vs time. Compared to the release from PLGA films, the release here is at the most biphasic. Most likely this is due to the absence of the erosion-controlled regime seen after day 14 for PLGA. The pDNA releases (after the initial burst) in a diffusion-controlled manner, slowly from day 2 to 28, as expected for a macromolecule releasing from a polymer matrix of moderate crystallinity.

PCL is a slow degrading polymer. The release of macromolecules from such systems is very limited. In general the release is diffusion controlled. The rate of release of proteins from PCL microspheres has been reported to be rather slow^{152,153}. Another study showing the release of lysozyme, from PCL films concluded that low diffusivity of the proteins (hydrophilic) and the high crystallinity of PCL are both critical factors that limit protein release. As far as we know no release of pDNA or complexes loaded in PCL polymeric films has been reported.

In case of PCL films also, there is no appreciable burst effect observed for complexed DNA. The argument for the lack of a burst effect is similar to that proposed for PLGA: specific ionic/ hydrophobic interaction between the PCL molecules and the positively charged PEI. The release rate beyond the burst is comparable to that observed for pDNA

For PCL, the amount of complexed PEI/DNA released at any time interval is not enough for transfection. This was confirmed experimentally. Clearly, a faster release is required from the polymeric film for transfection. So the strategy applied here is to create a porous structure in PCL films, and the diffusion of the drug is enhanced by these pores. Poly (ethylene glycol) (PEG) and Triacetyl citrate (TEC) were selected to plasticize the PCL, as well as to create pores on the PCL matrix as they leach out. This could enhance the release of PEI/DNA complexes from the films

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5.10.2 Effect of PEG/PVA and TEC on the release of DNA from PCL films:

Figure 5.4 Effect of PEG/PVA and TEC on the release of pDNA from PCL films

The graphs (Figure 5.4, Figure 5.5) are plotted as the pDNA release % Vs time. This data shows that the addition of PEG drastically alters the release profile of naked DNA from PCL films. Specifically, the burst effect is suppressed while the latter portion of the release is accelerated. In contrast, the addition of TEC has little or no effect on the naked DNA release profile.

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5.10.3 Effect of PEG/PVA and TEC on the release of PEI/DNA from PCL films:

Figure 5.5 Effect of PEG/PVA and TEC on the release of polyplexes from PCL films

For the complexes, the picture is different: for all practical purposes, the release profile of complexed DNA is not significantly altered by addition of either PEG or TEC. Reasons are explored below.

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The SEM picture below shows the presence of pores on the PEG films when compared to the PCL films. A previous study revealed a special phenomenon in PCL-PEG blends, where the PEG phase was dispersed as spherical droplets in the range of 2- 5 μm through out the PCL phase ¹⁵⁴. A similar morphology was observed in the current study. The micropores formed were spherical and were homogenously dispersed throughout the polymer films. The TEC film also shows pores, but to a smaller extent.

Figure 5.6 (a) PCL film on day 1 (surface)

Figure 5.6(b) PCL film on day 4 (surface)

Figure 5.6 (c) PCL film on day 7 (surface)

Figure 5.6 (d) PCL film on day 14 (surface)

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Figure 5.7 (a) PCL/PEG/PVA film on day 1 (surface)

Figure 5.7 (b) PCL/PEG/PVA film on day 4 (surface)

Figure 5.7 (c) PCL/PEG/PVA film on day 7 (surface)

Figure 5.7 (d) PCL/PEG/PVA film on day 14 (surface)

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Figure 5.8 (a) PCL/TEC film on day 1 (surface)

Figure 5.8 (b) PCL/TEC film on day 4 (surface)

Figure 5.8 (c) PCL/TEC film on day 7 (surface)

Figure 5.8 (d) PCL/TEC film on day 14 (surface)

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The effect of PEG on the release of naked pDNA is due to both porosity and to accelerated degradation of the PCL. The molecular weight drops by 5% by day 14 in PCL films. It drops by 32.6% in films loaded with PCL/PVA/PEG as shown by GPC data (Figure 5.9). The highly porous structure created interconnected channels in the leached films increased the diffusion of pDNA. The permeation of the pDNA was controlled by micro pores and interconnected channels formed by PEG (Figure 5.7). In contrast, the TEC-containing film shows less porosity (Figure 5.8). This could be due to much slower efflux of TEC (less hydrophilic than PEG) in to the release medium.

In contrast, the addition of PEG/PVA or TEC has no effect at all on the release of the complexed pDNA. Please see Figure 5.5. This implies that the hydrophobic interactions overwhelm any effect due to pore formation. The interaction holds the polyplexes in the polymer matrix, in spite of the pore formation and degradation of the PCL. All the released polyplexes were in the complex form as tested with and without heparin sulphate.

Films loaded with TEC have about 89% of DNA in the uncomplexed form. This could be due to the presence of citrate ions in it, which dissociates the complexes.

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Figure 5.9 GPC data for the drop in molecular weight for PCL, PCL/PVA/PEG and PCL/TEC films

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5.11 PEVAC films:

Figure 5.10 Release of pDNA and polyplexes from PEVAC films:

Ethylene vinyl co-acetate (EVAc) has been used as a model system for the fabrication of non-degrading controlled release systems. The presence of interconnecting pores in the matrix helps in the release of high molecular weight macromolecules. Pores are randomly situated in the matrix and communicate through narrow throats. Plasmid DNA dispersed in the EVAc films was continuously released for about a month. The DNA release from EVAc matrices consists of two phases: the initial burst phase (< 6 days) and the continuous and slow release phase. SEM picture given below show the presence of pores after 28 days. (Figure 5.11)

The above graph (Figure 5.10) shows the release of pDNA and polyplexes from PEVAC films. The polyplexes were not released from the polymer matrices as of day 28. This again demonstrates that pore formation does not help in the release of

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polyplexes. The $\text{CH}_2\text{-CH}_2$ interaction that occurs between the polymer matrix and the components of PEI which holds the PEI/DNA in the polymer matrix.

Figure 5.11 PEVAC blank film on day 28
(surface)

5.12 FT-IR Analysis:

We postulated above that the lack of a burst effect for the PEI-DNA complexes (and relatively slower release) from the 3 polymers is due to a predominant hydrogen bonding between the -O- groups in the polymers and in NH groups in PEI. To verify this, an FTIR experiment was carried out. Spectra were taken of polymer films with and without added PEI. Figures 5.12 and 5.13 show the FTIR spectra of PCL and PEI with and without the addition of PEI.

The main functional groups in ethylenimine unit of PEI's are CH_2 and NH . The IR spectra of PEI show the peak at 1453 cm^{-1} corresponding to in plane bending of CH_2 . The peaks for the bending vibrations of the NH group and the stretching vibration of the C-N groups of PEI can be seen at 1595 and 1114 cm^{-1} respectively. The C=O stretching band of PCL/PEVAC lies close to 1720 cm^{-1} . Overall, the IR spectra of PEI, PCL and PEVAC show peaks that are characteristic of their main functional groups. However, there are distinct differences between the IR spectra PEI/PCL and that of PEI or PCL alone. Similar differences were noted in case of PEVAC.

In case of PEI/PCL or PEI/PEVAC, the position of the peak for NH of PEI is shifted from 1595 to 1548 cm^{-1} . This could be due to the hydrogen bond formation between

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-O- of PCL/ PEVAC and NH of the PEI. This prevents the polyplexes release from PCL/PEVAC, showing a very slow release.

Based on these initial results, it was found that PEI is not a suitable vector for loading in the PCL and PEVAC films, if sufficient complex is to be released for transfection. TEC also is not effective since it appears to generate less pores, and the citrate ions in it dissociate the PEI/DNA before it is released. So further studies were done with lipofectamine loaded polymeric films and using gelatin as a porogen/ carrier phase.

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Figure 5.12 FTIR Analysis of PCL films with 0% and 10% PEI

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Figure 5.13 FTIR Analysis of PEVAC films with 0% and 10% PEI

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CHAPTER VI

PHASE II PREPARATION OF POLYMERIC FILMS, PDNA, POLYPLEXES, LIPOPLEXES LOADING AND RELEASE STUDIES – GELATIN STUDIES

In this section, we report on studies done with the PEI-pDNA polyplex, as well as a different complex, pDNA with a liposome (lipoplex). In addition, the use of gelatin as an additive was explored, as it provided a means of sequestering and stabilizing the lipoplex.

6.1 Preparation of PEI/DNA Complexes:

This is prepared in a similar way as in Chapter 6, Section 6.1.

6.2 Preparation of Lipofectamine/DNA Complexes (LPF/DNA)

pDNA was diluted from the stock solution using water. It was diluted to a working range of 28 μg in 100 μl (280 $\mu\text{g/ml}$). Lipofectamine was diluted using SFM. By keeping the DNA amount same, Lipofectamine is added at 8 times higher than pDNA (224 μg). An N/P (w/w) ratio of 8 is used in further studies. The complex was vortexed vigorously. The complexes were incubated at room temperature for 30 mins. No agglomeration was noted in the sample preparation.

6.3 Preparation of Gelatin:

Gelatin was prepared as a 10% and 20% solution in water (w/v) and autoclaved

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6.4 Incorporation of Complex into the Films

PLGA films:

A 10% (w/v) of PLGA solution was prepared using chloroform as a solvent. From this about 300 μ l were used for the film preparation. Additionally a 10% and 20% gelatin (w/w) to polymer was introduced to the polyplexes after incubation. The PEI/DNA complex and PLGA solution were mixed together in the volume ratio of 1:3 (100 μ l:300 μ l) and this emulsion was then mixed vigorously at 1800-2000 rpm. All the other steps were done as similar to Section 7.1.2. Blank films without gelatin were used as a control.

PCL films:

A 6.6% (w/v) PCL solution was made by dissolving the polymer in chloroform. 300 μ l of this solution was mixed with 100 μ l of LPF/DNA or pDNA in SFM. The ratio between the SFM and polymer was 1:3. PCL solution was added to the SFM and vortexed at 2200 rpm and cast as a film. Gelatin films were made with the addition of 10% and 20% gelatin (w/w) to the polymer.

PEVAC films:

12 % (w/v) of PEVAC solution was prepared using chloroform. 500 μ l of this solution was mixed with 100 μ l of SFM and vortexed. 10% gelatin films were prepared with a 10% gelatin solution (w/w) of polymer. The ratio between the SFM and polymer is 1:5. PEVAC solution was added to the SFM and vortexed at 2200 rpm and casted as a film.

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6.5 In vitro Release Study:

The films were placed in a 12 well cell culture plates. About 800 μ l of autoclaved water was used for the in vitro release studies. Plates were incubated at 37 deg Celsius at different time points (t=5, 9, 18, 23, 30) days. At every time point the water was removed and replaced with the fresh water. About 1% ampicillin was used as an antibiotic to avoid contamination. These studies were done with water instead of PBS, since the presence of PBS interfered with the transfection results (Chapter 7).

6.6 Quantification of the Release:

pDNA released from the films was quantified using EtBr as mentioned in section 3.2

Polyplexes were quantified using heparin sulphate as mentioned in section 3.2

6.7 Degradation study and SEM study:

These were done in a similar way as in Section 3.2

6.8 Dissolution Study:

The study was done as described in Chapter 5, section 5.7. Additionally to quantify the lipoplexes, oleic acid was used to decouple the pDNA from lipoplexes.

6.9 DSC studies:

Crystallinity studies of PCL and PEVAC polymers (pellets) were studied using differential scanning calorimetry (chapter 3, p62). The crystallinity was calculated using the following equation,

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$$C = \frac{\Delta H}{\Delta H_{100\%}} \times 100 \%$$

Where:

C = crystallinity (%)

ΔH = heat of semi-crystalline polymer (J/g)

$\Delta H_{100\%}$ = heat of fusion of the 100% crystalline polymer (J/g)

The crystallinity% of PCL was determined using a heat of fusion value of 100% crystalline PCL (139.5 J/g) ²³³. For PEVAC, it was determined using heat of fusion value of 100% crystalline polyethylene (293.6 J/g) ²³⁴.

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Results & Discussion:

6.8 Release of pDNA from PLGA (53/47), PCL and PEVAC films:

Figure 6.1 Release of pDNA from PLGA (53/47), PCL and PEVAC films

The in vitro release profiles from the three polymeric films (PLGA, PCL and PEVAC) is shown in Figure 6.1. A sustained release profile was obtained for the release of pDNA from all the three polymeric systems. About 50 % of the drug is released in the initial phase as a burst effect by day 5 in both PCL and PEVAC films. In case of PLGA films 35% is released. The total release of pDNA from PLGA films results from a combination of diffusion and erosion, whereas release from PCL is mostly diffusion. The GPC data given below (Figure 6.19, 6.4) do not show a significant MW-change in PCL films by day 23, whereas in the case of PLGA films a 90% weight loss was observed. This could be related to the faster

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degradation of PLGA when compared with the PCL. The overall higher release of pDNA from PCL is dominated by the early release, and must be attributed to the lower solubility of pDNA in the semi-crystalline PCL matrix.

6.9 Release of Polyplexes from PLGA films:

Figure 6.2 Release of polyplexes from PLGA films

The release of polyplexes shows the classical triphasic profile caused by a combination of diffusion and erosion controlled release (Figure 6.2). About 8% of the drug was released by Day 9. No burst release was noted in the initial stage. This could be related to the interaction occurring between the positively charged PEI and the negatively charged polymer matrix as explained before. (Section 5.9)

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Figure 6.3 PLGA (53/47) on day 30
(surface)

Figure 6.4 GPC data for PLGA 53/47 (0%,10%,20% gelatin) films

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6.10 Effect of additives on the release of pDNA and polyplexes from PLGA films:

To increase the amount of polyplexes and lipoplexes released from the polymeric films, use of a non-CH₂ containing polymer was considered as a medium into which the complex can partition; in addition, if the polymer is sufficiently hydrophilic, it may also act as a porogen. Gelatin was chosen for this purpose. Gelatin is the denatured form of collagen, and has good aqueous solubility. About 10% and 20% of gelatin (w/w) to polymer was used in the following study. In the previous section, it was found that release from PLGA films as well as from PCL and PEVAC films of PEI-pDNA complexes was retarded due to specific PEI-polymer interactions.

Figure 6.5 Effect of additives on the release of pDNA from PLGA films

The effect of additives on the release of pDNA and polyplexes is shown in figure 6.5 and 6.6. Films loaded with 20% gelatin release pDNA at an overall faster rate. About 40% of pDNA is released by day 5. The addition of 10% gelatin does not seem to increase the release of pDNA as compared to 0% gelatin films. In general,

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pDNA-loaded PLGA polymers (whether in the form of microspheres or nanospheres or polymeric films) show a high initial release rate. This could be due to the extremely hydrophilic nature of the pDNA that leads to low entrapment levels (undissolved pDNA mostly on the surface) and high initial burst release rates. Furthermore the addition of gelatin will increase the presence of pores on the surface (20% gelatin), which will increase the rate of release of pDNA.

Figure 6.6 Effect of additives on the release of polyplexes from PLGA films

Polyplexes released from PLGA films loaded with 0%, 10% and 20% are shown in figure 6.6. Films loaded with gelatin show higher initial release of polyplexes than films loaded with out gelatin. Films loaded with 20% gelatin show a high burst effect, about 20%, compared with 0% in films with the absence of gelatin. A triphasic behavior (burst, diffusion, degradation phase) is noted in the release profile of samples with 0% and 10% gelatin loaded PLGA films. For samples with 0% gelatin, a slow diffusion followed by degradation controlled release is seen from day 7 to day 30. A 56% drop in the molecular weight in week 1, was noted in Fig 6.4. The molecular weight drop increases to 90% in two weeks. In case of 10 % gelatin films, a burst phase is seen at day 5 (18%), followed by a

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slow diffusion /degradation controlled release from day 7 to day 30. After day 23, a sharp increase from 26% to 58% release was noted in films with 10% gelatin; where as an increase from 22% to 65% was noted in films with an absence of gelatin. This behavior is in contrast to the behaviour of films with pDNA. The difference in behaviour of the 10% and 20% gelatin films must be attributed to faster onset of degradation for the 20% gelatin film.

Figure 6.7 PLGA films on day 30 (surface) Figure 6.8 PLGA films with 20% gelatin on day 23 (surface)

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6.11 Release of Lipoplexes from PCL films:

Figure 6.9 Release of lipoplexes from PCL and PEVAC film

The release of lipoplexes from the polymeric films are shown in Figure 6.9. The early or burst release is suppressed for the lipoplexes, consistent with the relatively higher lipophilicity of the lipoplexes, and the corresponding higher solubility. The amphiphilicity of the lipoplexes helps to achieve a better dispersion as well as dissolution of the complexes. This results in a slower release of the complexes. The earlier release of lipoplexes from PEVAC compared with the PCL films is probably due to the relative solubilities of the lipoplexes in the two polymers, as well as to differences in porosities.

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Figure 6.10 PCL film on day 30
(surface)

Figure 6.11 PEVAC film on day 30
(surface)

Figure 6.12 PCL films with lipofectamine
on day 30 (surface)

Figure 6.13 PEVAC films with
lipofectamine and 20% gelatin on day 30
(surface)

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6.12 Effect of additives on the release of pDNA and lipoplexes from PCL Films:

Figure 6.14 Effect of additives on the release of pDNA from PCL films

Figure 6.14 shows the effect of gelatin on the % accumulated release of pDNA from the polymeric films. Films without gelatin release faster. Gelatin acts as porogen, and as the porogen leaches out of the film, it leaves pores behind. The formation of pores is seen in the SEM pictures (Figure 6.17, 6.20). However, the creation of pores does not accelerate the release of pDNA because the release is dominated by the burst release of undissolved pDNA. Most of the pDNA remains in the amorphous portion of the polymer, as undissolved pDNA. The solubility of the pDNA in the amorphous phase is quite low; hence there is a large burst observed. As gelatin is added, more of the pDNA partitions into the gelatin, as the solubility is higher in gelatin. Hence the burst effect is suppressed and subsequent release involves re-partitioning from the gelatin to the PCL

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phase, which also slows release. Surprisingly, however, the 10% gelatin loaded films release pDNA at an earlier stage than the 20% loaded films. About 90% of the DNA is released at the end of 30 days in 0% gelatin films. The films loaded with 10% and 20% gelatin release 55% and 40% at the end of a month. The addition of gelatin as a porogen increases the loading % (0% gelatin Vs 10% gelatin).

Lipoplexe release from the films loaded with the gelatin is shown below (Figure 6.15). Here the release of lipoplexes is in the following order 20%>10%>0% gelatin. 20% gelatin shows the highest 5-day release of about 39% as compared with 10% and 3% release from films loaded with 10% and 0% gelatin films. The addition of a hydrophilic polymer gelatin reduces the overall amount of hydrophobic polymer available for lipoplexes to dissolve, and this might lead to some lipoplex being ejected from the solution, the ejection being in the form of a burst effect. The magnitude of the burst release increases with the increase in the % gelatin. The figure 6.15 shows a slight increase in the release by day 23 for the 10% and 20% gelatin samples. However, the GPC data (Figure 6.18) does not show substantial differences in degradation between the 3 samples. The addition of gelatin functions only as a porogen as shown from the SEM picture (Figure 6.17). The earlier release of lipoplexes from the gelatin loaded films must be due to the creation of substantial pores around day 20-23, rather than due to the degradation effect.

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Figure 6.15 Effect of additives on the release of lipoplexes from PCL films

Figure 6.16 PCL film on day 30 (surface)

Figure 6.17 PCL film with 20% gelatin on day 30 (surface)

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Figure 6.18 Degradation profile for PCL (0%,10% and 20% gelatin)

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Figure 6.19 PCL film with lipoplexes on day 30 (surface)

Figure 6.20 PCL film with lipoplexes 20% gelatin on day 30 (surface)

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In general, literature reports show that the release of protein from PCL matrices is purely diffusion controlled. The release is in general very slow due to the semi-crystalline nature of PCL. A study by Lu ¹⁵³ et al. showed that the diffusion of proteins is controlled by the presence of pores on the PCL matrix. It was also found that the protein lysozyme could not directly diffuse through the films. The diffusion of protein was totally controlled by water filled micropores formed by the leaching of PEG. The permeation rate of proteins increased as an increase in the pore fraction. Similar results are observed here.

The presence of gelatin creates pores on the film. The pores were uniformly dispersed in the whole PCL matrix. The highly porous structure created interconnected channels in the leached films, which increased the diffusion of lipoplexes. In other words an increase in the pore fraction increased the permeation rate of lipoplexes from these porous films.

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6.13 Effect of additives on the release of pDNA and lipoplexes from PEVAC films:

Figure 6.21 Effect of additives on the release of pDNA from PEVAC films

Figure 6.21 shows a biphasic behavior. An initial burst release is noted followed by a slow continuous release. Shen et al found a slow continuous release of pDNA from the release of pDNA from EVAc discs. Another study by Jong¹⁶³ et al showed that about 60% of the pDNA was released in 24 hrs. The results from Figure 6.1 show that about 50% of the pDNA was released in 5 days. The difference could be that their studies were done in EVAc discs and rods in contrast to films. Also the formulations and DNA loading were different.

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In pDNA loaded films, the presence of additives does not show a significant, earlier release. This is probably because the addition of 10% gelatin is not sufficient to increase the dissolution or partitioning of the pDNA into the gelatin phase, so that the pDNA was largely still left undissolved in the matrix. The release is dominated by the lack of dissolution of the pDNA rather than the porosity or other factors.

Figure 6.22 PEVAC film on day 30 (surface)

Figure 6.23 PEVAC film with 10% gelatin on day 30 (surface)

In contrast to pDNA, the release of lipoplexes (Figure 6.24) from PEVAC clearly shows the effect of additives. The 10% gelatin loaded films release considerably faster than the films without gelatin. This again could be linked to the presence of pores, as well as to some partitioning into the gelatin phase. About 36% of the lipoplexes is released as the burst effect in the presence of gelatin. No burst release is noted with the films loaded with the absence of gelatin. The addition of 20% gelatin to the PEVAC polymer solution, resulted in an improper emulsion. Hence experiments with 20% gelatin was not continued further.

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Figure 6.24 Effect of additives on the release of lipoplexes from PEVAC films

The release of proteins from EVAc matrices, first demonstrated over 20 years ago²²⁹, is controlled by diffusion. The time course of the release can be modified by changing either the polymer or encapsulants. For example, manipulation of the internal pore structure of the matrix²³⁵, the molecular weight of the polymer within the matrix²³⁶, can change the release profile significantly.

There is a difference between the release profiles of pDNA and of the lipoplex (Figure 6.21 and Figure 6.24), showing the effect of additives. For pDNA, the release is dominated by a relative lack of solubility of the pDNA in the PEVAC, with or without gelatin. However, for the more lipophilic lipofectamine, higher solubility in the matrix without gelatin ensures lack of a rapid early release, whereas the addition of gelatin increases this release further by pore creation, as evidenced by SEM. (Figure 6.25, Figure 6.26)

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Figure 6.25 PEVAC film with lipoplexes and on day 30 (surface)

Figure 6.26 PEVAC film with 10% gelatin lipoplexes on day 30 (surface)

Figure 6.27 GPC data for PEVAC films (0%,10% gelatin)

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As far as we know, no previous study has been reported for release of PEI/pDNA complexes from PLGA film or for the loading and release of lipoplexes from PCL and PEVAC films.

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6.14 Effect of 10% gelatin on the release of pDNA from PLGA (53/47), PCL and PEVAC films

Figure 6.28 Effect of 10% additive on the release of pDNA from PLGA (53/47), PCL and PEVAC films

A comparison of pDNA release among the 3 polymers (Figure 6.28) shows that the early release is more rapid for PCL than for the other polymers, reflecting the relatively lower solubility of pDNA in the PCL matrix, presumably due to the higher crystallinity of PCL. DSC data given below (Figure 6.29 and Figure 6.30) show that PEVAC is about 8.9 % crystalline and PCL is about 62.3 % crystalline. It is calculated as explained in section 6.9. Since the pDNA can only dissolve or disperse in the amorphous phase of any polymer, the pDNA is more concentrated in the PCL amorphous phase, hence releases faster, as most of the pDNA is undissolved in the amorphous phase.

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Figure 6.29: DSC data for PEVAC films

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Figure 6.30: DSC data for PCL films

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6.15 Effect of 10% gelatin on the release of lipoplexes from PCL and PEVAC films

Figure 6.31 Effect of 10% additive on the release of lipoplexes from PCL and PEVAC films

Clearly, the effect of the gelatin is to accelerate the release of the lipoplexes from either matrix, the effect being more pronounced for PEVAC. The reason as explained earlier, is the creation of pores due to the leaching out of gelatin.

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6.16 Comparison between the release of polyplexes and lipopolyplexes from PCL films:

Figure 6.32 Comparison of release of polyplexes and lipopolyplexes from PCL films

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6.17 Comparison between the release of polyplexes and lipoplexes from PEVAC films:

Figure 6.33 Comparison of release of polyplexes and lipoplexes from PEVAC films

Figures 6.32 and 6.33, shows the release of polyplexes and lipoplexes from PCL and PEVAC films in the absence of gelatin. These figures clearly demonstrate the effect of the hydrophobic interaction on the release profiles. The lipoplexes which do not exhibit any hydrogen bonding interactions, show faster release from all polymer matrices, and substantiates the hypothesis discussed earlier (Section 5.12). The difference in the release rates of lipoplexes between these polymers could be due to the porosity as explained before. (Section 6.11)

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Studies were done on loading lipoplexes in PLGA films. Quantifications of the released complexes were performed till day 9. But as the polymer starts to degrade by day 14, quantification of the released complexes using oleic acid were hindered. We saw a visible white colored precipitation after the addition of oleic acid to the released samples. This could be due to the interference of the degradation products. Diluted samples also had the same problem. So it was decided not to continue with the study.

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6.18 Summary:

We now summarize all the effects observed with respect to the release of naked DNA, polyplexes and lipoplexes, from the three polymer classes: a degrading amorphous polymer (PLGA), a semi-crystalline, slower-degrading polymer (PCL) and a non-degradable, partially-crystalline polymer (PEVAC).

Naked DNA release:

- The release of naked DNA is dominated entirely by its hydrophilicity, and lack of significant solubility in any of the matrices studied so far (with or without additives). Most of the naked DNA is released in the early stage, as the undissolved DNA simply dissolves or leaches out into the release medium. The release is fastest from the highly semi-crystalline PCL.

Polyplex Release:

- The release of polyplex is dominated by the hydrogen bonding interaction.
- The release is very slow from non or slow-degrading polymers, and somewhat faster (classical triphasic) from PLGA.
- Although release is sustained over several days, the amount released is so low that transfection effects are not observed.

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Lipoplex Release:

- Lipoplex release is faster from PCL and PEVAC films, when compared to polyplex release. This is clearly due to the lack of hydrogen bonding.
- The lipoplex release is further enhanced by addition of gelatin, which creates pores, but does not cause any accelerated degradation of the matrix.
- Release of lipoplexes from PLGA was not measurable due to interaction between the lipoplex and the degraded polymer fragments.

Thus, in order to create a sustained release dosage form for complexed DNA (we ignore the naked DNA as being not efficient enough), the best candidates are slow- or non-degrading polymer systems, with small amounts of a hydrophilic polymer, such as gelatin. The best type of complex is the lipoplex, or pDNA complexed to a non-CH₂-containing entity. Such formulations can release the complexed DNA over a month or two, in sufficiently large amounts, and in the complexed state. Chloroform is a suitable solvent for the preparation of such matrices.

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CHAPTER VII

PHASE III TRANSFECTION STUDIES

In this section, we report on studies done with the released pDNA, polyplexes, and lipoplexes from the gelatin loaded films. The main goal of these studies was to establish the bioactivity of the released DNA: is all the released DNA still able to transfect cells? If not, how much is bioactive?

7.1 Preparation of COS-7 cells:

This is prepared in a similar way as in chapter 4, section 4.1.1.6

7.2 Loading of complexes to cells:

The released samples were collected and air dried for 4-5 hrs in a laminar hood to evaporate the release medium. The water needs to be removed since cells loaded with samples in water with an incubation time of 5 hrs caused significant cell death. This could be due to the lack of nutrients for the cells if water is used. Once dried the samples were replaced with fresh SFM. The drying of the sample affected the transfection efficiency of both the test and control samples by 10%. All of the samples were loaded at loading of 1 $\mu\text{g}/0.5\text{ ml}$ for PCL and PEVAC films. For PLGA films about 0.5 $\mu\text{g}/\text{ml}$ was used. Samples were allowed to incubate for 5 hrs. 2X medium (DMEM with 10% FBS) were used to replace the SFM after 5 hrs. Transfection results were analyzed after 48 hrs.

All transfection studies were done in duplicates.

7.3 Analysis of Luciferase expression:

This is studied as mentioned in chapter 4, section 4.1.1.6.

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7.4 Bioactivity of Polyplexes and Lipoplexes from polymeric films:

The bioactivity of the released polyplexes and lipoplexes from the polymeric films were determined before transfection. This is determined using heparin sulphate (HS) for polyplexes and oleic acid (OA) for lipoplexes. (Chapter 4, section 4.4 part II). If the complexes are active, no free pDNA should be detected with the absence of HS/OA.

For polyplexes:

Released samples from polyplexes loaded films + HS = DNA detected
 -----" ----- w/o HS = DNA not detected

For lipoplexes:

Released samples from lipoplexes loaded films + OA = DNA detected
 -----" -----w/o OA = DNA not detected

7.5 Transfection:

The transfection efficiency of the released pDNA/lipoplexes/polyplexes was calculated using the formula below,

$$\text{Transfection Efficiency} = \frac{\text{fg of luciferase expressed / mg of protein of total cell protein}}{\mu\text{g of pDNA}}$$

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The bioactivities of the released polyplexes/lipoplexes from the polymeric films were summarized below.

Table 5: Percentage complexed form of polyplexes from PLGA films

Time Points	% complexed form (polyplexes)		
Days	PLGA/0 %gelatin	PLGA/10 %gelatin	PLGA/20 %gelatin
Day 5	100	60	70
Day 9	100	-	67
Day 14	100	47	53
Day 23	100	40	50
Day 30	100	30	33

Table 6: Percentage complexed form of lipoplexes from PCL and PEVAC Films

Time Points	% complexed form (lipoplexes)				
Days	PCL/0 %gelatin	PCL/10 %gelatin	PCL/20 %gelatin	PEVAC/0 %gelatin	PEVAC/10 %gelatin
Day 5	100	70	90	100	100
Day 9	100	70	90	100	100
Day 14	100	100	90	-	70
Day 23	100	100	100	80	100
Day 30	100	80	80	80	0

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The following table shows the samples used for the transfection study

Table 7: Transfection Samples

Sample	Time points/Days	Amount released at each time point (μg)	% complexed form	Amount of transfection detected (fg/mg of protein)/ μg of pDNA released
PLGA/ 20% gelatin/DNA	Day 5	1.35	-	1×10^4
PLGA/20% gelatin/Polyplexes	Day 5	0.9	70	2×10^3
PCL/10% gelatin/ DNA	Day 5	6.2	70	1×10^4
PCL/20% gelatin/ DNA	Day 5	6.4	-	1×10^4
PCL/10% gelatin/ lipoplexes	Day 5	2.25	70	9×10^4
PCL/10% gelatin/lipoplexes	Day 23	3.8	100	- nil-
PCL/20 % gelatin/ lipoplexes	Day 5	6	90	5×10^5

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PCL/20% gelatin/lipoplexes	Day 23	3.23	100	- nil-
PEVAC/10% gelatin/DNA	Day 5	2.7		1×10^4
PEVAC/10% gelatin/lipoplexes	Day 5	6.5	100	1×10^5
PEVAC/10% gelatin/lipoplexes	Day 9	2.4	100	6×10^4

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Results & Discussion:

7.4 From PLGA films:

Figure 7.1 Release of polyplexes from PLGA films (20% gelatin)

The above graph shows the sample loaded, polyplexes (indicated by arrows) for transfection studies. The graph is plotted as the amount of pDNA (in complexed or uncomplexed forms) released at individual time intervals. For example, 1.1 μg of pDNA is released on day 5, while only about 0.15 μg is released between day 5 and day 9, and so on. The 5-day time point was selected as the concentration of released DNA is high enough to yield transfection. The released samples were treated with heparin sulphate to

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calculate the amount of the complexed form of polyplexes. At this time point about 30% of the released polyplexes were in the uncomplexed form (free pDNA).

7.4.1 Transfection of polyplexes Vs naked pDNA from PLGA films:

Figure 7.2 Transfection of released pDNA from PLGA films

Using the pDNA released on day 5, for PLGA/pDNA/20% gelatin showed a reasonable transfection compared with fresh pDNA controls. In case of polyplexes, however, about 30% of the released samples were in the uncomplexed form. This uncomplexed form could be related to the charge interaction occurring between the polyplexes and PLGA at high N/P = 4 (w/w) ratio. So the drop in transfection with the released polyplexes is due to the lower amount of polyplexes in the complexed forms. (0.7 μ g); it is also likely that the binding of the PLGA fragments (negatively charged) to the PEI affects the transfection results. This binding increases with increasing degradation. We were unable

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to show transfection at the remaining points since relatively low amounts were released, the % of uncomplexed form increased, followed by the effects of degradation products, as explained earlier.

7.5 From PCL and PEVAC films:

In case of PCL and PEVAC films, films loaded with gelatin were chosen, since with 0% gelatin, the amount of lipoplexes released was relatively small. For PCL films, samples loaded with 10% gelatin and 20% gelatin were tested on day 5 and day 23. In case of PEVAC, films with 10% gelatin were tested on both day 5 and day 9.

The figure below (Figure 7.3) explains the selection of samples (indicated as arrows). Here the samples are plotted as the amount of pDNA released at individual time intervals rather than accumulated pDNA Vs time. The released samples were treated with oleic acid for the detection of complexed pDNA. PCL films (10% gelatin) showed about 30% in the uncomplexed form (day 5) (Table 7). In comparison, 10% uncomplexed pDNA was released from 20% gelatin loaded PCL films on day 5. No uncomplexed pDNA was seen with PEVAC samples on day 5. In all cases, about 1.0 μg of the released lipoplexes was used for the transfection studies.

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Figure 7.3 Release of lipoplexes from PCL and PEVAC films

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7.5.1 Comparison of transfection results between PCL films:

Figure 7.4 Transfection of lipoplexes from PCL films (10% Vs 20% gelatin films)

The above picture shows the comparison between the transfection results between films loaded with 10% gelatin and 20% gelatin at the same time point (day 5). The higher transfection efficiency of the 20% gelatin samples is related to both the reduced transfection due to uncomplexed pDNA, as well as to the toxicity effect exhibited by the free forms of lipoplexes (Lipofectamine). About 10% of uncomplexed DNA was found with 20% gelatin loaded films compared to 30% uncomplexed form from 10% gelatin loaded films. From these data, we may infer that when the complexed form partitions into the gelatin phase, it is kept relatively stable.

Transfection with day 9 and day 18 samples were not possible since very low amounts of lipoplexes were released at those time points. Transfection done on day

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23 showed low values. At this time point the lipoplexes were in the complexed forms but unable to transfect. The released samples were in 100% complexed forms (as tested with oleic acid). The reason for low transfection is due to agglomeration of the released lipoplexes. Particle size study was done with the released samples showed the following (Table 8). The particle size increases to 174-176 nm in samples released from PCL/lipoplexes/gelatin films. Freshly formed samples showed a particle size of 59 nm. Lipoplexes particle size of 85 nm showed a drop in the transfection activity of 1×10^5 fg/mg. Lipoplexes with the particle size of 60 nm, showed an increase in the transfection to 1×10^7 fg/mg.

Table 8: Particle sizes of the released lipoplexes from PCL films

Sample	Particle size (nm)
Lipoplexes (Untreated)	59
PCL/10% gelatin/Lipoplexes	174
PCL/20% gelatin/Lipoplexes	176.5

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Figure 7.5 Transfection activity Vs particle size of LPF/DNA

Transfection experiments were not performed after day 23. The released complexes were aggregated and resulted in zero transfection on day 23. On day 30, PCL films released 0.27 μg , PCL /10% gelatin released 0.29 μg of lipoplexes. The amount of lipoplexes needed for transfection is 1 μg . In case of PCL/20% gelatin samples 1 μg of lipoplexes were released and 20% was in the decomplexed form. So transfection studies were not continued after day 23.

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7.5.2 Transfection of lipoplexes from PEVAC films Day 5 Vs Day 9:

Figure 7.6 Comparison of transfection between Day 5 and Day 9 from PEVAC films.

The above picture shows that there is a drop in the transfection efficiency over time. In the initial stage (day 5) the released complexes were 100% in the complexed form and as time increases the complexes begins to dissociate, affecting its stability and hence transfection.

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7.5.3 Transfection of lipoplexes Vs naked pDNA from PCL and PEVAC (Day 5, 10% gelatin) films:

Figure 7.7 Transfection of between pDNA and lipoplexes from PCL and PEVAC (10% gelatin films)

The above results show that samples with lipoplexes were able to show higher transfection than naked DNA as expected. In case of lipoplexes from PCL films about 30% was released in the uncomplexed form (as tested using the addition of oleic acid) whereas in case of PEVAC all of the complexes are released in the the complexed form. For comparison of results between the two, both of them are loaded at the same amount (1 μ g).

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A slightly higher transfection is noted with the PEVAC when compared to PCL, which could be linked to the fact that a greater percentage of the pDNA retains its complexed form when released from PEVAC.

It appears that partitioning into the gelatin phase partially decomplexes the polyplex and lipoplex, hence the released pDNA has lowered transfection at longer times.

For a sustained-release dosage form to function effectively, not only should sustained release of the DNA be achieved, but the formulations developed should ensure that much of the released DNA is in the complexed form, in order to achieve sustained transfection. In our results, we find that PEVAC (a non-degrading polymer) achieves this goal with lipoplexes, but not with PEI-DNA polyplexes, for reasons elucidated earlier. A 30- day sustained transfection by lipoplexed DNA is possible with PEVAC matrices, if the release of the lipoplexes is increased after day 9. Studies to determine why PCL/gelatin matrices lead to decomplexation should be undertaken: is it related to accelerated degradation that is happening in the gelatin-loaded PCL? Even a low extent of degradation of polyester-type matrices may be sufficient to effectively destabilize complexed DNA. This poses great challenges for the development of degradable gene carriers.

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CHAPTER VIII

CONCLUSIONS & FUTURE WORK

8.1 Conclusions:

In this work, it has been shown that pDNA, both naked and complexed with cationic agents, can be successfully incorporated into polymeric films. These forms of DNA can also be released from the films in a controlled manner, such that transfection of surrounding cells may be sustained over 1-2 months. Developing such a controlled-release system for DNA is novel, and understanding further the limitations of the polymeric matrices helps to design the optimum device for a given gene delivery application.

The major learning points from this study (which have not been reported previously) include;

- With naked pDNA, it is not possible to eliminate the burst effect, with the use of commonly used polymer matrices. To develop a truly sustained-release system for pDNA would involve the use of hydrophilic/hydrophobic polymer blends where the hydrophilic component attains levels of 40-50%.
- Polyplexes may be released in sustained fashion over 1-2 months, but the release rate is typically too slow to be of practical use; the release is dominated by the presence of the hydrogen bonding interactions between the PEI and the polymers used
- Lipoplexes can be formulated to useful sustained release forms with both degradable and non-degradable polymer matrices. However, relatively rapid-degrading polyesters such as 53/47 PLGA affect the stability of the complexed form, and its ability to transfect cells.

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- The best type of controlled-release system for sustained transfection is the lipoplex, or pDNA complexed to a non-CH₂-containing entity. Such complexes, when dissolved/dispersed in a gelatin-containing biostable polymer such as PEVAC, can release the complexed DNA over a month or two, in sufficiently large amounts, and in the complexed state. Chloroform is a suitable solvent for the preparation of such matrices.

8.2 Future Work:

There are some gaps in the above studies, which need to be explored, to have a better understanding of the sustained release of non-viral vectors (PEI/Lipofectamine) using these polymer matrices. These include the following,

1. PCL loaded lipoplexes (10% gelatin/20% gelatin) showed an increase in the lipoplexes on day 23. The GPC data does not show a significant drop in MW at that time point. This increase in the release could be related to the pore formation by gelatin rather than degradation. SEM studies need to be done more frequently from gelatin loaded films, especially around day 23, to confirm this.
2. The lipoplexes released on the day 23 from PCL films were in the complex form, but still unable to transfect. The reason needs to be explored further.
3. In case of PLGA films, with polyplex-loaded samples, higher degree of decomplexation are noted at the earlier time points (day 5). Using different N/P ratios may help to understand the high degree of decomplexation better.
4. Growing cells over PCL scaffolds loaded with lipoplexes could have an impact on the transfection (GAM- gene activated matrices). In this case the released samples will be readily available for the cells to uptake or the released samples will be more concentrated to show an increase in the transfection.

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5. We have used a high loading (28 $\mu\text{g}/100\text{ }\mu\text{l}$) in our studies. Perhaps other loading studies will help to have a good insight on the factors which affect the release of pDNA as well as complexes.
6. In all our studies reporter gene (pEGFP-*Luc*) was used. Once all the procedures and the formulations are optimized, an in-depth study involving the use of the therapeutic gene should be carried out.
7. Apart from gelatin, other hydrophilic polymers could be tried. Also other non-viral vectors need to be explored.

A major conclusion from this thesis work is that complexed pDNA is adversely affected by degrading polyester matrices. Formulations using a non-degrading matrix appears to be a better choice for sustained release of complexed pDNA.

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