Study of bioactivity of complexed DNA in biodegradable polymer films

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STUDY OF BIOACTIVITY OF COMPLEXED DNA IN
BIODEGRADABLE POLYMER FILMS

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**STUDY OF BIOACTIVITY OF COMPLEXED DNA IN
BIODEGRADABLE POLYMER FILMS**

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STUDY OF BIOACTIVITY OF COMPLEXED DNA IN BIODEGRADABLE POLYMER FILMS

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Dedicated To
My Beloved Parents

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List of Abbreviations

ADA-	Adenosine deaminase
AIDS-	Acquired Immune Deficiency Syndrome
BCA-	Bicinchoninic acid
BMP-	Bone morphogenic proteins
CFTR-	Cystic fibrosis transmembrane conductance regulator
DAB-	Butylenediamine
DAPI-	4',6-diamidino-2-phenylindole
DD-	Degree of deacetylation
DLS-	Dynamic Light Scattering
DMEM-	Dulbecco's Modified Eagle Medium
DMRIE-	1, 2-dimyristyloxypropyl-3-dimethylhydroxyethyl ammonium bromide
DNA-	Deoxyribonucleic acid
DOPC-	1, 2-dioleoyl- <i>sn</i> glycerol-phosphatidylcholine
DOPE-	Dioleoylphosphatidyl ethanolamine
DOPE:	1, 2-dioleoyl- <i>sn</i> -glycerophosphatidylethanolamine
DORIE-	1, 2-dioleyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide
DOTAP-	1, 2-diolyoxy-3-(trimethylammonio) propane
DOTMA-	1, 2-dioleoyloxypropyl-trimethylammonium chloride

List of Abbreviations

DSC-	Differential Scanning Calorimetry
EGF-	Epidermal growth factor
EGFP- <i>Luc</i> -	Enhanced green fluorescence protein-luciferase
EVAc-	Ethylene vinyl-co-acetate
FBS-	Foetal bovine serum
FESEM-	Field Emission Scanning Electron Microscopy
GAM-	Gene-activated matrices
GFP-	Green fluorescent protein
HA-	Hyaluronic acid sodium salt
HEK-	Human Embryonic Kidney
NLS-	Nuclear localization sequences
PAA-	Poly acrylic acid
PAMAM-	Polyamidoamine
PCL-	Poly (ϵ -caprolactone)
pDNA-	Plasmid DNA
PEG-	Poly (ethylene glycol)
PEI-	Polyethyleneimine
PI-	Propidium Iodide
PLA-	Poly (lactic acid)
PLGA-	Poly (lactic-co-glycolic) acid

List of Abbreviations

PLL- Poly (L-lysine)

PPI- Polypropylenimine

PVA- Poly (vinylamine)

PVP- Poly (vinyl pyrrolidone)

RNA- Ribonucleic acid

SCID- Severe combined immunodeficiency syndrome

SEC- Size Exclusion Chromatography

SFM-Serum free medium

siRNA- Small interfering RNA

UV- Ultraviolet light

Abstract

Sustained DNA delivery from polymeric films provides a means for enhanced and prolonged gene therapy. However, in the case of bioactive molecules such as pDNA, there are limitations on the achievable release profiles as well as on the maintenance of bioactivity over time. To address these issues, in this research project various polymeric formulations were developed in which DNA was incorporated. The polymeric system consists of a biodegradable semi crystalline polymer such as PCL blended with a hydrophilic polymer such as gelatin, hyaluronic acid (HA), PEG, PVP, or the sodium salt of poly acrylic acid (PAA). Release studies of pDNA and complexed DNA from different polymeric films were performed by quantifying the amount of DNA released at specific time points over a period of month, utilizing the fluorescence of ethidium bromide complexed to DNA. The studies show that the release of pDNA and complexed DNA from polymer film can be sustained for several days. In all the cases studied, a rapid initial *in vitro* release was obtained for pDNA, while the release of the complexed pDNA was slower, and sustained for a month. To rationalize the observed release profiles, polymeric films were characterized by SEC, SEM, pore size measurement and confocal fluorescence microscopy. Bioactivity of the released DNA was determined by *in vitro* cell transfection efficiency using COS 7 cell lines. Transfection was observed from released lipoplexes samples till day 9 from PCL film with lower MW gelatin and till day 18 in the case of PCL films with higher MW gelatin. The gene expression efficiency of released lipoplexes decreased over time due to a combination of size increase and cytoplasmic degradation. Of all the

Abstract

hydrophilic additives used, only gelatin was able to sustain the release of bioactive complexed pDNA over time. The behaviour of PCL-gelatin blends is rationalized on the basis of confocal microscopy of tagged gelatin and lipoplex/pDNA as well as evaluation of the degree of complexation and the size of the lipoplex.

CHAPTER I

1. Introduction

1.1 Background

Gene therapy has a very significant role to play in the future of modern molecular medicine in alleviation of many diseases which are currently incurable.¹ Gene therapy initially was projected for the treatment of inherited genetic disorders but is currently being studied in a wide range of diseases, including cancer, peripheral vascular disease, arthritis, and neurodegenerative disorders.

The main dispute in gene therapy is to develop approaches for delivering genetic material to the appropriate cells of the patient in a way that is specific, efficient and safe. To do this, special gene delivery vehicles (*vectors*) have been developed. Vectors generally fall into two categories: viral and non-viral.²⁻⁴ Currently, the majority of the vectors for human gene therapy treatments are of viral origin. Attention is now diverted towards non-viral systems due to safety concerns of viral vectors because they are difficult to produce and toxic (in particular immunogenic), as well as having a limitation in terms of the size of the inserted genetic materials.² However, non-viral vectors also have important disadvantages, including their low efficacy and transient gene expression.³ The poor efficiency of delivery and expression is the major drawbacks of non-viral vectors to the development of gene therapy and DNA vaccination.³ Now much attention is given to

design a new formulation of DNA with various substances such as lipid, protein, polysaccharide, peptide and polymer. But DNA delivery by all these systems is bolus without long term sustained release.

The treatment of a chronic disease is the major challenging issue of the gene therapy. The injected corrective gene reaches into the nucleus but does not integrate itself into the cell's DNA. Therefore the benefit from the corrective gene is short-lived. A repeated injection is necessary, sometimes as often as once every week for the therapy to be effective. Only adeno-associated and retro viral vectors can overcome this deficiency,⁵⁻⁶ but these vectors have other problems, such as potential immunogenicity and limited capacity for the size of the gene.⁷

Therefore, it would be more beneficial to the patient if the duration of action of injectable or implantable sources of corrected genes is extended. This may be achieved by sustaining the release of the gene vector over several days or months, because prolonged and continuous DNA delivery to tissues will enhance gene expression.⁸

Sustained release systems using biocompatible and/or biodegradable polymer include microspheres, nanospheres, and polymer matrices which provide an attractive alternative for long term delivery of variety of drug molecules such as proteins, peptides, oligonucleotides and DNA. So, in sustained release polymeric gene delivery system, DNA is either entrapped or dispersed into the polymer matrix or adsorbed on to the surface. So encapsulation of DNA into polymeric system can be used to control of DNA release. DNA may be condensed with cationic polymers or cationic lipids to

decrease particle size, protect DNA from degradation, promote interaction with cell membrane and facilitate endosomal release before encapsulation (Figure 2.5). Complexing agents help to maintain the stability of DNA complexes during polymer processing. Sustained release polymeric gene delivery system offers many advantages over conventional delivery systems:⁹⁻¹⁰

- Sustained delivery of plasmid DNA at the tissue site offers a source for continual gene uptake and thereby prolonging the gene expression
- Increased amounts of plasmid DNA (pDNA) uptake
- They are well protected before being released
- Site specific delivery can be achieved by simple implantation or direct injection
- Repeated drug administration is not necessary

1.2 Problem Statement

Although in recent years sustained release systems have been successfully employed to deliver proteins and macromolecules, polymer based DNA delivery system have not been fully explored. Now either synthetic or natural biodegradable and non-biodegradable polymers have been investigated for sustained delivery systems. But due to large size and polar nature of DNA, encapsulation of plasmid DNA molecules in a hydrophobic matrix will be a great challenge.

The reported DNA encapsulation processes lead to some degradation of DNA during encapsulation and also in the course of polymer degradation.

Figure 1.1: Schematic of DNA condensation and incorporation of DNA in polymer Matrix

However, the system delivers DNA as a bolus without a long term sustained release. To date, there is no reported sustained-release dosage form for release of pDNA or complexed DNA that has good bioactivity and prolonged action of the released DNA. Prolonged and continuous DNA delivery to tissues enhances the duration of gene expression compared to more traditional delivery methods (Injection). In addition, sustained release systems typically deliver vector locally reduce the toxicity to the nontarget cells, fewer physician visits, and overall reduction in the cost of disease management.

1.3 Objectives

The goals of the studies are as follows:

- ❖ To develop an implantable sustained-release system for gene delivery using polymers
- ❖ To understand the factors those govern the bioactivity and release of complexed and pDNA from polymers.

1.4 Outline of This Work

In order to achieve the above goals, the work undertaken is outlined as follows:

- ❖ Standardization and characterization of lipoplexes
- ❖ To formulate polymeric films with incorporation of DNA (pDNA and complexed DNA; model and therapeutic) into polymeric matrices for controlled release/sustained-release of DNA
- ❖ Quantitative study of the release of pDNA and complexed DNA (lipoplex) from various polymer matrices
- ❖ To characterize the polymeric films, study in *vitro* release characteristics of the DNA, and characterize the DNA released from the polymeric films
- ❖ To evaluate the in *vitro* transfection efficiency of the released DNA from polymeric films
- ❖ To study the mechanism of release DNA (pDNA and complexed) from polymer

Figure 1.2: Overview of project

CHAPTER II

2. Literature Review

2.1 Gene Therapy

Gene therapy is a newer approach to treat diseases based on modifying a person's genes toward a therapeutic goal. This approach is to cure both inherited and acquired disorders by adding, replacing, repairing, or regulating genes.

Gene therapy, as first proposed 1972 by Friedman and Roblin,¹¹ 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. Viral vectors currently in use include retrovirus, adenovirus, adeno-associated virus, herpes simplex virus, papilloma virus, and others.¹² However, safety concerns regarding their use in humans make nonviral delivery systems (Electroporation, Gene gun, cationic liposomes, cationic polymer, etc.) an attractive alternative. Nonviral vectors are particularly suitable with respect to simplicity, ease of large-scale production and lack of specific immune response. Recently, various novel nonviral vectors have been developed that approach viruses with respect to transfection efficiency. In 1990, Blaese & Anderson, who were the first to insert successfully the adenosine deaminase (ADA) gene into T-lymphocytes of an ADA-deficient patient.¹³

This was the first approved human gene therapy protocol for the correction of an ADA deficiency.¹³ ADA-deficient patients suffer from severe immunodeficiency and resultant infections because of a mutation in the adenosine deaminase gene. Rosenberg *et al.*(1993) were used retroviral vectors in a human cancer gene therapy trial.¹⁴⁻¹⁵

There are two types of Human Gene Therapy:

2.2 Germline Gene Therapy

Germline gene therapy involves the genetic modification of germ cells that will pass the change on to the next generation.¹⁶

2.3 Somatic Gene Therapy

Somatic gene therapy is the manipulation of gene expression in cells that will be corrective to the patient but not inherited to the next generation. Somatic gene therapy can be split in to two categories:¹⁷

2.3.1 *Ex vivo* Approaches

In the *ex vivo* approach, cells would be extracted from the patient and genetic material inserted into cells grown in *vitro*. Then transfected cells are selected, expanded and reintroduce into a patient.^{1, 17}

2.3.2 *In vivo* Approaches

In the *in vivo* approach, gene therapy treats cells in the individual without removing them. Recombination-based approaches *in vivo* are particularly uncommon, because of very low probability of DNA constructs recombination effect.¹⁷

2.4 Clinical Applications of Gene Therapy

Earlier, plasmid-based gene therapy was attempted to correct inheritable disorders resulting from a single gene defect such as severe combined immunodeficiency disease (SCID), cystic fibrosis, etc.¹⁸⁻²² In 2002, scientists described the successful gene-therapy-based cure for SCID.¹⁸⁻¹⁹ The Chinese drug regulatory agency approved the first gene therapy product for head and neck squamous cell carcinoma, which will be marketed under the trade name Gendicine.²³ Currently, diseases with complex etiologies such as cancer and neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease are being targeted. In addition, Hemophilia A and B, infectious diseases, vascular diseases, DNA vaccines for malaria, AIDS, and many other diseases are in development. DNA vaccines have been also used to prevent allergic response squamous cell carcinoma, which will be marketed under the trade name Gendicine.²³ Currently, diseases with complex etiologies such as cancer and neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease are being targeted. In addition, Hemophilia A and B, infectious diseases, vascular diseases, DNA vaccines for malaria, AIDS, and many

other diseases are in development. DNA vaccines have also been used to prevent allergic response.⁷

Table 1. Diseases for Gene Therapy⁷

Table 1.1 Acquired Diseases⁷

Disease	Defect	Incidence	Targets
Cancer	Many causes, including Genetic and environmental	1 million /year in USA	Variety of cancer-cell types, liver, brain, pancreas, 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

Table 1.2 Genetic Diseases⁷

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 and 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

2.5 Gene Delivery Systems

The main challenging aspect of gene therapy is to develop approaches for delivering therapeutic genetic material to the appropriate cells of the patient in a way that is specific, efficient and safe. Gene that is delivered must be entered into the nucleus to produce protein molecule. A protein molecule maintains the primary therapeutic effect by killing of cells in case of tumour therapy or preservation of cell in the case of neurogenerative disorder.

Gene therapy may hold the potential to revolutionize modern molecular medicine, provided that appropriate nucleic acid delivery systems ('vector systems') are available. Vectors can be divided into two major groups, namely viral vectors derived from natural viruses and nonviral, synthetically manufactured vectors.²⁴⁻²⁶

2.5.1 Viral Vectors

Since naturally viruses deliver their genes into host cells, they present good candidates for the development of effective gene delivery. Adenoviral vectors retroviral vectors and have been most commonly used in clinical trials.²⁷ Viruses in general are highly efficient regarding cellular uptake and intracellular delivery of therapeutic genes to the nucleus.²⁸⁻²⁹ So, few viral particles are enough for the transduction of cells. Gene transfer activity of the viral vectors also strongly depends on the type of virus used for transduction.²⁹

Generally, viral transfection systems show high transfection efficiency. However, they show serious drawbacks, such as its difficulty to produce and toxicity

(in particular immunogenic), as well as having a limitation in terms of the size of the inserted genetic materials.³⁰ For the above reasons, attention is now diverted toward developing non-viral systems for DNA delivery.

2.5.2 Nonviral Vectors

Nonviral vectors can be divided into two major categories, physical and chemical. In physical methods, plasmids enter into cells through electroporation, sonoporation, or particle bombardment. In chemical methods, lipids, polymers, or proteins are used to complex with DNA, condensing it into particles, negative surface charge on the DNA and facilitate internalization by the cell.³¹⁻³² These packages can also help in targeting specific cell populations, efficient internalization and intracellular trafficking, and provide protection against nuclease activity, significantly increasing the gene transfer efficiencies.

2.5.2.1 Gene Transfer of pDNA by Needle Injection

Simple injection of plasmid DNA without additional help from either a chemical agent or a physical force directly into a tissue is able to transfect cells. Local injection of plasmid DNA into the lungs, liver, skin, muscle, leads low level of gene expression.³³⁻³⁴ Gene transfer by needle injection method is attractive due to simplicity and lack of toxicity of pDNA. The broad ranges application of application of DNA mediated gene transfer may not be possible because DNA is large in size and hydrophilic, is kept out of the cells by physical barriers, such as blood endothelium,

the interstitial matrices, etc. Degradation of DNA by intra- and extracellular nuclease activities reduces the chances of intact and functional DNA entering into the nuclei.³³⁻

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2.5.2.2 Electroporation

The application of controlled electric fields to facilitate cell permeabilization, was first described in the 1960.³⁶ The last few years have seen a rapid progress in electroporation-mediated *in vivo* gene transfer which involves injection of DNA followed by pulsed electric field treatment. A number of tissues have been examined including liver, skin, tumors and muscle.³⁷⁻³⁹ Generally, most reports showed 10- to 1000-fold differences between DNA injection and DNA injection followed by electroporation. It should be noted that different tissues may respond differently to electroporation due to the differences in their local biological environment. Thus, electroporation parameters need to be optimized individually for each gene transfer protocol.

2. 5.2.3 Gene Transfer by Particle Bombardment (Gene Gun)

Particle-mediated gene transfer was initially developed to deliver genes to plant cells and later was extended to gene delivery to mammalian cells.⁴⁰⁻⁴¹ Currently, this technology has been largely used for gene transfer to the skin due to its physical accessibility. This approach has been used for successful gene transfer into a number of different tissues. This direct injection techniques are simple and relatively safe.

However, gene transfer efficiency is poor and a low level of stable integration of the injected DNA. This property is particularly harmful in the case of proliferating cells, and would necessitate repeated injections.⁴²

2.5.2.4 Synthetic Vectors

A numbers of synthetic vectors primarily based on cationic lipids and polymers have been developed to carry plasmid DNA. The number of studies have demonstrated that the biocompatibility and utility of synthetic vectors can be improved by the incorporation of hydrophilic polymers and targeting ligands.⁴³⁻⁴⁴

Two main types of synthetic vectors are currently are use in therapeutic applications. Polyplexes are formed by the self-assembly reaction between positively charged polycations such as polyethyleneimine (PEI) and negatively-charged DNA, while lipoplexes are formed with lipids such as DOTAP.⁴⁵⁻⁴⁶ A large numbers of clinical studies have been initiated using synthetic vectors for delivery of therapeutic genes. Recent studies have showed that intratumoral administration of DNA/lipid mixtures is safe and can produce clinically significant responses. However, the transfection efficiency of synthetic vectors is poor compared to viral vectors and will need to be improved significantly to enable successful development in the clinics.

Polyplex

Cationic polymers are commonly used as a non-viral vector that can be used to increase gene delivery and transfer to target cells *in vivo*. The most important advantages of polymeric gene carriers are versatility of physicochemical properties

and easy manipulation.⁴⁷ Several types of polymers have been examined with respect to their ability to offer protection from nuclease degradation, deliver to target cells, and increase the transfection efficiency of plasmid DNA.⁴⁷⁻⁴⁸ Cationic polymers can condense with plasmid DNA more efficiently than cationic liposomes and this would be beneficial in controlling the tissue distribution of plasmid DNA complexes. The most widely studied polymers for gene therapy include polyethyleneimine (PEI) and poly-L-lysine (PLL), poly-L-ornithine, chitosan, starburst dendrimer and other novel synthetic polymers.⁴⁵

Poly-L-lysine (PLL)

The first polycation to be employed for gene delivery was poly-L-lysine conjugated with asialoorosomucoid for hepatocellular gene targeting.⁴⁹ PLL are polypeptides with the lysine as the repeat unit and linear structure. Biodegradable property of poly-L-lysine is very useful for *in vivo* application. PLL are rapidly bound to plasma proteins and cleared from the circulation.⁵⁰⁻⁵¹ A positively charged complex is formed by the interaction of cationic PLL and anionic DNA molecules, which can interact with the negatively charged cell surface and undergo rapid internalization.⁵²⁻⁵⁵

Polyethyleneimine (PEI)

Polyethyleneimine (PEI) was first introduced by Behr in 1995, and has become one of the gold standards of nonviral gene delivery.⁵⁶ Polyethyleneimine is a cationic polymer capable of delivering DNA molecules into cultured mammalian cells as charge complexes.⁵⁷⁻⁵⁸ PEIs offer a significantly more efficient transfection and

protection against nuclease degradation than other polycations, e.g., PLL, due to their high charge density and more efficient complexation.^{57, 59} It was found that low molecular weight (10 kDa) moderately branched polymer showed efficient delivery with low toxicity in comparison with high molecular weight commercial PEI.⁵⁷ The high amount of positive charges, results in a high toxicity of PEI polymers. The toxicity and non biodegradability of polymers are limiting factors, especially for its *in vivo* use.^{56, 59} It was demonstrated that linear PEI 22 kDa, e.g., ExGen™ 500 (Euromedex, France), displays excellent transfection efficiency.^{56, 60} Recently Linear PEI has been reported to mediate a cell-cycle-independent nuclear entry of plasmid DNA. This finding is of particular importance in the therapy of slowly-dividing tissues.⁵⁶ The transfection efficiency and cytotoxicity of PEI are directly proportional to its molecular weight.⁶¹ Cytotoxicity of PEI reduced by synthesis of PEI with graft copolymers such as linear poly (ethylene glycol),⁶²⁻⁶³ incorporation of low molecular weight PEI, and PEI glycosylation.⁶⁴

Chitosan

Chitosan is a non toxic, natural biodegradable polymer. It is an unacetylated derivative of the second most universally abundant biopolymer, chitin (cellulose being the most abundant).⁶⁵ The main sources of chitin are the shell wastes of shrimp, lobster, krill, and crab.⁶⁵ The degree of deacetylation (DD) is an important property of chitosan which affects the biodegradability and immunological activity.⁶⁶⁻⁶⁷ Due to its good biocompatibility, biodegradability and low toxicity; it has been widely used in pharmaceutical research and in industry as a carrier for drug delivery and as

biomedical material for artificial skin and wound healing bandage application.^{66, 68-69} Most recently chitosan has been extended to the field of gene delivery and results show that it is a suitable material for efficient nonviral gene delivery.⁷⁰⁻⁷¹ Mumper was the first to propose to deliver gene into cell using chitosan as a vector and reported that particle size of the polyplex depends on the molecular weight of chitosan.⁵³

Depending on the degree of deacetylation and molecular weight, chitosans are capable of forming small (<100 nm), rather stable, toroidal complexes with plasmid DNA and provide protection for the complexed DNA against DNase degradation that is comparable to PEI.⁷²⁻⁷⁴ Other researchers also reported that effect of molecular weight of chitosan, DNA concentration, salt concentration, pH of the media and charge ratio of the complex.⁷³ For example, Liu and Yao⁷⁵ reported that chitosan and trimethylated chitosan oligomers proved to be nontoxic on several types of cells in contrast to DOTAP that decreased viability to 50%. Small (1.2 kDa) and very large chitosan polymers lead to low levels of reporter gene expression.

However, in the molecular weight range between 30-170 kDa chitosan polymers provide levels of gene expression that are comparable to PEI.⁷¹ Intratracheal administration of chitosan- and PEI (25 kDa) polyplexes to mice *in vivo* revealed similar distribution patterns, however transfection efficiency was approximately 10 fold lower for chitosan.⁷¹ However, high doses of chitosan can cause hypocholesterolemia in humans, thereby limiting its applications.⁷⁶

Poly Vinyl Amine (PVA)

PVA was another type of polycation studied for nonviral gene delivery. Molecular weight of the PVA mainly affects the dimensions of DNA complexes formed with PVA.⁷⁷

Collagen

Collagen was the one of the first polymers to deliver DNA in tissue engineering applications. It was involved in the delivery of plasmids encoding for bone morphogenic proteins (BMPs) and human parathyroid hormone genes fragment. Collagen Type 1 was used and the collagen-DNA mixtures were termed gene-activated matrices (GAM).⁷⁸

Gelatin

Gelatin is a natural polymer, derived from collagen, and is commonly used for pharmaceutical and medical applications because of its biodegradability⁷⁹⁻⁸¹ and biocompatibility in physiological environments.⁸²⁻⁸³ It was also used to form nanoparticles (200 nm) containing DNA.⁸⁴ Circulating properties of the nanoparticles can be enhanced easily by modifying the amine residues of basic amino acids with PEG derivatives.⁸⁴⁻⁸⁵ These nanoparticles were internalized by nonspecific endocytosis pathway and able to deliver their content in the peri-nuclear region within 12 h. The transfection efficiency of PEGylated gelatin nanoparticles was significantly higher than that for classic gelatin nanoparticles.⁸⁶ Recently, Kommareddy *et al*⁸⁷ found an efficient in vivo expression of a pDNA encoding for the soluble form of the extracellular domain of vascular endothelial growth factor receptor-1 (VEGF-R1 or

sFlt-1) after intravenous administration in female nude mice bearing orthotopic MDA-MB-435 breast adenocarcinoma xenografts.⁸⁷ PEG-modified gelatin-based nanovectors are promising systems for an efficient gene delivery vehicle in solid tumor.

Dendrimers

Dendrimers have a number of identifying properties which differentiate them from other polymers. High density of terminal groups contribute to the molecules surface characteristics and offer multiple attachment sites and determine the molecular volume which is important for the ability to sequester other molecules within the core of the dendrimer.⁸⁸ These key properties are playing an important role in terms of the potential use of these materials in drug and gene delivery.⁸⁹ The first consideration of dendrimers as molecules for gene delivery focused on the PAMAM dendrimers.⁹⁰ Low polydispersity can lead to consistent gene delivery and a clinically reliable formulation.⁴⁵ The cationic amino acid residues in the polymeric structure of PANAM dendrimers can help in DNA condensation and endosome release. Partially degraded PAMAM dendrimers are described to have more flexible structures than intact dendrimers and therefore to interact more efficiently with DNA.⁹¹

Partially hydrolyzed polyamidoamine dendrimers were found to be more effective gene transfer agents than branched PEI in the *in vivo* transfection of the carotid artery of rabbits, but less effective than branched PEI in transfecting the lungs of mice on intratracheal administration.⁵³

Peptide Mediated Gene Delivery

Positively charged cationic peptides interact with the negatively charged phosphate backbone of DNA through electrostatic interactions.⁹² Peptides are known to enhance the condensation of DNA to small particles with variable shapes, namely rods, toroids and spheroids, and finite size conformation,³² mainly depending on DNA size.⁹³⁻⁹⁴ Furthermore, the net positive charge exhibited by peptides complexes permit them to interact with cell membranes and internalize into the cell, both in vivo and in vitro systems^{92, 95-97} and allowing nuclear gene delivery and expression.⁹⁸⁻⁹⁹ Peptides–DNA complex also protect DNA from cytosolic nucleases degradation and extend the half-life of the targeted nucleic acid, which is known to be of 50–90 min for plasmid DNA.¹⁰⁰

Figure 2.1: Cationic polymers most frequently used for nucleic acid delivery¹⁰¹

Cationic Liposome

Liposomes are composed of one or more closed, concentric phospholipid bilayer membranes surrounding internal aqueous compartment. Basically cationic liposomes are formed by sonication, heating or membrane extrusion of a mixture of cationic lipids and neutral helper lipids such as DOPE. Among cationic lipids currently in use are DOTMA, DOTAP and DC-Chol, etc. Negatively charged or classical liposomes have been used as drug carrier. Problems associated with the efficiency of nucleic acid encapsulation lead to development of positively charged liposomes which are prepared with cationic lipids (able to interact spontaneously with negatively charged DNA) to form clusters of aggregated vesicles along the nucleic acid and work as a DNA carrier.¹⁰²

A large number of cationic lipid/liposome systems have been developed to improve the transfection efficiency of plasmid DNA. The first successful *in vitro* transfection with cationic lipid was described by Felgner *et al* in 1987.¹⁰² To date; various cationic lipids have been synthesized and tested for their transfection efficiency as well as toxicity.¹⁰²⁻¹⁰³ Theoretically, there is no strict size limitation on DNA and almost 100% of DNA can be recovered in complexed form. The electrostatic binding to negatively- charged cellular membranes followed by endocytotic uptake is the driving force for lipoplex to introduce genes into cells. Finally, the complexation of cationic liposomes with DNA may offer some protection for DNA against physical forces and enzymatic digestion. Currently, cationic liposomes are widely employed *in vitro* and several liposomal formulations have

undergone clinical evaluation as vectors for gene therapy in cancer¹⁰⁴ and cystic fibrosis.¹⁰⁵ Cationic lipids, used for gene therapy are generally consisting of three basic domains: a positive charged head group, a hydrophobic chain, and a linker which joins the polar and non-polar regions.^{102, 106} The positively charged head group is necessary for binding and complexation of nucleic acid phosphate groups. The hydrophobic and polar domains of cationic lipids may have dramatic effects on both transfection and toxicity levels. Linker group is also an important determinant of the chemical stability, biodegradability, and transfection efficiency of the cationic lipid.¹⁰⁷⁻¹⁰⁸ The linker group provides sites for the introduction of novel side chains to enhance targeting, uptake and trafficking. The order in which the lipid and the DNA are mixed is critical.¹⁰⁹ 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.¹¹⁰

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.

Generally smaller particles are favored for both *in vitro* and *in vivo* applications.^{102, 111} Recent data of Ross demonstrated that the efficiency of transfection

with cationic liposomes-DNA complexes (lipoplexes) increased with increasing lipoplex size.¹¹² Furthermore, by using large lipoplexes, the degradative properties of serum may be minimized. DNA is protected from nuclease degradation to various extents when it complexed with lipids.¹¹³ Lipid mixing and the endosomal membrane is a crucial part of the mechanism of endosomal release.^{25, 114} Lipids capable of promoting intermembrane lipid exchange (e.g. unsaturated PEs, unsaturated acyl chains and hydroxylated head groups) increase the ability of lipids to disrupt the endosomal membrane.¹¹⁴ Dioleoylphosphatidyl ethanolamine [DOPE] is particularly useful for cationic liposomes because of its ability to form non-bilayer phases and promote destabilization of the bilayer.¹¹⁵

The disadvantages of cationic lipids are their toxicity and decreased activity in the presence of serum.¹¹⁶ LipofectinTM mediated transfection was inactivated by serum.¹¹⁶ Furthermore, 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. Newer cationic lipid formulations are available that exhibit decreased toxicity, .e.g. the inclusion of a helper lipid (DOPE or cholesterol) in new formulation reduces the effective charge ratio required to deliver oligonucleotides into cells and permits delivery in the presence of high serum concentration.¹¹⁶⁻¹¹⁷ The amount of serum protein, bind to liposome/DNA complexes can be reduced by increasing the dose of liposome/DNA complex administered intravenously. This reduction in protein binding produced a corresponding increase in circulation times (4 minutes to over 80 minutes).

Some *in vivo* studies have revealed that the gene transduction responses were short lived and transient.¹¹⁸⁻¹¹⁹

Figure 2.2: Chemical structure of cationic lipids used for Gene delivery: DC-Chol: 3- β -[N-(N', N'dimethylaminoethanol) carbarmoyl] cholesterol; DOTMA: 1, 2-dioleoyloxypropyl-trimethylammonium chloride; DOTAP: 1, 2-diolyoxy-3-(trimethylammonio) propane DORIE: 1, 2-dioleoyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide; DMRIE: 1, 2-dimyristyloxypropyl-3-dimethylhydroxyethyl ammonium bromide¹²⁰

Figure 2.3: Chemical structures of neutral lipids **Figure 2.4:** Lipoplex mediated transfection and endocytosis

Table 2.1 Advantages and Limitations of Current Nonviral Gene Delivery**Systems**¹²²

Method	Route of Gene Delivery	Advantages	Limitations
Needle injection	Intratissue	Simplicity and safety	Low efficiency
Gene gun	Topical	Good efficiency	Tissue damage in some applications
Electroporation	Topical Intratissue	High efficiency	Limited working range; need for surgical procedure for nontopical applications
Hydrodynamic delivery	Systemic Intravascular	High efficiency, simplicity, effectiveness for liver gene delivery	Extremely effective in small animals; surgical procedure may be needed for localized gene delivery
Ultrasounds	Topical Systemic	Good potential for site specific gene delivery	Low efficiency in vivo
Cationic lipids	Topical Systemic Intratissue Airway	High efficiency in vitro; low to medium high for local and systemic gene delivery	Acute immune responses; limited activity in vivo
Cationic polymers	Topical Systemic Intratissue Airway	Highly effective in vitro; low to medium high for local and systemic gene delivery	Toxicity to cells; acute immune responses
Lipid/Polymer hybrids	Intratissue Systemic Airway	Low to medium high efficiency in vitro and in vivo; low toxicity	Low activity in vivo

Table 2.2 Characteristics of the Most Commonly used Gene Transfer Vectors

Vector	Capacity for DNA carrying/kb	Duration of expression	Inflammatory response	In vivo gene delivery efficiency
Retrovirus	<5	Stable	Low	Low
Adenovirus	<8	Transient	High	Moderate/Effective
Adeno-associated virus	<5	Stable	Low	Low
Liposome	unlimited	Transient	Low	Low
Molecular conjugate	<10	Transient	Low	Low

2.6 Comparative Study of Common Transfection Agents

There are variety of factors including dose, N/P ratio, confluency of cells, composition of incubation medium, mode of complex formation and time of incubation affects on in vitro transfection efficiency of a polymer or lipid formulation. Therefore, by considering each of these factors and their influence on complex properties, cellular uptake, toxicity and transfection efficiency, optimal formulation techniques would be established. Table 2.3 shows results of a comparative study of efficiency and toxicity of several commercially available transfection agents (Data from) at optimized N/P ratios on Cos-7 cells.³⁰ It is clear that lipid formulations overall showed a higher toxicity than polymers tested. Among the latter linear PEI

ExGenTM 500 and the dendrimer formulation SuperfectTM show superior transfection efficiency compared to PEI 25 kDa (Aldrich) with moderate toxicities. Among lipids only LipofectamineTM exhibits high transfection efficiency (higher than any of the tested polymers).

Table 2.3 Comparison of Reporter Gene expression and Toxicity of Different Transfection Systems¹²³

Transfection system	DNA dose (µg/well)	Luciferase expression % of ExGen TM 500	Cellular protein % of control
Polymers			
ExGen TM 500	0.75	100	79
PEI (25 KDa), Aldrich	0.75	39	85
Superfect TM (dendrimer formulation)	0.75	134	84
Lipid Formulations			
Lipofectin TM	0.4	40	63
Lipofectamine TM	0.4	176	61
Cellfectin TM	0.4	8	67

2.7 Cellular Barriers and Targeting

Gene vectors have to overcome a number of extracellular and intracellular barriers for successful gene delivery until the carried nucleic acids reach their final destination, the nucleus (Figure 2.5). An efficient gene delivery system will protect the nucleic acid from degradation, target the appropriate cell population, be efficiently internalized by the cell and avoid the degradative pathway, and ultimately localize to the nucleus. Various methods have been used to overcome the extracellular and intracellular barriers for effective gene delivery, including complexation of DNA with cationic polymer/lipids, attachment with targeting ligands and Electroporation.

2.7.1 Extracellular Barriers

Many nonviral vectors are generated using positively charged lipids or polycations resulting in particles with a net positive surface charge. Cationic surface charge is the major problem associated with this type of vectors, which leads to numerous unspecific interactions with e.g. vessel endothelia, cellular blood components, and plasma proteins such as albumin, fibronectin, immunoglobulin, complement factors or fibrinogen.⁶² These lead to very short plasma half lives for complexes with a high cationic surface charge.⁶²

For intravenous administration, complexes were rapidly cleared from the circulation and mainly accumulated in the liver and to a lesser extent in organs with

fine capillary beds like skin, muscle and intestine.¹²⁴ The interaction of blood components and their subsequent entrapment in the lung capillary bed with the complexes may induce aggregation or dissociation of the complexes, which may limit bioavailability and internalization.^{62, 124} Lipoplexes and polyplexes have showed an increase in size to 1 – 2 μ m within minutes after delivery, most likely the result of an interaction with serum components.¹¹⁶⁻¹¹⁷ Serum components (albumin, heparin, lipoprotein, or specific opsonins), which associate with the complex governed by the surface charge density and surface morphology of complexes, can make the complex susceptible for clearance by macrophages.^{17, 125} Complex formulations can provide an enhanced stability against degradation and may allow for a more efficient interaction with the negatively charged endothelial cell; however, non-cationic or less cationic formulations may be required to avoid accumulation in the reticuloendothelial system.^{17, 125} Variations in the charge ratio can be used as part of a passive targeting strategy. For lipoplexes delivered intravenously, different formulations (e.g., charge ratio) may be used to target the lung, liver, blood cells, or tumors.¹²⁵ The non-specific interactions of liposomes¹²⁶ and polymers,⁶² reduced by incorporating PEG into the lipoplex or polyplex, thus tend to divert the liposomes from the lung and allow transfection of distal solid tumours.⁶²

Another method, which has been used to achieve gene targeting, is the attachment of targeting ligands to lipoplexes and polyplexes. As such a variety of targeting ligands have been used to increase uptake in specific cell types, including galactose, or asialoorosomucoid moieties for increased uptake by hepatocytes and

mannose moieties for targeting the liver macrophages.¹²⁷⁻¹²⁸ Folate or transferrin ligands for uptake by cells expressing the folate or transferrin receptor and cytoskeleton specific ligands for targeting injured cells from within which the cytoskeleton is exposed on cell injury.^{62, 129} Gene delivery to the lung epithelium is severely limited by purulent infective sputum, a normal feature of the cystic fibrosis lung and also by normal mucus.¹³⁰⁻¹³¹ Sustained release systems are one of the great approaches to overcome the difficulties associated with clearance from the tissue.¹³² Sustained DNA release with implantable PLG scaffold led to significantly greater physiological responses than scaffold implantation of bolus delivery of DNA.¹³³ Sustained delivery system has the potential to release larger quantities of DNA over a longer period of time, compared to delivered by a single injection.

2.7.2 Intracellular Barrier

After cellular association of nonviral vectors (Lipoplex and polyplexes) with a net positive charge may interact non-specifically with the negatively charged cell surface to the target cells, particles are internalized by endocytosis, receptor-mediated endocytosis, macropinocytosis, phagocytosis or related processes.¹³⁴⁻¹³⁵ Gene transfer complexes are internalized and mostly found in intracellular vesicles such as endosomes.¹³⁶⁻¹³⁷ Entrapment in endosomes is thought to be associated with degradation of the complexes upon endosomal acidification.¹³⁵ Therefore; subsequent release of particles into the cytoplasm represents a major bottleneck to gene delivery. So main efforts have focused on the effecting the release of particles from the

endosome before the gene is destroyed within this organelle. A combination of both receptor mediated uptake ligands and endosomal disrupting peptides have been used in lipoplexes and polyplexes to improve the gene transfer across both the extracellular and intracellular barrier.^{135, 138}

Receptor-mediated gene delivery is a promising approach to create a specific interaction with the cell surface and to target a particular internalization pathway.¹³⁵ Molecular conjugates of synthetic materials are being developed and are composed of two domains: a DNA binding domain and a receptor-binding domain. The DNA binding domain is composed of a cationic polymer or a cationic lipid, which self-assembles with DNA. The receptor-binding domain is often attached to functional groups on the cationic polymer or lipid prior to complexation with DNA;¹³⁴ however, complexes can be initially formed and subsequently coupled to a ligand.^{129, 135} The main function of the receptor-binding domain is to direct the DNA/molecular conjugate complex to a receptor and guide the complex through the internalization pathway that leads to endosomal entrapment. Receptor mediated endocytosis a powerful tool for controlling uptake because of the diversity, abundance of membrane bound receptors and the high efficiency of internalization. The targeting ligand attachment to the lipoplex has been shown to increase transfection by 1000 fold as compared to lipid with no targeting ligand.^{129, 134, 138} Targeting ligands are use for receptor mediated uptake and an endosomolytic moiety for endosomal escape has resulted in improvements in gene transfer although the constructs are rather complex entities.^{134, 138}

The gene transfer from the endosome to the cytoplasm once effected then resulted in the gene encountering a further barrier the nuclear membrane that is regarded as one of the most significant intracellular barriers to efficient transfection.^{114, 139-140} The lipids interaction with the endosomal membrane is thought to facilitate escape of the DNA to the cytoplasm prior to its degradation in the lysosome in the lipoplex mediated delivery. It is not well understood, some lipoplexes preferentially fuse with the early endosome while others fuse with the late endosome.¹¹⁴ pH sensitivity of liposomes can take advantage of the acidification process to facilitate the release of plasmids into the cytoplasm prior to lysosomal degradation.⁵² Release of the DNA from the complex may occur at the wall of the endosome.^{52, 114} Secondary and tertiary amines materials, that are protonated at acidic pH, and not at neutral pH like primary amines, can act as proton sponges that buffer the decrease in pH and ultimately cause DNA release into the cytoplasm. The proton sponge effect is supposed to result from the protonation of amino moieties on the polymer as the pH decreases inside the endocytic vesicle. The influx of counter ions, which occurs to maintain electroneutrality, induces osmotic swelling and rupture of the vesicle membrane.¹⁴¹⁻¹⁴² Endosomal disrupting peptides (fusogenic peptides) can be covalently incorporated into DNA/lipid¹⁴³ and DNA/cationic polymer¹⁴⁴⁻¹⁴⁵ complexes to enhance escape from the endosome. The transport of DNA from the cytoplasm to the nucleus may be the most significant limitation to successful gene transfer.^{114, 146} In addition to this limitation, the size of DNA is problematic for crossing into the nucleus.¹⁰⁰ The degradation of un-complexed plasmid DNA within the cytoplasm can occur in hours.

So, it could badly limit the total amount of plasmid DNA that can be transported into the nucleus.¹⁰⁰ The nuclear pores permit free diffusion entry of only small particles (less than approx. 70 kDa).¹⁴⁷⁻¹⁴⁸ Transport of plasmid DNA to the nucleus occurs during cell division, when the nuclear envelope breaks down, or independently of cell cycling, through pores in the nuclear membrane.^{59, 149} Although transfection efficiency has been shown to depend on mitosis there is also evidence that the nuclear pores act as a size-exclusion barrier.¹⁰¹ Oligonucleotides or plasmids (small DNA fragments) are enter the nucleus by passive diffusion while larger fragments are transported through the nuclear pore complex in an energy-dependent manner.¹⁵⁰⁻¹⁵¹ NLSs, short peptide sequences with 5 – 25 amino acids, redirect intracellular protein transport to the nucleus.^{143, 147, 152-153} These sequences can be incorporated into complexes with cationic lipids,¹⁴⁵ cationic polymers,¹⁵⁴⁻¹⁵⁵ or can be directly linked to the plasmid.^{152, 156} For the transfection of non-dividing endothelial cells, lipoplex transfection showed that 5% of the cells testing positive for transfection; whereas, the incorporation of a NLS resulted in more than 80% of the cells testing positive.¹⁴⁵

Figure 2.5: Schematic generalized representation of delivery of a DNA-based therapeutic using a viral or nonviral DNA delivery vector (1) complexation and/or entrapment of DNA-based therapeutic with DNA delivery vector; (2) interaction of DNA-based therapeutic-vector complex with cell membrane; (3) cellular internalization via receptor- or non-receptor-mediated endocytotic pathways; (4) endosomal breakdown; (5) cytoplasmic release of DNA-based therapeutic-vector complex or DNA-based therapeutic alone (Cytoplasm is the site of action for antisense oligonucleotides, aptamer, ribozymes, DNazymes, and cytoplasmic plasmid DNA expression systems); (6) dissociation of DNA-based therapeutic from vector; (7) nuclear translocation of viral vectors or DNA-based therapeutics. (Nucleus is the site of action for transgenes in plasmids for gene therapy, siRNA generating plasmids, and antigenic oligonucleotides.)⁴

2.8 Polymeric Gene Delivery System for Controlled Release

Polymeric gene delivery systems provide an attractive alternative for long term delivery of variety of drug molecules such as proteins, peptides, oligonucleotides and DNA. Polymer matrices have been used to increase DNA resistance to degradation by nucleases, increase plasmid DNA uptake compared to bolus delivery methods, and provide controlled dosing. In addition, sustained DNA release can prolong foreign gene expression, thereby reducing the need for repeated dosing, which is a significant advantage for long-term gene therapy.¹⁰ A number of different polymeric biomaterials are available that provide continuous, controlled release of molecules. In 1976, Langer and his coworkers introduced the first ethylene-vinyl acetate copolymers (EVAc) polymer composites systems, appropriate for the controlled release of proteins.¹³² Now a day's various types of polymer matrices have been explored for DNA delivery, with poly (lactic-co-glycolic acid) (PLGA) systems being the most common.⁸ Controlled delivery of plasmid DNA has been reported with chemically synthesized polymers such as poly (vinyl pyrrolidone), poly (vinyl alcohol), poly (ethylene-co-vinyl acetate), and poly (D, L-lactide-co-glycolide).^{8, 157}

However, DNA encapsulation efficiencies can be low with PLGA delivery vehicles and encapsulation efficiency can be improved by introducing amphiphilic molecules to increase the association of DNA with the hydrophobic polymers.^{8, 133, 157} More important, the DNA damage that has been observed as result of PLGA degradation and the degradation products are thought to damage encapsulated DNA.¹⁵⁸ More porous PLGA structures help to reduce accumulation of degradation

products in the implant interior, thereby mitigating damage to pH-sensitive molecules like DNA.¹⁵⁸ A substitute to PLGA-based delivery systems is to encapsulate DNA in more hydrophilic polymer matrices, such as hydrogels. Hydrogels are much better than PLGA for less damaging of DNA and high encapsulation efficiencies.^{133, 159}

Hydrogels based on naturally occurring biopolymers, including alginate, chitosan, collagen and gelatin have shown transfection of cells *in vitro* and/or *in vivo* with released DNA.¹⁵⁹ Hydrogels represent an improvement as a class of biodegradable polymeric materials with excellent biocompatibility, which can swell by taking up large amounts of water without dissolving to allow protracted localized gene expression, and improve the pDNA release kinetic.¹³³ However, the DNA release from biopolymer hydrogels is mainly by ionic exchange or enzymatic degradation, which can make it difficult to control the DNA dosing and/or can reduce the ability to sustain release for long periods. Synthetic hydrogels can provide broader control of the release characteristics, but the gelation conditions and the chemical environment must be carefully selected to limit DNA damage. Transfection has been found with DNA released by hydrolytic degradation of succinimidyl crosslinked poly (ethylene glycol) (PEG) gels, hydrogels formed by emulsion with triblock copolymers of PEG and poly (lactic acid).^{133, 160} Thermosensitive hydrogels are a newer class of hydrogels that have many advantages over “traditional” hydrogel because they can exhibit sol-gel transition at both room and elevated temperatures. These changes corresponding to swelling or deswelling of the polymer, can be controlled to release of encapsulated pDNA in response to external temperature changes.¹⁶⁰⁻¹⁶¹

Poly (ϵ -caprolactone) (PCL) is semi crystalline, biocompatible and biodegradable polyester with a low melting point (57° C) and a low glass transition temperature. It degrades slowly relative to the PLA/PLG polymers. PCL is rubbery at room temperature, which contributes to the very high permeability of PCL for many therapeutic drugs, especially in the contraceptive field. PCL exhibits certain desirable characteristics for drug-delivery applications, including biodegradability, biocompatibility, commercial availability, etc.¹⁶²⁻¹⁶⁴

Studies on the release of macromolecules from PCL are limited. In general the release is diffusion controlled. The rate of release of proteins from PCL microspheres has been reported to be rather slow.¹⁶⁵ Another studies 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.¹⁶⁵ No studies of the incorporation of DNA and its release from PCL have been reported to date.

Figure 2.6: Synthetic polymers for gene delivery¹²⁰

2.9 Controlled Release of Plasmid DNA

The delivery of plasmid DNA encoding a therapeutic protein exhibits a promising substitute to the outright delivery of the protein. The inherent chemical stability of DNA facilitates the application of conventional methods established for the controlled release of proteins toward achieving controlled gene delivery. However, the success of therapeutic gene delivery needs a system that both facilitates cellular uptake of DNA and maintains gene expression. The development of controlled release systems for the delivery of pDNA may offer various advantages over parenteral administrations.^{8, 133} Firstly, Encapsulation of plasmid DNA in controlled release systems could protect DNA from rapid *in vivo* degradation and sustained delivery of

plasmid DNA at the tissue site may offer a source for continual gene uptake thereby prolonging the duration of expression. 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. Earlier ethylene vinyl co-acetate (EVAc) has been widely used as a model system for the fabrication of non-degrading controlled release systems.^{132, 166-167} An essential point regarding the EVAc release systems is that drugs ($M > 300$) cannot actually diffuse through the polymer phase but require the formation of a porous network of channels to be released. Therefore, at low drug loadings, complete entrapment of isolated drug particles within the polymer will prevent sustained release except for those particles on the outer surface.^{54, 64}

Recently much attention has been focused on biodegradable, biocompatible polymers such as PLGA, for the encapsulation of proteins and peptides and for genes.¹⁵⁸ PLGA was selected since it is compatible and biodegradable polymer that is approved for *in vivo* applications.¹⁵⁸ It provides the release rates ranging from a few days to more than 60 days, with the polymer composition and polymer composition regulating the release. Also the DNA integrity can be affected by degradation of the polymer to lactic acid and glycolic acid.¹⁶² Sustained release and delayed release were achieved with hydrogels composed of PEG and lactic acid segments based on the number of lactic acid units.¹⁶⁸ Recently Nie *et al* fabricated PLGA/chitosan foams with plasmid DNA and found the effect of chitosan on *in vitro* release of plasmid

DNA, cell attachment, cell viability and *in vitro* expression.¹⁶⁹ PLGA/chitosan porous composite foams provide sustained DNA release for more than 60 days period. They also observed that chitosan help to slow down the disintegration of PLGA matrix and consequently retarding the release of DNA from the polymer matrix. PLGA/chitosan matrix also shows luciferase expression upto 7 days period.¹⁶⁹ Collagen based materials release plasmid DNA for times ranging hours to days.¹⁷⁰ HA (hyaluronan) based hydrogels also release the DNA, the rate of release can be controlled by the extent of cross linking.¹⁷¹⁻¹⁷²

Leong and co-workers¹⁷³ have fabricated nanospheres with natural polymers like collagen and chitosan and some 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. Labhasetwar and Levy¹⁷⁴ 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 82% cumulative DNA release of the encapsulated DNA over 17 days under *in vitro* physiological conditions. The gene expression efficiency of the nanospheres in tissue culture was two to five orders of magnitude greater than the gene expression with the same amount of plasmid DNA in solution. Apart from nanospheres DNA was delivered from polymeric scaffolds. Shea

*et al*¹³³ have used an implantable PLG scaffold delivering 500 µg of DNA. Transfection was noted in cells within and adjacent to the scaffolds. Ethylene vinyl acetate discs implanted intravaginally induced gene expression for 28 days.¹³³ Another study by Jong *et al* using EVAc rods showed that the DNA was released without degradation and it retained the ability to transfect cells *in vitro*.¹⁰ 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 µg of DNA during a month long period with majority of the release occurring in the first one week.

Klugherz *et al*¹⁷⁵ 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 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*¹⁷⁶ 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.

Therefore, by virtue of its large size and physical properties, controlled release of plasmid DNA provides many challenges. It is important to determine whether plasmid DNA can be incorporated and continuously released from a controlled release system without degradation or loss of plasmid bioactivity. However, the plasmid integrity tends towards 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 compare to the open or linear conformations.¹⁷⁷ The degradation minimized by 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.¹⁷⁸ 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.¹⁵⁸ To accomplish an effective therapy using a matrix containing non-condensed pDNA, high amounts of the plasmid are required.¹⁷⁹ Condensing with cations needs a low amount of pDNA, thus increasing its encapsulation efficiency. They also help to increase the incorporation efficiency.¹⁷⁹ 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 with cationic lipids or polymers in solution with

the condensing agents and immobilized to the polymers which can facilitate the uptake and transfection *in vitro* and *in vivo*.^{120, 180-181} The presence of complexing agents can maintain the stability of DNA complexes during polymer processing.^{55, 182} Dunne *et al* 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.¹⁸³ Bielinska *et al* found that polyamidoamine dendrimer complexes released from phosphatidyl glycerol coated PLGA and collagen membrane were slow and yielding transfection *in vitro* comparable to controls bolus transfection. Six- to eight fold enhancements in transfection was found *in vivo* relative to plasmid DNA delivery.¹⁸⁴

Interestingly they found that the DNA release was faster than other investigations and released DNA was able to transfect in HEK 293 cells *in vitro*.¹⁸⁵ 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.¹⁸³ Capan *et al* produced PLGA microspheres loaded with pDNA /PLL. It was found out that supercoiled pDNA was degraded in the uncomplexed pDNA loaded microspheres.¹⁸² 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 accomplished 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 microspheres reported the sustained release of the complexes and the released DNA from the complexes were able to transfect the CHO cells *in vitro*.¹⁸⁶ 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 pDNA with PLL was capable to maintain the functional activity of pDNA, which seems to be affected during microspheres preparation.

Howard *et al* studied the formulation and characterization of a PLGA microparticle carrier containing PEI polyplexes for the deposition of intact polyplexes in intestinal lymphoid tissue.¹⁸⁷ 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, zero order release was found with the DNA only encapsulated system. 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 shows that the microparticle carrier or the polyplexes released have followed a migratory pathway

from the intestine to peripheral sites.¹⁸⁷ Oster *et al* 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 and the transfection efficiency in non-phagocytic cells was elevated compared to pDNA.¹⁸⁸

Sacks *et al* found similar rates for DNA and Lipofectamine/DNA complex and he also reported that the pDNA released from the pDNA-LIP (from collagen films) was in a condensed form along with higher transfection capability than that observed with pDNA released from pDNA–collagen films.¹⁸⁹ Whereas Scherer *et al*¹⁹⁰ observed a long-term expression and sustained release profile of polyplexes (PEI-DNA) from the protective polymer (PROCOP). Scherer *et al*¹⁹⁰ 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 released slowly 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 DNA allow its use at low dose while retaining the transfecting capacity in the context of carrier –mediated gene delivery. Lei *et al*¹⁹¹ investigated the DNA delivery from the PEG hydrogels crosslinked with

metalloproteinase (MMP) degradable peptides. They found that PEI/DNA polyplexes were encapsulated into MMP degradable PEG hydrogel without changing the hydrogel mechanical properties. Polyplexes are remain 65% active in post encapsulation. They observed that cumulative transgene expression increased through the 21 days period in clustered cells and cumulative transgene expression was plateau after 7 days of culture in homogeneously seeded cells. Rives *et al*¹⁹² fabricated layered porous poly (lactide –co-glycolide) (PLG) scaffolds for in vivo plasmid DNA delivery. They observed that incorporation efficiency of plasmid DNA significantly increased relative to scaffold form without layers and transgene expression over a period of 1-2 weeks with peaked expression level for first few days and then declined. Saul *et al*¹⁹³ investigated the sustained transgene expression of polyplexes (PEI/DNA) from spheres- templated fibrin scaffolds and achieved different transgene expression profiles based on the spatial distribution of polyplexes in the scaffold. Polyplex embedded within the scaffolds showed peak expression at 7-9 days and linear expression for 21-29 days, whereas scaffolds with surface coated polyplexes showed peak expression at day 5 and linear expression through 15 days.

2.10 Summary

The main dispute in gene therapy is to develop approaches for delivering genetic material to the appropriate cells of the patient in a specific, efficient and safe manner. Appropriate gene vectors can be crucial for gene transfer. Viral carriers are naturally evolved to infect cells and transfer their genetic materials into the host cells. However,

viral vectors are difficult to produce and can be toxic (in particular immunogenic), as well as having a limitation in terms of the size of the inserted genetic materials. Non-viral vectors, such as polymers and cationic lipids, have been developed as gene carrier molecules to overcome these problems. Non-viral vectors are advantageous due to the capability of large production with acceptable costs and the low immune response that enables repeated administration. In order to achieve 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.

Although the current non viral carriers are less efficient in their transfection activity than viral vectors, biomaterials-based gene delivery offers any advantages over more conventional methods. 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 need 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. Also it was found that the addition of complexing agents delayed the release from PLGA microspheres.

So the main challenge for the successful design and implementation of polymer based gene delivery system is maximizing the extent and duration of transgene expression. There are very few studies that report on the nature of the released pDNA complexes from polymer films that are of relevance to its transfection efficiency. Clearly, it is worthy of releasing the pDNA in its complexed state in order to maintain the plasmid DNA bioactivity as well as the improvement of transfection efficiency.

CHAPTER III

3. Materials and Methods

3.1 Materials

The dual vector pEGFPLuc, encoding a fusion protein of enhanced green fluorescent protein (EGFP) and luciferase from the firefly *photinus pyralis*, was purchased from Clontech (supercoiled, 6.4 kb). 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 is maximum at 488 nm and emission is at 507 nm. It is a cotransfection marker that normalizes transfection efficiencies by fluorescence microscopy of living cells or by a standard luciferase assay. It was propagated according to the standard method using a Qiagen Plasmid Giga Kit (Qiagen, Hilden, Germany). JM 109 Competent cells were used for the transformation of the foreign pDNA and purchased from Promega. COS - 7 cells is an African green monkey kidney fibroblast-like cell line suitable for transfection by vectors requiring expression of SV40 T antigen. It is a transformed cancerous cell line. It was purchased from ATCC. All the transfection experiments were done using COS-7 cells. Cell culture plates (24 well, 96 well) were purchased from Costar.

Kanamycin Sulphate was selected as antibiotic for the pDNA and used at a concentration of 50 µg/ml. Dulbecco's Modified Eagle Medium (DMEM) with 4.5g/L Glucose and L-Glutamine and it was used in the cell culture experiments. These

chemicals and all the remaining cell culture reagents like antibiotics, trypsin, etc were purchased from GIBCO.

LipofectamineTM reagent is a 3:1 (w/w) liposome formulation of the polycationic 2, 3-dioleoyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane filtered water. It is suitable for the transfection of DNA into eukaryotic cells. DAPI is a blue-fluorescent nucleic acid stain preferentially stains dsDNA and was used for DNA labeling. Lipofectamine and DAPI were purchased Invitrogen.

Sodium was dissolved in water with a pH of 7.4 at a concentration of 1 mg/ml. Ethidium Bromide is a fluorescent dye used for DNA quantification. It is a dark red, crystalline, non-volatile solid, moderately soluble in water, which fluoresces readily with a reddish-brown color when exposed to ultraviolet light (UV). 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. Foetal bovine serum (FBS) was heat inactivated in the lab. Gelatin is made from porcine skin (Type A). These chemical were purchased from Sigma.

Polycaprolactone (PCL) ($C_6H_{10}O_2$)_n is a non-toxic, biodegradable polymer and has an average $M_n \sim 80,000$ as measured by GPC. Low molecular weight gelatin (type A, medium bloom: 175, M_n 40,000) and high molecular weight gelatin (type A, high bloom: 300, M_n 50,000-100,000) were purchased from Sigma.

Polyvinylpyrrolidone (PVP) is a hydrophilic polymer (Av. Mw 10,000). Polyacrylic acid Sodium salt (PAA) is a hydrophilic polymer with Mw 5100 by GPC. Hyaluronic acid (HA) sodium salt (Mw 1.63×10^6) is composed of repeating dimeric units of glucuronic acid and N-acetyl glucosamine. It was used to make PCL/HA blend. PI is an intercalating agent and a fluorescent molecule. It was used for DNA labeling. All these chemicals were purchased from Sigma-Aldrich.

Luciferase Reporter Assay Kit, provides a firefly luciferase substrate formulation (Substrates A and B) and cell lysis buffer. This is meant for measuring luciferase expressed by transfected cells. QuantiLim Recombinant Luciferase is 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. These chemicals were purchased from BD Sciences.

Poly Ethylene Glycol (PEG 4000) was used as a plasticizer and its Mw Average is 3000 and it was purchased from TCI Chemicals, Tokyo. Micro BCATM Protein Assay 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 (0.5-20 µg/ml). Used for the quantification of total cell protein. It was purchased from Pierce Biotechnology, Rockford, IL, USA. Oregon Green® 488 Gelatin is fluorescence conjugate of gelatin. It was used for gelatin release studies and purchased from Molecular Probes. Fluorescence Mounting Medium (Dako) was used to reduce the fading of immunofluorescence during microscopy and it was purchased from Dako, USA

3.2 Methods

3.2.1 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 (LB) medium, isolated by alkaline lysis and followed by anion exchange chromatography using the Giga Qiagen kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). The isolated plasmid DNA was dissolved in a small volume of TE buffer (10 mM Tris HCl; 1 mM EDTA, pH 8). The concentration of plasmid DNA in solution was determined from the UV absorbance at a wavelength of 260 nm and the ratio of the absorbance at wavelengths of 260 and 280 nm (A₂₆₀/A₂₈₀) was measured for evaluation of plasmid purity to be between 1.8 and 2.0.

3.2.2 Preparation of Lipoplexes for Optimization of Complexes

LipofectamineTM reagent was obtained from Invitrogen Life technologies as a stock solution 2mg/ml in water. pDNA was diluted to 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 is 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 was 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.

3.2.3 Preparation of Lipofectamine/DNA Complexes (LPF/DNA) for Incorporation into the Polymeric Films

pDNA was diluted to a working range of 28 μg in 100 μl (280 $\mu\text{g}/\text{ml}$) from the stock solution using water. Lipofectamine was diluted using serum free media (SFM). It was added 8 times higher than pDNA (224 μg), since N/P (w/w) ratio of 8 was 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.

3.2.4 Preparation of Solutions

The concentrations of the polymers used for film preparation and average molecular weight of the polymers are tabulated in Table 3.1.

Table 3.1 Polymers Concentrations for Film Preparation

Polymers	Solution Concentration	Required amount for film preparation	Molecular Weight (Dalton)
PCL	6.6% (w/v) in chloroform	300 µl	Average Mw = 80,000
Polyvinylpyrrolidone (PVP)	50% (w/v) in water and sterilized by 0.2 µm filter	30% (w/w) of PCL	Average Mw = 10,000
Polyacrylic acid Sodium salt (PAA)	50% (w/v) in water and sterilized by 0.2 µm filter	50% (w/w) of PCL	Average Mw = 5,100
Hyaluronic acid (HA) sodium salt	5 mg/ml in water and sterilized by 0.2 µm filter	2.5% (w/w) of PCL	Average Mw = 1.63 x 10 ⁶
Poly ethylene glycol (PEG)	5% (w/v) in water and sterilized by 0.2 µm filter	5% (w/w) of PCL	Average Mw = 3000
LMW gelatin	10% (w/v) in water followed by autoclave sterilization	10% (w/w) of PCL	Average Mw (~ 175 bloom) = 40,000
HMW gelatin	10% (w/v) in water followed by autoclave sterilization	10% (w/w) of PCL	Average Mw (~ 300 bloom) = 50,000-100,000

3.2.5 Incorporation of pDNA and Lipoplex into the Films

PCL Film: A 6.6% (w/v) PCL solution was made by dissolving the polymer in chloroform. 300 μ l of this solution was mixed with 200 μ l of LPF/DNA or pDNA in SFM. The ratio between the SFM and polymer was 2:3. PCL solution was added to the SFM and vortexed at 2200 rpm and casted as a film.

PCL/Gelatin Film: PCL film with Gelatin was made with the addition of 10% gelatin (w/w) of the PCL.

PCL/PVP Film: PCL film with PVP was made with the addition of 30% (w/w) of the PCL.

PCL/Sodium salt of PAA Film: PCL film with sodium salt of PAA was made with the addition of 50% PAA sodium salt (w/w) of the PCL and 2.5% (w/w) gelatin was added as an emulsion stabilizer.

PCL/HA Film: PCL film with HA was made with the addition of 2.5 % HA (w/w) of the PCL and 1 % (w/w) PVA was added as an emulsion stabilizer.

PCL/PEG Film: PCL film with PEG was made with the addition of 5% PEG (w/w) of the PCL and 1% (w/w) PVA was added as an emulsion stabilizer.

3.2.6 Drying of Films

All the films were dried in the laminar hood for 3 days, for the evaporation of the solvent. The specific concentrations selected here based solely on the quality of

the emulsion obtained. The choice of chloroform was mandated by pDNA activity retention compared to solvents such as dichloromethane or acetone.

3.2.7 *In vitro* Release Study

The *in vitro* release profile of DNA from polymer films were determined in autoclaved distilled water. 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°C and released medium was assayed at stipulated time intervals (5, 9, 18, 23, 30 days) by microplate fluorescence reader. At every time point the water was removed and replaced with the fresh autoclave distilled water. About 1% Ampicillin was used as an antibiotic to avoid contamination.

3.2.8 Micro plate Fluorescent Reader

Multi Detection Micro plate Reader (Bio-Tek, FL_X 800) was used to quantify released pDNA from Lipoplexes (Lipofectamine/DNA). All the samples were measured in a 96-well dark bottom plates (used to minimize the background effect) from Costar. For each assay 50 μ l of each sample solution was added to individual well in a 96-well plate. pDNA was tagged with ethidium bromide and fluorescence intensity of each well was measured with a microplate fluorescence reader equipped with Ex: 485 nm and Em: 590 nm.

3.2.9 Quantification of the Released pDNA/LPF-pDNA Complexes

The DNA concentrations were determined from unknown samples by comparison to the standard curve. The pDNA loaded film samples were collected and quantified directly using ethidium bromide. In the case of films loaded with complexed pDNA, the pDNA had to be disassociated from LPF before quantification. Sodium oleate was used for the dissociation of lipoplexes. Oleic acid was prepared as a 10 mg/ml stock solution. From this, about 5 μ l was added to the lipoplexes. These complexes were incubated for about 4 hours at 37 $^{\circ}$ C. To the decomplexed samples, about 60 μ l of ethidium bromide (80 μ g/ml) was added and mixed well and the fluorescence intensity was measured. Appropriate blank solutions of PCL with sodium oleate at different intervals were used to get the final value. Another determination of free pDNA (without the decomplexing step) was also carried out on the released lipoplex samples, to quantify the amount of uncomplexed and complexed pDNA at each time point.

3.2.10 Preparation of Oregon Green® 488 Gelatin Solution

5 mg Oregon Green® 488 Gelatin conjugate was supplied in lyophilized form in 5 ml phosphate-buffered saline, pH 7.2 (PBS). Gelatin conjugate was dissolved in 5 ml distilled water to give 1mg/ml concentration in PBS.

3.2.11 Gelatin Release Studies

Fluorescence labeled gelatin (Oregon Green® 488) release studies from gelatin loaded PCL films were determined in autoclaved distilled water. The films were placed in a 12 well cell culture plates. About 800 µl of autoclaved water was used for the *in vitro* release studies. Plates were incubated at 37 °C and released medium was assayed at stipulated time intervals (0, 3, 5, 9, 18, 23, 30 days) by microplate fluorescence reader. At every time point the water was removed and replaced with the fresh autoclave distilled water.

3.2.12 UV Spectrophotometer

UV spectrophotometer was used to check physical integrity of DNA and quantification of its concentrations. This was also used for Protein quantification from cell lysates (BCA kit). 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. Protein samples were quantified at 560 nm using the UV-1700 Pharmaspec (Shimadzu).

3.2.13 Zeta Potential

Zeta potential measurement of LPF/pDNA complexes were performed by using a Nano Zetasizer, NS (Malvern Instruments) instrument bearing integrated size and zeta modules, equipped with a 10-miliWatt helium neon laser at a wavelength of 633 nm and the Malvern PCS software. Samples were measured using disposable cuvettes at 25 °C at a fixed scattering angle of 90°.

3.2.14 Particle Size Measurement

Particle size measurement of LPF/pDNA complexes was performed by photon correlation spectroscopy and electrophoretic mobility, respectively on a Nano Zetasizer, NS (Malvern Instruments) instrument bearing integrated size and zeta modules, equipped with a 10-miliWatt helium neon laser at a wavelength of 633 nm and the Malvern PCS software. Samples were measured in glass cuvettes at 25 °C at a fixed scattering angle of 90°. Samples were diluted in PBS, which was filtered through a 0.2 µm membrane.

3.2.15 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.

3.2.16 Scanning Electron Microscopy (SEM)

Field emission scanning electron microscopy (FESEM, JEOL JSM- 6340) and scanning electron microscopy (SEM, JEOL-5310) were used to analyze the surface morphology of the polymeric films at accelerating voltage 5.0 kV. The sample films were dried and mounted on a metal holder by using a double sided copper tape. Samples were coated with platinum (Fine auto Coater JEOL JSM- 6340) for FESEM and with gold for SEM before observation.

3.2.17 Pore Size Measurement by Image Analysis

Image analysis software (ImageJ, Wayne Rasband), which is commercially available from NIH was used to determine the mean pore size and distribution within the polymeric film. FESEM image was used to measure the mean pore size. To obtain the pore size distribution, measurements were also performed manually by first adjusting the contrast and threshold of each image so as to clearly focus on the pores of the top layer and then by moving the cursor to the various edges of the pores so as to identify their relative positions in the polymer film.

3.2.18 Confocal Laser Scanning Microscopy

The fluorescence images were acquired with a confocal fluorescence microscope (Leica TCS SP5, Heidelberg, Germany) with proper excitation laser wavelengths. Confocal imaging was performed at room temperature with an argon laser using a $\times 40.0$ objective (HCX PL APO CS 1.25 Oil UV).

3.2.19 Fluorescence Imaging of pDNA, Lipoplex and Gelatin onto Polymer Films

The special distribution of pDNA, lipoplexes and gelatin onto polymer films was determined by confocal fluorescence microscope (Leica TCS SP5) with an UV laser (405 nm excitation) to induce the blue fluorescence of DAPI and emission laser wavelength is 460 nm. Green fluorescence of Oregon Green 488 gelatin was induced

by the 488 nm excitation and detected as 575–640 nm wavelengths. The images were taken by sequential scan and images were later processed and analyzed by LAS AF software. Polymeric films with and without fluorescent labeled gelatin (Oregon Green® 488 Gelatin) were prepared as described above by emulsion technique for the incorporation of pDNA and lipoplexes. However, prior to formation of lipoplexes, DNA was labeled with blue fluorescent dye, (DAPI). For confocal imaging, dry polymer films were washed with PBS and then imaged immediately on a Leica confocal fluorescence microscope.

3.2.20 Size Exclusion Chromatography (SEC)

Size exclusion chromatography (SEC) was used to study the degradation of polymer films. 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 for overnight. Samples were prepared at a concentration of 1mg/ml. These samples were filtered by 0.22 μm filters and then filled into 2 ml vials sealed for auto- injection. Polystyrene standard was used to calculate average molecular weight and chloroform was used as a mobile phase.

3.2.21 Cell Culture

All the cell culture works were done in the cell culture lab, using the equipments like laminar hood, CO₂ incubator, Liquid nitrogen tank and Fluorescent microscope.

3.2.22 Inverted Fluorescent Microscopy

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

3.2.23 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). About 100 μ l of prepared lipoplexes were topped up with 300 μ l of fresh SFM (DMEM) .This was added drop wise to each well .The cells were incubated with complexes at 37 °C in a 5% CO₂ incubator for 5 hrs unless otherwise stated. Media was replaced with 0.5 ml of 2X medium and left overnight .The medium was changed with 1X medium, the next day.

Transfection efficiency was determined after 48 hrs using the Luciferase Assay Kit (BD Sciences) as described below:

3.2.24 *In Vitro* Bioactivity of Released DNA from Polymer Films

The released samples of pDNA/ lipoplexes from the polymer films were collected and air dried in the laminar hood for a few hours. 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. On the following day, the medium was removed and the air dried samples were treated with SFM (0.5 ml). This was added to the cells. Luciferase activity was determined after 48 hrs transfection using the Luciferase Assay Kit (BD Sciences). The activity was normalized to the total protein content.

3.2.25 Analysis of Luciferase Expression

After removal of medium, cells were washed twice with PBS and harvested from culture dishes by scraping in the presence of cell lysis buffer. After the addition of 100 μ l lysis buffer, the plate was incubated at 37 °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. The Sirius luminometer (Berthold systems) measures standard glow chemistry (Luminescence). 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 at 60⁰C 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 per mg of cell protein. The cell toxicity also measured by absorbance at 560 nm and it is calculated as follows, Cells transfected with pDNA were used as a control.

$$\text{Relative Toxicity (\%)} = \frac{\text{Protein Amount of Control Cells} - \text{Protein Amount of Transfected Cells}}{\text{Protein Amount of Control Cells}} \times 100$$

3.2.26 Cellular Tracking of pDNA/Lipoplexes by Confocal

Fluorescence Microscopy

Cos-7 cells (1×10⁵ cells/well) were cultured in a six-well culture dish for 24 h in 1 ml of DMEM medium supplemented with 10% fetal bovine serum (FBS) (10% FBS DMEM) and grown overnight to approximately 80% under normal growth conditions under a humidified atmosphere of 5% CO₂ in air. On the following day, the medium was removed and the air dried released samples were treated with fresh SFM (0.5 ml). This was added to the cells. The cellular entry of (PI)-labeled pDNA/

complexes were monitored by confocal fluorescence microscope (Leica TCS SP5). First, the cells treated with samples were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. After wash twice with PBS, the coverslips were mounted on glass microscope slides by using a drop of antifade solution, diazabicyclooctan (2.5% w/v). Then the samples were observed under CLSM with an argon laser (488 nm excitation) to induce the green fluorescence of Oregon green and their emission was observed using a band filter (515–565 nm). Red fluorescence was induced by the 546 nm excitation and detected as 575–640 nm wavelengths.

CHAPTER IV

4. Results and Discussion

4.1 Optimization of Lipofectamine/DNA complexes

To optimize the lipoplexes, complexation of DNA by lipofectamine was evaluated by ethidium bromide exclusion assay, gel electrophoresis, zeta potential for measurement of charge of the complexes, particle size and transfection efficiency followed by toxicity.

Figure 4.1 shows the effect of LPF/DNA (w/w) ratios on the zeta potential of lipofectamine/DNA complexes. The negative potential at a LPF/DNA ratio of 0 is due to the negative charges from unbound DNA. The zeta potential increases to a maximum of between +31.3 and +57.3 mV at the LPF/DNA ratios between 2 and 12.

Figure 4.2 shows the effect of LPF/DNA (w/w) ratio on the size of the lipoplexes. Generally the particle size of pDNA is 105 nm. The particle size drops to 59 nm at the LPF/DNA ratio (w/w) of 6. This shows that the pDNA is being condensed with the addition of lipofectamine. However, particle size increases from 58.6 nm to 62.85 nm with the increase of w/w ratio from 6 to 12.

Figure 4.1: Effect of LPF/DNA (w/w) ratio on the charge of the LPF/DNA complexes

Figure 4.2: Effect of LPF/DNA (w/w) ratio on effective particle size of LPF/DNA complexes

Figure 4.3: Transfection activity vs. cellular toxicity of LPF/DNA complexes

Figure 4.3 shows the transfection activity and cellular toxicity of lipoplexes of different LPF/DNA (w/w) ratios. From the figure 4.3, it could be noted that transfection efficiency drops after the w/w ratio of 8 due to a higher overall positive charge. The toxicity also increases with w/w ratio. So, from the above studies a LPF/DNA ratio of 8 (w/w) was selected as a standard for the complex preparation for the film loading part.

4.2 *In vitro* Release Studies of pDNA and Complexed DNA from Polymer Films

These studies focus on polymer blends, with incorporation of pDNA and complexed DNA. The main objective is to understand, and thereby modulate, the release of these genes from blended systems. The basic biodegradable polymer, PCL, is hydrophobic, and is not expected to act as a good reservoir for DNA. Addition of more hydrophilic polymers (PAA, PVP, PEG, HA and gelatin) was expected to partition the DNA into a more friendly environment.

4.2.1 Release of pDNA from PCL/PAA and PCL/PVP Films

The release profile of pDNA from various polymer films is shown in figure 4.4. The release profile of pDNA from PCL film shows biphasic behavior with a burst effect at day 5 interval, followed by slower release over 30 days. However the addition of sodium salt of Polyacrylic acid (PAA) and Poly vinyl pyrrolidone (PVP) does not decrease the burst effect. Figure 4.5 shows the release data, plotted as percentage (%) of initial loading of pDNA from various polymer films. About 50% of the pDNA is released in the initial phase as a burst effect by day 5 from PCL film and release percentage increases from about 50% to 90% from day 5 to day 9. However the release profile shows almost 100 percent of pDNA release at the end of the month.

For PCL films with PAA, PCL films with PVP, burst effect is also observed with an increase of release percentage of pDNA about 45% to 70% from day 5 to day

9 in PCL-PAA film and 46% to 66% in PCL-PVP films. Release profile shows about 80% of pDNA release at the end of the month.

Figure 4.4: Release of pDNA from polymer films

Figure 4.5: Release percentage (%) of pDNA from polymer films

The burst release of pDNA at day 5 is due to low solubility of pDNA in the polymer matrix. The pDNA is hydrophilic and does not dissolve appreciably in the hydrophobic polymer (PCL) matrix; additionally, there may be some repulsion between the negatively charged pDNA and slightly-negatively charged PCL. The addition of PAA and PVP does not change the burst effect as compared to pDNA release from PCL film alone. Generally, the addition of a hydrophilic polymer suppresses the burst effect, due to the greater solubility of the pDNA in the hydrophilic component. Thus the lack of an effect of the addition of PAA and PVP is unexpected. We believe that this is likely due to the large size of the supercoiled pDNA that prevents it from partitioning readily into any of the hydrophilic polymers (PVP, PAA), and quite likely exists as a separate third phase. (As is well known, polymer-polymer miscibility is also affected by size of each polymer). For PVP and for PAA, the overall release is somewhat slower than for PCL indicating perhaps that a small amount of partitioning may have occurred. Pore formation does not explain the slower release seen with either polymer (Figures 4.9 and 4.10).

4.2.2 Release of Lipoplexes from PCL/PAA and PCL/PVP Films

The release profile of complexed DNA (lipoplexes) from various polymer films is shown in figure 4.6. In contrast to the pDNA loaded films, no burst effect is observed with the lipoplex loaded samples. The release of lipoplexes is generally lower than that of pDNA. However, the addition of PAA and PVP increases the burst release, although only by a small amount.

Figure 4.7 shows the percentage of lipoplex release from various polymer films. About 3% of the lipoplex is released in the initial phase by day 5 from PCL film, but release shows about 8% of lipoplex release at the end of the month. Overall, there is much lower release of lipoplexes over 30 days compared to pDNA release, as expected. The lower release of lipoplex compared to pDNA is generally due to higher solubility of lipoplex in the hydrophobic polymer (PCL) matrix.

However, all these hydrophilic additives do modulate the release of lipoplexes, which are condensed forms of the pDNA, where the pDNA is associated with the positively charged sections of the liposome and therefore more hydrophobic compared to the “naked” pDNA. Thus the lipoplex dissolves more readily in PCL and shows no burst effect. Upon addition of the hydrophilic polymer (PVP, PAA), lipoplex release is increased. The reason for the increased release is due to the addition of large amount of hydrophilic polymer (30% and 50% of PVP and PAA respectively, see Table 3.1 in chapter III) reduces the PCL phase that is available for dissolving the lipoplex; hence some of the lipoplex becomes dispersed, rather than dissolved. Following the burst, the PAA sample still shows faster release of lipoplex. We are not sure the reasons for this faster release; pore formation is low for the PCL/PAA film (Figure 4.9), hence it cannot be invoked to explain this result.

Figure 4.6: Release of lipoplexes from polymer films **Figure 4.7:** Release percentage (%) of lipoplexes from polymer films

4.2.3 FESEM Analysis of PCL/PAA and PCL/PVP Films

The morphology of the films was studied to understand the mode of release of pDNA and complexed DNA (lipoplex) from polymer films. Figure 4.8 shows the surface morphology of PCL film at different time interval. The figure shows the few larger gaps and few numbers of pores on the polymer surface and their non-uniform distribution.

Figures 4.9 and 4.10 show the morphology of PCL film with PAA and PCL film with PVP respectively. Figure 4.9 shows the few numbers of pores on the film surface (Figure 4.9B) and figure 4.9C shows the cross sectional view of the PCL/PAA film. Figure 4.10 shows the appearance of pores on the film surface (Figure 4.10B) and cross sectional view also shows the large number of pores (Figure 4.10C). The PVP films show significantly larger number of pores than either the PCL or PCL-PAA film. This difference does not seem to have any bearing on the observed release rates.

Figure 4.8: PCL film surface on day 0 (A), PCL film surface on day 9 (B), PCL film surface PCL film on day 23 (C), cross sectional view of PCL film on day 23 (D)

Figure 4.9: PCL/PAA film surface on day 0 (A), PCL/PAA film surface on day 9 (B), cross sectional view of PCL/PAA film on day 9 (C)

Figure 4.10: PCL/PVP film surface on day 0 (A), PCL/PVP film surface on day 9 (B), cross sectional view of PCL/PVP film on day 9 (C)

4.2.4 Degradation Studies of PCL/PAA and PCL/PVP Films by Size

Exclusion Chromatography

Degradation studies of different polymer films were evaluated by size exclusion chromatography. Figure 4.11 shows the molecular weight changes with specific time intervals. Molecular weight changes of three different polymeric films do not show major difference from day 5 to day 30.

Figure 4.11: Degradation profile of PCL/PAA and PCL/PVP Films

4.2.5 *In vitro* Release Studies of pDNA and Complexed DNA from PCL/PEG and PCL/HA Films

Two other hydrophilic additives, PEG and hyaluronic acid (HA) were also evaluated for their effects on release. Figure 4.12 show the release profile of pDNA

from PCL, PCL/HA (2.5% of high molecular weight HA) and PCL/PEG (5% of a low molecular weight PEG) films. Qualitatively, the effect of adding smaller amounts of these two hydrophilic polymers is similar to the addition of PVP and PAA, although in detail there are some differences. The release profile shows a biphasic behavior, a burst phase followed by diffusion-controlled phase. The release is dominated by the burst release (50% by day 5) of undissolved pDNA. The films loaded with HA and PEG release around 50% and 28% of pDNA, respectively, at the end of 30 days.

Figure 4.12: Release percentage (%) of pDNA from polymer films

The addition of HA and PEG does not suppress the burst release of pDNA from PCL films. Generally, the addition of a hydrophilic polymer suppresses the burst effect, due to the greater solubility of the pDNA in the hydrophilic component, but for

the same reasons as given for PAA and PVP, these additives also do not show much of an effect on the burst. Here also, there is very little partitioning into the PEG or the HA (possibly due to the smaller amounts of these two polymers or due to pDNA-polymer immiscibility), and the pDNA quite likely exists as a separate third phase. Again, there is slower release following the burst as seen for PVP and PAA addition.

It is clear that HA and PEG also create pores on the film, and these pores on the film surface are created by leaching out of the additive (along with the pDNA). SEM pictures of the PCL film with HA (Figure 4.15B) and PCL film with PEG (Figure 4.16B) given below show the presence of pores on the surface of polymer films as early as day 9. Again, pore formation does not appear to account for the release behaviour seen, so there is likely to be some partitioning or association of the pDNA into either PEG or HA.

The figure 4.13 shows the effect of HA and PEG on the release profile of lipoplexes from PCL films. In contrast to the pDNA loaded films, no burst effect is observed with the lipoplex loaded samples. From the figure it is clear that the effect of HA and PEG is to increase the release of lipoplexes from PCL films and the higher accumulated release was observed for PCL film with PEG film. The figure 4.14 shows the effect of HA and PEG on the released percentage of lipoplexes from PCL films. The addition of HA and PEG increases the release of lipoplexes. PCL/HA and PCL/PEG shows around 15% of release at day 5 as compared with 3% from PCL film alone. Thus all the additives used (PAA, PVP, HA and PEG) appear to have very similar effects on release of both pDNA and lipoplex.

Figure 4.13: Release of lipoplexes from polymer films

Figure 4.14: Release percentage (%) of lipoplexes from polymer films

The PEG and HA amounts added are low (5% and 2.5% respectively), hence observed effect on lipoplex release must be due also to the creation of pores, as seen in figures 4.15 and 4.16. In this instance, these two additives behave somewhat differently from PAA.

4.2.6 SEM Analysis of PCL/PEG and PCL/HA Films

Figures 4.15 and 4.16 show the morphology of PCL film with HA and PCL film with PEG respectively. Figures (4.15B and 4.16B) show the appearance of pores on the film surface and figures (4.15C and 4.16C) show the pores in the cross sectional area of the films. Pores are uniformly dispersed in the whole matrix. Figures show that PEG creates larger number of pores compared to HA.

Figure 4.15: PCL/HA film surface on day 0 (A), PCL/HA film surface on day 9 (B), cross sectional view of PCL/HA film on day 9 (C)

Figure 4.16: PCL/PEG film surface on day 0 (A), PCL/PEG film surface on day 9 (B), cross sectional view of PCL/PEG film on day 9 (C)

4.2.7 Degradation Studies of PCL/PEG and PCL/HA Films by Size

Exclusion Chromatography

Degradation studies of different polymer films were evaluated by size exclusion chromatography. Figure 4.17 shows the molecular weight changes with specific time intervals. Molecular weight changes of three different polymeric films do not show major difference from day 5 to day 30.

Figure 4.17: Degradation profile of PCL/HA and PCL/PEG Films

4.2.8 Effect of Gelatin on the Release pDNA and Lipoplex from the PCL Films

4.2.8.1 Release of pDNA from PCL/Gelatin Films

The effect of gelatin (additive) on the release profile of pDNA from PCL films is shown in figure 4.18. The release profile shows a biphasic behavior, a burst phase followed by diffusion-controlled phase. The release is dominated by the burst release (50% by day 5) of undissolved pDNA.¹⁹⁴ The films loaded with low molecular weight and high molecular weight gelatin, release 62% and 45% of pDNA at the end of 30 days.

Figure 4.18: Effect of gelatin on the release percentage of pDNA from PCL films

Clearly, gelatin shows effects qualitatively different from all of the other hydrophilic additives (PEG, PVP, PAA and HA). Although there is still a large burst release due to undissolved pDNA,¹⁹⁴ the burst is reduced by addition of 10% of low or high-MW gelatin. This is due to an association of pDNA with gelatin or “partitioning”. Either by association of pDNA with gelatin or by actual dissolution into the gelatin phase, the burst is reduced. At the conditions of mixing, gelatin is positively charged ($pI = 8.0$, $pK_{a1} = 5.2$, $pK_{a2} = 11.5$)¹⁹⁵ and hence may interact electrostatically with the pDNA. Beyond the burst, the release of pDNA is slower from the PCL with higher-MW gelatin. Either the strength of association is higher for higher-MW gelatin (higher number of charges along the backbone) or diffusion through the high-MW gelatin phase is slower.

4.2.8.2 Release of Lipoplexes from PCL/Gelatin Films

The figures 4.19 and 4.20 show the effect of gelatin on the release profile of lipoplexes from PCL films. In contrast to the pDNA loaded films, no burst effect is observed with the lipoplex loaded samples.¹⁹⁴

Figure 4.19: Effect of gelatin on the release of lipoplexes from PCL film

From the figure it is clear that the effect of gelatin is to increase the release of lipoplexes from PCL films and substantially higher accumulated release was observed for PCL film with higher molecular weight gelatin. In this regard, the addition of 10% gelatin does lower the amount of PCL available for dissolution of lipoplex, but it is also more likely that gelatin associates with the lipoplex (see confocal images, below) and leaches out with the associated lipoplex. Again the extent of association of the

lipoplex is probably higher for high-MW gelatin. Subsequent release may be facilitated by the pores.

One small additional detail may be noted here. Around day 23, the release is somewhat accelerated for both gelatin-containing films. This may be due to a sudden increase in pores or pore size around this time period (Figures 4.22 and 4.23).

Figure 4.20: Effect of gelatin on the release percentage of lipoplexes from PCL films

None of the features seen above is attributable to PCL degradation, as the SEC data (Figure 4.21) does not show significant difference of molecular weight from day 5 to day 23.

A study by Lu *et al* showed that the diffusion of proteins is controlled by the presence of pores in the PCL/PEG matrix. It was 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 leaching of PEG.¹⁶⁵ Here we find that the effect of hydrophilic additives (PEG, HA, PVP and gelatin) is two-fold: one to decrease the PCL phase available for dissolution of the lipoplex, and second to create pores to facilitate diffusion subsequently.

Pore creation by leachants does not have an effect on naked pDNA release from PCL films, as this is dominated by the burst of undissolved pDNA in the matrices. Gelatin is clearly the exception to this in that it associates with the pDNA and suppresses its burst release, effectively acting as a higher-solubility phase for pDNA compared to PCL. The association of gelatin with lipoplex or pDNA has an effect on transfection of the released DNA, as we will see later in section 4.3.

4.2.9 Degradation Studies of PCL/gelatin Films by Size Exclusion

Chromatography

Figure 4.21 shows the degradation profile of PCL and PCL/gelatin films. Molecular weight change of three different polymeric films does not show major difference from day 5 to day 30. From the figure (Figure 4.21) it is clear that the release of pDNA and complex DNA from the polymeric film is mostly due to diffusion rather than due to bulk degradation, with solubility or partitioning also playing a key role.

Figure 4.21: Degradation profile of PCL/Gelatin Films

4.2.10 FESEM Analysis of PCL/gelatin Films

Figures (4.22B and 4.22C) show the appearance of pores on the film surface of PCL/LMW gelatin and figures (4.23B and 4.23C) show the appearance of pores on the film surface of PCL/HMW gelatin; here the number of pores has increased significantly on day 23. Figures (4.22D and 4.23D) show the pores in the cross sectional area of the films.

The leaching of gelatin creates pores on the film. The pores are uniformly dispersed in the whole PCL matrix. As explained above, the pore formation has an effect on subsequent release (following burst) of the lipoplex, but less so for pDNA.

Figure 4.22: PCL/LMW gelatin (10% w/w) film surface on day 0 (A), PCL/LMW gelatin (10% w/w) film surface on day 9 (B), PCL/LMW gelatin (10% w/w) film surface PCL film on day 23 (C), cross sectional view of PCL/LMW gelatin (10% w/w) film on day 23 (D)

Figure 4.23: PCL/HMW gelatin (10% w/w) film surface on day 0 (A), PCL/HMW gelatin (10% w/w) film surface on day 9 (B), PCL/HMW gelatin (10% w/w) film surface PCL film on day 23 (C), cross sectional view of PCL/HMW gelatin (10% w/w) film on day 23 (D)

4.2.11 Pore Size Measurement of PCL/gelatin film by Image Analysis

The mean pore size and distribution of pores on the surface of the PCL/gelatin film were measured by image analysis (ImageJ software) to understand the higher substantial burst release for the higher molecular weight gelatin film.

Figure 4.24 and 4.25 show the mean pore size distribution of PCL/LMW gelatin film and PCL/HMW gelatin film respectively. From the figures it could be noted that the presented pores over 450 μm for PCL/LMW gelatin film and 700 μm for PCL/HMW gelatin film. From the figures 4.24 and 4.25, it is also noted that majority of pores created on the films surface are of 150-200 μm and > 400 μm respectively.

The difference in pore sizes may help to explain the substantially higher overall release from PCL/HMW gelatin films. Thus gelatin clearly behaves differently from other hydrophilic additives in influencing the release of pDNA and of the lipoplexes. To explore the reasons for this as well as for the observed differences in transfection efficiencies, we examined the association of gelatin with pDNA and with lipoplex

Figure 4.24: Pore Size distribution of PCL/LMW gelatin film

Figure 4.25: Pore Size distribution of PCL/HMW gelatin film

4.2.12 Fluorescence Imaging Studies of PCL/Gelatin Film

When a bioactive molecule is incorporated in a polymer film, it may be either dispersed or dissolved in the matrix. When pDNA or its complexed form, a lipoplex, is incorporated in PCL, it is likely to be dispersed, since PCL is hydrophobic. However, when gelatin is blended with PCL, then the distribution of pDNA or the lipoplex may be different. First, to characterize the distribution of pDNA, lipoplexes and gelatin in the PCL film, fluorescence imaging studies were performed by confocal fluorescence microscopy. Plasmid DNA was labelled with DAPI, a blue-fluorescent nucleic acid stain which preferentially stains dsDNA. Gelatin was labeled with Oregon Green ® 488 Gelatin, while the lipoplex was prepared with DAPI-labeled pDNA. Figures 4.26A and 4.26B show the pDNA and lipoplex distribution in a PCL film with fluorescent labeled gelatin (green) respectively. From the figures, it is clear that both pDNA and lipoplex tend to associate substantially with the gelatin phase.

Figure 4.26: Confocal images of PCL film with labeled pDNA and labeled gelatin (A), PCL film with lipoplex prepared with labeled DNA and labeled gelatin (B)

This association is responsible for the unique effects of gelatin on release. For the pDNA, the association translates to effective partitioning into the gelatin phase, hence a lower burst and slower release is seen. For the lipoplex, the association leads to faster release when the gelatin leaches out with the associated lipoplex. In other words, when release from the matrix is dominated by the amount of undissolved pDNA, the release is slowed down by introduction of the gelatin phase into which the pDNA “dissolves”. On the contrary, when the introduced gelatin has a lower solubility (but still some degree of association) for the lipoplex than the PCL, it accelerates the release of the lipoplex by a combination of reducing the PCL phase and leaching out with associated lipoplex. These differences for gelatin contribute to the enhanced transfection seen from PCL-gelatin matrices, as will be discussed in section 4.3.

4.2.13 Gelatin Release Studies

The main objective of this study is to understand the leaching behaviour of gelatin separately from the PCL film and compare the release profile of gelatin with pDNA and lipoplexes. Figure 4.27 shows the comparative release profile of pDNA and gelatin. From the figure it could be noted that around 60 % of pDNA in gelatin phase at day 30. Figure 4.28 shows the comparative release profile of lipoplex and gelatin. From the figure it could be noted that around 31% of lipoplex in gelatin phase at day 30. From the figures, it is also clear that most of the gelatin leached out from the PCL film by day 23.

Figure 4.27: Comparison of pDNA and gelatin release from PCL films

Figure 4.28: Comparison of LPF and gelatin release from PCL films

4.2.14 Summary

The main features of the release studies and observed effects from the various formulations are summarized as follows:

- i. Hydrophilic additives such as PAA, PVP, HA and PEG are not good phases for pDNA to partition into, hence no effect is seen on the burst of pDNA by these additives. Gelatin is different in that it associates with the pDNA, effectively increasing its partitioning into the gelatin phase, thus retarding the release.
- ii. For lipoplex release, the additives (HA, PEG, PAA, PVP) all increase the release of lipoplex from PCL, as desired. However, the effect of gelatin although similar, is still different in that there is a contribution to the release of lipoplex, of the gelatin that leaches out with associated lipoplex.

In these studies it has been shown that the burst effect of lipoplexes is very low and better controlled release is obtained from lipoplexes. Release from pure PCL matrices is in fact too slow to be of practical use. The addition of hydrophilic polymers (PEG, PVP, PAA, HA and gelatin) all increase the release rate and sustains the release over a 30-day period. The quality of the released lipoplex (in its ability to transfect cells) may be different, and this is discussed in the next section.

4.3 Transfection Studies

The bioactivity of the released DNA was determined by *in vitro* cell transfection efficiency using COS 7 cell lines. Transfection efficiency of released DNA (pDNA/ complexed DNA) from polymer films was observed by inverted phase contrast microscope. The transfection is quantified by using enzyme (luciferase) activity, normalized using the total protein content of cell extracts. Analysis of luciferase expression was mentioned earlier (Chapter III)

The following table shows the amount of transfection for different samples

Table 4.1 Amount of transfection (fg/mg of protein) of released DNA (released DNA / time interval) from different polymer films

Samples	Transfection Amount (fg/mg of protein)			
	Lipoplex			pDNA
	Day 5	Day 9	Day 18	Day 5
PCL/PAA	2.32E+04	-	-	-
PCL/HA	7.40E+04	-	-	-
PCL/LMW gelatin	1.00E+06	8.35E+05	-	1.50E+04
PCL/HMW gelatin	9.75E+05	7.32E+05	1.13E+05	1.07E+04

Transfection efficiency of released DNA from polymer films was studied for all intervals as same as released intervals. The figure 4.29 shows the transfection

efficiency of released lipoplexes on day 5 from PCL/HA and PCL/PAA film; however the transfection is not measurable from day 9 onwards. The transfection efficiency of released lipoplexes from PCL/PAA film is relatively lower than the released lipoplexes from PCL/HA film, even though the lipoplexes released were substantially in the complexed state (at least on day 5, see Table 4.2 on page 102).

Figure 4.29: Transfection of released lipoplexes/time interval from PCL film

Figure 4.30: Transfection of released lipoplexes/time interval from PCL/Gelatin film

Figure 4.31: Cumulative transfection of released lipoplexes from PCL/gelatin

The figure 4.30 and figure 4.31 shows the transfection of released lipoplexes/time interval and the cumulative amount of transfection by released lipoplexes from PCL/gelatin film respectively. In particular, lipoplexes released from PCL films show no transfection at all, even on day 5. Compared to HA and PAA, the addition of either LMW gelatin or HMW gelatin substantially increases the transfection efficiency of released lipoplexes from PCL films. This difference is due to a favorable gelatin-lipoplex interaction, as discussed below. When we compare the high and low-MW gelatin, it appears that on day 9 only, the transfection efficiency of released lipoplexes from PCL film with LMW gelatin is higher than with the released lipoplexes from PCL/HMW gelatin. In general, however, the transfection efficiency decreases over time of residence in all the polymer films.

In order to determine the reasons for the differences in transfection efficiencies of the released lipoplexes, the extent of de-complexation and the particle size of the released lipoplexes were determined. From the particle size data (Table 4.3), it is clear that the major reason for decreased transfection with time of release is aggregation of the released complexes, which reduces nuclear penetration. On the other hand, we find that there is not much difference in size of the released complexes from the two PCL/gelatin samples, showing that the lowered transfection efficiencies in this case are mainly due to de-complexation. So, the higher transfection seen with the LMW-gelatin on day 9 is due to the increase in the percentage (%) of the complexed form. On day 9, about 85% was complexed in PCL/LMW when compared with 80% in PCL/HMW gelatin (Table 4.2).

Figure 4.32: Transfection of released pDNA vs. lipoplexes from PCL/gelatin films

Figure 4.32 shows the comparative transfection efficiency of pDNA and lipoplex released from PCL film with gelatin on day 5. Transfection efficiency of pDNA is much lower than lipoplexes as expected. This is mainly due to the repulsion of negatively-charged DNA and negatively-charged cell membrane and the consequent lower ability to penetrate the cell membrane.

4.3.1 Analysis of Complexed form and de-complexed form of Released Lipoplexes

In order to find out why there are differences in transfection efficiencies of the released lipoplexes from different films, we quantified the extent of de-complexation of the released lipoplexes for different films. Sodium oleate was used to decouple the

lipoplexes (as described in chapter III). Another determination of free pDNA (without the decomplexing step) was also carried out on the released lipoplex samples, to quantify the amount of uncomplexed pDNA at each time point. By subtraction, the amount of complexed pDNA was obtained.

Table 4.2 Percentage (%) of complexed form of released lipoplexes at each time point

Samples	Complexed form (%)		
	Day 5	Day 9	Day 18
PCL/lipoplex	100%	100%	100%
PCL/PAA/lipoplex	88 %	58 %	-
PCL/HA/lipoplex	100%	79%	-
PCL/LMW gelatin/lipoplex	79 %	85 %	90 %
PCL/HMW gelatin/lipoplex	72 %	80 %	86 %

4.3.2 Comparison of Transfection Efficiency of Released Lipoplexes from Films and Lipoplexes incubated at Different time points

In order to understand further the reasons for the differences in transfection between the PCL and the PCL-gelatin films, we postulated that the differences might be due to instability of lipoplexes. To test this hypothesis, we incubated lipoplexes for different time periods in buffer. Figure 4.33 shows the comparative transfection efficiency of lipoplexes incubated in the PBS buffer and the lipoplexes released from polymeric films at different time points. The data does show lowered transfection with incubation time, which contributes partially to the lowered transfection of the released lipoplexes, but other factors (such as aggregation of the released lipoplexes) may also be responsible.

The released lipoplexes are decomplexed, to some extent, as shown in Table 4.2. However, the degree of complexation is still relatively high on day 18 (90%). Thus the decomplexation cannot by itself explain the decreased transfection over time. We believe this decreased transfection over time of release could be largely due to agglomeration of the released lipoplex, perhaps due to aggregation occurring inside the polymer matrix even before release. To verify this, the particle size of the released lipoplexes from PCL/gelatin films up to day 18 and lipoplex stored for 18 days in PBS were compared (Table 4.3).

Figure 4.33: Comparison of lipoplexes released from PCL films with lipoplexes incubated at different time points

Table 4.3 Particle Size of Lipoplexes (lipoplexes incubated in PBS vs. Released from polymer films)

Samples	Particle Size (nm)			
	Day 0	Day 5	Day 9	Day 18
Lipoplex (incubated in PBS)	60	120	160	180
PCL/lipoplex	-	440	530	590
PCL/LMW gelatin/lipoplex	-	460	515	700
PCL/HMW gelatin/lipoplex	-	420	530	710

It was observed that the particle size of the released lipoplexes from PCL film increases to 590 nm from 440 nm, while those released from PCL/ LMW gelatin films increases to about 700 nm (from 460 nm) and to about 710 nm (from 420 nm) from PCL/HMW gelatin films, by day 18. The size increases thus range from about 35% to 70%, which are substantial. In contrast, lipoplexes incubated in buffer for the same length of time, show an increase in size from about 60 nm on day 0 to about 175 nm on day 18 (Table 4.3). Thus, one of the factors for reduced transfection of the released lipoplexes over time is increased size. However, this factor does not explain why the released lipoplexes from PCL/gelatin films continue to show some transfection while those released from PCL films (which are actually smaller in size) do not transfect at all. Thus neither aggregation nor decomplexation levels explain why gelatin-containing films release more bioactive lipoplexes.

4.3.3 Cellular Tracking of pDNA/Lipoplexes by Confocal

Fluorescence Microscopy

In order to verify the effect of size (aggregation) on transfection, a study was undertaken to determine the intracellular localization of pDNA and lipoplexes at different time points. The cell nucleus was labeled with DAPI staining (blue colour). Confocal laser spectroscopy was used to track the lipoplex and/or pDNA (as described in chapter III).

Figure 4.34 and 4.35 shows the intracellular distribution of day 5 released lipoplexes and gelatin after 2 hrs and 24 hrs post transfection respectively. The figure

4.34 clearly shows the red fluorescence (PI labeled DNA) in the cell cytoplasm (Figure 4.34c) and green fluorescence of gelatin also seen in the cell cytoplasm (Figure 4.34d). So, lipoplexes and gelatin both are internalized through the cell membrane and after 2 hrs post transfection, lipoplexes and gelatin are present at or near the region of the cell nucleus. The merged image (Figure 4.34e) shows the colocalization of lipoplex and gelatin in the cytoplasm. Figure 4.35 shows that the cellular distribution of particles after 24 hrs post transfection. We now see red and green fluorescence within the cell nucleus (White arrow indicates the presence of red fluorescence in figure 4.35c), indicating that these gelatin-associated particles, can still enter the nucleus. In vitro size measurement of the released lipoplexes on day 5 shows particles of around 460 nm. Since the nuclear pores allow free diffusion entry only for small particles (≤ 50 nm), this unexpected entry of the larger particles (~ 460 nm) into the nucleus may occurred either because the particles inside the cytoplasm are in fact much smaller than 460 nm, or such particles may have entered during the cell division when the nuclear membrane temporarily breaks down.¹⁹⁶⁻¹⁹⁷ We cannot also rule out disassociation of the lipoplex prior to nuclear entry.

The extent of co-localization of gelatin with the lipoplex in the cytoplasm is remarkable. It appears that the lipoplex is associated with the gelatin extensively after cellular entry. The confocal experiment was done by sequential scanning and there was reasonable difference of excitation wavelengths (λ_{ex} of blue fluorescence is 405 nm, λ_{ex} of green fluorescence of gelatin is 488 nm and λ_{ex} of red fluorescence is 546 nm). For lipoplexes released from a PCL matrix, figures 4.36 and 4.37 show the red

fluorescence (PI-labeled DNA) in the cell cytoplasm and near the nuclear membrane but the fluorescence does not reach into the nucleus. In comparison, figure 4.38 and 4.39 show the cellular distribution of pDNA released from PCL/gelatin film. Red fluorescence was detected in the nucleus (Figure 4.39c). Figure 4.40 and 4.41 shows the cellular tracking of day 9 released lipoplexes and gelatin from PCL/gelatin film after 2 hrs and 24 hrs post transfection respectively. Red fluorescence and green fluorescence of gelatin both are observed in the cell nucleus (Figures 4.41c and 4.41d).

The cellular distribution of day 18 released lipoplexes and gelatin also shows the faint red and green fluorescence in the cell nucleus (Figures 4.43c and 4.43d) and cellular distribution of other intervals released lipoplexes were also studied but the intensity of fluorescence was very low and seen mostly in the cell cytoplasm (figure not shown here). From the intracellular trafficking pictures, it appears that gelatin-associated lipoplexes enter the cytoplasm in larger numbers and some of them penetrate the nucleus to show transfection. In contrast, fewer of the lipoplexes released from PCL (without any gelatin association) appear to be in the cytoplasm after 24 hours of incubation, and none appear to penetrate the nucleus.

Thus gelatin-associated lipoplexes appear to survive in the cytoplasm long enough to allow for some to enter the nucleus. This protection afforded by gelatin has been previously observed in gelatin particles with incorporated plasmid DNA.¹⁹⁸ In that study; smaller gelatin nanoparticles entered the cells by non-specific endocytosis, and then survived endosomal attack in the cytoplasm, as observed by visualization of

the tagged particles in confocal microscopy. The reason for this protection is not entirely clear, although it is likely that the endosomal pH of 5-5.5 is not low enough to degrade gelatin (measured pH of our gelatin solutions is around 4.7), and the lipoplexes are most likely to be coated with a layer of gelatin.

Figure 4.34: Intracellular distributions of released lipoplexes and gelatin at day 5 from PCL/gelatin film (after 2hrs post transfection); a) Bright fields image of the cells, b) Blue fluorescence indicates the nucleus, c) Red fluorescence indicates the localization of lipoplexes, d) Green fluorescence indicates the localization of gelatin, e) Merged image of all fluorescence

Figure 4.35: Intracellular distributions of released lipoplexes and gelatin at day 5 from PCL/gelatin film (after 24hrs post transfection); a) Bright fields image of the cells, b) Blue fluorescence indicates the nucleus, c) Red fluorescence indicates the localization of lipoplexes (white arrow indicates the presence of red fluorescence in the nucleus), d) Green fluorescence indicates the localization of gelatin, e) Merged image of all fluorescence

Figure 4.36: Intracellular distributions of released lipoplexes from PCL at day 5 (after 2hrs post transfection); a) Bright fields image of the cells, b) Blue fluorescence indicates the nucleus, c) Red fluorescence indicates the localization of lipoplexes, d) Merged image of all fluorescence

Figure 4.37: Intracellular distributions of released lipoplexes from PCL at day 5 (after 24 hrs post transfection); a) Bright fields image of the cells, b) Blue fluorescence indicates the nucleus, c) Red fluorescence indicates the localization of lipoplexes, d) Merged image of all fluorescence

Figure 4.38: Intracellular distributions of released pDNA and gelatin at day 5 from PCL/gelatin film (after 2 hrs post transfection); a) Bright fields image of the cells, b) Blue fluorescence indicates the nucleus, c) Red fluorescence indicates the localization of pDNA, d) Green fluorescence indicates the localization of gelatin, e) Merged image of all fluorescence

Figure 4.39: Intracellular distributions of released pDNA and gelatin at day 5 from PCL/gelatin film (after 24 hrs post transfection); a) Bright fields image of the cells, b) Blue fluorescence indicates the nucleus, c) Red fluorescence indicates the localization of pDNA, d) Green fluorescence indicates the localization of gelatin, e) Merged image of all fluorescence

Figure 4.40: Intracellular distributions of released lipoplexes and gelatin at day 9 from PCL/gelatin film (after 2hrs post transfection); a) Bright fields image of the cells, b) Blue fluorescence indicates the nucleus, c) Red fluorescence indicates the localization of lipoplexes, d) Green fluorescence indicates the localization of gelatin, e) Merged image of all fluorescence

Figure 4.41: Intracellular distributions of released lipoplexes and gelatin at day 9 from PCL/gelatin film (after 24 hrs post transfection); a) Bright fields image of the cells, b) Blue fluorescence indicates the nucleus, c) Red fluorescence indicates the localization of lipoplexes (white arrow indicates the presence of red fluorescence in the nucleus), d) Green fluorescence indicates the localization of gelatin, e) Merged image of all fluorescence

Figure 4.42: Intracellular distributions of released lipoplexes and gelatin at day 18 from PCL/gelatin film (after 2hrs post transfection); a) Bright fields image of the cells, b) Blue fluorescence indicates the nucleus, c) Red fluorescence indicates the localization of lipoplexes, d) Green fluorescence indicates the localization of gelatin, e) Merged image of all fluorescence

Figure 4.43: Intracellular distributions of released lipoplexes and gelatin at day 18 from PCL/gelatin film (after 24 hrs post transfection); a) Bright fields image of the cells, b) Blue fluorescence indicates the nucleus, c) Red fluorescence indicates the localization of lipoplexes (white arrow indicates the presence of red fluorescence in the nucleus), d) Green fluorescence indicates the localization of gelatin, e) Merged image of all fluorescence

From the above confocal studies, a few key features are noted:

- i) Released lipoplexes from PCL/gelatin film reached into the nucleus and expressed protein (quantification data shown before). Lipoplexes maintain bioactivity upto day 18, but its efficiency decreases with increasing time period. Lipoplexes released from other films (PCL, PCL with other additives) do not transfect beyond day 5 or 9. So, gelatin helps to maintain the bioactivity of released lipoplexes from the PCL film. Lipoplexes are associated with gelatin and such lipoplexes appear to be in the cytoplasm in larger numbers, with some of them reaching the nucleus, in spite of their large size. Thus gelatin association of the lipoplex appears to protect them from endosomal/lysosomal degradation. At this point we are not sure of the mechanism for this protection, and further studies are planned to address this.
- ii) Released pDNA from PCL/gelatin film at day 5 is also able to transfect the cell. pDNA is negatively charged and interact with the positively charged gelatin, resulting the net positive surface charge of the complexes that allow to internalize through the negatively charged cell membrane.
- iii) Released lipoplexes from PCL film alone do not show any transfection on days 5, 9 and 18. These lipoplexes are still in the fully complexed form (Table 4.2). However, confocal studies show very few lipoplexes inside the cytoplasm, and none at all in the nucleus. We believe this is most likely due to endosomal/lysosomal degradation of the larger particles. In general,

the presence of gelatin (possibly as part of the lipoplex) appears to improve cellular penetration and/or survival in the cytoplasm for these complexes.

CHAPTER V

5. Conclusions and Future Suggestions

5.1 Conclusions

In these studies, we were able to incorporate both pDNA and complexed DNA with cationic agent (lipofectamine) into polymeric films. The DNA release from the polymer matrix is sustained for over a period of 1 month. These controlled release systems for DNA are novel, therefore further understanding of the limitations of the polymeric matrices helps to design the optimum device for a given gene delivery application.

The major conclusions of this work are as follows:

- 1) Various hydrophilic polymers were blended with PCL for incorporation of DNA, lipoplexes, and studied for the resulting sustained release.
- 2) The release profile of pDNA and complexed DNA (lipoplex) from the polymer matrix shows a sustained behavior over a period of 1 month.
- 3) Different release profiles were observed for pDNA and lipoplexes. For pDNA the release is dominated by lack of solubility of the pDNA in the PCL matrix, and burst release was observed. pDNA is hydrophilic and does not dissolve appreciably in the hydrophobic polymer (PCL) matrix; additionally, there may be some repulsion between the negatively charged pDNA and slightly-negatively charged PCL. For lipoplexes, the burst effect is very low, and better controlled release is obtained from lipoplexes. The lipoplex release from the

PCL is too low for practical use; however addition of hydrophilic polymers enhances the release of lipoplexes.

- 4) To obtain a more useful profile, the role of hydrophilic polymer (PVP, PAA, HA, PEG and gelatin) was studied. All the additives except gelatin do not suppress the early burst of pDNA. The suppression by gelatin is modest but significant. For lipoplex release, all additives increase the release of lipoplex over time. All these additives give sustained release of lipoplex over a month. Only PCL/gelatin films appear to give sustained release of bioactive lipoplex.
- 5) Lipoplexes released from the PCL/gelatin film were in the complexed form and also able to transfect the cells. However, the extent of transfection decreases with time.
- 6) Gelatin helps to maintain the bioactivity of released lipoplexes from the PCL film due to interaction between lipid and gelatin. After day 18, lipoplexes are unable to transfect the cells.
- 7) Aggregation, not decomplexation, is the limiting factor in controlled release of lipoplexes over time. With increasing residence time of lipoplex in the polymer matrix, lipoplexes tend to aggregate, and the released lipoplex reflects this by increased size. This increased size allows for cellular but only reduced nuclear penetration by the lipoplex.
- 8) In these studies, it was not possible to estimate the exact extent of partitioning of the lipoplex between the hydrophobic and hydrophilic phases; based on the observed association of lipoplex with gelatin (by confocal microscopy), it is

assumed that the lipoplexes partition substantially into the gelatin phase. Within this phase (and within the PCL phase), it appears that the lipoplex continues to aggregate over time, presumably due to unfavorable interactions with the polymer.

The detailed studies have shown that polymer blends may be used to obtain useful sustained release of complexed pDNA (lipoplex) from polymer films. The released lipoplexes are also able to transfect the cells.

5.2 Future Suggestions

The next phase of the study will mainly focus on the improvement of efficiency of the released lipoplexes from PCL/gelatin film for longer period of time.

Study of other alternative sustained release formulations.

So, the major future studies can be summarized as follows.

- i) Gelatin helps to maintain the bioactivity of released lipoplex. The exact mechanisms by which gelatin stabilizes the lipoplexes need to be elucidated, with spectroscopic studies.
- ii) More experiments will be needed to increase the transfection efficiency of the released lipoplexes after day 18, since released lipoplexes are unable to transfect although the lipoplexes are still in complexed form. In other words, decreasing aggregation of lipoplexes within the partitioning phase is critical to long-term sustained transfection.
- iii) Factors that decrease aggregation of lipoplex should be determined; for example, coating the lipoplex with different polyelectrolytes might shed some light on their stability over time. The use of other blend components may also be helpful; for example, does release from completely hydrophilic matrices (e.g. hydrogels with varying crosslink densities) yield complexes with sustained transfection ability.
- iv) We have used reporter gene (pEGFP-*Luc*) for our all studies. After optimization of all the procedures and the formulations, the release of therapeutic gene should be studied.

5.2.1 Experiments Proposed for Objective 1

Cellular uptake studies with fluorescence tagged complexes and fluorescence labeled gelatin by FACS may be helpful to find out the effect of gelatin.

5.2.2 Experiments Proposed for Objective 2

Since enough complex was available on day 23, but unable to transfect, it may be due to the aggregation of lipoplexes, which then are unable to crossing into the nucleus. The aggregation of lipoplexes inside the polymer matrix is reduced by addition of additives during lipoplex incorporation into the polymer. Lipoplex also tends to aggregate upon interaction with serum proteins, causing premature release of DNA from the complex and degradation by nucleases which interfere with cellular uptake and nuclear transport. So covering the lipoplex surface with hydrophilic polymer can be helpful to reduce the aggregation of lipoplex. PEG containing liposome are precluded the aggregation and increase their stability.

5.2.3 Experiments Proposed for Objective 3

Coating the lipoplexes with different polyelectrolytes could be helpful to reduce the aggregation and increase the bioactivity of the lipoplexes. This approach can allow the formation of compact particles with low aggregation. Recently it was reported that coating the polycation/DNA complexes with polyanions can diminish the particle aggregation.¹⁹⁹ Zaitsev *et al* ²⁰⁰ recently proposed that polyvinyl sulphate

(PVS) coated PEI/DNA complexes are much better to overcome the colloidal instability and reduce the aggregation.

5.2.4 Experiments Proposed for Objective 4

Therapeutic genes are used to replace the abnormal or the disease-causing genes. The selection of therapeutic genes depends on particular disease, such as VEGF gene for number of cardiovascular diseases, including myocardial ischaemia and peripheral artery disease, CFTR gene for cystic fibrosis, IL2RG for SCID or "bubble boy" disease, etc.

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List of Publications

Posters and Presentations

- 1) **D. Mondal**, Y. Ramgopal and S. S. Venkatraman; Role of gelatin on the controlled release of complexed DNA from Polycaprolactone (PCL) films, ICMAT 2007, July 2007, Singapore.
- 2) Y. Ramgopal, **D. Mondal** and S. S. Venkatraman; Release Studies of Lipofectamine - DNA from Biodegradable and Bio stable Polymer Films, 3^d MRS-S Conference on Advanced Materials, February 2008, Singapore.

Journal Articles

- 3) Y. Ramgopal, **D. Mondal**, S. S. Venkatraman, W. T. Godbey; Sustained release of complexed and Naked DNA from polymer films, Journal of Biomedical Materials Research: Part B - Applied Biomaterials, 85(2), 496-503, 2008.
- 4) Y. Ramgopal, **D. Mondal**, S. S. Venkatraman, W. T. Godbey, G. Y. Yuen; Controlled release of complexed DNA from polycaprolactone film: Comparison of lipoplex and polyplex release. Journal of Biomedical Materials Research: Part B - Applied Biomaterials, 89 (2), 439-447, 2009.

List of Publications

- 5) **D. Mondal**, S. K. Tiwari, S. S. Venkatraman; Sustained release of complexed DNA from films: study of bioactivity and intracellular tracking. (Accepted, Biointerphases)

Appendix

Ionic Species Calculation of Gelatin type A

Gelatin type A has an isoelectric point, $pI=8.0$, and its pKa values are, $pKa_1=5.2$ and $pKa_2=11.5$. Thus, the different species of gelatin can be represented as follows:

The equilibrium between the cationic and amphoteric species of gelatin can be expressed as:

Thus K_{a_1} can be expressed as:

$$K_{a_1} = \frac{(H^+) (NH_3^+GelCO_2^-)}{(NH_3^+GelCO_2H)} \quad (3)$$

Thus, the concentration of the different species of gelatin can be expressed in terms of its ionization constant and pH

$$\frac{(NH_3^+GelCO_2H)}{(NH_3^+GelCO_2^-)} = 10^{pK_{a1}-pH} \quad (8)$$

$$\frac{(NH_2GelCO_2^-)}{(NH_3^+GelCO_2^-)} = 10^{pH-pK_{a2}} \quad (9)$$

The total concentration of gelatin can be expressed as:

$$1 = (NH_3^+GelCO_2^-) + (NH_3^+GelCO_2H) + (NH_2GelCO_2^-) \quad (10)$$

Dividing Eq. (10) by $(NH_3^+GelCO_2^-)$:

$$\frac{1}{(NH_3^+GelCO_2^-)} = 1 + \frac{(NH_3^+GelCO_2H)}{(NH_3^+GelCO_2^-)} + \frac{(NH_2GelCO_2^-)}{(NH_3^+GelCO_2^-)} \quad (11)$$

Thus, the fraction of amphoteric species is:

$$(NH_3^+GelCO_2^-) = \frac{1}{1 + \frac{(NH_3^+GelCO_2H)}{(NH_3^+GelCO_2^-)} + \frac{(NH_2GelCO_2^-)}{(NH_3^+GelCO_2^-)}} \quad (12)$$

and expressed in terms of ionization constant and pH :

$$(NH_3^+GelCO_2^-) = \frac{1}{(1 + 10^{pK_{a1}-pH} + 10^{pH-pK_{a2}})} \quad (13)$$

The fraction of cationic species is:

$$(NH_3^+GelCO_2H) = \frac{10^{pK_{a1}-pH}}{(1 + 10^{pK_{a1}-pH} + 10^{pH-pK_{a2}})} \quad (14)$$

and by difference the fraction of anionic species is:

$$(NH_2GelCO_2^-) = 1 - (NH_3^+GelCO_2^-) - (NH_3^+GelCO_2H) \quad (15)$$

pH of Gelatin solution in water = 4.7

Percentage (%) of cationic species is 76% and amphoteric species is 24%

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