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IMPLEMENTATION AND EVALUATION OF A MOLECULAR STRATEGY FOR DELIVERY OF NUCLEIC ACID BASED THERAPEUTICS

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Major Professor's Signature

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Date

IMPLEMENTATION AND EVALUATION OF A MOLECULAR STRATEGY FOR DELIVERY OF NUCLEIC ACID BASED THERAPEUTICS

By

Felicia Codrea

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ABSTRACT

IMPLEMENTATION AND EVALUATION OF A MOLECULAR STRATEGY FOR DELIVERY OF NUCLEIC ACID BASED THERAPEUTICS

By

Felicia Codrea

Although "gene therapy" is a concept that was introduced more than there decades ago, the successes of this method are below the expectations. The bottleneck of gene therapy is gene delivery. The current non-viral methods used for transfection possess low transfection efficiency and are not suitable for therapeutic use, showing high toxicity.

A new macromolecular scaffold that combines the properties of cationic polymers and cationic lipids was synthesized in only two steps. It contains a lipophilic part, like lipids, and a charged amine moiety, like cationic polymers. A polymeric backbone was synthesized through a Michael addition reaction, starting from 2(5H)-furanone and cysteine. After purification through membrane fractionation, the backbone was functionalized with N, N dimethylethylenediamine and octylamine. The ability of this new carrier to mediate transfection of plasmid encoding for green fluorescent protein in vitro was tested on COS 1 cells. The transfection conditions were optimized and an efficiency of transfection of 6.59 % was achieved. The evaluation of the cytotoxicity of this new carrier proved that the carrier is non-toxic. The cells proliferated up to confluence even when the concentration was ten times higher than what was identified as optimum for transfection.

ACKNOWLEDGEMENTS

This thesis tells the story of one chapter from the cutting edge research carried out in Dr. Hollingsworth's laboratory. One of the luckiest days of my life was in September of 2002 when I met Dr. Rawle Hollingsworth. I will always be thankful for his time, the most precious currency. I just hope that the future will prove the time that he invested in me worthwhile. After spending three years in his laboratory, I have a different perspective of "the chemistry of life".

My deepest and sincere appreciation goes to Dr. John LaPres. His friendly attitude encouraged me to speak openly about various ideas and concerns and his door was always open for me. I would like to express my gratitude to Dr. Charles Hoogstraten, for his guidance and suggestions that lead me on the right pathway. Dr. Shirley Owens from the Confocal Laser Microscope facility was always there for me.

This thesis is dedicated to all my friends that help me to go through ups and downs, here at MSU. I was always happy to count many smiles and encouraging words around me. Dana was the "sister" I never had. Living together made us more than friends – closer to family. Dr. Maria Zavodszky has been the Romanian island in the department, a person who always understood and helped me with both personal and professional issues.

Modern communication technology makes long distance relationship easier and more effective. Throughout these years, I had constant encouragement from my brother (the best gift my parents ever gave me). My parents are particularly thanked for their life-

lasting support and for teaching me the ABCs of science. I wish I inherited some of my grandmother's genes, especially the ones related to the hard-working skills, strong will and kindness.

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List of Abbreviations

CMV – cytomegalovirus

NLS – nuclear localization signal

RES – reticuloendothelial system

RISC – RNA-induced silencing complex

RT – reverse transcriptase

LTR – long terminal repeats

HSV – herpex simplex virus

AAV – adeno-associated viruses

DOPE – dioleoylphosphatidyl ethanolamine

DOPG – 1,2-dioleoyl-*sn*-glycerol-3-(phosphor-*rac*-(1-glycerol))

DOTMA – [2,3-bis(oleoyl)propyl]trimethyl] ammonium chloride

DOTAP – 1,2-diacyl-3-trimethylammonium propane

DOPC – dioleoylphosphatidyl choline

DMRIE –1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide

DOSPA – 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate

DOGS – dioctadecyl amido glycyl spermine

SUV – small unilamellar vesicles

LUV – large unilamellar vesicles

GUV – giant unilamellar vesicles

MLV – multilamellar vesicles

PEG – polyethylene glycol

PTD – protein transduction domain

PEI – Polyethyleneimine

PAMAM – polyamidoamine

NLS – nuclear localization signal

EPR – enhanced permeability and retention effect

NMR – nuclear magnetic resonance

FT-IR – Fourier Transform infra red spectroscopy

EDC – 1-ethyl-3-dimethylaminopropylcarbodiimide

FITC – fluorescein isothiocyanate

MEF – mouse embryonic fibroblasts

PBS – phosphate buffer saline

DMEM – Dulbecco's Modified Eagle Medium

DCCD – dicyclohexylcarbodiimide

MTT –3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

Chapter 1

Introduction

1.1 Drug delivery

Although the development of efficient drugs has recorded remarkable progress during the last century, one of the most challenging issues in modern pharmacology remains their efficient delivery to the target. The goal of developing drug delivery systems is to simultaneously increase the therapeutic activity of the drugs and reduce their toxicity and side effects. Drug delivery is an interdisciplinary research area, comprising findings from biology, chemistry, biochemistry, physics, pharmacology, and physiology. The development of effective drug delivery systems that can transport a drug safely to the target is the "holy grail" of current pharmaceutical research[1].

1.2 Gene therapy

Solving the DNA structure (Waston, Crick and Franklin, 1953) opened a new era in science[2]. The understanding of how the genetic material is stored, passed to new generations and what is encoded offered a better understanding of function of the genes and the effects of mutations. French Anderson[3], considered "the father of gene therapy", used the term to describe the delivery of a gene(s) to cure diseases. If one could replace the bad copy of a gene with a good one, the cause of the disease would be treated, as opposed to the effects of it as in traditional medicine. The completion of the human

genome project also has a great impact on identification of gene function and selection. In particular the identification of genes that are involved in diseases has an immediate application. This leads to development of *nucleic acid based drugs* which include plasmids, antisense oligonucleotides, aptamers, ribozymes, DNAzymes and small interfering RNA (siRNA). Oligonucleotide based therapies target either the DNA and in this case is called antigene therapy, or target the RNA and it is called antisense therapy.

The spectrum of diseases that could be treated using this approach is broad. Gene therapy was initially introduced to treat genetic diseases like cystic fibrosis, severe combined immunodeficiency (SCID/ADA), hemophilia, sickle cell anemia, and Duchenne muscular dystrophy[4] to mention only a few. In 1990 the first protocol for gene therapy that targeted the adenosine deaminase deficiency obtained the FDA approval. The concept was later extrapolated to treat acquired diseases such as several types of cancer, neurodegenerative diseases like Alzheimer's and Parkinson's[5], cardiovascular diseases (restenosis, arteriosclerosis) and infectious diseases (AIDS, hepatitis B). For cancer treatment, gene therapy is applied as replacement of missing tumor suppressor gene and/or inhibition of oncogenes[6]. The first gene therapy product was approved to the market, in China, in 2003. It is a cancer drug, which using a viral method interferes with the tumor suppressor gene p53[7], thus stimulating apoptosis.

Although very appealing, introducing foreign DNA into cells is a more difficult task than it was first thought. To date, Achilles' heel of gene therapy remains gene delivery[8]. After the completion of the Human Genome Project, the challenges of gene therapy are not identification of genes that need to be replaced, but the effective delivery of nucleic acids into the nucleus of cells.

Gene therapy can interfere with gene expression either *in vivo* or *ex vivo*. The *ex vivo* approach assumes that malfunctioning cells are collected from the patient, or a compatible donor, to prevent rejection by the immune system if foreign cells are used, and manipulate them outside the body. The genetic material is introduced into cells *in vitro*, followed by insertion of cells back into the patient. This approach is suitable for diseases that affect the blood system, since cells from blood or bone marrow can be removed from the patient and grown outside the body in culture media. When the genetic disease is localized to a tissue like lung in case of cystic fibrosis, or skeletal muscle in case of Duchenne muscular dystrophy, the *in vivo* approach is more appropriate. This is a better alternative when cells cannot grow in culture in sufficient quantities or when the re-implantation process is difficult. The major challenge of *in vivo* gene therapy is that there is no safe and efficient gene delivery system[8].

1.2.1 Nucleic acid based therapeutics

Recent advances in molecular biology and molecular genetics have resulted in a new concept in treating diseases. Intracellular delivery of genetic material is the key step in gene therapy[9]. Plasmids are high molecular weight circular double stranded DNA which contains the gene of interest (Figure 1). Besides the gene of interest, the promoter and the enhancer sequence are compulsory for regulating gene expression. The promoter is essential in initiation of transcription; it contains the recognition sites for the RNA polymerase. A commonly used promoter is extracted from cytomegalovirus (CMV). The enhancer is introduced into the plasmid construct for the same purpose that exists in endogenous DNA, to enhance the production of the gene of interest. One hundred times

more protein can be obtained using a proper enhancer[10]. At a molecular level, plasmids can be considered prodrugs since they require transport into the nucleus where the transcription/translation machinery of the cell synthesizes the protein that the gene encodes for. The problems with delivery of plasmid DNA to cells are related to some properties of plasmid DNA. First, the size of plasmid DNA is 3-30 Kb, too big to cross the cellular membranes. The charge of DNA is highly negative due to the phosphate backbone. The nucleic acids are susceptible to enzymatic degradation both outside and inside the cell. One of the major challenges for the plasmid molecule is to enter the nucleus. In dividing cells nuclear entry takes advantage of the disruption of the nuclear membrane. This is why it is much easier to transfect cells in logarithmic growth, in cell culture. In non-dividing cells, the plasmid DNA should pass through the nuclear pore. There are proteins responsible for transport through nuclear pores and they contain nuclear localization signal (NLS), usually a sequence of basic amino acids.

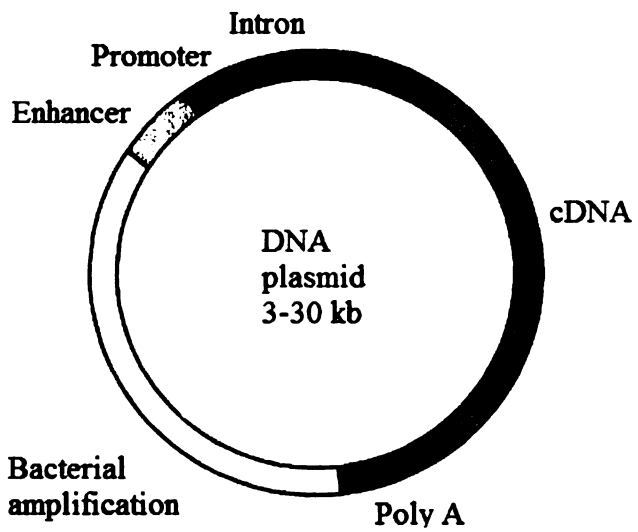


Figure 1. A schematic representation of plasmid with sequences needed for effective expression and replication. (modified from Walther et al., 1996)

The discovery of non-conventional drugs like RNA interference (RNAi)[11] or antisense RNA[12], where the oligonucleotides are the therapeutic agents, has expanded the concept of gene therapy from introducing genetic material into cells to introduction of molecules that have the ability to disrupt gene expression. Currently gene therapy means not only the replacement of a dysfunctional gene, but use of nucleic acid transfer, either RNA or DNA, to treat or prevent a disease. Novel drugs like RNA interference and antisense RNA introduced new challenges in drug delivery. The cellular delivery of nucleic acids raises the same problems as the delivery of other drugs: the danger of clearance of the drug due to the reticuloendothelial system (RES) and difficulty to cross the cellular membrane. In the absence of a carrier molecule, even small oligonucleotides are not able to diffuse through the hydrophobic cellular membranes due to the high negative charge of the phosphate backbone. Besides the fact that the nucleic acids should avoid the immune system, the enzymatic degradation of the nucleic acids is a problem both outside and inside the cells[13]. A vector to transport the nucleic acids to target is required.

Oligonucleotides are short segments of single stranded DNA that are used for disruption of one protein's function. Antisense oligonucleotides are designed to interact in a complementary fashion with a determined sequence of mRNA and inhibit its translation into protein (Figure 2).

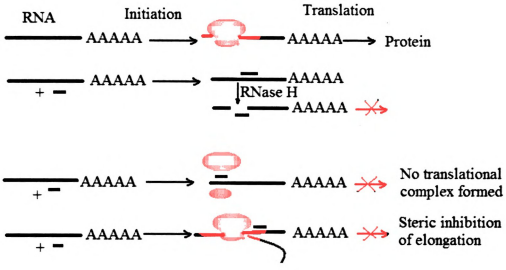


Figure 2. Mechanism of action of antisense oligonucleotides. Antisense oligonucleotides (*blue*) form a hybrid with their RNA target (*black*). There are at least three ways that the formation of the oligonucleotide: RNA duplex might prevent translation of a mRNA resulting in protein production. **1)** The oligonucleotide: RNA duplex may form a substrate for endogenous RNase H, leading to mRNA cleavage. **2)** The oligonucleotide: RNA duplex may prevent the productive assembly of the ribosomal complex (*red*) preventing translation. **3)** The oligonucleotide:RNA duplex may arrest a ribosomal complex already engaged in translation leading to a truncated protein *(green).* (modified from Dagle et al., 2001)

The prevention of the protein synthesis can occur at various levels, inducing degradation of mRNA, inhibition of ribosome binding or arrest of translation. For example, if the antisense sequence is designed to interact with the mRNA at the spliceosome level the inhibition of protein expression will be accomplished[14]. Another alternative is to degrade the message by activating the RNAse H, the ribonucleotide

responsible for mRNA degradation, thus altering the synthesis of the protein that that specific mRNA encoded for[15]. In designing the oligonucleotides, one important factor is the length, which usually varies from 12 to 28 bases. If the sequence is too short, it may lose in specificity. It may self-hybridize or achieve secondary and tertiary structure if too long.

One advantage of using oligonucleotide based gene therapy is that the size of the prodrug is diminished compared to the plasmid DNA. Another advantage is that, depending what process is targeted to be inhibited, the requirement for nuclear localization is not necessary. One of the major disadvantages consists of their high instability; the single stranded oligonucleotides are very susceptible to enzymatic degradation. To increase the oligonucleotides stability, several modifications have been proposed. The most common are the replacement of the phosphodiester backbone with phosphorothioate, peptide-nucleic acids (PNA)[16] or 2-O'-methyl modifications to name just a few. Replacing the oxygen atom with sulfur in phosphorothioate molecules confer greater stability, since these molecules are not substrates for ribonuclease. The methyl phosphonate modification is beneficial since it introduces hydrophobicity, thus enhanced diffusion through cellular membranes.

Antigene oligonucleotides have a portion of a gene as a target, resulting in a triplex formation[17], and thus the inhibition of the transcription process (Figure 3). The nuclear delivery of antigenes is compulsory.

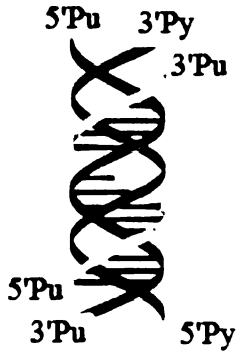


Figure 3. Triplex forming oligonucleotides binding in the major groove of the duplex. (modified from Dagle et al., 2001)

To achieve a greater stability of oligonucleotides inside the cellular milieu, chimeric molecules have been synthesized. RNA/DNA systems consists of 25 nucleotides targeted to the gene of interest, connected through a 5 bp GC clamp and two hairpin ends of T loop. To increase the stability the RNA is 2'-O-methyl modified[18] (Figure 4).

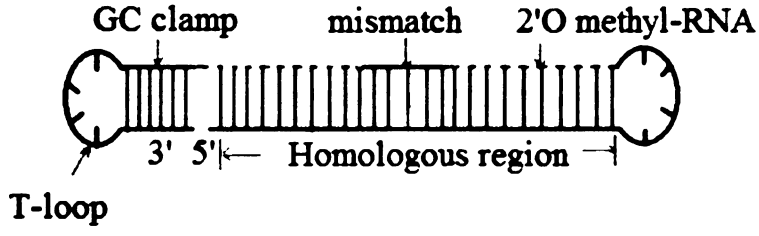


Figure 4. Diagrammatic structure of RNA/DNA chimera. (modified from Liang et al., 2002)

Other versions of nucleic acid therapeutics include ribozymes, DNAzymes and aptamers. Ribozymes are RNA molecules that have catalytic activity, cleaving the mRNA molecules[19]. Two common types of ribozymes used for inhibiting the expression of

specific genes are hairpin ribozymes and hammerhead ribozymes. Similarly, the DNAzymes are analogs of ribozymes but they have increased stability because the ssRNA backbone is replaced by the dsDNA. Zang and collaborators injected DNAzymes directed against VEGF into tumor to inhibit angiogenesis[20].

Aptamers are either single stranded or double stranded oligonucleotide molecules that interact with specific molecular targets. Aptamers targeted against the coagulation factor IXa[21] and HIV1 trans activation responsive element[22] prove the successes of this technology.

Small interfering RNAs (siRNA) are short fragments of oligonucleotides, 21-23 bp with 2 overhanging bases on the 3' end. They degrade the mRNA that encodes for a specific protein so that the protein cannot be synthesized. After administration, the siRNA molecules integrate into the RNA-induced silencing complex (RISC), where the antisense sequence binds to the complementary target mRNA (Figure 5). The mRNA is degraded by a nuclease similar to RNase H. The RISC complex is still being studied intensively, since its function and structure are not completely clear yet. The siRNAs can also be used as primers for the generation of new dsRNA by RNA-dependent RNA polymerase (RdRp). If longer sequences of dsRNA are delivered to the cells, those are cut by an enzyme called DICER into the 21-23 oligonucleotide segments[23]. Among the advantages of using siRNA instead of plasmid DNA to interfere with gene expression are the size of siRNA and its site of action into the cells'. The relationship between the size of the drug and the efficiency of the delivery is well known: the bigger the size the more difficult the uptake[24]. SiRNA delivery to cytosol should to result in more efficient interference with gene expression since translocation to the nucleus is an extremely inefficient process. In

this case, the integration into the host cell genome is not an issue. SiRNA, being dsRNA, also has better resistance to ribonuclease.

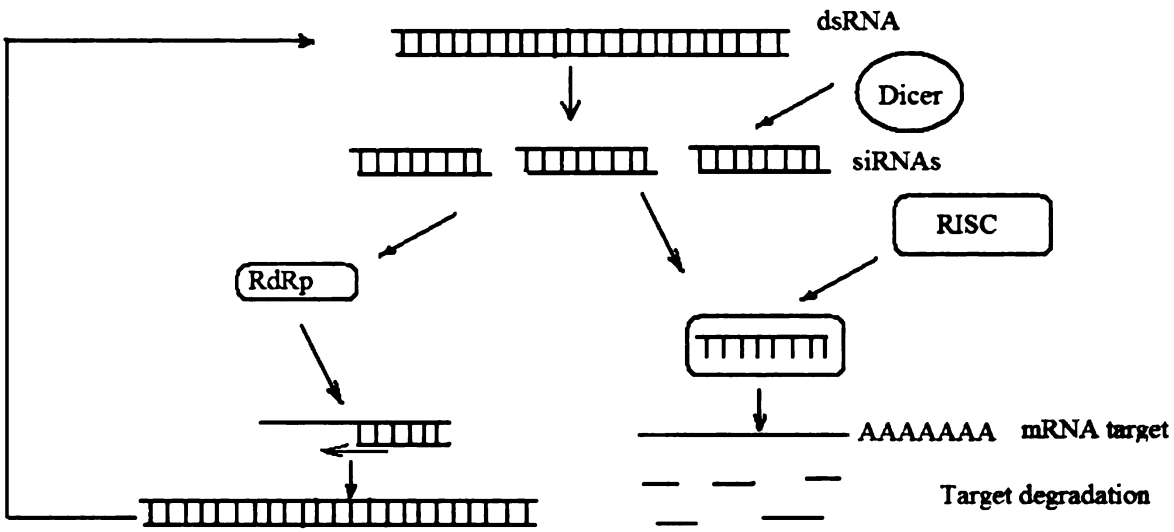


Figure 5. Overview of RNAi pathway. Intracellularly synthesized or exogenously administered dsRNA is cleaved by the enzyme Dicer into 21–25 nucleotide siRNAs. siRNAs become associated with the RNA-induced silencing complex (RISC), which uses the antisense strand of the siRNA to bind to and cleave the target mRNA. The siRNAs can also be used as primers for the generation of new dsRNA by RNA-dependent RNA polymerase (RdRp). This newly formed dsRNA can then also serve as a target for the Dicer enzyme. (modified from Shuey et al., 2002)

Several variants of generating the siRNA are known[25]. Long dsRNA can be delivered to cells and rely on the DICER enzyme to cut it into fragments of 21-25 oligonucleotides long (Figure 6). *In vitro* chemical synthesis, or *in vitro* transcription of optimum fragments of siRNA are other alternatives. *In vivo* synthesis of short hairpin RNA inserted into a plasmid and transfected to cells in culture is another option.

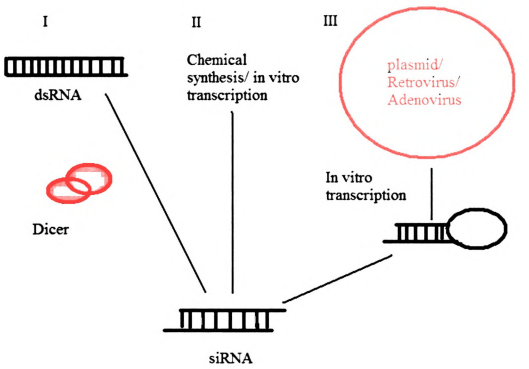


Figure 6. Gene silencing by RNA interference (RNAi). RNAi is triggered by siRNAs, which can by generated in three ways. (I) Long double-stranded RNA molecules are processed into siRNA by the Dicer enzyme; (II) chemically synthesized or *in vitro* transcribed siRNA duplexes can be transfected into cells; (III) the siRNA molecules can be generated *in vivo* from plasmids, retroviral vectors or adenoviruses. (modified from Kurreck, 2003)

1.2.2 Nucleic acid delivery techniques

1.2.2.1 Naked DNA

It was shown that direct injection with naked DNA induces gene expression. When naked DNA solution in 5 % sucrose was injected into skeletal muscle of mice, the reporter gene that was encoded in the plasmid DNA was expressed for several days, even

weeks[26]. This type of expression is accessible only to a limited number of organs, and the muscle is one of them. Recently, efficient delivery of naked DNA to kidney, after intravenous administration, was reported [27]. The exogenous DNA was detected extracellularly 10 minutes after administration at the target organ, and after 30 minutes, intracytoplasmatic and intranuclear. The reporter gene β-galactosidase was detected for up to 35 days. According to the official site of the Journal of Gene Medicine, as of October 2005, 16 % of gene therapy clinical trials involve naked DNA[28].

1.2.2.2 Physical methods

Physical methods like electroporation[29,30], microinjection, sonication[31,32], and biolistic particle delivery require high equipment cost. Electroporation consists of creating pores into cellular membranes under an electric field. The method is well known for delivering nucleic acids *in vitro*, but the cell mortality is very high. Nucleofection[33] is a new technique based on electroporation. The genetic material is delivered directly to the nucleus[34].

Particle bombardment was first introduced in late 1980s, and consists of gold microparticles coated with DNA. An inert gas under high pressure is used to deliver the DNA coated particles *in vivo*. The drawback of this method is that the gold particles cannot be eliminated and remain in the body[35]. This technique could find immediate application to deliver nucleic acid to skin, superficial tumors, and muscle or *ex vivo* gene therapy. Electrical or mechanical techniques for nucleic acid delivery are in general

difficult to be setup in a clinical environment, require specific training of the personnel, and are very invasive.

1.2.2.3 Viral vectors

The best systems that deliver genetic material into cells remain viruses[36]. Viral vectors are biological systems derived from naturally evolved viruses capable of transferring their genetic materials into the host cells. Viral vectors are modified viruses, tailored for the designed purpose. In recombinant viruses [37], the genes responsible for virus replication are substituted by the gene(s) of interest (i.e., therapeutic genes). This is illustrated in Figure 7.

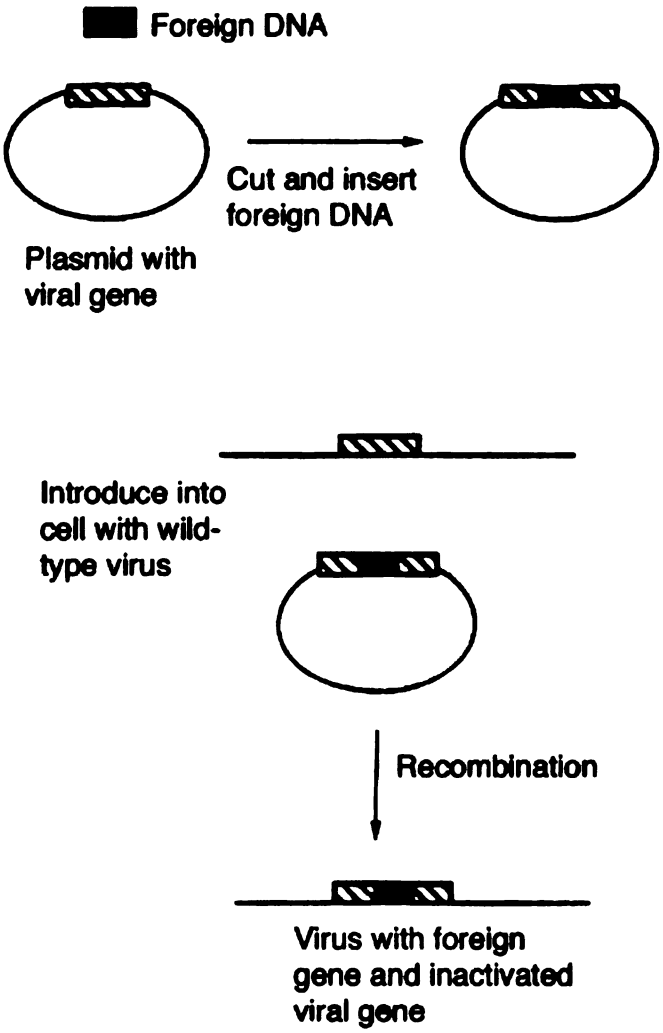


Figure 7. Introduction of foreign DNA into the HSV-1 genome by recombination. (modified from Latchman 2001)

Viruses have evolved genetically to infect cells. However, their use as drug delivery agents has several drawbacks because during the same evolutionary time line, higher organisms developed immune mechanisms for defense to combat the viral infection[38]. For example, even for viruses depleted of their genome, the viral capsids include proteins that are toxic or highly immunogenic. Also, they have limited loading

capacity and may be a carcinogenic hazard due to insertional mutagenesis[39]. Drug delivery viral vectors include retroviruses, adenoviruses, adeno-associated viruses, herpes simplex viruses and lentiviruses[40].

Retroviruses have their genome expressed as RNA. Their name "retro" comes from using an enzyme, reverse transcriptase (RT) to convert their genetic information into DNA in the infected cell. Three proteins are encoded in their genome, *gag, pol* and *env*, with LTR (long terminal repeats) at both ends. The LTR are important in integration of viral genome into host's genome[41]. One severe disadvantage in using retroviruses for gene therapy comes from their ability to infect cells during a specific phase of the cell cycle – mitosis[42]. This may be a drawback in cancer therapy, since most tumor cells contain cells that are in the resting phase – Go. By replacing the viral genes with the gene(s) of interest, modified retroviruses can be used as gene carriers.

Lentiviruses are retroviral vectors that can infect both dividing and non-dividing cells[43]. The best known lentivirus is the human immunodeficiency virus (HIV), which contain coding sequences for six other proteins besides *gag, pol* and *env*. One of them, the *tat* protein, is responsible for transport to the nucleus in non-dividing cells.

The advantage of using adenoviruses as gene carriers is that the adenovirus genome consists of double stranded DNA[44], and it can infect both dividing and non-dividing cells[45]. Also, by depleting the coding sequence of the viral genome responsible for integration of viral genome into the host genome, no integration of the genetic material is acquired[46].

Herpex simplex virus (HSV) is a double stranded linear DNA virus, which is modified in order to be used for gene delivery. It has a large genome, approximately 152 kb, encoding 81 viral genes[47], with 43 coding sequences being non essential for replication *in vitro*. The possibility of insertion of multiple genes, about 30 kb of foreign genes, makes HSV one of the largest viral vectors[48]. Another advantage of HSV is the ability to infect non-dividing cells[49].

Adeno-associated viruses (AAV) are ss DNA viruses which infects both dividing and nondividing cells. They are named AAV because they require a helper virus for replication, which can be either adeno-virus or herpes simplex virus. Their genome encodes for two genes, *cap* and *rep*, which are flanked by inverted terminal repeats that mark the end of the genome[50]. The *cap* gene encodes viral capsid, the coating protein, whereas the *rep* gene is responsible for proteins involved in replication and integration into the host's genome. If integration into the host's genome occurs, the end result will be stable transfection, which enables persistent gene expression. The integration into the human genome occurs at a specific location in chromosome 19[51]. If the *rep* and *cap* genes are replaced with therapeutic genes, AAVs can be used as viral vectors to transport DNA at the desired location. One problem arises though: if AAV is *rep* negative, the integration in the host genome occurs randomly, generating insertional mutagenesis. Another drawback of AAV is that the capacity of the virus is small, only 4 kb.

Alternatively, non-viral methods for gene delivery have been developed. Non-viral vectors can avoid many of the problems encountered with viral vectors. Among the advantages of using non-viral vectors for gene delivery are the ability to be administered repeatedly with little or no immune response, production at large scale with high

reproducibility at acceptable costs and generally good stability in time. The main disadvantages are low transfection efficiency and toxicity.

1.2.2.4 Chemical methods

The association of DNA with a cationic carrier helps by compacting the DNA and reducing the negative charge. Cationic carriers (polymers or lipids) can be used to ferry nucleic acids to cells and also to counterbalance the negative charges that appear due to the phosphate backbone. The interaction between the nucleic acids and cationic carriers results in a condensed form of DNA that shows better stability against nucleases[52].

1.2.2.4.1 Cationic lipids

Chemical methods to improve the DNA delivery into cells rely on development of positively charged vehicles, the most popular ones being liposomes. Anionic liposomes also have been used, but their efficiency is very low. Divalent metal ion solutions help to bring together the anionic liposomes and plasmid DNA into a ternary complex[53]. One example of a mixture of anionic lipid 1,2-dioleoyl-*sn*-glycerol-3-(phosphor-*rac*-(1-glycerol) DOPG, and neutral lipid dioleoylphosphatidyl ethanolamine DOPE[54], was used to deliver a plasmid encoding for green fluorescent protein into CHO-K1 cell line, in the presence of Ca^{2+} ions.

Liposomes are colloidal vesicular structures formed from (phospho) lipid bilayers, and can be used as drug delivery agents since they provide a cavity where the drug is

protected. Felgner[55] and collaborators, in 1987, introduced liposomes as DNA delivery vectors. They showed that the DNA interacts spontaneously with [2,3-bis(oleoyl)propyl]trimethyl] ammonium chloride (DOTMA) and a neutral lipid dioleoylphosphatidyl ethanolamine (DOPE). Practical experience demonstrated the importance of the neutral lipids into efficiency of transport. They observed that DNA loaded liposomes fuse with COS7 cells in culture and that the complex mediates the nucleic acid delivery into cells. In Figure 8 are presented the chemical structures of some molecules that are able to form cationic liposomes, the molecules known as cationic lipids[56], along with some neutral lipids[57] (Figure 9).

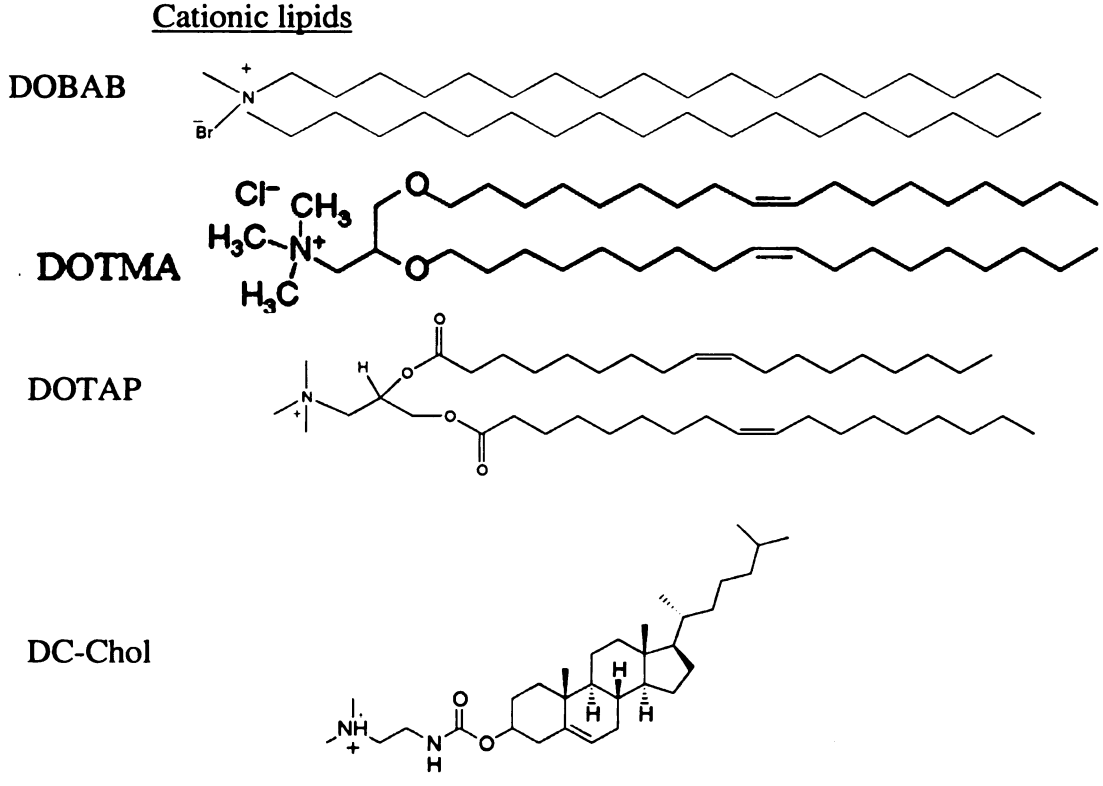


Figure 8. Structures of cationic lipids used as materials for gene therapy. DOTMA: [2,3-bis(oleoyl)propyl]trimethyl] ammonium chloride; DODAB: dioctadecyldimethyl ammonium bromide; DOTAP: 1,2-diacyl-3-trimethylammonium propane; DC-Chol: 3[*N*-(*N'*,*N'*-dimethylaminoethane)-carbamoyl] cholesterol.

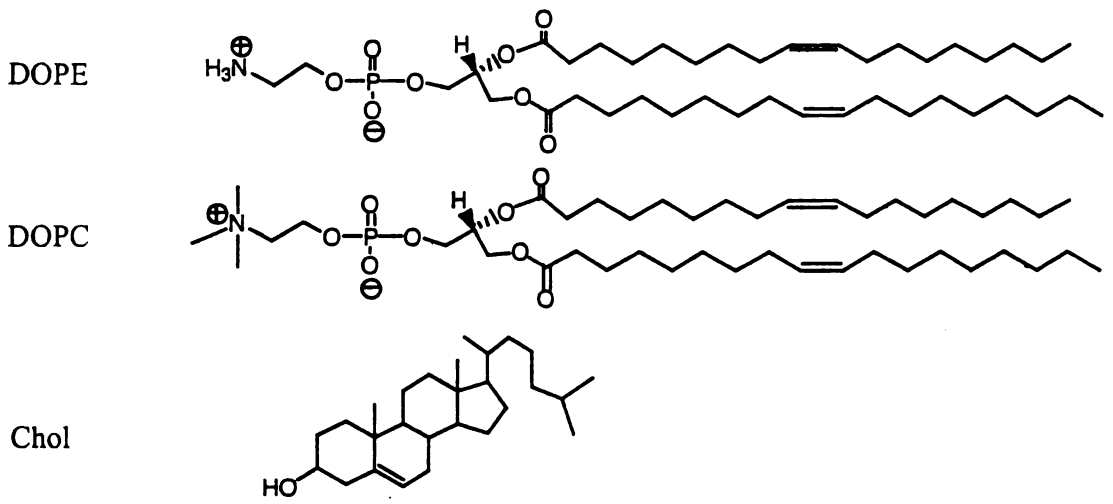
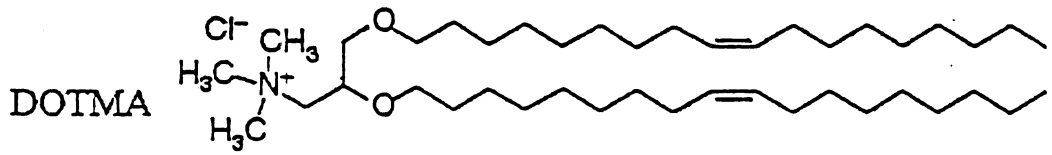
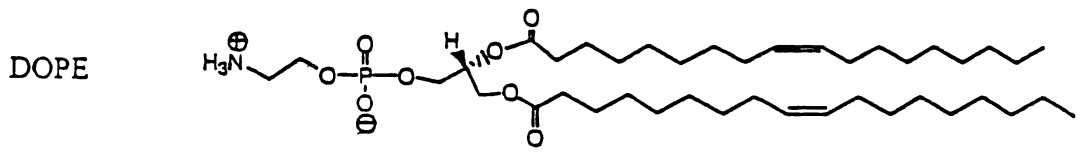


Figure 9. Structures of neutral lipids used as materials for gene therapy. DOPE: dioleoylphosphatidyl ethanolamine; DOPC: dioleoylphosphatidyl choline; Chol: cholesterol. (modified from Segura et al., 2001)

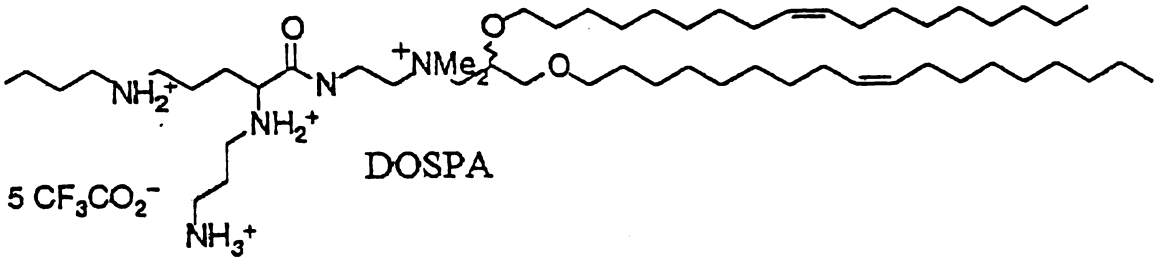
The commercially available transfection reagent Lipofectin from Invitrogen contains a 1:1 mixture of DOTMA and DOPE[58]. Other commercially available products for this purpose are Lipofect-AMINE reagent, a 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE)[59] (Figure 10).

Lipofectin





Lipofectamine



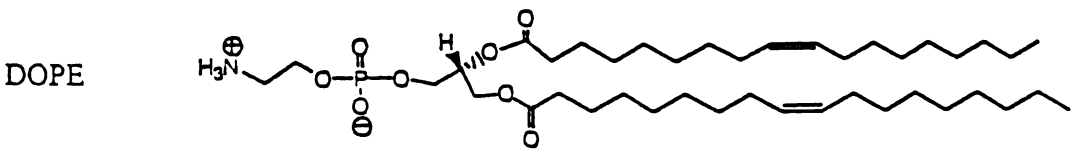


Figure 10. Structures and compositions of some commercially available cationic lipids formulations for nucleic acid transfer Lipofectin and Lipofectamine. (adapted from Schuber et al., 1998)

If we analyze the structures of the most commonly used lipids we notice that all possess the same elements: a head group connected by a linker to a hydrophobic anchor[60] (Figure 11).

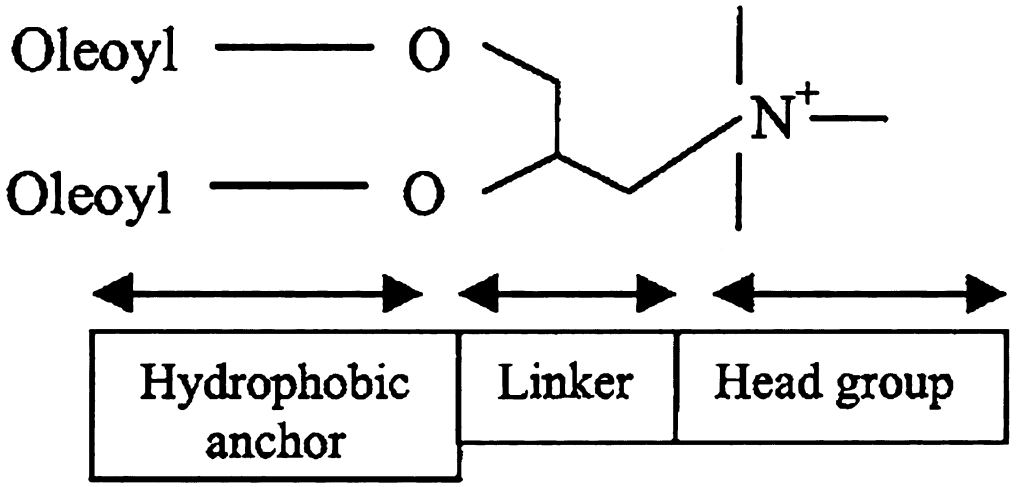


Figure 11. Graphic representation of cationic lipids. (modified from Chesnoy et al., 2000)

The choice of lipid in cationic lipids is between double chain hydrocarbons and cholesterol[61]. Single chain hydrocarbons form micelles in solution and are known as surfactants. Their application in gene delivery is limited by their toxicity[62]. Cholesterol was used successfully as a hydrophobic anchor and it was found to confer rigidity to the lipid bilayer. Two examples of cationic lipids containing cholesterol residues as a hydrophobic anchor are DC-cholesterol and GL-67.

Double chain hydrocarbons are the most popular hydrophobic residues used in cationic lipid formulations. Saturated acyl chain lengths vary from 10 carbons (lauryl), 12 (myristoyl), 14 (palmitoyl) and 16 (stearoyl). Oleoyl residue (C18:1) is the most commonly used unsaturated hydrocarbon chain, although two double bonds (C18:2 lynoleyl) and three double bonds (C18:3 lynolenyl) are also common. The unsaturated acyl chain is thought to enhance membrane fluidity. One of the drawbacks of using molecules that contain double bonds is their limited shelf life, since the double bonds can be oxidized easily.

The linker is the part of the molecule that connects the hydrophobic anchor with the head group. The linker group is an important determinant of the (bio) degradability of the cationic lipid; depending on the chemical bond that connects the linker, the catalytic activity of various hydrolytic enzymes like esterases or peptidases can degrade the lipid. For the cationic lipids that contain two hydrocarbon chains, the most commonly used linker molecule consists of a glycerol residue (e. g., DOTMA, DOTAP). When cholesterol derivatives are responsible for hydrophobicity, a spacer of 3-6 carbon atoms is inserted between the linker and the head group[63].

A lot of variation in structure and properties can be introduced using various cationic head groups. Quaternary ammonium groups, primary, secondary or tertiary amine groups introduce the positive charge that is responsible for electrostatic interactions with the highly negatively charged phosphate backbone of the DNA molecule. For example, monocationic molecules like N-[1-(2,3-dimyristyloxy)propyl]-N,N-dimethyl-N-(2-hydroxyethyl) ammonium (DMRIE) and N-[1-(2,3 dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), as well as polycationic molecules like dioctadecyl amido glycyl spermine (DOGS), and 2,3-dioleyloxy-N-[2 (sperminecarboxamido) ethyl] - N , N, -dimethyl-1 - propanaminiumtrifluoroacetate (DOSPA) are used extensively. The multication groups, like "T-shaped" spermine, are more efficient than the monocation ones.

As a summary, cationic lipids are amphiphilic molecules, with double hydrocarbon chains or cholesterol derivatives responsible for the hydrophobic part, while the hydrophilic part can carry various charged groups. Various combinations are possible and some examples are presented in Table 1[64]. Some of the acronyms in the table are

mentioned in the previous paragraph while DC-chol stands for 3[*N*-(*N'*,*N'*-dimethylaminoethane)-carbamoyl] cholesterol and Lipid 67 is cationic lipid amphiphile (GL-67) consisting of a cholesterol anchor linked to a spermine head group[65].

Table 1. Cationic lipids classified according to structural properties

Hydrophilic part	Hydrophobic part	
	double chained lipids	cholesterol derived lipids
monocationic	DMRIE, DOTMA	DC-chol
polycationic	DOGS, DOSPA	Lipid 67

Liposomes are colloidal vesicular structures formed from (phospho) lipid bilayers, and can be used to some extent as drug delivery since they provide a cavity where the drug is protected. Depending on size, liposomes are known as small unilamellar vesicles (SUV, <100 nm), large unilamellar vesicles (LUV, 100-500 nm) and giant unilamellar vesicles (GUV, >1 μm)[66]. Considering lamellarity, liposomes can be unilamellar or multilamellar vesicles (MLV, 0.1-10 μm)[67]. Liposomes have been used as carrier systems since they confer some advantages of delivery, compared to naked drugs. Hydrophobic drugs are difficult to be administered intravenously, but they can be solubilized into the liposome's phospholipids bilayer. Also, hydrophilic drugs can be encapsulated into vesicle's cavity (Figure 12).

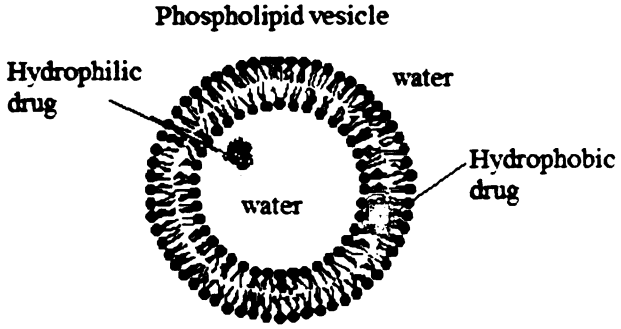


Figure 12. Liposome structure. The phospholipid bilayer can transport hydrophobic drugs while hydrophilic drugs can be encapsulated inside the aqueous core.

It was shown that liposomal drug formulation is beneficial for increasing circulation time, since the vesicle protects the drug from enzymatic degradation. In a slow release mode, the concentration of the therapeutic agent is constant over a longer period of time. The active dose that the tissue is exposed to is also smaller, diminishing the toxic effect of certain drugs. For example, it is well known that, the anticancer drug doxorubicin has side effects on heart muscle. By encapsulating the doxorubicin into a liposome the direct exposure of heart to the cytotoxic drug is diminished. DOXIL is a liposomal formulation of doxorubicin that is manufactured by Alza Corporation. Doxil was approved in June 1999 for treatment of Kaposi's sarcoma. Another advantage of liposomal drug delivery is the ability to direct the drug to a specific target.

Four types of liposomes are known: conventional liposomes, cationic liposomes, targeted liposomes and stealth liposomes (Figure 13). Conventional liposomes, also known as "naked liposomes", consist only of a phospholipid bilayer and are either neutral

or negatively charged. They are not chemically modified, so nothing protects them from phagocytes. These formulations usually carry drugs that target macrophage cells (Kupffer cells) in the liver or spleen. For example, delivery of antimicrobial agents loaded into conventional liposomes to macrophages is a potential application in case of an infection[68]. To control the circulation time of liposomes, they were chemically modified with polyethyleneglycol (PEG)[69, 70]. The PEG chains become hydrated and thus provide steric protection, blocking the enzymes and nucleophiles, and also limiting diffusion of encapsulated drug into liposome, thus creating a "stealth liposome"[71]. A half-life of approximately 48 hours in terms of circulation time was achieved in humans for PEG-ylated liposomes.

Cationic liposomes have both interior and exterior surfaces positively charged. They were designed for delivery of genetic material. The positive charges from the cationic lipid interact with negatively charged nucleic acid molecules, thus condensing the DNA into a more compact structure. The complex ensures DNA protection and promotes cellular internalization. Cationic liposomes can successfully deliver their load but they are not stable, have low loading capacity, are subjected to leaking and have a limited storage life[72].

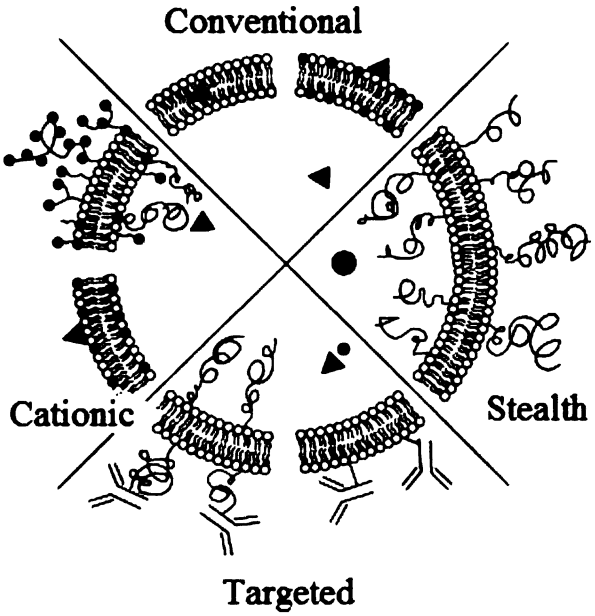


Figure 13. Schematic representation of four major liposome types. Conventional liposomes are either neutral or negatively charged. Sterically stabilized ('stealth') liposomes carry polymer coatings to obtain prolonged circulation times. Immunoliposomes ('antibody targeted') may be either conventional or sterically stabilized. For cationic liposomes, several ways to impose a positive charge are shown (mono-, di- or multivalent interactions). (modified from Storm, 1998)

Targeting liposomes to different organs or tissues was achieved by using specific antibodies or antibody fragments (like Fab' or single chain antibodies). These liposomes are thus also known as immunoliposomes. These fragments are attached to liposomes' surface to promote target site binding. The primary application of targeted liposomes is the delivery of cytotoxic anticancer drugs. A variation of immunoliposomes coated with PEG, to increase their circulation time, is also possible. The antibody fragments can be

attached directly to the liposome surface (which may provide steric hindrance to antigen binding), or to PEG molecules (which do not give a steric hindrance problem)[73] (Figure 14). Targeted liposomal gene delivery can be achieved also by attaching different fragments that bind to extracellular receptors, facilitating receptor-mediated endocytosis. It is known that cancer cells can be targeted using folate receptor[74,75,] transferrin receptor [76,77,78]and LDL receptor. By chemically attaching these kinds of ligands to liposomes, preferential tissue or tumor targeting can be achieved.

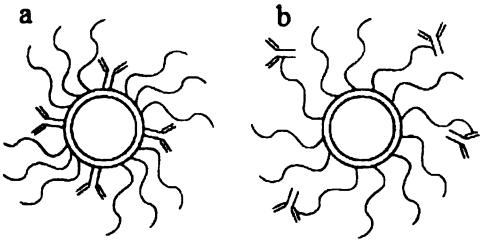


Figure 14. Immobilization of antibody on PEG-liposomes by (a) direct coupling to the liposome surface and (b) coupling to the terminal ends of the PEG chains. (modified from Klibanov et al., 1992)

1.2.2.4.2 Cationic polymers

Another fairly successful way to deliver cargo into cells are the so called "trojan peptides" [79, 80]. Natural polypeptides like HIV-1 TAT protein, Antennapedia homeodomain and the herpes simplex virus 1 DNA binding protein VP22 have been shown to enter cells when added to culture media. These proteins contain a motif, the protein transduction domain (PTD) that is rich in basic amino acids lysine and arginine

and is responsible for internalization. Highly cationic peptide sequences like polyarginine[81] and polylysine [82] (Figure 15) have been synthesized after this model and were successful in nucleic acid delivery but have limitations including iterative synthesis, and small size associated with low capacity of protection the genetic material from nucleases.

Polyethyleneimine (PEI) (Figure 15) was shown to successfully transfer nucleic acids into COS1 cells, but one of its major limitations is the cytotoxicity [83]. Other chemical methods use specifically designed activated dendrimers that possess precisely defined size and shape[84,85]. Dendrimers have a tridimensional spherical architecture, with branches radiating from a central core and terminating with charged amino groups. One of the advantages of synthesizing dendrimers is their monodispersity. An example of a widely used dendrimer is polyamidoamine (PAMAM) [86] (Figure 16). Their tedious synthesis and low protection capacity doesn't make them the best candidates for gene delivery. Examples of commercially available sixth generation dendrimeric structures are Superfect and Polyfect (Quiagen, Valencia, California) used for in vitro gene delivery.

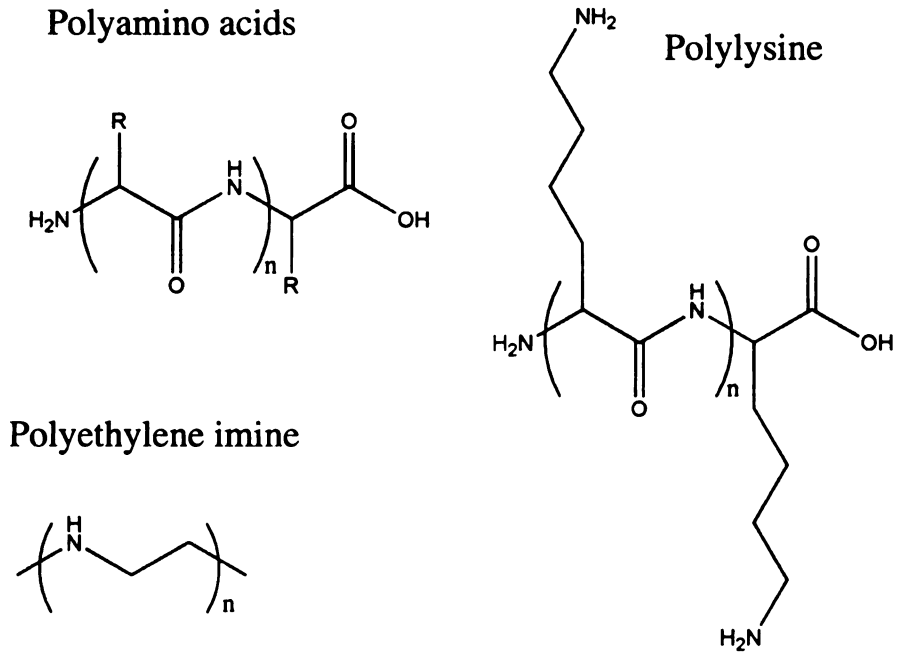


Figure 15. Cationic polymers used for gene delivery (polylysine, polyethyleneimine). (modified from Segura et al., 2001)

Polyamidoamine (PAMAM)

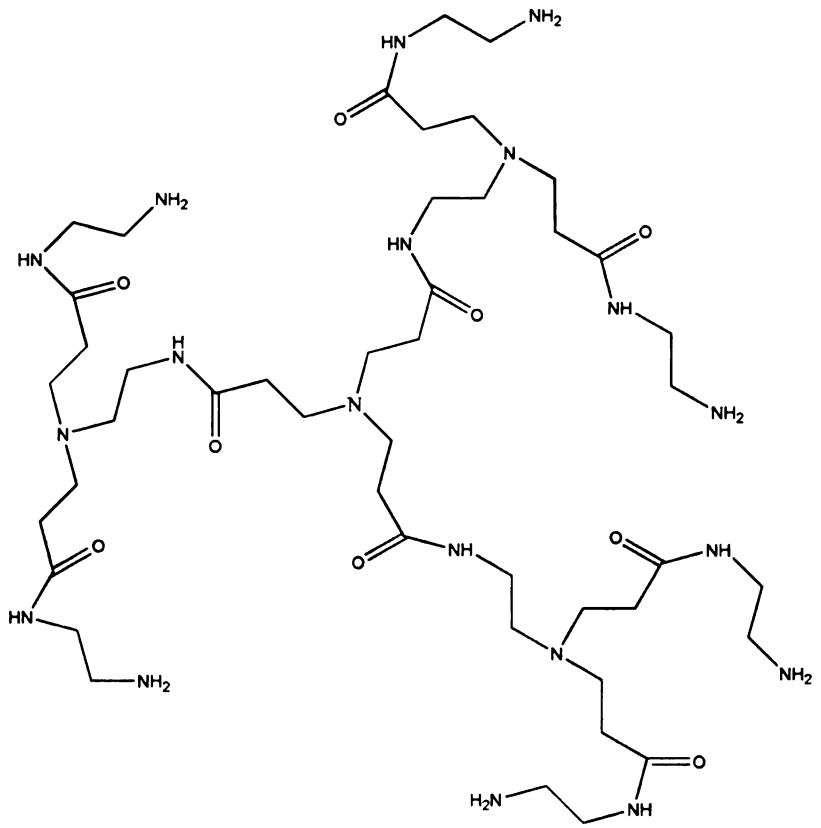
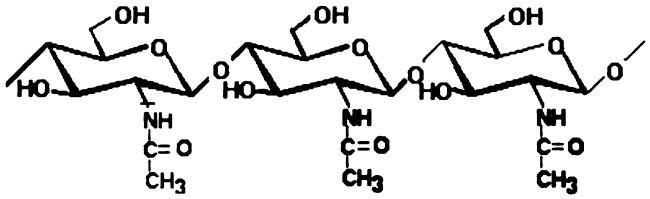


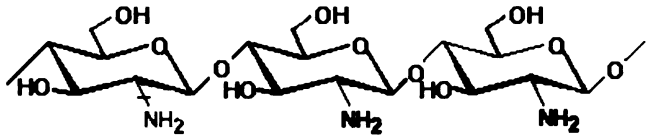
Figure 16. Cationic polymers - polyamidoamine structures. (modified from Segura et al., 2001)

Natural polymers have been used also for gene delivery because they are biodegradable and ensure better compatibility with cells. One example is chitosan[87], obtained from deacetylation of chitin which is found in crab's exoskeleton (Figure 17). At an appropriate ratio, the positively charged chitosan interacts electrostatically with negatively charged DNA and generates a positively charged complex that mediates the nucleic acid uptake into cells[88]. One of the limitations in using chitosan for gene therapy

in humans is that chitosan was reported to cause hypocholesterolemia[89]. In addition its structural complexity makes its purification and functionalization difficult to accomplish. Significant "batch-to-batch" variations depend on the source.



Chitin



Chitosan

Figure 17. Structures of chitin and chitosan.

(modified from http://dalwoo.com/chitosan/structure.htm)

1.3 Mechanism of uptake

The association by electrostatic interactions between nucleic acid therapeutics and the cationic carriers results in complexes that ensure a tight compaction and protection of the DNA. Subsequently, these positively charged complexes bind to the cell surface and are taken up by endocytosis. Once the endosome is formed and the cargo crosses the cellular membrane, the hydrophobic site of lipids acts synergistically with polycationic residues to release the complex from the endosome. The efficient escape from the endosome results in release of the nucleic acids into the cytosol, followed by nuclear

uptake. The interaction between drug and carrier should be strong enough to allow a complex to be formed yet weak enough to enable the drug to dissociate from the complex, once it has reached its target. The final product, the protein expressed after the reporter gene allows the evaluation of this multistep transfection process. Yokoyama[90] proposed a model for how a cationic carrier accomplishes the task to deliver plasmid DNA to cells. The cationic carrier-DNA complex is able to adhere to the cell surface through electrostatic interactions, followed by endocytosis of the complex. Once the complex or the DNA released from the complex escapes from the endosome, it translocates to the nucleus and allows transcription to initiate. A graphic representation of this model is presented in Figure 18.

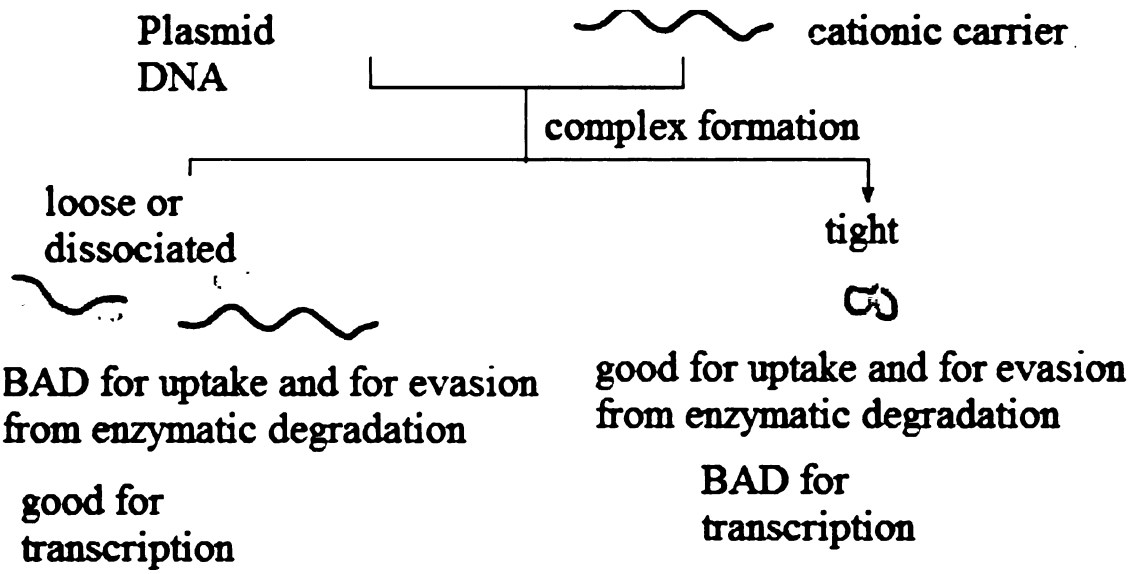


Figure 18. The dilemma of the DNA – polymer complex: how tightly should DNA be complexed? For the cationic DNA-polymer complex a tight complex will be favorable for cellular uptake and provide protection against enzymatic degradation, and a loose complex will allow release of DNA from the complex in order to be transcribed. (Modified from Yokoyama, 2002)

1.3.1 External barriers

In order to reach the target, the active component of a drug must overcome several biological barriers. The immune system has developed evolutionarily to guard against foreign material spreading throughout the body. One of the most common ways of systemic administration is intravenous injection. The interactions between the therapeutic agent and blood compartments, serum proteins and other cellular elements like erythrocytes cannot be disregarded. If the DNA carrier complex interacts with serum proteins (opsonins) that mark the complex for clearance by the reticuloendothelial system, it is easy to realize that once destroyed, the DNA will not get to the target and obtain the desired therapeutic effect. The liver and the kidney are the organs responsible for detoxification of the organism, and after degradation of the complex, these are the places where the therapeutic DNA ends up.

1.3.2 Internal barriers

An efficient gene delivery system should protect the nucleic acid from degradation, both outside and inside the cell.

For both viral and non-viral vectors, the intracellular barriers consist mainly of cellular membranes, intracellular trafficking, and the nuclear membrane. Cellular entry, internalization, is considered to be achieved mainly through endocytosis, though other alternatives like membrane fusion have also been considered.

In case of plasmid DNA complexed with either cationic polymers or cationic lipids, the endocytotic mechanism is considered responsible for cellular uptake[91]. For

cationic lipid complexes, the lipoplexes, an alternative mechanism, membrane fusion, was also proposed[92] (Figure 19).

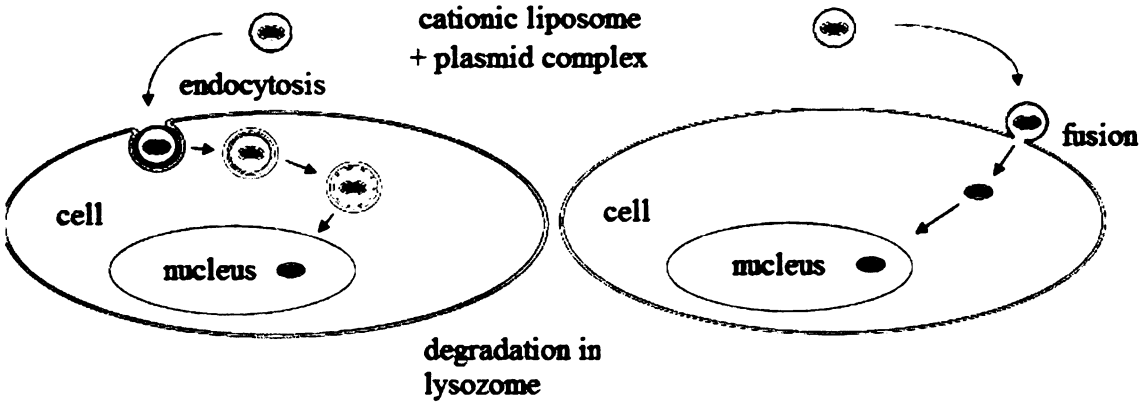


Figure 19. Cell entry mechanisms for cationic liposome/DMA complex. Endocytosis versus membrane fusion[93]. (modified from Smyth Templeton, 2002)

Once the free DNA or DNA complex enters the cell, in order to be efficient it has to reach its final target, the cell nucleus. For some eukaryotic cells, the distance from cellular membrane to the nucleus can range from a few μm, up to 100 μm in larger cells. The cytoplasm is not a bag that contains the cellular organelles and metabolic products but is a complex system, with a highly branched network of microfilaments, microtubules and intermediate filaments. The cytoplasm's consistency resembles a gel-sol system more than an aqueous solution. It was reported that protein concentration inside the cytosol is up to 100 mg/mL [94]. Lucaks and collaborators examined DNA mobility through the cytoplasm, and they found that large DNA fragments are not able to passively diffuse to the nucleus. When DNA fragments of about 2000 bp and macromolecules larger than 2000 kDa were microinjected in HeLa cells and fibroblasts, their movement through cytoplasm was not detectable. This result suggests that plasmid DNA movement through

cytoplasm by physical diffusion is very limited[95]. It was not determined if the efficient transport of free DNA to the cell nucleus takes place using the cytoskeleton network and molecular motors[96].

After internalization of the DNA complex via endocytosis, several alternatives are possible for the fate of the DNA. One is that the DNA complex is entrapped into the endosome, and is never released. Another alternative is that the early endosomes mature into late endosomes, the DNA escapes from the endosome by membrane destabilization and enters the cytoplasm. Once in the cytoplasm the plasmid DNA has to dissociate from the complex and find its way to the nucleus[97] before being degraded by cytoplasmic nucleases. It was suggested that the plasmid DNA is released from the cationic polymer complex[98], but not from cationic lipid complex[99], through transfer reactions. The cytoplasm contains DNA-binding proteins and RNA-binding proteins that interact with free DNA/RNA. This interaction could promote stabilization of the DNA and protection from nucleases. It is also possible that this DNA protein interaction should mediate interaction of nucleic acids with microtubules or actin-based motors[100]. It is known that free DNA does not bind motor proteins[101]. When entry to the nucleus is facilitated by disruption of the nuclear membrane the timing is important also. The DNA should be able to survive enzymatic degradation until the appropriate phase of the cell cycle, mitosis.

Zabner and collaborators found by gold electron microscopy that in the case of cationic lipid mediated DNA delivery the lipid/DNA complex accumulates in the perinuclear region[102]. Huang and collaborators tried to prove the hypothesis that the lipid/DNA complex should escape from the endosome before mixing with the lysosome. When lipid/DNA complex was microinjected into the nucleus, the gene expression failed

to occur, since the DNA was not accessible to the transcription complex. The third alternative for the fate of DNA/carrier complex, if the complex is entrapped into the lysosome the DNA will be degraded by the lysosomal enzymes, and the expected therapeutic effect will fail to happen.

1.3.2.1 Endosomal escape

Most of the cationic lipid based methods for nucleic acid delivery consist of mixture of lipids. The lipid complex contains also a neutral amine, a hexagonal phase forming lipid, dioleoylphosphatidylethanolamine (DOPE), which is expected to facilitate the escape from the endosome by membrane destabilization at acidic pH[103]. It was shown that at lower pH a phase transition from lamellar to inverted hexagonal phase resulted in destabilization of endosome membrane with release of the nucleic acid cargo into the cytoplasm. When the neutral lipid DOPE was replaced by DOPC the effect of destabilization of endosomal membrane was not seen. The structure of DOPE with a small polar head and a large hydrophobic tail allows it to form micelle like structures, inverted hexagonal phase[104] (Figure 20). Cholesterol had also been used as a co-lipid or neutral lipid, but the complexes formed with cholesterol were less efficient compared to DOPE containing carriers[105].

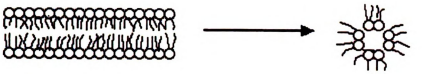


Figure 20. Phase change of lipid structure from lamellar phase to hexagonal phase. (modified from Monkkonen et al., 1998)

Safinya and collaborators studied the structure of complexes formed between cationic lipids and nucleic acids[106]. X-ray diffraction studies had shown that one possible structure is similar to a sandwich, with nucleic acids in between sheets of cationic lipid bilayers. A model is presented in Figure 21. Under cellular conditions, due to differences in intracellular compartments, some lipids undergo phase transitions. One common structure that is adopted by the neutral lipid DOPE at low pH is the inverted hexagonal phase. This phase transition may play a role in DNA release from the complex.

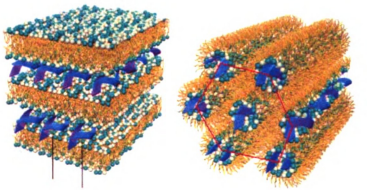


Figure 21. Condensed multilamellar lipids incorporating DNA and hexagonal arrangement of lipids with DNA. An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery. (modified from Koltover et al., 1998)

Xu and Szoka proposed a model for destabilization of the endosomal membrane in which the anionic lipids that face the cytoplasm from the endosome, through a flip-flop mechanism will pair with cationic lipids from the cationic liposome/DNA complex (Figure 22). This pairing results in displacement of the DNA from the cationic lipid and release of the DNA into the cytoplasm. Displacement of the cationic lipid from the DNA prior to its entering the nucleus is critical for gene expression[107].

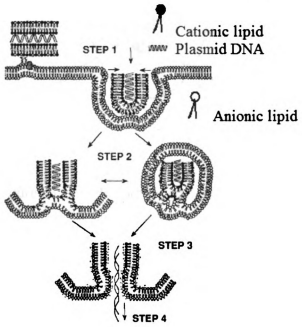


Figure 22. Mechanism of uptake and release of plasmid DNA from the complex. (Step 1) After electrostatic interaction with the cell membrane, cationic liposome/DNA complexes are endocytosed. (Step 2) In the early endosome, membrane destabilization results in anionic phospholipid flip-flop. (Step 3) The anionic lipids diffuse into the complex and form a charge neutral ion pair with cationic lipids. (Step 4) The DNA dissociates from the complex and is released into the cytoplasm. (modified from Xu et al., 1996)

In case of cationic polymers, carriers that contain many amine groups like polyethylene imine (PEI) or other polyamines, the proton sponge mechanism was proposed to be responsible for escaping the endosome[108] (Figure 23).

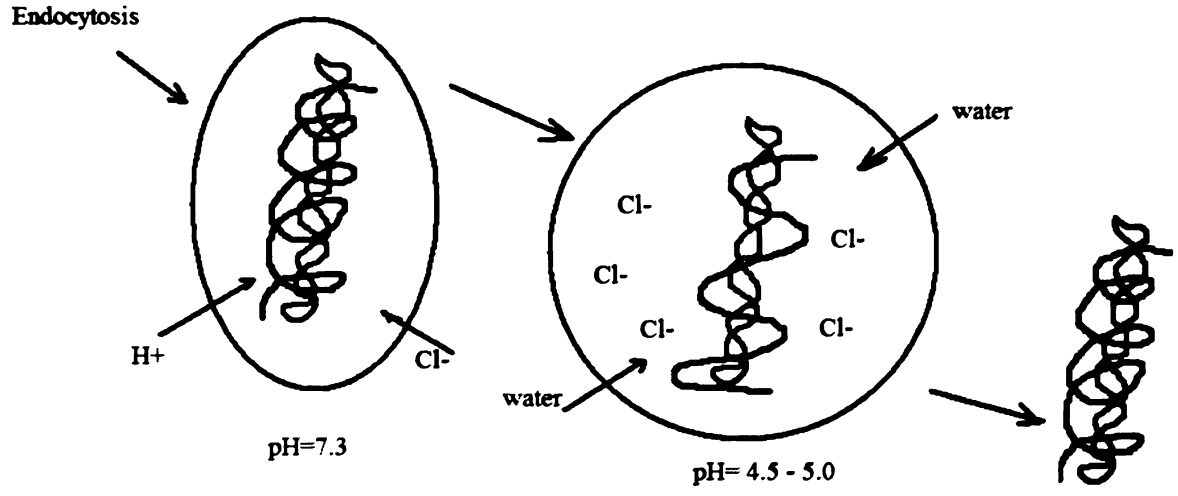


Figure 23. The PEI proton sponge hypothesis. (modified from Remy et al., 1998)

The cationic polymers have the capacity to buffer the influx in protons that acidifies the endosome from the pH 7.4 – 7.5 to 4.5 – 5.0. The influx of protons brought by endosomal ATPase is coupled with an influx of chloride ions. The influx of water is also increased and the result is an increase in osmotic pressure inside the endosome. The endosomal compartment swells and bursts, releasing its content into the cytoplasm[109]. Interestingly, if for cationic lipids the dissociation of the complex prior to entry to the nucleus is required, in the case of cationic polymers, it was shown that gene expression was obtained even when complexes cationic of polymer/DNA were injected into the nucleus[110].

1.3.2.2 Nuclear entry

Nuclear entry represents the major barrier for nucleic acid therapeutics. In case of small molecules, oligonucleotides can passively diffuse through the nuclear membrane. In case of plasmid DNA there are two alternatives to reach the nucleus, one is to pass through nuclear pores and the other is to take advantage of the nuclear membrane disruption during mitosis. Since 1980, it is known from microinjection experiments, that nuclear membrane represents a major limitation to gene expression. Plasmids microinjected into the nucleus were able to express the gene whereas the plasmids microinjected into the cytoplasm did not express the gene[111], and not more than 0.1 – 0.001 % of cytosolic microinjected plasmid DNA was transcribed. In another microinjection experiment, with the same number of plasmid copies injected in the cytoplasm and the nucleus, the gene expression for plasmid microinjected into cytoplasm was about 3 % of what was reported for plasmids microinjected directly into nucleus[112]. These experiments suggest that passing the nuclear membrane is the rate-limiting step in gene expression. It is well known that the nuclear membrane had been developed evolutionarily to enclose the genetic material and keep it safe from foreign material that could result in mutations. Recently Ludtke's group reported that the plasmid DNA is localized into cytoplasm in divided cells, even after microinjection into the nucleus[113]. Thus, the exclusion of foreign genetic material from the nucleus is another limiting factor in achieving the desired gene expression, and should be considered in addition to enzymatic degradation. One example of expressing plasmid in dividing cells is the expression of reporter gene in cells that look like twins (Figure 24), a clear indication that the plasmid origin is from the mother cell that had divided into two daughter cells[114].

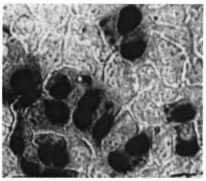


Figure 24. BNL.Cl-2 hepatocytes transfected with nuclearLacZ/PEI appear as twins. (modified from Zuber et al., 2001)

The transfer of plasmid DNA to the nucleus is regulated by the nuclear pore complexes, which restrict passive diffusion to molecules no larger than 40 – 50 kDa. The passage of larger molecules through nuclear pores is an active process, requires energy, ran-GTP gets hydrolyzed to Ran-GDP, a protein complex is formed from importin α/β, proteins that contain nuclear localization signal (NLS) bound to the plasmid DNA[115]. A schematic representation of a possible nuclear import mechanism is presented in Figure 25.

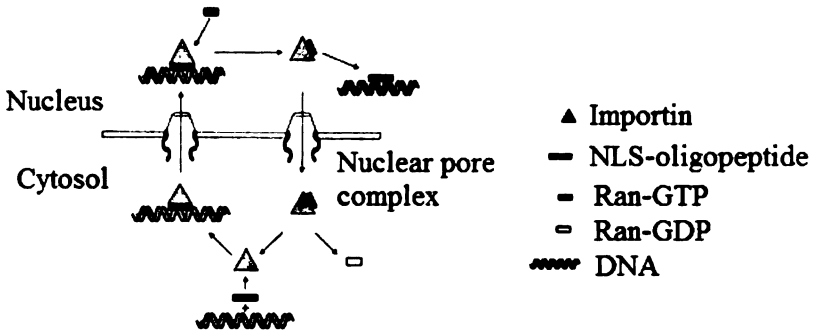


Figure 25. Hypothetical mechanism of nuclear import of plasmid DNA by importin transport receptors. DNA is covalently attached to a NLS or bound to a NLS containing protein, such as transcription factor. The complex binds to importin in the cytoplasm and translocates into the nucleosol. Following nuclear entry, the importin-Ran GTP complex is recycled back to the cytoplasm where Ran-GTP is displaced from the complex upon the hydrolysis of GTP. (modified from Lechardeur et al., 2002)

Chapter 2

Design of novel carriers for nucleic acid delivery

The percentage of clinical trials for gene therapy involving viruses to deliver the nucleic acids decreased from 72.4 % in October 2000 to 50 % in October 2005. Charts presenting vectors used in clinical trials in 2000 and 2005 are presented in Figure 26 and Figure 27.

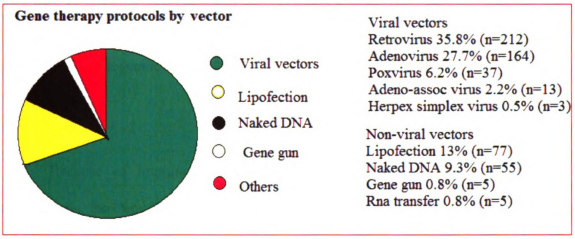


Figure 26. Overview of vectors used in clinical trials in October 2000. (adapted from http: / /www.wiley.co.uk/genetherapy / clinical /)[116].

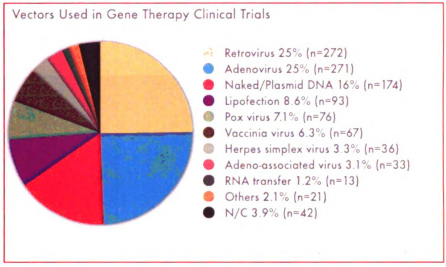


Figure 27. Overview of vectors used in clinical trials in October 2005. (adapted from www.wiley.co.uk/genmed/clinical)[117]

Because of immunogenic responses, toxicity, and the threat of insertional mutagenesis, the trend has been to switch to develop safer, non-viral methods. Non-viral vectors can avert many of the problems encountered with viral vectors. Among the advantages of using non-viral vectors for gene delivery are the ability to be administered repeatedly with little or no immune response, the production at large scale with high reproducibility at acceptable costs and generally good stability in time. The main disadvantages are low transfection efficiency and toxicity.

As described above, there is a plethora of drug delivery systems; however, each is limited by significant shortcomings. Cationic liposomes can successfully deliver their load but they are not stable, have low loading capacity, leakage is not uncommon and

limited storage life[118]. A commercially available dendrimer product, SuperFect (Qiagen, CA), is commonly used for transfections, but it can cause cell lysis (Figure 28).

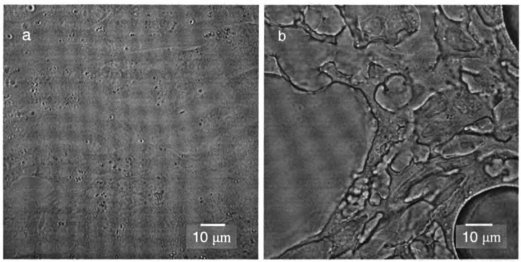


Figure 28. Transmitted images of MEF cells (a) control, (b) treated with SuperFect. The cells are destroyed by SuperFect treatment.

The limitations of using short peptides for gene delivery come from the iterative synthesis, small size associated with low capacity of protection the genetic material from nucleases. The use of natural polymers is limited by the fact that their structural complexity makes their purification and functionalization difficult to accomplish. They have significant "batch-to-batch" variations, depending on the origin. The current non-viral methods used gene delivery show low transfection efficiency and are not suitable for therapeutic use. Development of new delivery agents that have better compatibility with cells, exhibiting lower toxicity and also better efficiency is necessary for turning the gene therapy into a successful therapeutic method.

There are a few features that were found to be important in delivering nucleic acids. The **molecular weight** is one of them. Drugs that have molecular weight less than 40 Kda are susceptible to clearance from the systemic circulation due to renal filtration. If gene therapy targets a tumor, the enhanced permeability and retention effect (EPR) can be used to achieve higher drug concentrations at the tumor site. The advantage of coupling the drugs with high molecular weight polymers consists of accumulation of the complexes at the tumor site due to the EPR effect[119]. This effect is characteristic of tumor tissues, is also known as passive targeting. There are two aspects related to this effect. First, the tumor vasculature allows the macromolecules with a MW of 50 kDa or even higher to enter the tumor. Second, the lymphatic system, that is responsible for the drainage of macromolecules from normal tissues, is not as efficient in tumors. Not only can macromolecules reach the tumor site, but also they remain there for a longer period of time (more than 100 hours), since the removal of macromolecules from the tumor site is not as efficient[120]. Low molecular weight compounds are able to reenter systemic circulation, since they can diffuse through the biological barriers. A schematic representation of the EPR principle is presented in Figure 29.

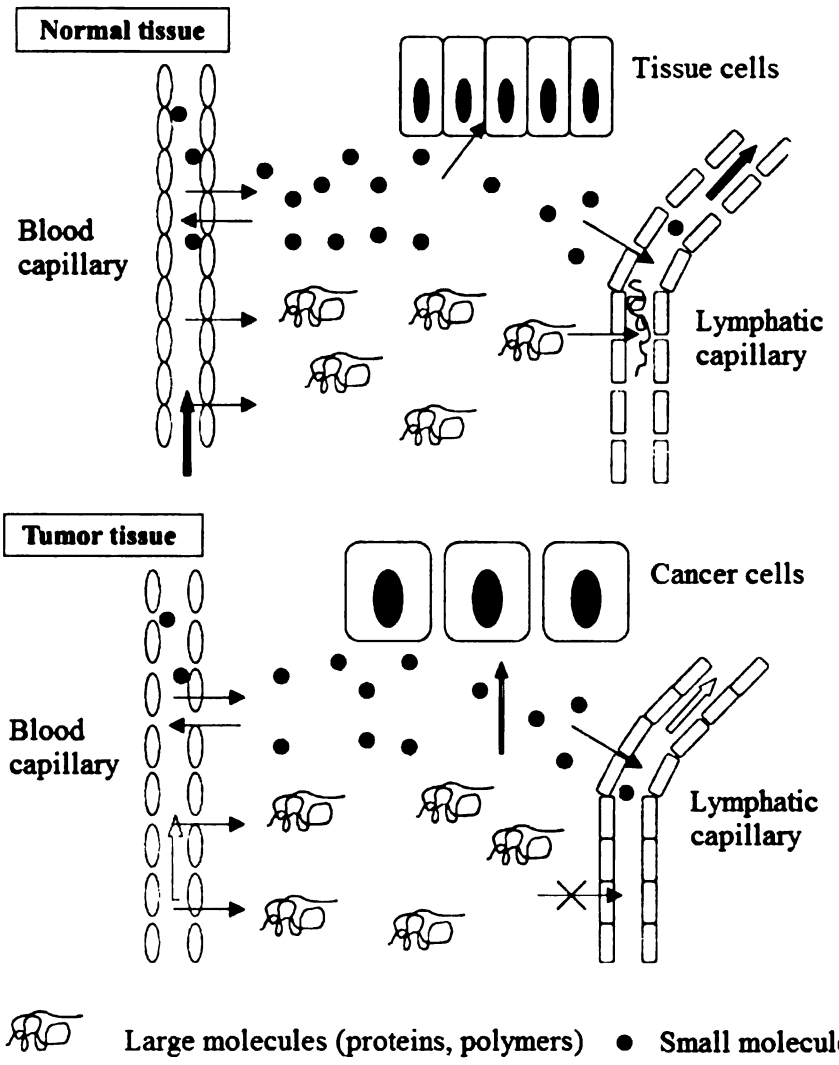


Figure 29. Schematic illustration of the EPR effect principle. Angiogenesis and enhanced vascular permeability of tumor capillaries and impaired or missing lymphatic clearance of macromolecules result in accumulation of macromolecules (polymers) in tumor tissue[121]. (modified from Ulbrich et al., 2004)

Another important characteristic of molecules that mediate nucleic acid delivery is **charge**; positive charge seems to be important. Cationic polymers like polyethyleneimine,

polyarginine, polylysine, cationic liposomes of various compositions and natural polymers like chitosan and DEAE dextran, all have in common the positive charge. It was reported that anionic liposomes were also used to deliver nucleic acids to cells, but they were successful only when they formed a ternary complex with calcium ions.

Hydrophobicity is another characteristic that some of the non-viral vectors have in common. Cationic lipids contain a cationic head and a lipid tail connected by a linker[122] (Figure 30). It is believed that the hydrophobic tail mediates membrane fusion. The ratio between the charged part and the non-polar part is about ranges between (30-40):(70-60).

Figure 30. General representation of a cationic lipid. (modified from Trenchant et al., 2004)

If we analyze the structures of the most commonly molecules used as transfection reagents, we see that there is a lot of diversity among the structures of efficient delivery systems, various functional groups seems to play a role in efficient delivery of genetic material to cells. Three physico-chemical features are important for delivery: **charge** (cationic polymers and cationic lipids have positive charge), **hydrophobicity** (cationic lipids have non-polar functionalities) and **size**. Size of the efficient drug delivery systems can vary from 1 kDa (small peptides like polyarginine, polylysine, and cationic lipids) to 25 kDa (Polyethyleneimine) and up to 500 kDa (DEAE-dextran, chitosan). The goal of gene therapy is to develop a vector that can be used to deliver nucleic acids to treat

human diseases. Biocompatibility of the carriers and their metabolic products plays a major role in developing safe solutions to this issue.

Preliminary studies in our lab resulted in development of a platform that permits incorporation into a single molecule of different functional groups. The properties that were shown to be important for nucleic acid delivery are charge (positive charge) and hydrophobicity. A schematic representation of a molecular scaffold that allows incorporation of various side chains is presented in Figure 31. Our laboratory developed a synthetic scheme for a biodegradable platform that can be functionalized via amidic bonds with molecules that contain positive charges and molecules that have a hydrophobic side chain. The platform consists of a polymeric backbone that that contains free carboxylic groups. The relative proportion in which these specific moieties are introduced is easy to control. By controlling the reaction conditions (temperature, reaction time) the molecular weight of polymers can be tailored at will. Membrane fractionation can be used to obtain fractions of molecules that fall in a particular range. The biocompatibility issue is addressed by having biodegradable linkages – the amide bond is a substrate for many proteases. This mechanism may be involved in releasing the nucleic acids from the carrier, by degradation of the polymer.

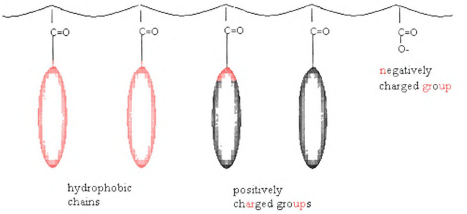


Figure 31. Model for the general platform that allows incorporation of hydrophobic chain, and charged groups in the same molecule.

The elements of design of a novel non-viral nucleic acid delivery platform are molecular weight, hydrophobicity, positive charge and biocompatibility, and biodegradable linkages. A fine balance of all these elements would ensure the success in delivering plasmids to cells.

Chapter 3

Materials and methods

All chemical materials were obtained from Sigma-Aldrich Company and were analytical grade, unless otherwise noted. NMR measurements were made on a Varian VXR 300 MHz Spectrometer with deuterated water as a solvent. The FT-IR spectra were collected with a Nicolet 710-FT-IR spectrometer. The images for the evaluation of the uptake of antisense oligonucleotides mediated by polymer I and the characterization of the uptake mechanism of polymer II were collected using a Zeiss LSM 5 Pascal laser confocal microscope. For the evaluation of efficiency of transfection of plasmid DNA mediated by polymer II and the cytotoxicity of polymer II a Nikon inverted microscope was used. The plasmid encoding for green fluorescence protein, pEGFP-C2 was a gift from Dr. John Lapres' lab.

3.1 Synthesis of polymer I

A polymeric material that is positively charged has been synthesized. The synthesis takes place in two steps. First a polymeric backbone is synthesized according to the reaction scheme presented in Figure 33. After purification of the product, the second step consists of functionalization of the polymeric backbone. The reaction scheme of backbone functionalization is underlined in Figure 36.

Cysteine (0.01 moles, 1.21 g) and sodium hydroxide (.012 moles, 0.48 g) were dissolved in warm Mili-Q water (2 ml) in a 50 ml round bottom flask. The reaction mixture was kept on ice. After adding the 2(5H) furanone (0.01 moles, 0.84 g) cooled on ice, the solvent was removed with a rotary evaporator at 70°C for three hours. It is important that the water is removed for good yield of the Michael addition reaction. The reaction mixture was heated on an oil bath at 100°C overnight, followed by 7 hours at 140°C. The reaction was monitored by ^{1}H NMR. After the polymeric backbone was purified, the second step of the reaction was done as follows.

The high molecular weight fraction (described below) of polymeric material ($1x10^{-4}$ moles, 0.02 g) was dissolved in 1ml of Milli-Q water and 1-ethyl-3-dimethylaminopropylcarbodiimide (EDC) ($2 x10^{-4}$ moles, 0.0041 g) was added. The pH was adjusted (final pH=3) using hydrochloric acid. A corresponding $1 x10^{-4}$ equivalents of N,N-dimethylethylenediamine (0.0113 g) was added to the mixture. The reaction mixture was incubated at room temperature for 24 hours. The reaction was stopped by adding sodium carbonate ($1 x10^{-4}$ moles, 0.0135 g). The polymeric material was further purified.

Purification of the backbone polymer was done using biogel p10 exclusion chromatography. The polymeric material from the first reaction step (0.2 g) was dissolved in Milli-Q water (1 ml), and then loaded into the biogel p10 column (3x80 cm) with water as eluant. Five ml of polymeric fractions were collected and 0.2 ml samples were loaded into a 96 well plate. Absorbances were measured using a Quant Universal Microplate Spectrometer at a wavelength of 220 nm. High, medium and low molecular weight polymer fractions were identified and collected together, following the absorbance

reading. The solvent was removed with a rotary evaporator and the polymeric fractions were lyophilized for 24 hours. Another technique used for polymeric materials purification was selective precipitation using acetone/water solvent systems. The backbone polymer (0.2 g) was dissolved in minimum amount of Milli-Q water (0.5 ml), followed by addition of acetone in excess (2 ml). The mixture was left in the vial for two hours. The higher molecular weight polymeric material, the precipitate, was collected and the procedure was repeated.

3.2 Synthesis of polymer II

A polymeric material that is positively charged but also contains hydrophobic side chains has been synthesized. The synthesis takes place in two steps. First a polymeric backbone is synthesized according to the reaction scheme presented in Figure 33. After purification of the product, the second step consists of functionalization of the polymeric backbone. The reaction scheme of backbone functionalization is underlined in Figure 37.

The polymeric backbone was obtained using equimolar amounts of cysteine methyl-ester treated with furanone and sodium bicarbonate dissolved in minimum amount of water. The use of cysteine methyl-ester is beneficial because the methyl ester has a better water solubility than cysteine. The water was removed using a rotor evaporator, heating the reaction mixture at 70^0C for one hour. The reaction mixture was further heated at 70^0C for 24 hours. The reaction progress was monitored by NMR. The ester was hydrolyzed further using NaOH until the solution achieved the pH=11. After one hour the reaction mixture was treated with acetic acid, until the solution achieved pH=4.

In order to obtain molecules that fall into a nominal molecular weight range, membranes are used to fractionate the compounds by size; the polymeric backbone was fractionated using a membrane with a 12-14 kDa cut-off. The high molecular weight fraction was used in the second step of the reaction – the coupling of carboxylic groups with amines. The second step of the reaction consists of functionalization of the polymeric backbone by coupling the carboxylic groups with either octylamine or N, N dimethylethylenediamine. In either case the reaction take place in the presence of the coupling reagent 1-ethyl-3-dimethylaminopropylcarbodiimide (EDC), at pH=5. For one equivalent of polymeric backbone were used 0.66 equivalents of octylamine and 0.33 equivalents of N,N-dimethylethylenediamine, in the presence of 2 equivalents of coupling reagent. The reaction was stopped after 24 hours by adding sodium bicarbonate. The excess reagents are removed by equilibrium membrane size fractionation (membrane cut-off 3500 Da). The resulting product (polymer II) has a molecular weight higher than 20 kDa.

3.3 Labeling of polymer II with Fluorescein isothiocyanate (FITC)

One fraction of the polymer II was labeled with fluorescein isothiocyanate (FITC). One mg of polymer was lyophilized and after the water was removed it was mixed with 0.1 mg FITC in 200 μL pyridine. The coupling of the fluorescent label took place at room temperature for 4 hours. The pyridine was removed under a nitrogen flow and the sample was dissolved in 1 mL water. The removal of the free FITC was performed using a reverse phase (C18) column, SEP-PAK cartridge. The elution of the sample was performed using mixtures of water:methanol in various ratios (4:1, 3:1, 2:1 and 1:1). The

FITC labeled polymer II was collected in the fraction eluted with 4:1 water:methanol mixture.

3.4 Cy5 labeled antisense oligonucleotide treatment

The ability of polymer I to deliver Cy5 labeled oligonucleotides to MEF cells was tested by laser multi channel fluorescence scanning confocal microscopy. Mouse embryonic fibroblasts (MEF), passage 8-12, were grown in DMEM media supplemented with non-essential amino acids, heat inactivated fetal bovine calf serum (10 %) and Hepes at 37^0C, 5 % CO_2. MEF cells were plated at low density (5x 10^5 cells) on cover slips in 6 well plates. After 24 hours, one cover slip was flipped over a microscope slide which had 5 μL of the following mixture: 4 μL of polymeric solution (1 mg/mL) and 1 μL of Cy5 labeled antisense oligonucleotide solution (0.1 mg/mL) dissolved in PBS. Control cells with no treatment and positive control cells were treated with 1 μL of antisense oligonucleotide solution (0.1 mg/mL dissolved in phosphate buffer saline – PBS) diluted with 4 μL PBS. Confocal microscopy images of cells treated with solutions containing polymer I in various concentrations in combination with Cy 5 labeled antisense oligonucleotide were collected. The microscope settings were as follows: laser 633 nm, the first dichroic mirror (HFT) 488/543/633nm, the second dichroic mirror (NFT) 635 nm, long pass filter (LP) 650 nm for collecting the Cy5 signal. Images collected from this experiment are presented in color.

3.5 Evaluation of plasmid DNA transfection mediated by polymer II

COS1 cells (African green monkey kidney cells) were grown in Dulbecco's Modified Eagle Medium (DMEM) media that was supplemented with non-essential amino acids (1 %), heat inactivated fetal bovine calf serum (10 %), penicillin/streptomycin (1 %), and glutamine (1 %). Cells were grown for 24 hours at 37°C and 5 %CO_2.

COS1 cells were grown in 96 well plates at initial seeding density of 2000 cells/well followed by treatment with polymer II DNA complexes using the commercially available plasmid encoding for green fluorescent protein gene, pEGFP-C2 (Clontech, CA). Figure 32 presents the map of pEGFP-C2 plasmid[123], containing the gene encoding for GFP along with promoter region pCMV, origin of replication and kanamycin/neomycin resistance gene. Various sites for restriction enzymes are specified.

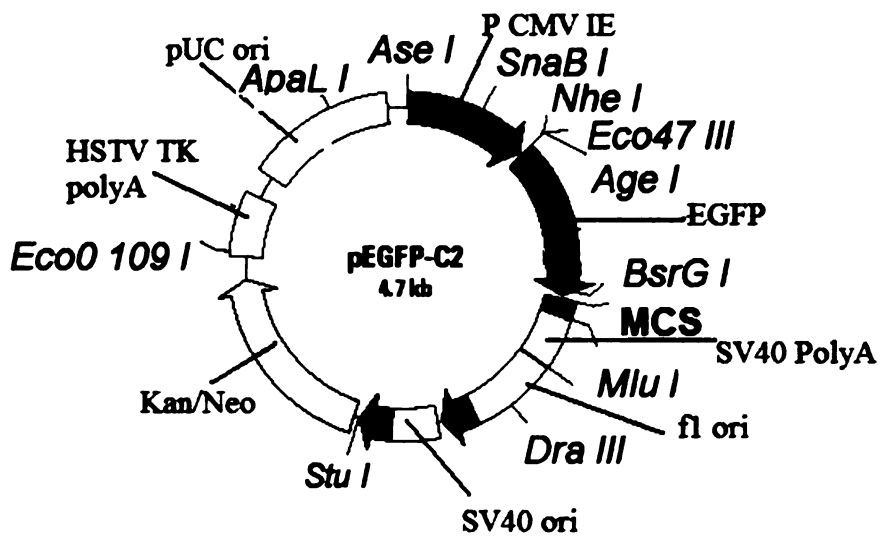


Figure 32. pEGFP-C2 encodes a red-shifted variant of wild-type GFP.

The plasmid DNA was diluted in serum free media at a concentration of 0.01μg/μL, and either 100 ng, 200 ng or 300 ng were distributed in a 96 well PCR plate. Various amounts of polymer II solution containing 1, 2, 3, 4, 5 or 6 μg polymer II were added to each well. Control wells with no carrier and positive control wells with commercially available transfection reagent Lipofectamine 2000 (Invitrogen, CA) were set up. The manufacturer's protocol suggested that 0.5 μg of Lipofectamine 2000 be used for each well of a 96 well plate. The Lipofectamine 2000 was diluted in 25 μL serum free media 20 minutes prior to each treatment. The homogeneity of each mixture was ensured by pipetting up and down with a multichannel pipettor, followed by capping the 96 well plate and vortexing at high speed for 30 seconds. The mixtures were allowed to incubate at room temperature for 20 minutes. Then the samples were diluted with serum free media, to a total volume of 200 μL. The media was removed from the 96 well plate containing the cells, followed by the distribution of the mixtures from the PCR plate. The cells were incubated at 37^0C for 6 hours followed by changing of media with normal growth media. The efficiency of transfection was evaluated by capturing images with an inverted microscope at 72 hours after treatment. Phase and fluorescence images of cells under various treatment conditions were collected with a Nikon inverted microscope.

3.6 Effects of polymer II treatment on cells viability and proliferation

COS1 cells were grown in 96 well plates at initial seeding density of 2000 cells/well followed by treatment with polymer II DNA in concentrations varying from 0, 0.5 μg/mL, 1 μg/mL, 2.5 μg/mL, 5 μg/mL, 7.5 μg/mL, 10 μg/mL, and 100 μg/mL. Two parallel sets of experiments were set up, one using 100 ng of plasmid DNA and the other

using 200 ng of plasmid DNA. The effects of polymer II on cell growth and proliferation were compared with the commercially available reagent Lipofectamine 2000 treated cells. COS 1 cells were treated with complexes of Lipofectamine 2000 (0, 0.5 μg/mL, 1 μg/mL, 2.5 μg/mL, 5 μg/mL, 7.5 μg/mL, 10 μg/mL, and 100 μg/mL) and either 100 ng or 200 ng plasmid. The complexes were allowed to incubate for 20 minutes and the cells were treated in a similar manner that was previously described in Section 3.5. Phase contrast images of cells under various treatments were collected with a Nikon inverted microscope 72 hours after treatment.

3.7 Investigation of the uptake mechanism of FITC labeled polymer II

The uptake mechanism of FITC labeled polymer II to COS 1 cells was investigated. COS1 cells were seeded at 10000 cells/well 24 hours prior treatment, in 35 mm tissue culture dish, on cover slips. They were incubated at 37^0C, with 5 % CO2. After 24 hours, one cover slip was flipped over a microscope slide which had a droplet of 10 μL of stock solution of FITC labeled polymer II (0.1 mg/mL). To inhibit the ATP synthesis, the cells were incubated with 100 μM solution of DCCD (stock solution in ethanol) for 15 minutes. After the incubation period, the cover slip was flipped over a microscope slide which had a droplet of 10 μL of stock solution of FITC labeled polymer (0.1 mg/mL). The uptake of fluorescently labeled polymer was analyzed in the two situations by confocal laser fluorescence microscopy within the next 30 minutes. The slides were analyzed by confocal microscopy immediately after treatment using an 100x oil objective. The microscope settings for collecting the FITC signal were as follows: laser 488 nm, the first dichroic mirror (HFT) 488 nm, the second dichroic mirror (NFT)

545 nm, and band pass filter (BP) 505-530 nm. Images in this thesis are presented in color.

Chapter 4

Results and discussion

4.1 Principle

Our laboratory developed a synthetic method that allows incorporation of different moieties into a molecular scaffold. Since our goal is to develop molecules that could be used to deliver nucleic acids into cells, we are interested in functionalizing the scaffold with groups that carry positive charges, as well as hydrophobic groups.

The synthesis of macromolecular compounds consists mainly of two steps. These are illustrated in scheme presented in Figure 33. First, a backbone that carries free carboxylic groups is synthesized. The thiol group of the amino acid cysteine reacts with the double bond of the lactone 2(5H)-furanone via a Michael addition mechanism. The head to tail polymerization reaction occurs via opening the resulting γ lactone, resulting in a linear polymer backbone with alternating thio-ether and amide linkages. The free carboxylic group from cysteine provides the negative charge and allows functionalization using one or more reagents in order to achieve the desired properties.

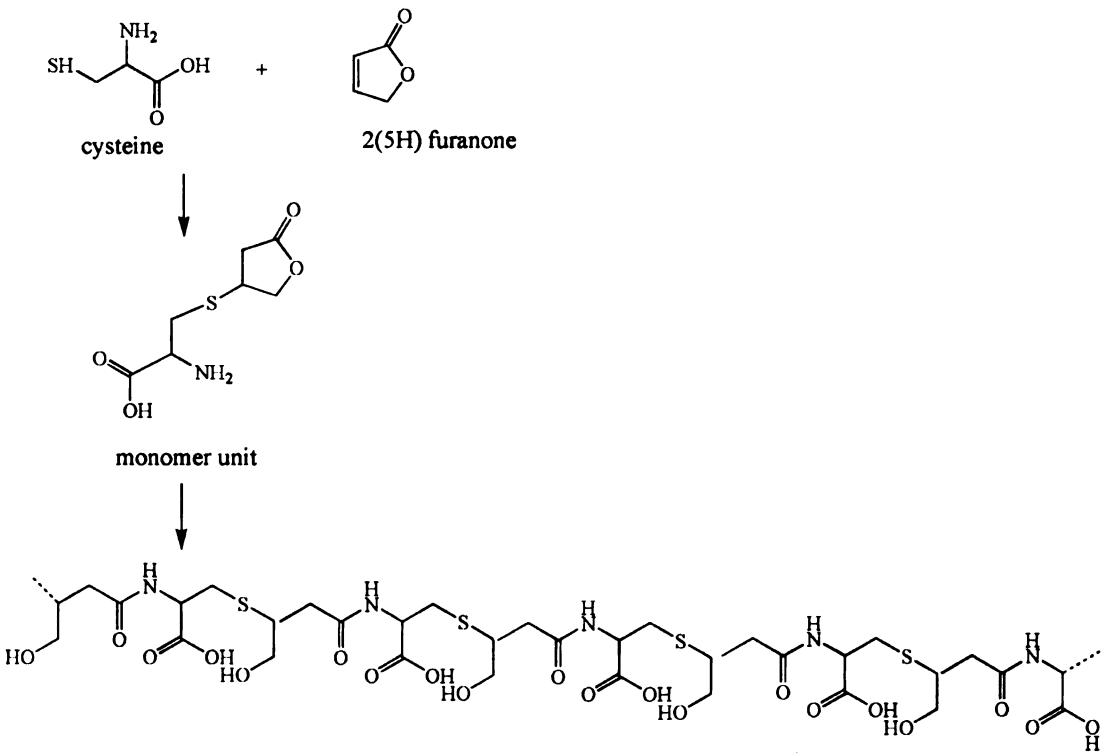


Figure 33. Polymerization of cysteine with furanone via a Michael addition mechanism leading to synthesis of backbone polymer.

The reaction was monitored by NMR. In Figure 34 is presented the spectra of starting material consisting of cysteine and furanone. The signal from C2 of furanone is around 6 ppm, C3 gives a signal at 7.7 ppm while C4 gives a signal at 5 ppm. Analyzing cysteine, the CH_2 gives a signal at 3.0 ppm, while the CH has a signal at 3.7 ppm. After the reaction was completed, the signals from furanone beyond 5.0 ppm disappeared (Figure 35), consistent with the opening of the lactone ring. The signals from the amino acid are labeled (c) for CH at 4.5 ppm and (d) for CH_2 at 3.0 ppm. The CH_2 next to hydroxyl group from the former lactone ring is labeled (b) and gives a signal at 3.5 ppm,

while the CH_2 labeled (a) gives a signal at 2.2 ppm. Also, the peaks are broader indicating that the product is polymeric.

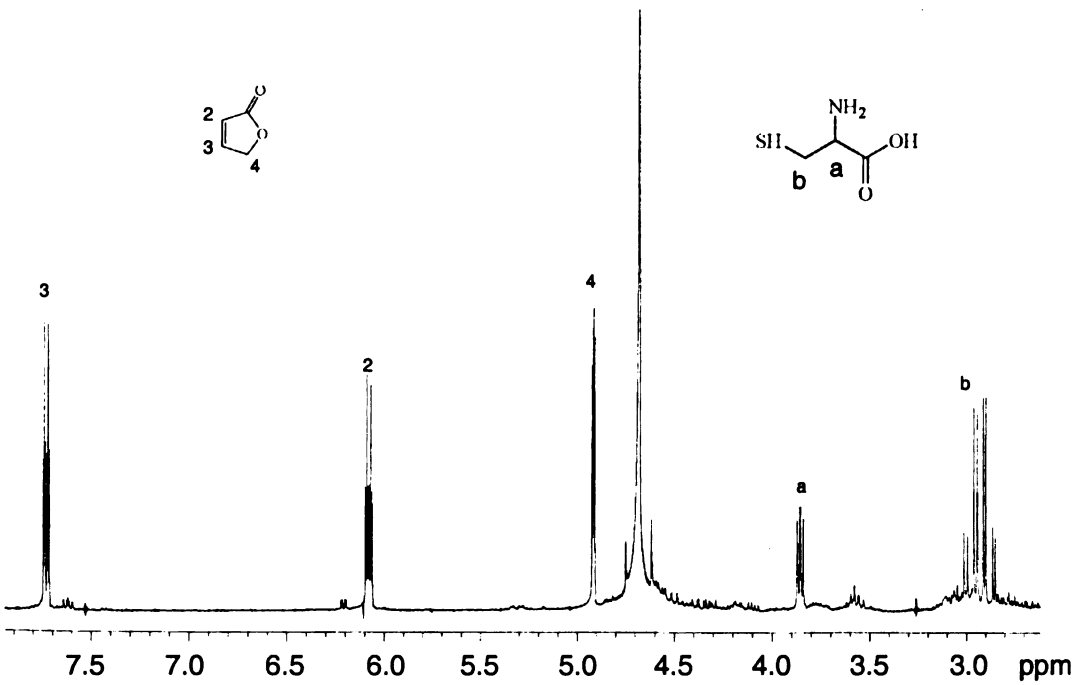


Figure 34. The ^1H NMR spectra of the cysteine and furanone mixture before polymerization

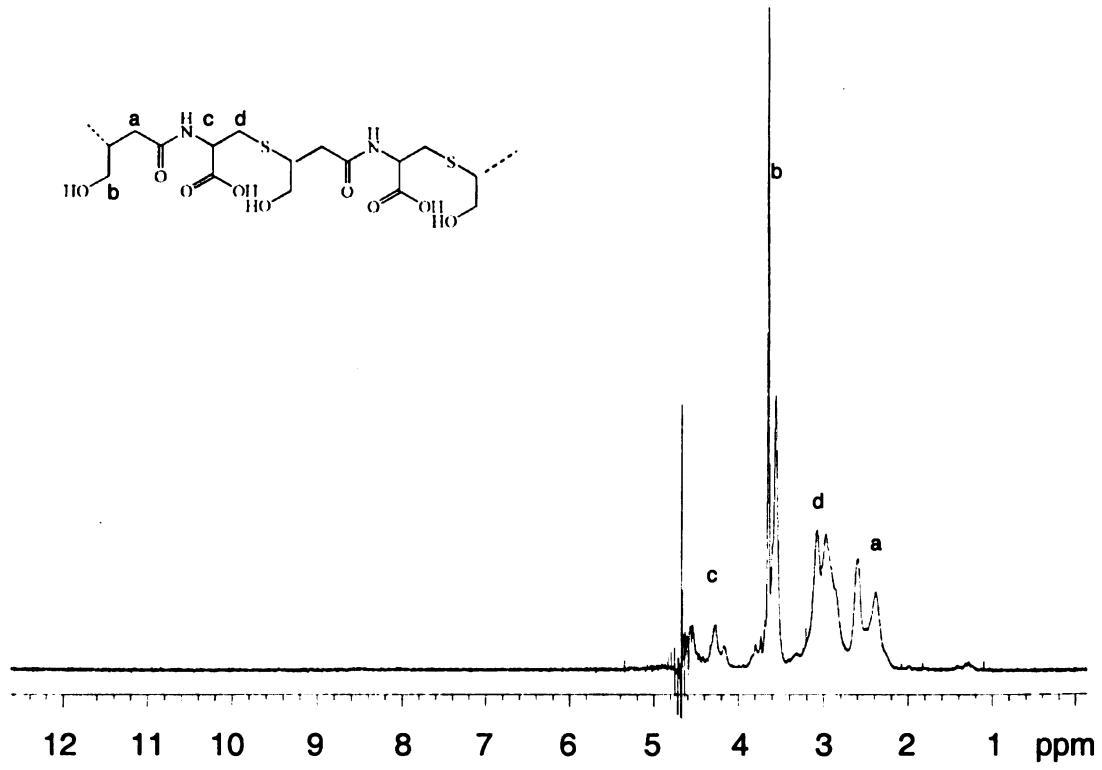


Figure 35. The ^{1}H NMR spectra of the polymeric backbone. The signals from the starting material disappeared.

In the second step, a pendant group is added to the carboxylic groups. The best candidates are amines to form amidic bonds. If N,N-dimethylethylenediamine is used to functionalize all the free carboxylic groups the final product is a polymer that is positively charged. In Figure 36 is presented the reaction scheme that leads to this product. In further discussion, this product will be referred as polymer I.

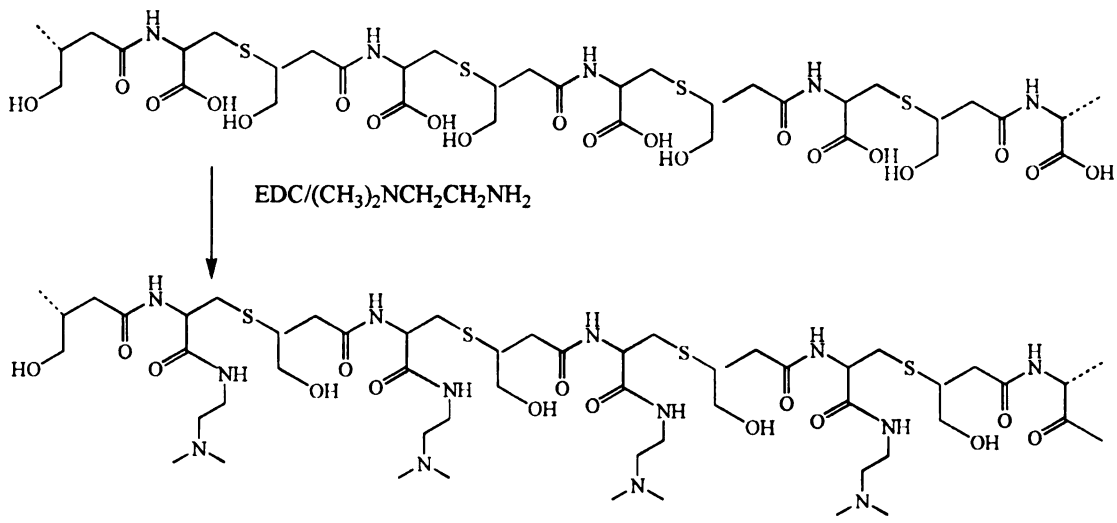


Figure 36. Functionalization of the free carboxylic groups with N,N-dimethylethylenediamine resulting polymer I.

If a mixture of amines is used to couple the free carboxylic groups, , the final product can be engineered to contain moieties at a desired proportion by controlling the composition of the mixture. Besides the positive charges introduced by the N,N-dimethylethylenediamine, amines that carry a non-polar residue will introduce hydrophobicity in the final product. If octylamine is used besides the N,N-dimethylethylenediamine for coupling the free carboxylic groups, the ratio in which the two amines are added is important. Cationic lipids are very common non-viral nucleic avid delivery vectors. This class of molecules has a positive to hydrophobic chain ratio of about 35:65. This ratio was mimicked at a macromolecular level, and a polymeric material that contains 66 % hydrophobic chains introduced by octylamine and 33 % positive charges introduced by N,N-dimethylethylenediamine was synthesized. The

synthetic scheme underlying the coupling reaction is presented in Figure 37. The polymer with this composition will be referred as polymer II.

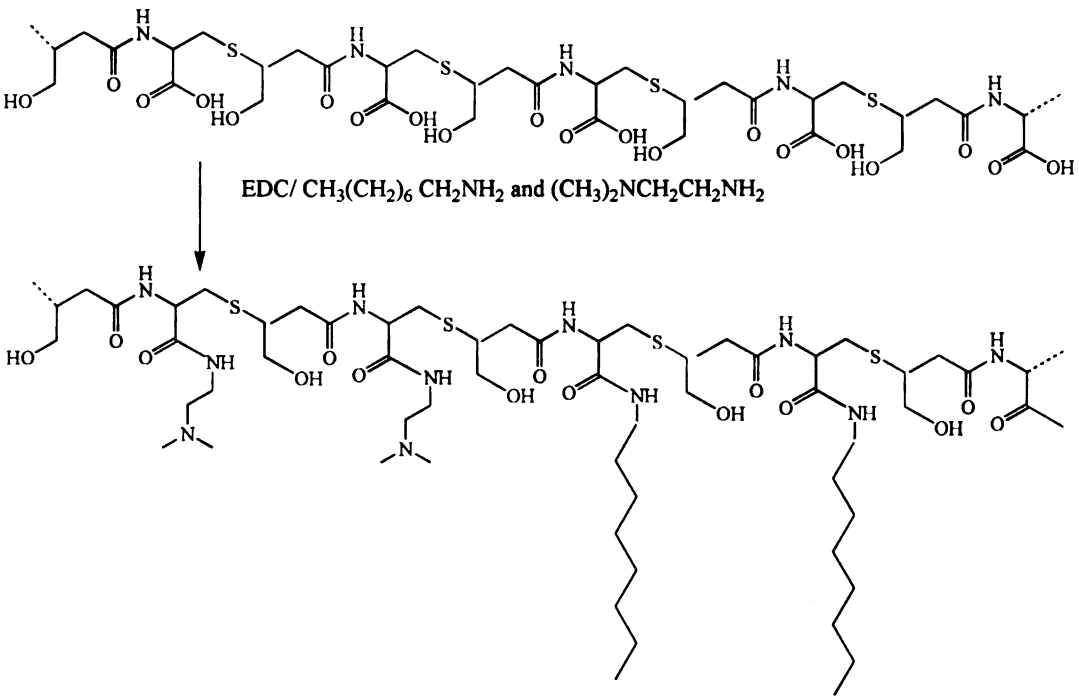


Figure 37. Functionalization of the polymeric backbone by coupling amines to the free carboxylic groups. Polymer II contains 33 % positive charges and 66 % hydrophobic side chains.

In the FT-IR spectrum of the polymer II presented in Figure 38 the broad signal at 3300 cm^{-1} indicates the presence of -OH and -NH, signals at 2972 cm^{-1}, at 2798 cm^{-1} and 2720 cm^{-1} indicate the presence of -CH_2, and -CH stretches, while the signals at 1637 cm^{-1} were attributed to carbonyl group.

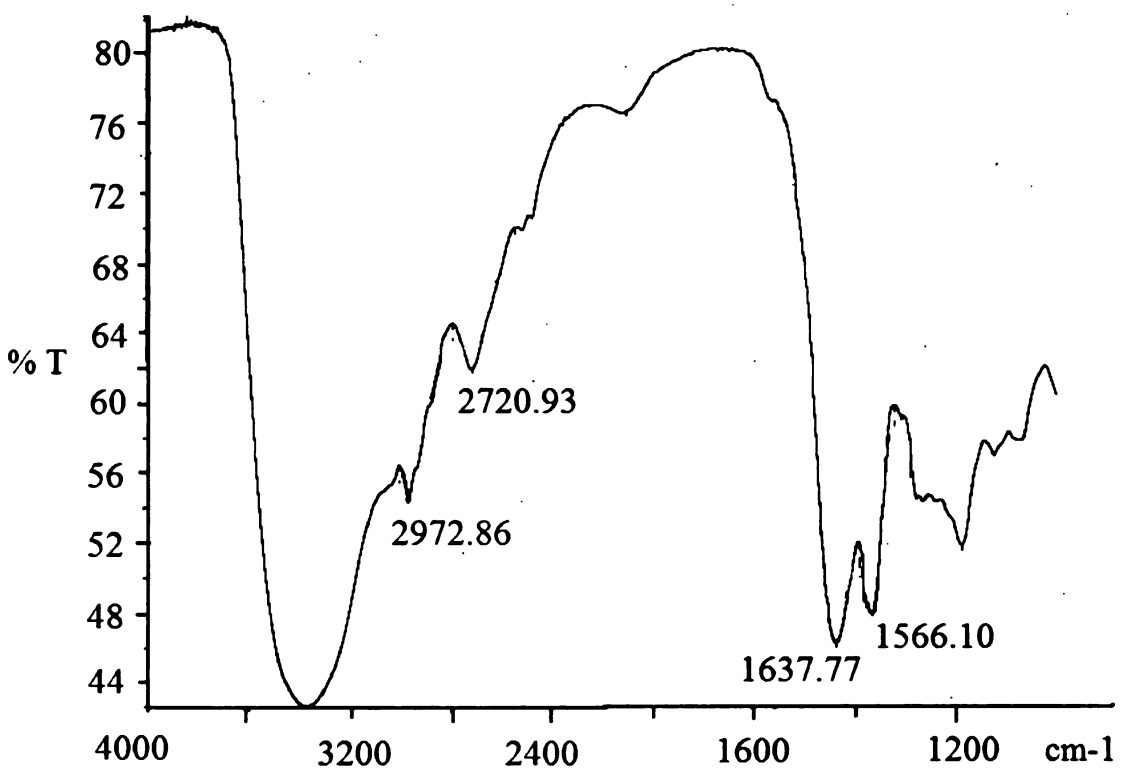


Figure 38. FT-IR spectrum of polymer II. The carbonyl group gives signal at 1637 cm^{-1}, the -OH and -NH at 3300 cm^{-1}, $-CH_2$, and -CH stretches are present at 2720 cm^{-1} and 2972 cm^{-1}.

4.2 Evaluation of the performance of polymers *in vitro*

4.2.1 Evaluation of the ability of polymer I to deliver antisense oligonucleotide to MEF cells

The membranes of mammalian cells typically have a net negative charge. Because of this, due to the highly negative charge of the phosphate backbone, introducing RNA into cells is a challenging task. To test the efficacy of the new molecular systems at the transfer of oligonucleotides of the sizes used for interference of gene function by the antisense strategy, transfer of the antisense oligonucleotide 5'-GCG CGG GGA GCA

AAA GCA C-3' from the hTR subunit of human telomerase RNA[124] into mouse embryonic fibroblast cells was attempted. To aid visualization, the 5' end was labeled with the fluorophore Cy5, which emits at 670 nm when it is excited with a laser operating at 633 nm.

The antisense delivering capacity of a polymer I having all the carboxylic groups coupled with N, N dimethylethylenediamine was tested in MEFs. Control experiments with MEF cells (Figure 39 (A)), and cells treated only with antisense oligonucleotide solution in PBS were set up. The cells were treated with polymer I at various concentrations (0.1 g/mL, 0.01 g/mL, 0.001 g/mL, or 0.0001 g/mL) and Cy5 labeled oligonucleotides. The amount of antisense oligonucleotide was constant through the experiment (1 μL of stock solution (0.1 mg/mL) freshly prepared in PBS). The ability of the highly positively charged polymeric materials to deliver their cargo to the nucleus was successful in each case Figure 39 (C, D, E, and F). Antisense oligonucleotides in the absence of a carrier are excluded from the cells, demonstrating that naked antisense oligonucleotide constructs are unable to enter the cells Figure 39 (B). Series of images (36 μm apart) of MEF cells treated with 1mg/mL polymer I and 1 μL of stock solution (0.1 mg/mL) of Cy 5 labeled antisense oligonucleotides confirmed that t polymer I and the fluorescent label are inside the cells Figure 40.

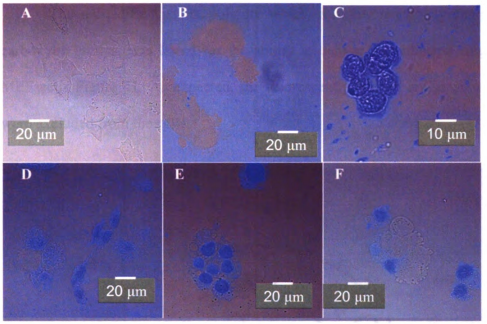


Figure 39. MEF treated with polymer I at various concentrations (A). Control – MEF no treatment. (B). MEF with no carrier (Cy5 antisense oligonucleotide in PBS). (C, D, E, F) MEF and antisense oligonucleotide with various concentrations of polymer I solution: C (0.1 g/mL), D (0.01 g/mL), E (0.001 g/mL), F (0.000 1g/mL).

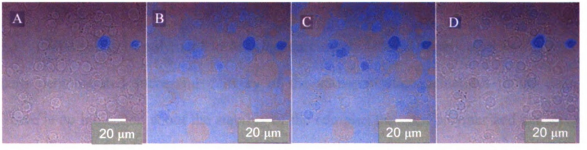


Figure 40. Series of confocal images through a group of MEF cells treated with polymer I (1 mg/mL). The Z series consists of 4 images; the distance between images is 36 μm. The scale bar is the same in all images, 20 μm.

The unmodified parent polymer which is highly negatively charged due to the carboxylic groups, was tested for the ability to deliver the antisense oligonucleotide to MEF cells Figure 41. As expected, the fluorescently labeled antisense oligonucleotides were excluded from the cells.

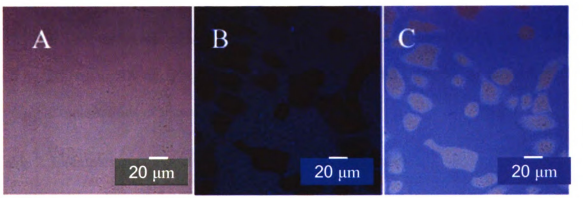


Figure 41. MEF cells treated with Cy5 labeled antisense oligonucleotides in combination with a solution of backbone polymer. A. Bright field image showing the MEF cells. B. The fluorescent image. C. An overlay image of the transmitted and fluorescence image. The drug was not delivered inside MEF cells.

The ability of polymer I to deliver fluorescently labeled oligonucleotides to MEF cells demonstrated successful delivery of the antisense oligonucleotides to the cells. There is no indication about the stability of the complex between polymer and antisense oligonucleotide; in order to disrupt gene expression the nucleic acids should dissociate from the complex formed between the antisense oligonucleotides and the positively charged carrier.

4. 2. 2 Evaluation of plasmid DNA transfection mediated by polymer II

The polymer II, which contains 66 % hydrophobic side chains and 33 % positively charged amine groups, was tested for the ability to transfect COS1 cells with plasmid encoding for GFP, pEGFP-C2. Several combinations varying the amounts of polymer II and the amounts of plasmid DNA were tested. Examples of ratio polymer II:plasmid DNA that were tested are presented in table 2. The amount of plasmid DNA varied from 100 ng, 200 ng to 300 ng. The amounts of polymer II used for transfection varied from 1 to 6 μg. Combinations of these quantities were tested.

Table 2. Examples of combinations of polymer II:plasmid ratios

10:1	20:1	30:1	40:1	50:1	60:1
10:2	20:2	30:2	40:2	50:2	60:2
10:3	20:3	30:3	40:3	50:3	60:3

The commercially available transfection reagent Lipofectamine 2000 was used as a positive control. The commercially available transfection reagent was also tested with 100 ng, 200 ng or 300 ng plasmid DNA. That translates into Lipofectamine 2000:plasmid DNA ratios of 5:1, 2.5:1or 5:3.

The image of COS1 cells before treatment is presented in Figure 42.

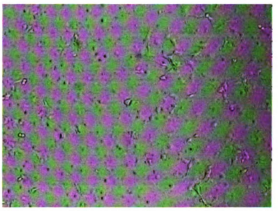


Figure 42. COS 1 cells before treatment, phase contrast image.

As soon as 24 hours after treatment some cells exhibited green fluorescent protein expression. One specific combination, the one with 20:1 polymer II DNA ratio was identified Figure 43.

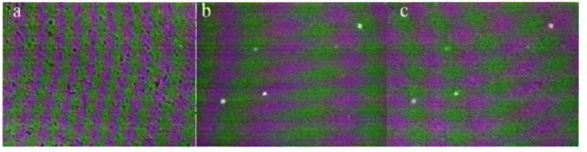


Figure 43. COS 1 cells transfected with plasmid encoding for green fluorescent protein, transfection mediated by the polymer II at 24 hours after treatment. (a) the phase contrast image and (b) the corresponding fluorescent image, (c) the overlay of the phase contrast image and the fluorescent image.

The efficiency of transfection was tested by visualizing, under the fluorescence mode, the cells that were able to express the green fluorescent protein, 72 hours after treatment. Control cells with no treatment are presented in Figure 44. As expected, the COS 1 cells do not exhibit fluorescence, since the cells do not contain the gene encoding for green fluorescent protein.

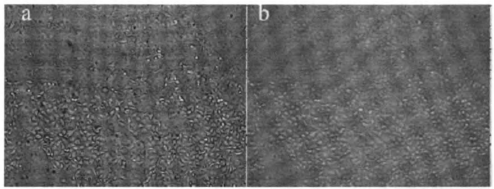


Figure 44. COS 1 cells – no treatment, (a) the phase contrast image and (b) the corresponding fluorescent image, at 72 hours after treatment.

At 72 hours after treatment, the COS1 cells treated with carrier solution at a polymer II:DNA ratio of 20:1 exhibited the highest efficiency of transfection. A few examples are presented in Figure 45. Some cells were able to express the green fluorescent protein.

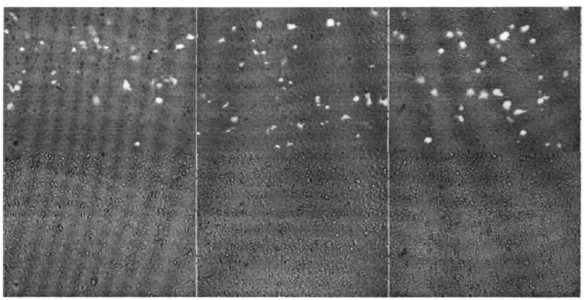


Figure 45. The upper panel represent the overlay of the phase contrast image and the fluorescent image of cells treated with a 20:1 of polymer II:DNA ratio, the bottom panel the corresponding phase contrast images of the upper panel at 72 hours after treatment.

Other conditions that exhibited cells fluorescing green were the 20:2 polymer II:DNA ratio, presented in Figure 46, and 20:3 polymer II:DNA ratio, presented in Figure 47. The efficiency under these conditions is lower when compared to the 20:1 polymer II:DNA ratio condition. Other conditions did not exhibit any fluorescence.

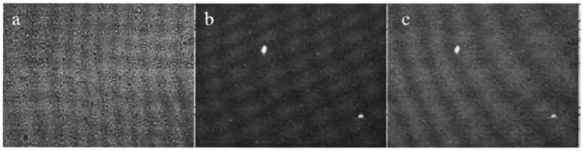


Figure 46. COS1 cells – (a) the phase contrast image (b) the corresponding fluorescent image, and (c) the overlay of the phase contrast image and the fluorescent image at 72 hours after treatment with a polymer II:DNA ratio of 20:2.

Figure 47. COS1 cells – (a) the phase contrast image and (b) the corresponding fluorescent image, and (c) the overlay of the phase contrast image and the fluorescent image at 72 hours after treatment with a polymer II:DNA ratio of 20:3.

Analyzing Table 2, where the condition treatments are presented, one would expect that 20:1 polymer II:DNA ratio, 40:2 polymer II:DNA ratio and 60:3 polymer II:DNA ratio would give similar results. When these conditions where compared (Figure 48) only the 20:1 ratio had a positive outcome, while the other two did not have any fluorescent cells, thus no efficient transfection of the plasmid encoding for GFP.

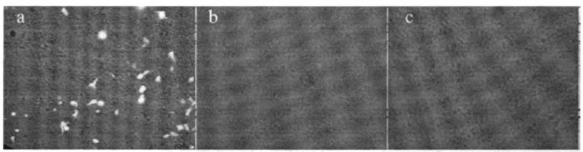


Figure 48. Comparison of the overlay of the phase contrast image of three treatment conditions (a) 20:1 polymer II:DNA ratio, (b) 40:2 polymer II:DNA ratio, and (c) 60:3 polymer II:DNA ratio.

The images of cells transfected with Lipofectamine 2000 are presented in Figure 49, 50, and 51 respectively, where the same amount of Lipofectamine 2000 was used in combination with 100 ng, 200 ng and 300 ng plasmid DNA respectively. It seems that in all conditions, the efficiency was similar and not affected by the amount of plasmid DNA used.

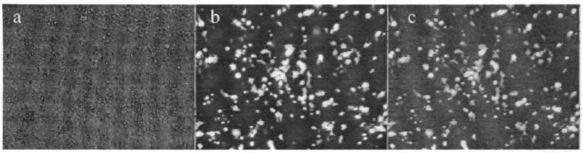


Figure 49. COS1 cells (a) the phase contrast image, (b) the corresponding fluorescent image, and (c) the overlay of the phase contrast image and the fluorescent image at 72 hours after treatment with Lipofectamine 2000 and 100 ng plasmid.

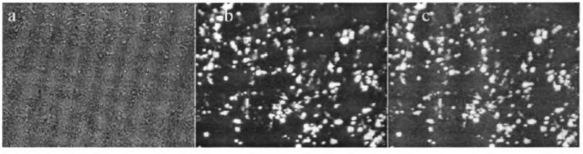


Figure 50. COS1 cells (a) the phase contrast image, (b) the corresponding fluorescent image, and (c) the overlay of the phase contrast image and the fluorescent image at 72 hours after treatment with Lipofectamine 2000 and 200 ng plasmid.

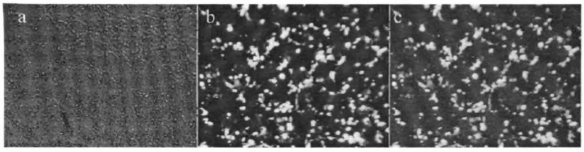


Figure 51. COS1 cells (a) the phase contrast image, (b) the corresponding fluorescent image, and (c) the overlay of the phase contrast image and the fluorescent image at 72 hours after treatment with Lipofectamine 2000 and 300 ng plasmid.

When the cells were examined under the fluorescence mode at a lower magnification, the area that can be visualized at once is increased. For the condition that was identified that exhibit the highest transfection efficiency, 20:1 ratio polymer II:DNA, several fields are presented in Figure 52. The distribution of cells that exhibit fluorescence in any field is homogenous.

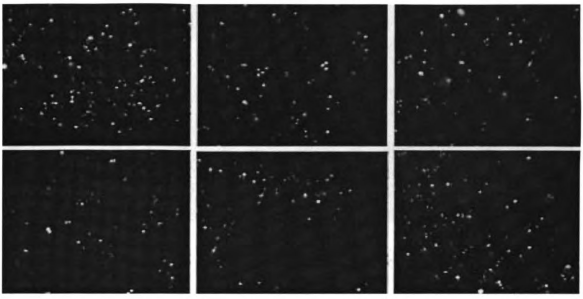


Figure 52. COS1 cells 72 hours after treatment with 20:1 ratio polymer II:DNA at a lower magnification under the fluorescence mode. The distribution of cells exhibiting fluorescence is even.

When the COS1 cells treated with Lipofectamine 2000 and 100 ng plasmid DNA were examined at lower magnification, some fields showed very high fluorescence, while other fields were completely dark, since no cells were fluorescing on these areas (Figure 53). This result may seem unexpected, but a very reasonable explanation exists. It is well known that the actual non viral transfection reagents are toxic, causing cell lysis. If a cell is lysed, its content is released outside the cell and the cell dies – it is not able to grow and proliferate. The reason that no cells are exhibiting fluorescence on the right side of figure 51 is that there are no cells there.

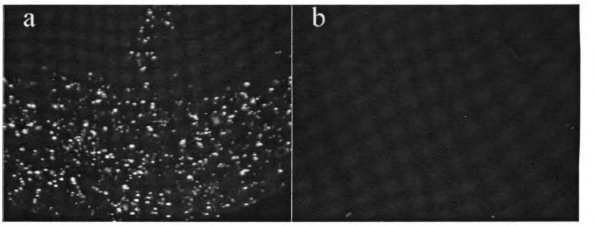


Figure 53. COS1 cells 72 hours after treatment with Lipofectamine 2000 and 100 ng plasmid at a lower magnification under the fluorescence mode, (a) many cells exhibit fluorescence and (b) no cells are fluorescent.

The efficiency of fluorescence can be qualitatively identified using the lower magnification of the microscope under the fluorescence mode. The conditions that were identified to exhibit fluorescence 20:1 polymer II:DNA ratio, 20:2 polymer II:DNA ratio, and 20:3 polymer II:DNA ratio were examined and the images are presented in Figure 54. Again, the highest efficiency is achieved by the 20:1 polymer II:DNA ratio condition.

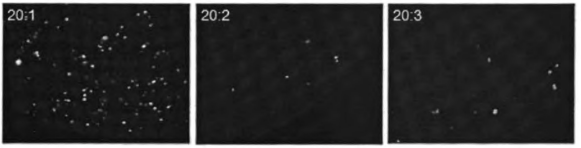


Figure 54. COS1 cells 72 hours after treatment with 20:1 polymer II:DNA ratio, 20:2 polymer II:DNA ratio, and 20:3 polymer II:DNA ratio at a lower magnification under the fluorescence mode.

The efficiency of transfection was expressed as the percentage of the ratio of the number of cells that express GFP over the total number of cells in one field. The mean of several fields was taken into account for each treatment condition. The highest efficiency for the polymer II is observed when the ratio polymer II:plasmid was 20:1 and is 6.5 %. In comparison, the treatment using Lipofectamine 2000 in a 5:1 ratio exhibited 52.14 % transfection efficiency (Figure 55).

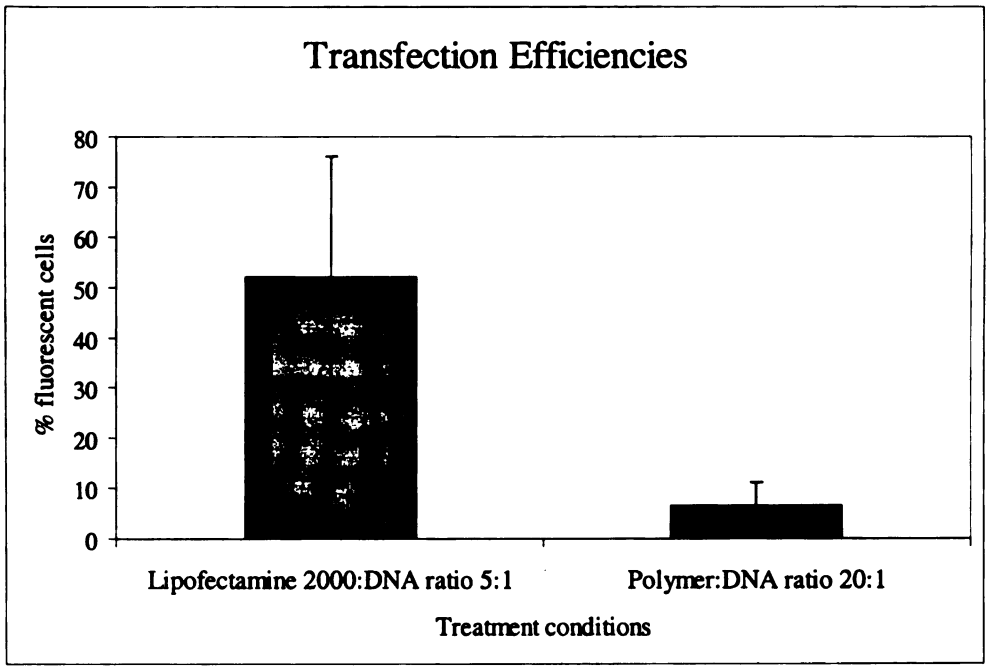


Figure 55. Evaluation of GFP expression 72 hours after treatment. Comparison of polymer II with Lipofectamine 2000 treatment.

When different polymer II:plasmid DNA combinations were compared, the results are consistent with qualitative examinations of images in figures 43, 44, 45 and 52. The highest transfection efficiency was obtained when the polymer II:plasmid DNA ratio was 20:1, followed by a 20:3 ratio and a 20:2 ratio. The quantitative results are plotted in Figure 56.

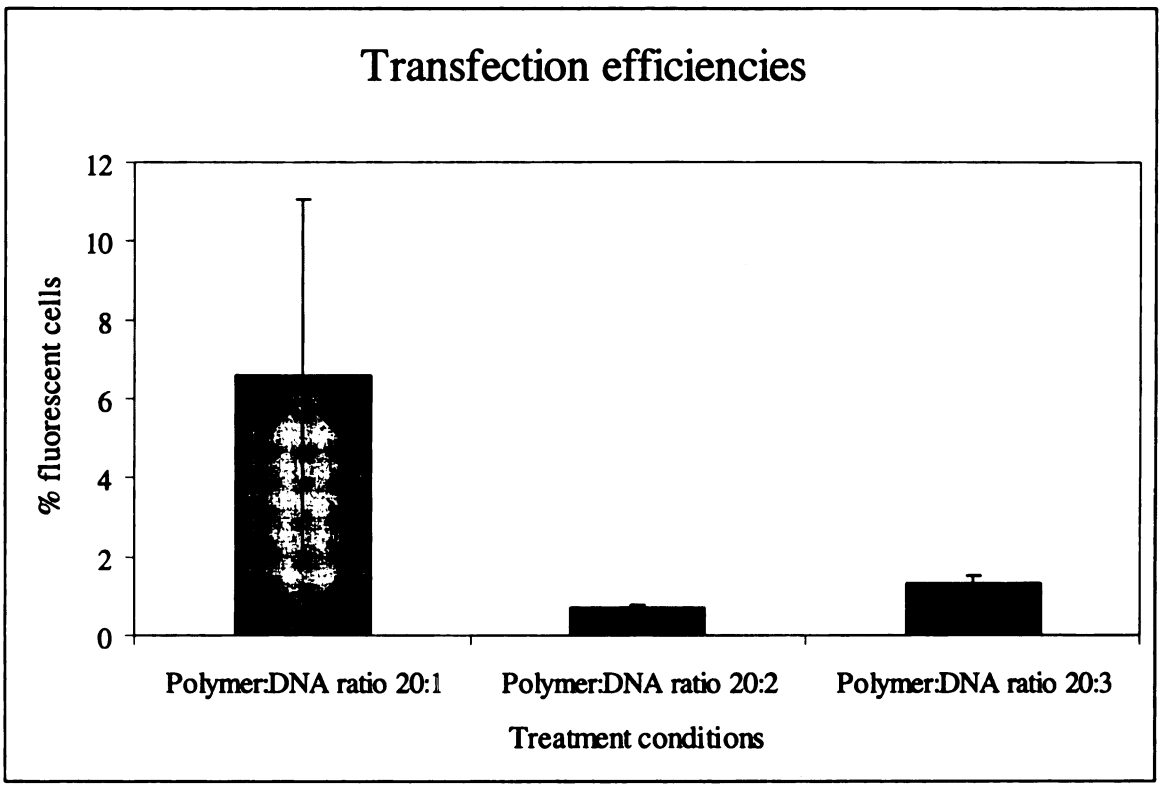


Figure 56. Evaluation of GFP expression 72 hours after treatment. Comparison of different polymer II treatments.

4.2.3 Effects of polymer II treatment on cell viability and proliferation

The impact of the carriers on the physiology and structural integrity of the cells are important aspects of the evaluation of their performance in transfection. This includes cell viability and ability to proliferate. To evaluate the cytotoxic effect of the newly synthesized carrier on COS 1 cells, various concentrations of carrier were tested. The COS1 cells were grown in a 96 well plate and treated with polymer in concentrations varying from 0, 0.5 μg/mL, 1 μg/mL, 2.5 μg/mL, 5 μg/mL, 7.5 μg/mL, 10 μg/mL, and 100 μg/mL. Two parallel sets of experiments were set up, one using 100 ng of plasmid

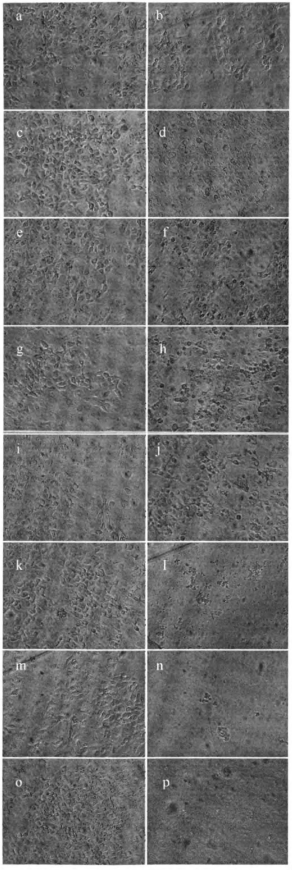
DNA and the other using 200 ng of plasmid DNA. Please recall that from the efficiency of transfection experiment, the combination that exhibited the highest transfection efficiency was when 100 ng plasmid DNA were delivered by 2 μg polymer. This translates into a final polymer II concentration of 10 μg/mL in a 200 μL volume per well in a 96 well plate. A concentration 10 times higher was tested for the toxic effect on cells. Lipofectamine 2000 was again chosen as a positive control. It was used in the same concentrations as the polymer, with two parallel sets using either 100 ng or 200 ng of plasmid DNA. The manufacturer recommended a 2.5 μg/mL concentration to be used for a 96 well plate set up.

A very common assay to evaluate the cytotoxicity that some treatments might have on cells is the MTT assay. This assay is controversial, since it does not involve a direct measure of the number of cells, but rather a measurement of the mitochondrial dehydrogenase activity. The dehydrogenase enzyme cleaves the tetrazolium rings of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) resulting in dark blue crystals. Those crystals are able to accumulate only in intact cells. To evaluate the activity of the enzyme, the cells are lysed and the formazan crystals are dissolved resulting in a colored solution. The absorbance of the solution can be correlated with the number of healthy cells.

We decided to use a more direct way to describe the effect of different treatments on the ability of cells to grow and proliferate. At a time point of 72 hours after treatment we collected phase contrast images of the cells using an inverted microscope. We performed a qualitative analysis, looking at cells morphologies under different treatment conditions when compared with controls. When analyzing the phase contrast image of the

control cells (Figure 57 a), which had no treatment, we observed that they reached confluence and proliferated covering the entire well surface. The same phenotype was observed for the cells that were treated only with plasmid DNA (Figure 57 b). The cells treated with polymer II of various concentrations (Figures 57 c to 57 o) also proliferated, the cells under this treatment being no different in morphology from the control cells. In contrast, cells treated with Lipofectamine 2000 of the same concentrations as polymer II (Figures 57 d to 57 p) exhibited a large reduction in growth. In some areas, only a few cells were seen to grow, leaving a lot of the available area bare. Lipofectamine 2000 treatment had a highly negative impact on the viability of the cells interfering with their capacity to grow and proliferate.

Figure 57. Phase contrast images of COS1 cells with treatments of polymer II or Lipofectamine 2000 at various concentrations. (a) control, no treatment, (b) treated with 100 ng plasmid DNA no carrier, (c) treated with 0.5 μg/ml polymer II, (d) treated with 0.5 μg/ml Lipofectamine 2000, (e) treated with 1 μg/ml polymer II, (f) treated with 1 μg/ml Lipofectamine 2000, (g) treated with 2.5 μg/ml polymer II, (h) treated with 2.5 μg/ml Lipofectamine 2000, (i) treated with 5 μg/ml polymer II, (j) treated with 5 μg/ml Lipofectamine 2000, (k) treated with 7.5 μg/ml polymer II, (l) treated with 7.5 μg/ml Lipofectamine 2000, (m) treated with 10 μg/ml polymer II, (n) treated with 10 μg/ml Lipofectamine 2000, (o) treated with 100 μg/ml polymer II, (p) treated with 100 μg/ml Lipofectamine 2000.


a
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A quantitative analysis was performed by counting the total number of cells present in each image. The means of the total number of cells from 9 images collected for each treatment condition are plotted in Figures 58, and 59. The influence of polymer II on the ability of cells to proliferate was evaluated. A concentration 10 times higher than the one that was identified to mediate transfection was tested. The averages of total number of cells in 9 images that were collected for the same treatment are plotted in Figure 58. In contrast to the results obtained from the Lipofectamine 2000 treatment, even at highest concentration tested, the number of cells that are present in the images is comparable to controls, indicating little or no deleterious impact on growth. The total number of cells is steady, throughout the experiment, under various polymer II concentrations. The same trend is noticed when Figures 58 and 59 are compared. These two figures differ only by the amount of plasmid DNA that was transported. Again, the amount of plasmid DNA tested to be transported does not influence the ability of cells to grow and proliferate.

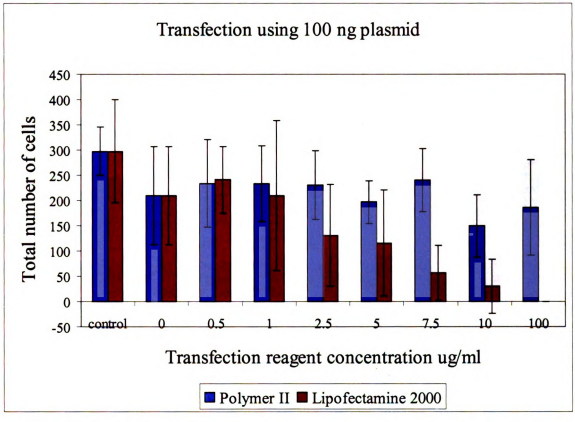

Figure 58. Total number of cells grown under treatment with polymer II or Lipofectamine 2000 and 100 ng plasmid.

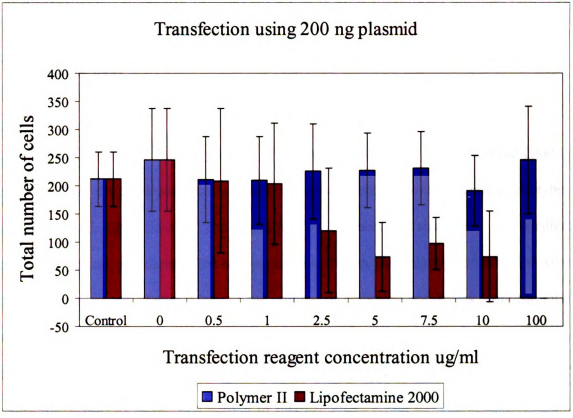

Figure 59. Total number of cells grown under treatment with polymer II or Lipofectamine 2000 and 200 ng plasmid.

Figure 58 represents also the total number of cells that were counted for the treatment with Lipofectamine 2000 at different concentrations and 100 ng of plasmid. At a concentration of 2.5 μg/ml of Lipofectamine 2000 the total number of cells was significantly smaller compared to the control condition. As the concentration of Lipofectamine 2000 increases, the number of cells that are able to grow and proliferate decreases. At the highest concentration tested (100 μg/mL) no intact cells were observed; all of them had been lysed. The same trend is seen when 200 ng of plasmid DNA was used and various concentrations of Lipofectamine 2000 (Figure 59), leading to the

conclusion that the amount of plasmid DNA that is to be transported does not influence the ability of cells to grow and proliferate, the toxic effect that is seen is due to the Lipofectamine 2000.

From Figure 57, the morphology of the cells treated with Lipofectamine 2000 is seen to be very different when compared to non-treated cells. Three different phenotypes were identified. One class of cells exhibits a difference in size: they are smaller, condensed, shrunk and darker. The number of cells that exhibit this type of behavior were counted and reported as percentage of the number of cells that are shrunk from the total number of cells in a field. Averages from 9 different images describing the same condition treatment were plotted in Figure 60. At a Lipofectamine 2000 concentration of 2.5 μg/mL about 30 % of cells belong to this category. When the Lipofectamine 2000 concentration was increased to 7.5 μg/mL and 10 μg/mL 100 % of the total number of cells were shrunk. For the polymer II treated cells, the number of cells that fall into this category is very small, under 2 %, for all the concentrations tested (Figure 60).

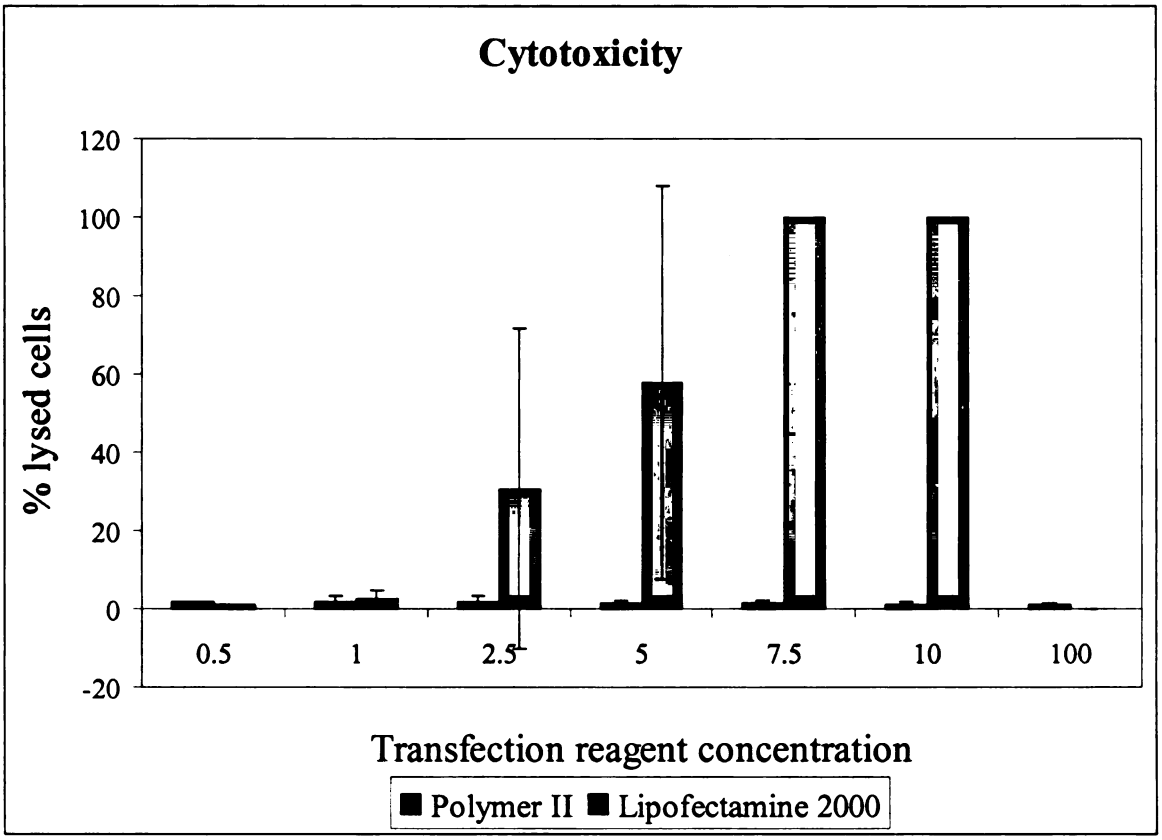

Figure 60. Percentage of the number of cells that are lysed under Lipofectamine 2000 or polymer II treatment.

Another class of cells is represented by cells that are larger than the control cells. The number of cells that exhibit this type of behavior were counted and reported as percentage of the number of cells that are large from the total number of cells from a field. Averages from 9 different images describing the same condition treatment were plotted in Figure 61. The percentage of cells that fall into this category is very small, under 1 %, and is present only at low Lipofectamine 2000 concentrations.

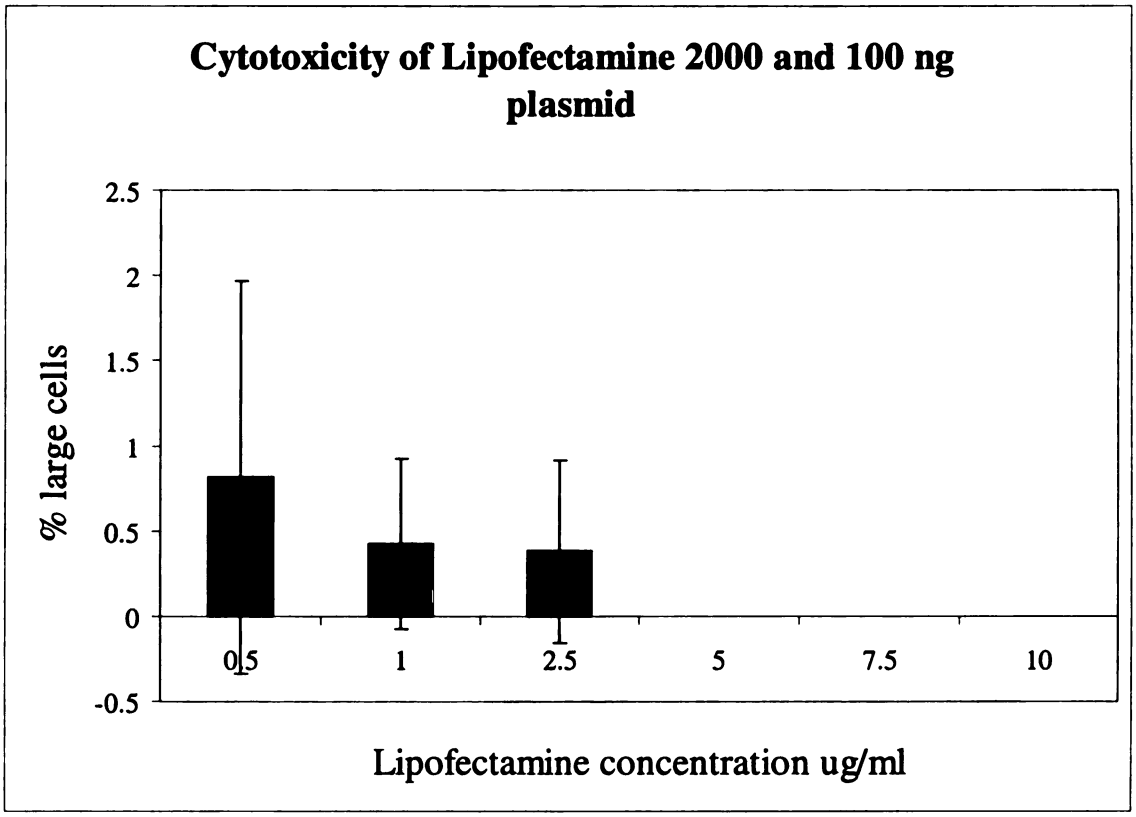

Figure 61. Percentage of the number of cells that are larger under Lipofectamine 2000 treatment.

The third phenotype consists of cells that are totally lysed. This situation was encountered only for the Lipofectamine 2000 treatment at a concentration of 100 μg/mL (Figure 57 p). In comparison with Lipofectamine 2000 treatment at this concentration, the cells treated with polymer II were able to grow and proliferate at a rate comparable to control cells (Figure 57 o). The amount of plasmid DNA does not influence the cell growth.

The results presented show that the polymer that we designed and tested has potential to be used as a nucleic acid carrier. Under the treatment with polymer II of various concentrations the cells are still growing and proliferating, covering the entire surface available, up to confluence. Even at a concentration 10 times higher that the one that was identified to mediate transfection, the ability of cells to grow and proliferate is not influenced.

4.3 Characterization of the mechanism of uptake

Characterization of the mechanism of uptake of polymer-complexed nucleic acids into cells is possible by analyzing confocal images of cells treated with FITC-labeled polymer. Two types of transporters are known: passive transporters that do not require energy (e.g., glucose transport), and active transporters that rely on ATP hydrolysis to drive the transport (e.g., sodium/potassium pump). It is generally accepted that macromolecules are taken up by endocytosis[125, 126]. In this process, the cellular membrane pinches-off enclosing the molecule.

To determine if the uptake is an active or a passive phenomenon ATP synthesis was inhibited using a classic ATP synthesis inhibitor, the dicyclohexylcarbodiimide (DCCD). This molecule reacts with a critical aspartic acid residue in the F_0 subunit of the ATP synthase molecule (Figure 62), forming a covalent adduct, thus impeding the proton flux necessary for ATP synthesis. The c subunit of F_0, also known as proteolipid, because of its hydrophobicity subunit, has a hairpin structure with two transmembrane α-helices and a connecting loop. One α-helix includes the Asp residue whose carboxyl reacts with

DCCD. Mutation studies have shown that this DCCD-reactive carboxyl, in the middle of the bilayer, is essential for H^+ transport through F_0 [127].

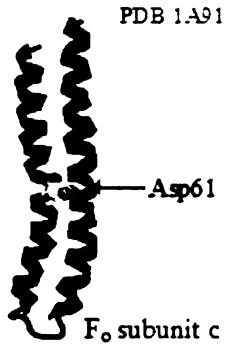

Figure 62. Critical aspartic residue in the c subunit of the F_0 [128].

N, N'-dicyclohexylcarbodiimide (DCCD) inhibits proton transport in endocytotic vesicles. The half-maximum inhibition of proton transport is observed at 10 μM DCCD after 30 min[129]. Sabolic and Burckhardt demonstrated that a 0.1 mM DCCD solution inhibits the proton transport for a 30 minute period of time and that ATP synthesis is lowered to 3 % compared to control cells with no treatment[130].

COS1 cells were treated with N, N'-dicyclohexylcarbodiimide (DCCD) as described in Section 3.7. The confocal microscope settings were established using a solution of FITC labeled polymer Figure 63. The control COS1 cells, with no treatment, are presented in Figure 64.

Figure 63. FITC labeled polymer solution.

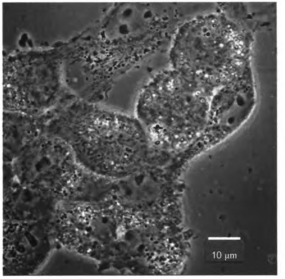

Figure 64. COS1 cells, no treatment.

The images recorded for the FITC labeled polymer treated cells are presented in Figure 65 and 66. Immediately after treatment, rounded vesicles including the fluorescent dye are visible at the periphery of the cells (Figure 65). Ten minutes after the FITC labeled polymer II treatment the polymer had already begun to accumulate in the nuclei of the cells, which are more brightly stained than the corresponding cytosol (Figure 66).

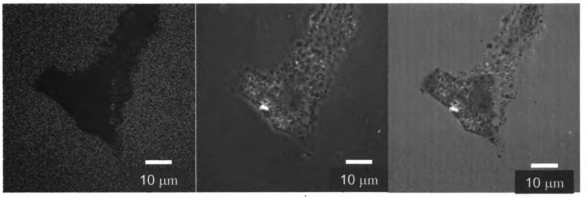

Figure 65. COS1 cells treated with FITC labeled polymer II, immediately after treatment.

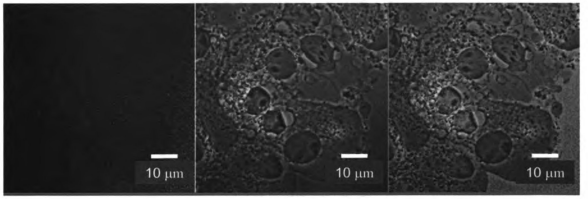

Figure 66. COS1 cells treated with FITC labeled polymer II. A confocal slice of several COS 1 cells 10 minutes after treatment.

To determine if the uptake mechanism requires energy, the ATP synthesis was blocked by using DCCD treatment. The cells were pre-incubated for 15 minutes with 100 μM DCCD solution, followed by the treatment with FITC labeled polymer II. Images of the COS 1cells under this treatment condition are presented in Figure 67. The images are recorded 10, 15 and 20 minutes after treatment respectively.

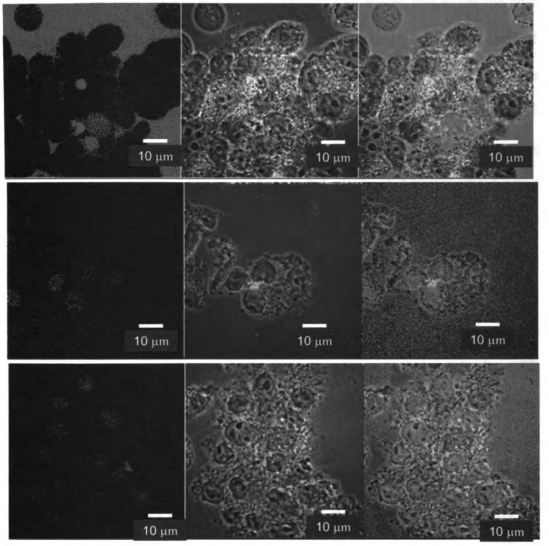

Figure 67. COS1 cells treated with FITC labeled polymer II in the presence of DCCD inhibitor. Images recorded 10, 15 and 20 minutes after treatment respectively.

In Figure 65, rounded vesicles are present at the periphery of the cellular membrane. In this particular situation, the COS1 cells were treated with FITC labeled polymer II. The vesicles present at the cellular membrane encapsulate the labeled polymer II suggesting that endocytosis is responsible for the transport of fluorescently labeled polymer II inside the cells. When the cells were pre-incubated for 15 minutes with the inhibitor of proton transport the fluorescently labeled polymer II accumulated in the

nucleus of some cells 10 minutes after treatment. That means that either the attempt of inhibition of the ATP synthesis with DCCD was not successful or the transport of FITC labeled polymer II does not rely on ATP resources. This result suggests several explanations. Maybe an alternate pathway besides endocytosis is responsible for the transport of the fluorescently labeled polymer II inside the cell. Some temporary disruption of the cellular membrane or fusion of the hydrophobic side of polymer II with the cellular membrane might be responsible for the efficient transport. Polymer II was designed to have 66 % hydrophobic side chains introduced by octylamine. Possibly a spontaneous phase transition at the cellular membrane favorized the formation of vesicles. Another explanation may be that the inhibition of the proton transport with DCCD was not effective, allowing the COS 1 cells to further synthesize ATP. It is possible that this particular cell line, COS 1 cells, does not have that critical aspartic acid that is important for proton transport, so that the inhibitor does not have the substrate to bind. Another alternative might be that the endogenous energetic resources of the cells, the ATP that was present in cells at the moment of incubation with DCCD were sufficient to allow cell survival and endocytosis of fluorescently labeled polymer II. Further studies, using different endocytosis inhibitors, or investigation of the efficiency of the uptake at 4°C should shed more light on the mechanism of uptake.

4.4 Conclusions

A macromolecular platform that can incorporate functional groups at a desired proportion was developed. The synthetic strategy consists only of two steps: the synthesis of a polymeric backbone and its further functionalization. The functionalization of the backbone relies on amide bond formation. This has two advantages. First, the chemistry behind this reaction is well known, as is used at a large scale in any laboratory that synthesizes peptides. Second, the peptide bond is a substrate for proteases, thus ensuring polymer degradation. Two significant representatives that can mediate the transport of nucleic based therapeutics were synthesized, characterized, and their in vitro performance was evaluated.

Polymer I, which is positively charged, was effective in delivering Cy5 labeled antisense oligonucleotides into the nuclei of MEF cells. The positive control, MEF cells treated with Cy 5 labeled antisense oligonucleotide solution with no carrier, showed the fluorescent dye excluded from the cells.

Polymer II, which contains hydrophobic side chains along with positive charges, was tested for the ability to transport pEGFP, a plasmid that encodes for green fluorescent protein to the nuclei of COS1 cells. This carrier not only efficiently mediates transfection, but also does not affect the structure of the cells.

After performing the tests that were presented we have an in depth characterization of materials that are able to deliver nucleic acids into mammalian cells. The characterization of the material covers the physico-chemical characterization, the optimization of conditions for transfection of plasmid encoding for GFP into mammalian

cells, comparison of cytotoxicity of this material to other commercially available transfection reagents, and preliminary characterization of the uptake mechanism.

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