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Development and Characterization of Polymeric Nanoparticles for Gene Delivery

by

NARESH ARELLY

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Committee Approval

To the Graduate Faculty:

The members of the committee appointed to examine the thesis of Naresh Arelly find it satisfactory and recommend that it be accepted.

_____

Guang Yan, Ph.D
Major Advisor

_____

James Bigelow, Ph.D
Committee Member

_____

Rene Rodriguez, Ph.D
Graduate Faculty Representative

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Abbreviations

AAV	Adeno associated virus
BCA	Bicinchoninic acid
$CaCl_2$	Calcium chloride
CaP	Calcium phosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
GFP	Green Fluorescent Protein
HaCaT	Cultured Human Keratinocyte cells
HEK293	Human Embryonic Kidney 293 cells
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazoliumbromide)
PBS	Phosphate Buffered Saline
PEG	Polyethylene glycol
PEI	Polyethylenimine
PLA	Poly(lactic acid) / Polylactide
PLGA	Poly(lactic-*co*-glycolic acid)

PVA	Polyvinyl alcohol
RNA	Ribonucleic acid
SEM	Scanning Electron Microscope
SDP	Size Distribution Processor

Chapter 1

INTRODUCTION

1) Background:

The previous 2-3 centuries have been the golden era for science in discovery of remedies for many diseases such as pain, fever, malaria, polio and diabetes etc. Especially the growth in the last 3-4 decades has been remarkable. Researchers have been working in invention of new drugs for curable diseases. With the new inventions and formulations of these drugs, pharmaceutical world has reached new heights in recent years. Although scientists have found many new drugs for various diseases, there are still many more diseases for which the development of drugs is still in far from ideal. Among those, Cancer and HIV are two main diseases that have been causing death for many people each year. More than 1 million people in the US are diagnosed with cancer each year and at least 500,000 deaths from cancer are projected to occur.[1] Many medications have been found to alleviate cancer symptoms but there is still no drug to eradicate it completely. With the increase of cancer deaths year by year, researchers are putting more emphasis on the development of new medication for the diseases. One possible way to cure such diseases is by replacing the mutated genes with functional genes- a concept is known as Gene Therapy.

Gene therapy was initially thought of as a method to correct single gene defects in genetic disorders. But it has tremendous potential for the future of cancer treatment too. It is a rapidly evolving concept for the treatment of different types of cancer. There are several different approaches of gene therapy for cancer treatment.

These approaches include:

1) Strengthening of the immune response against a tumor
2) Administration of functioning tumor suppressor gene to repair cell cycle defects
3) Delivery of suicidal gene to kill the cancer cells.[2]

Earlier, viral vectors were used to deliver gene inside the cell. Viruses are highly evolved biological machines that easily gain access to host cells and exploit the cellular machinery to facilitate their replication. Adenovirus and retrovirus were used as vectors for gene delivery. However, viral protein could cause severe immune response in humans. In1999, an adverse patient reaction to an adenovirus vector during a clinical safety study was observed. Again in 2000, retrovirus induced a lymphoproliferative disorder in 2 of 11 patients while treating fatal immunodeficiency disorder using a gene therapy. These setbacks made researchers think to develop a non-viral vector which would be safer in deliver of gene without the immune response from viral vectors. Several approaches have been used, though all with the same purpose, which is to protect therapeutic gene from external hazards throughout its journey from the external cellular fluid to nucleus of the target cell. Nanoparticles have the flexibility of controlling the particle size and the release of DNA, they do not insert the gene inside the chromosome like viral vectors and they have low immunogenicity too. With all these, nanoparticles have become more promising vectors than virus to deliver gene into the cells. Many polymers are used as nanoparticles in gene delivery. Poly lactic- co-glycolic acid (PLGA) is a polymer used for gene delivery as it is biocompatible and biodegradable. PLGA-PEG

(Poly Ethylene Glycol), PLGA-PEI (Polyethylenimine) and PLGA-Chitosan are a few of PLGA associated nanoparticles being used for gene delivery recently.

2) Concept of Gene therapy:

Gene therapy is defined as the transfer of nucleic acids to somatic cells or germ cells of a patient such that ultimately the expression of the gene encoded by the transferred nucleic acids can give rise to the desired therapeutic effect.[3] In gene therapy, the therapeutic DNA is usually enclosed within a vector which delivers the genes to the targeted cell. If the vector successfully invades the cell and delivers the gene to the nucleus, then the DNA is translated to mRNA which eventually produces the therapeutic protein, thus achieves either preventing or curing the disease. As compared to conventional medicine and treatment methods, gene delivery offers unique possibilities to treat genetic diseases such as Parkinson's disease, fatal enzyme deficiencies, Alzheimer's disease etc.[4] It was believed earlier that- gene therapy was a treatment for hereditary single-gene defects.[5] Lately, gene therapy targets have been changed to the acquired diseases such as cancer,[2] neurodegenerative disorder,[6] cardio-vascular disease.[7]

Gene therapy is not a new concept; scientist were speculating about the possibilities of gene therapy at least 30 years ago, but many technical obstacles prevented the gene therapy studies at that time. While some of these obstacles have been resolved, others remain, along with new problems. In 1999, gene therapy underwent a major setback when an 18 year old patient, who was going through a clinical trial of gene therapy, died because of severe immune response due to viral vector.[8] In late 2000, gene therapy gained back momentum due to other methods of gene delivery such as non-viral vectors

and physical methods. Though there are concerns with gene therapy, main justification for developing gene therapy is the number and the severity of genetic diseases. There are 2000-3000 familiar genetic disorders for which the cause is some specific genes.[9] Also, about 2 percent of infants suffer from genetic disorders.[10] For many of these diseases, including some of severe childhood diseases, the genes that cause the diseases have been identified and for a few of these diseases, copies of normal genes are available through use of recombinant DNA technology. Gene therapy is possible only for those diseases in which the defect genes have been identified and the normal genes have been isolated and cloned.

3) Types of gene therapy:

Currently, the gene therapy programs can be divided into two kinds: those where the gene transfer occurs inside the patient (*in vivo* transfer), and those where the gene transfer occurs outside the patient (*ex vivo* transfer).[11]

In vivo gene therapy relies on the cells ability to uptake the DNA. Researchers hope to package the normal functioning gene in a way that will permit cells to accept it readily, allowing the gene transfer to occur perhaps by injection.

In *ex vivo* transfer, targeted cells are taken out from patient. The normal functioning gene would be inserted into cells in the laboratory. Then the cells with the newly transferred gene would be implanted back into the patient.

in vivo ex vivo

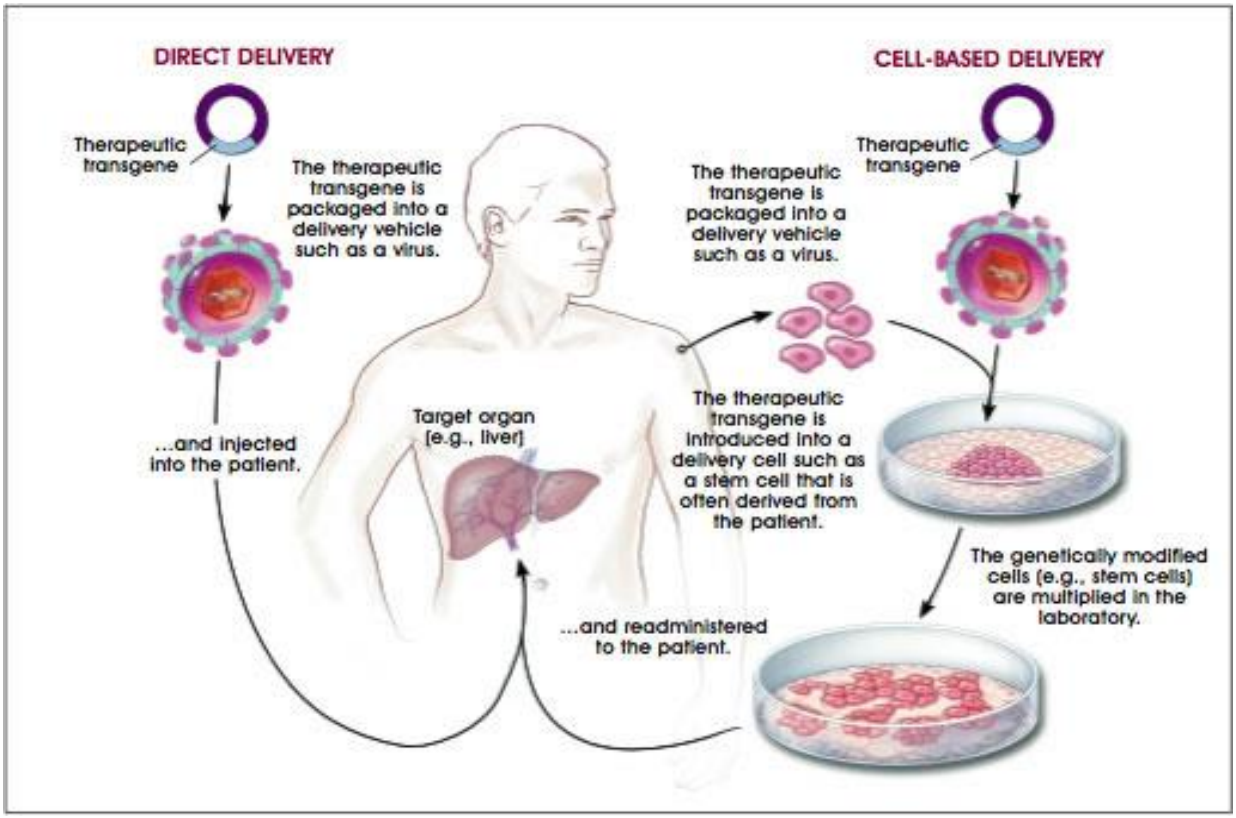

Figure 1.1: in vivo& ex vivo gene therapy

Source: http://stemcells.nih.gov/StaticResources/info/scireport/images/figure111.jpg

4) Challenges of gene therapy:

Despite the best efforts from researchers, there has been very limited success in gene therapy. This is because; introducing a gene to a cell is a difficult task in the gene therapy. Gene therapy is a complicated and advanced method that it works only if we can deliver a normal gene to a large number of cells in a tissue and they have to be the correct cells in the correct tissue. Delivering gene to wrong tissue would be inefficient and could cause the health problems for patient. Once the destination is reached by gene, it must be

turned on to produce therapeutic protein encoded by the gene. Gene delivery and activation are the biggest obstacles facing by gene therapy researchers.

The main problem with gene therapy is the DNA itself due to its easy degradable nature inside the body. This is because of the presence of various kinds of nucleases inside the body. So, a vector is needed to protect the gene and efficiently deliver the gene to the target cell. In early stage, viral vectors seem to be promising as they can efficiently deliver the gene directly inside the nucleus resulting in high transfection efficiency. However, viral vectors might insert the gene at random locations of host chromosomes which can lead to development of cancer and they might cause immune response too. There are some other risks too with gene therapy using viral vectors. The most obvious risk is that the viral vectors will act like viruses; they may keep or recover their ability to cause infections. Fortunately, most of the viral vectors under investigation either do not cause disease in humans or have been genetically rendered harmless. Most vectors are being modified further, both to increase their effectiveness and to add levels of safety against any unwanted viral activity. A second, more complex risk is that gene therapy will stimulate a person's immune system in a way that either decreases the effectiveness of the therapy or makes it difficult to perform future therapy.

Non-viral vectors do not pose those problems in gene therapy but their transfection efficiency is less compared to viral vectors. This is because- they deliver the DNA inside the cytosol and most of the DNA is degraded before reaching nucleus. Some of the diseases are due to mutation of more than one gene. Gene therapy should also address this issue too. One more barrier to gene therapy is, sometimes the required replacement gene is not available.

5) Gene delivery methods:

There are mainly three different types of methods to transfer the gene into body. They are

1) Viral vectors.
2) Non-viral vectors.
3) Physical methods.

Ideally a gene therapy vector would target a specific tissue with high transduction efficiency and a stable, regulated gene expression without any side effects or immunogenic responses. Earlier, viral vectors seem to be promising as they can efficiently deliver the gene directly inside the nucleus resulting in high transfection efficiency. However, viral vectors can invoke immune response and insert the gene at random locations of host chromosomes which can lead to other side effects.[12]

In contrast to viral vectors, non-viral delivery systems possess a much reduced biosafety risk by nature. It is therefore not surprising that this area has been the target for intensive research and the development of vehicles.[13] Nanoparticles have low immunogenicity, do not insert the gene inside the host chromosome, sustained release of gene and have the flexibility of controlling the size. Because of the controlled size, they are well endocytosed by the cells which might result in higher cellular uptake of entrapped DNA.[14] These characteristics of nanoparticles have made them more promising than viral vectors. Some examples of the most popular nanoparticles for gene delivery are liposomes, and cationic polymers. Various liposome compositions have been attractive materials for non-viral vector development. Cationic liposomes as well as neutral/zwitterionic liposomes have been widely experimented. Additionally, polymer

systems such as dendrimers, hyper branched polymers and polymeric nanoparticles are under investigation.

Coating of liposomes and polymers with polyethylene glycol (PEG) chains can significantly change the properties of liposomes, for instance protect the recognition by the host immune defense system. Poly lactic-co-glycolic acid (PLGA) is a USA Food and Drug Administration (FDA) approved bio-compatible and biodegradable polymer which has been extensively used to produce suture reinforcement, skin replacement materials, meshes, etc.[15] Therefore, developing a PLGA Nano formulation is encouraging for more efficient, sustained and safer delivery of gene. However, the two properties that severely hampered the use of these non-viral vectors have been the generally modest gene delivery efficiency and their short-term expression capacity.

The third method to deliver gene into cell is physical method. Electroporation is one of physical methods which has gained popularity in last 5 years.[16] Though the transfection efficiency of this method matches to viral vectors, this method is not as much popular as viral and non-viral vectors.

5.1) Viral vectors:

Viruses are highly evolved biological machines that effectively gain access to host cells and exploit the cellular components to help their replication. These viruses are modified genetically to remove their capability to produce infection.[12] This can be done by removing all or some of the coding regions of the viral genome. But the sequences such as terminal repeat which are required to pack the vector genome into virus capsid or unify the vector DNA into host chromosome are intact.

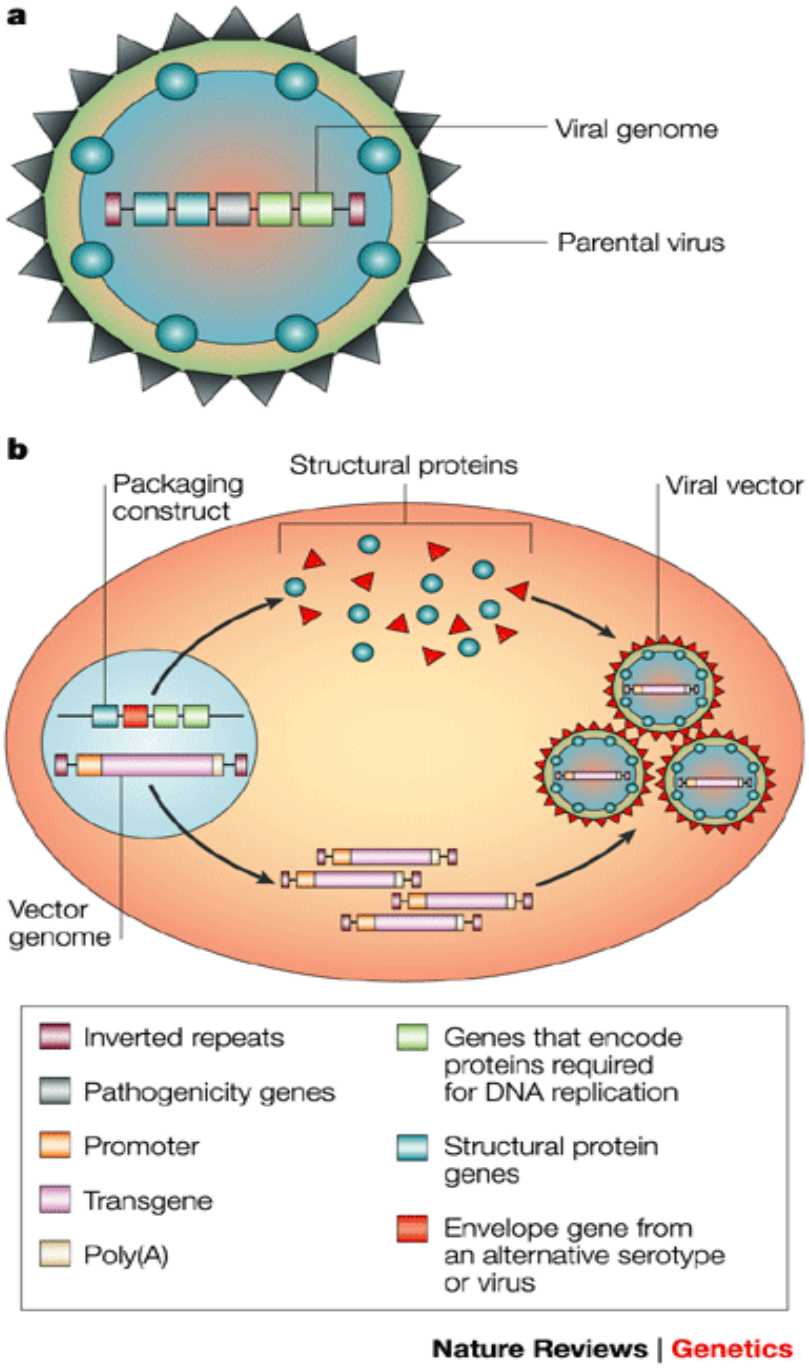

Figure 1.2: Converting a virus into a vector

a) Generic viral vector b)Process of preparing a viral vector

After preparation, these viral vector particles are purified and quantified to separate the vector particles from cellular components. In early time, density gradient centrifugation method was used for this process- but that is tiresome and sometimes it might damage vector particles. Later, column chromatography method was developed and it reduced the problems associated with density gradient method.[17] Both RNA and DNA viruses have been used as vectors for gene delivery. RNA viruses can produce long-term gene expression, but sometimes they can be undesirable due to the random stimulation of therapeutic gene into the host chromosome. In this regards, DNA viruses can be safer, but they can only produce a transient gene expression.[18]

Retroviruses:

The most commonly used RNA viral vectors are retro viruses[19] which have linear, single stranded genomes of 8-11 kilo-bases. Once these vectors are delivered and entered into target cells, RNA-genome is transcribed into linear double stranded DNA and integrated into host chromatin. This efficient integration of genome into host chromatin is the useful property of retro viral vectors. Though this integration does not guarantee a stable gene expression, it is an effective way to maintain the genetic information. Lentiviruses and Spumviruses are a few of retro viruses. One of the biggest disadvantages to use retroviruses is their infectivity is limited to dividing cells. The small size of retrovirus genome also limits their usefulness for gene therapy involving delivering of genes greater than 8-11 kbs.

Adenoviruses:

Another type of viral vectors is adenoviral vector.[20] Adenoviruses are non-enveloped DNA viruses that are about 70-90nm in diameter and whose capsid is composed of pentons and hexons. Most commonly used human adenoviruses genomes consist of a liner 36 kilo-base double stranded DNA molecule. These vectors are taken up by the cells by interaction of viral protein and specific receptors of the cell. Once inside the cell, the capsid dissociates and the DNA is delivered to the nucleus by nuclear pore. One of the major advantages of adenoviral vectors is, they provide gene therapy for wide variety of cell types. They transfer gene to both dividing and non-dividing cells. But the problem with adenovirus vectors is transient gene expression because these are replication deficient viruses and they do not integrate into host chromosome. So these are not suitable for long-term and chronic disorders.

Adeno Associated Virus (AAV):

AAVs are small (25 nm), non-enveloped viruses which packages a 5kb, linear single-stranded DNA genome. Currently, these are among the most frequently used viral vectors for gene therapy.[21] The unique life cycle and its ability to infect both proliferating and quiescent with relentless expression have made these AAVs an attractive vector. Other advantages of these vectors are lack of pathogenicity, stable integration and low immunogenicity. The major disadvantages are variations in infectivity of AAV among different cell types and also the size of the genome.

Herpes Simplex Virus (HSV):

Herpes Simplex Virus (HSV) is also been used as a vector for gene delivery. HSVs are enveloped, linear double stranded DNA 150 kb in length with an overall diameter of 180-200nm. These vectors can transfect non-dividing cells and have latent behavior which can be exploited to achieve long term transgenic expression. Wide host range is also one of the main advantages of these vectors. Some of the unique features of these vectors include the ability to transport retrogradely in the neuron and across the synapses, which has made them promising for neural gene delivery. But, the large size of this virus has led to its use lagging behind other smaller viral vectors.[22] Also, its ability to replicate lytically in brain might cause encephalitis, which has led to fears its potential safety in humans.

5.2) Non-viral vectors:

Though viral vectors are best vehicles to deliver gene to nucleus, problems such as immune response, oncogenic effects made them questionable to use *in vivo*. One example is, adenovirus vector causing the death of 18-year old Jesse Gelsinger who participated in a gene therapy clinical-trial at the University of Pennsylvania in Philadelphia. [23]

Concerns and problems with viral vectors make non-viral or synthetic vectors an attractive alternative. Moreover, non-viral vectors have advantages in terms of lack of immunogenicity, simplicity of use, ease of large scale production and low toxicity. However, main drawback with non-viral vectors is their low transfection efficiency and transient gene expressions. Non-viral gene delivery systems rely on normal cellular

uptake. Many obstacles must be overcome before these vectors reach cell surface. The easiest way to reach the target cells is Local administration.[24]

Different types of non-viral vectors are:

Naked DNA

Lipoplex (Liposome-DNA complex)

Polyplex (Polymer-DNA complex)

Peptide-DNA complex

Naked DNA:

The easiest way to deliver gene using non-viral vectors is direct injection of naked DNA. Clinical trials carried out following intramuscular injection of naked DNA. However, the expression has been very low compared to other methods. Nuclease degradation could be accountable for these low levels of transfection.

Lipoplexes:

To improve the delivery and expression of DNA, it must be protected from damage by endosomes/lysosomes and nucleases. For this, lipoplexes were invented that have the ability to protect DNA from degradation during transfection. Liposomes are one of the most promising non-viral vectors which were first reported by Felgner in 1987. DNA can be covered with lipids as liposomes or micelles. When DNA is complexed with liposomes, it is called as lipoplex. Though, there are three types (cationic, anionic and neutral) of liposomes available, cationic liposomes are very useful due to their positive charge, which can easily form complex with negatively charged DNA. High cationic liposome/DNA ratio is essential to achieve better gene delivery efficiency[25] which results in higher gene expression. The possible explanation is higher amount of positive charges

may condense the DNA molecule and protect the DNA from enzyme degradation. Lipoplexes can be used in gene transfer to cancer cells where these gens activate tumor suppressor genes and decrease the oncogenes activity. Nowadays, lipoplexes are being used to transfer gene to respiratory epithelial cells, which might be a treatment for cystic fibrosis. Nevertheless, liposomes have some drawbacks that include instability in the plasma, formation of complex with the plasma protein by the cationic liposomes, sensitivity to the external parameters such as temperature, and toxicity of cationic liposomes. [26]

Polyplexes:

Complexes of polymer with DNA are called polyplexes. While forming complex, these cationic polymers condense DNA to a relatively small size which may be crucial for gene transfer as smaller particle size would be favorable for good transfection efficiency. Many effective polyplexes have reached the efficiency equal to viral vectors, though many more particles per cell are required.[27]

The main difference between polyplexes and lipoplexes is that- polyplex cannot release DNA directly into cytoplasm as lipoplex does. Possible reason for this could be that, polyplexes do not have any hydrophobic domain. So, they cannot destabilize the endosome by direct interactions with endosomal membrane. The release of polymer/DNA complexes from endosomes is a main barrier to gene transfer, because in the endosome the DNA has to be protected from the acidic environment of it. For better transfection efficiency, the DNA must be released from the late endosome stage to avoid degradation by lysosomal enzymes. If the vehicle successfully reaches cytosol, it will release the gene; in cytosol most of the gene is degraded by nucleases. Only a few

percentage of the delivered gene is able to reach nucleus and can be translated to therapeutic protein. Therefore, endosomal escape is important for high efficiency of transfection. Many first generation cationic polyplexes like polylysine were ineffective in endosomal escape and had low transfection efficiency. Second generation polyplexes such as polyethylenimine (PEI) could manage endosomal disruption by acting as proton sponges. PEI is a very good effective gene transfer vehicle, especially for respiratory tract without any harmful effects for other tissues. [28]

Various polymers are used as vectors in gene delivery. They are:

PLGA (poly (lactic-co-glycolic acid)):

PLGA is an FDA-approved polymer for therapeutic human use and is biocompatible and biodegradable.[29] PLGA is highly soluble in a vast range of solvents. However, its characteristics depend on various factors like lactide-to-glycolide ratio, molecular weight, storage temperature, etc. The degradation period of PLGA depends on the monomer ratio used in its production, that is, the required time for degradation is lower for PLGA with higher content of glycolide units.[30] Once inside the body, PLGA goes through very slow hydrolysis due to its polyester nature. During this process, PLGA degrades to lactic acid and glycolic acid that are biocompatible, and can be easily removed from the body by citric acid cycle.[31] As PLGA has a very slow biodegradable rate, it can deliver the gene for a very long period of time, thus may allow longer gene expression.

PEI (Poly ethylenimine):

PEI is considered as one of the most efficient and cost-effective polymers for gene delivery and it is one of the most densely charged polymers. [32] This polymer forms complexes with DNA as a result of cooperative electrostatic interactions between the

ammonium groups of the polycation (PEI) and phosphate groups of the DNA. As the net charge of PEI-DNA complex is positive, the complex can easily be attached to the phospholipid bi-layer of the cell, and then taken up by cells through endocytosis. PEI can act as endosomal buffering system because the charge density of its cationic groups is pH dependent. Thus, DNA can be released from the endosome without degradation which is called as endosomal escape.[33] The relatively high efficiency of PEIs is believed to come from a high amine density and buffering capacity.

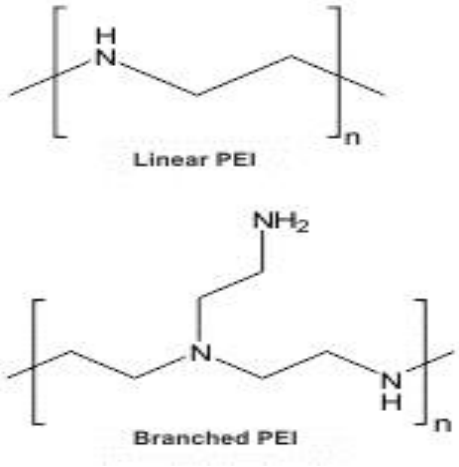

Figure 1.3.Polyethylenimine

Source: http://bi.tbzmed.ac.ir/JournalIssues/AllIssues/Volume1Issue1/4201111.aspx

Several researchers demonstrated that all the DNA chains are condensed by PEI to form DNA-PEI polyplexes when the ratio of nitrogen from PEI to phosphate from DNA (N:P) is above 3, but the ploylexes have a higher in vitro transfection efficiency only when N:P >10. [34] At high N/P ratios, positive net charge of PEI-DNA complex increases which improves cell interaction and enhances the cellular uptake. [35] However, PEI is very toxic due to its non-biodegradable nature.[36] Toxicity is depends upon the molecular weight of

the PEI chain. However, higher molecular weight PEI (i.e., 25kDa) also showed higher gene delivery efficiency compared to lower molecular weight PEI (i.e., 2kDa).[37]

Biodegradable and biocompatible polymer PLGA particles have been used together with PEI derivatives.[38] PLGA nanoparticles have negative charge that results in poor transport through mucosal barriers. To enhance permeability these particles can be modified by a cationic agent like PEI. It has been shown that PLGA-PEI complex retain DNA in tissues and enhance gene transfer.

Peptide-DNA complex:

Though, lipoplexes and polyplexes are two main categories of non-viral vectors, peptide based gene delivery is also gaining importance in recent times. Peptide based gene delivery systems which have many viral characteristics such as membrane fusion and nuclear localization are still in developing stage. There are some doubts on the electrostatic interactions between the plasmid and peptide which are not stable enough under normal physiological conditions. Peptides can be structurally engineered to provide efficient cell-specific targeting, endosome lysis and nuclear localization which cannot be effectively done by lipoplexes and polyplexes.[39] But the gene delivery based on peptide-DNA complex is still in its early stages with limited research results.

5.3) Physical methods:

The physical methods of gene delivery include gene gun (Biolistics), hydrodynamic injection, needle injection, electroporation, magnetic field mediated gene transfer, ultra sound etc.[40]

Electroporation is one of main physical techniques of gene delivery that uses a pair of electrodes to produce electricity at a certain voltage and frequency of electric pulses. Nanopores in the plasma membrane of the cells which are created by the electric field of electrodes, enables transfer of gene. But the disadvantage with this method is that- damage of cells might occur because of heating if the applied electric field is too high. If the electric field is low, it does not cause any nanopores on the plasma membrane of the cell. So, it is very important to maintain an optimum electric field in this method. Additionally, electrodes range is very limited. Thus, it is difficult to transfer gene to large areas of cell. Also, surgery needs to be done to place electrodes.

Another physical method for gene transfer is 'gene gun' method. In this method, heavy metal particles (gold or tungsten) which are coated with plasmid DNA are propelled into cells. It was first used in plant cells and later used on mammalian cells.[41] In this method, plasmid DNAs are deposited on gold or tungsten nanoparticles. Cells are permeabilized by small beads of these biocompatible particles when they are accelerated by the gene gun device using compressed helium gas and forced out on to the cells. The nanoparticles penetrate 1-5 millimeters into the cell due to their velocity and release DNA into the cell. Main drawbacks of this method is, metal particles might deposit in the body and might pose problems in long term. Other drawback is low efficiency of particles to reach entire tissue due to low penetration.

5.4) Nanotechnology in gene delivery:

Recently, nanoparticles have gained lot of importance in gene delivery, because they are relatively safe and easy to prepare.[42] Newly, nanoparticles prepared using

different polymers such as polyethylenimine, chitosan[43] and gelatin[44] are being investigated as non-viral gene deliver vectors. Achieving safe and sustained gene transfection are the two main reasons to use these vectors.[45] Nanoparticles are defined as particles having a size of diameter in between 1 to 1000 nm. Because of their size, nanoparticles are effectively endocytosed and cellular uptake would be higher. DNA entrapped in PLGA nanoparticles is released slowly with the hydrolysis of polymer. This slow release of DNA would be effective in achieving controlled gene expression in the target tissue. This sustained release of DNA is shown to be effective in bone regeneration, which is useful to repair bone fractures.[46]

Advent of nanotechnology has opened new gateways for effective and safe delivery of therapeutic genes. Delivering genes by nanoparticles has several advantages. Mainly, transfection efficiency is dependent on the size of the vehicle and it is very high with nanoparticles. Nanoparticles can easily be used to target specific cell. It can be achievable by passive or active targeting. Nanoparticles which have been gaining the researcher's interest in recent times are, PLGA-DNA, chitosan-DNA, PLGA-chitosan-DNA, PLGA-chitosan-cholesterol-DNA, PLGA-PEI-DNA, PLGA-PEI-cholesterol-DNA etc.

Chapter 2

Evaluation of PEI-DNA-PLGA nanoparticles

1) Introduction:

In comparison to viral vectors, more efforts have been shelling out in recent years on the development of non-viral vectors because of some death accidents in clinical trials using viral vectors.[47] But non-viral gene delivery techniques still remain behind viral vectors due to the mean number of gene copies needed to transfect a cell by non-viral techniques. Despite this limitation, plasmid-mediated transfection of non-viral vector gene delivery has the major advantage that it does not raise the concerns of biological vectors for human therapy. Thus, much effort is presently devoted to develop non-viral vectors. There are many non-viral vectors such as cationic lipids, polycationic polymers,[48] Polymeric micelles[49] and nanoparticles.[50] These non-viral vectors have several advantages, including stability, safety, ease of manipulation, low cost and high flexibility. Sustained gene expression has been achieved in polymer-mediated systems and biodegradable nanoparticles can be used to deliver genes due to their safe and controlled actions.[51] Other advantages of nanoparticles include their stability and ease of uptake into cells by endocytosis and their ability to target specific tissues and organs.[52]

Polyethylenimine (PEI) is a cationic polymer with the highest cationic-charge-density potential. Every third atom is amino nitrogen that can be protonated. Linear polyethylenimines contains all secondary amines, whereas branched PEIs contain primary, secondary and tertiary amines. PEI is one of the most efficient polycations used for gene delivery.[53] PEIs exhibit a high positive charge density in aqueous solutions and

are considered to be promising vehicles for delivery of negatively charged molecules of DNA for *in vitro* and *in vivo* applications.[54]

PEI's high transfection efficiency comes from high amine density and its substantial buffering capacity virtually at any pH.[55] Because of cooperative electrostatic interactions between the amine groups of PEI and phosphate groups of DNA, PEI forms complexes with DNA.[56] Using different methods to characterize the surface charge of the DNA/PEI polyplexes formed under various conditions, it was found that nearly all the DNA chains are condensed by PEI to form the DNA/PEI polyplexes when the molar ratio of nitrogen from PEI to phosphate from DNA (N:P) reaches ~ 3. But they have a high *in-vitro* gene transfection efficiency only when N:P $\geq$10.[34]

As mentioned above, high transfection efficiency and cytotoxicity of PEI depends on the degree of branching, molecular weight, cationic charge density and buffering capacity. High molecular weight branched PEI has been shown to have high transfection efficiency compared to low molecular weight branched PEIs.[57] Although the transfection is superior, cell viability is shown to be less and its cytotoxicity is still a severe problem. Toxicity level goes higher as the molecular weight of PEI increases. Therefore, there have been many efforts to combine PEI with other polymers to enhance transfection efficiency while lowering its toxicity.

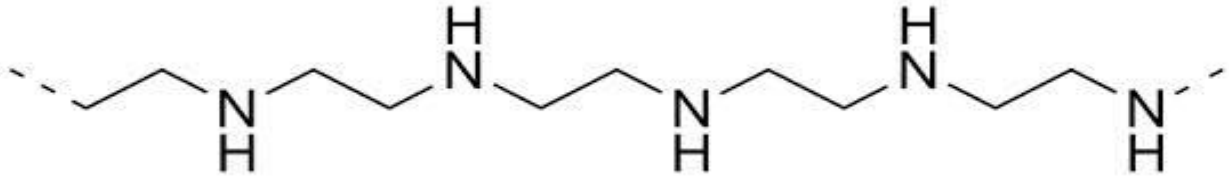

Figure 2.1 Linear PEI

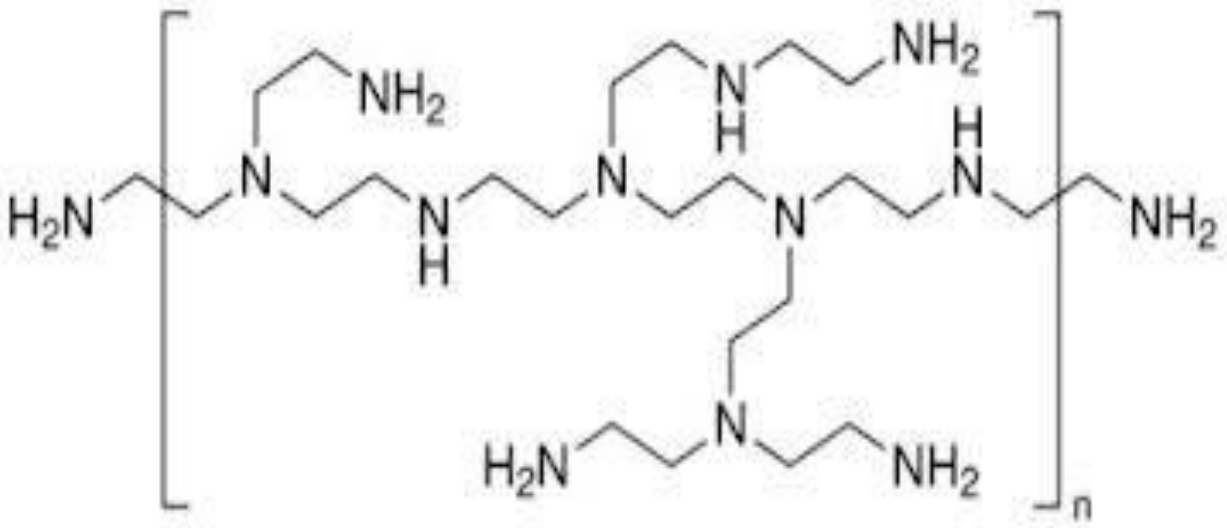

Figure 2.2: Branched PEI

In recent times, biodegradable and biocompatible polymer poly (lactic-co-glycolic acid) (PLGA) particles are being used together with PEI derivatives.[58] These biodegradable nanoparticles are available for delivering genes and degradation at a specific site.[59] By changing the molecular weight of PLGA or ratio of glycolic acid to lactic acid ratio, the degradation time can be varied from days to years. Because of their sustained action, PLGA nanoparticles are suggested to be good gene delivery carriers. It was discovered in a previous study[60] that the transfection efficient of PLGA-DNA nanoparticle was very low. Thus the purpose of the study in this chapter was to improve the transfection efficient of PLGA-DNA nanoparticles with the addition of PEI into the nanoparticles. Encapsulation of DNA condensed by PEI into PLGA nanoparticles has been shown to enhance gene transfection.[61] However, there is lack of more detailed evaluation of the PEI-DNA-PLGA nanoparticles in gene delivery, which has been addressed in this chapter.

2) Materials:

2.1) Chemicals:

Linear polyethyleimine (1.3kDa and 2kDa molecular weight) and branched polyethylenimine (25kDa molecular weight) (Sigma-Aldrich), Poly [lactide-co-glycolide] (PLGA) 50:50 with viscosity 0.95-1.20 dL/g and 0.24-0.54 dL/g in HFIP (Durect Corporation, Pelham, AL), poly(vinyl alcohol) 87-90% hydrolyzed with average molecular weight 30-70kDa, D-mannitol, glacial acetic acid (Spectrum Chemical Mfg. Corp., Gardena, CA), sodium hydroxide, tris base(Fisher scientific, Fair Lawn, NJ), agarose LF^{TM} (Amresco, Solon, OH), ethidium bromide, boric acid (Acros Organics, NJ), dichloromethane HPLC grade, cholestryl chloroformate (Alfa Aesar, Ward Hill, MA), chloroform HPLC grade (EMD Chemicals, Gibbstown, NJ), MTT dye (Sigma-Aldrich), (dimethyl sulfoxide, acetone, isopropyl alcohol (BDH chemicals,West Chester, PA), hydrochloric acid 6.00N (Ricca Chemical Company, Arlington, TX), DPBS (Lonza, Walkersville, MD), Quant-iT^{TM} Picogreen dsDNA reagent (Invitrogen Eugene, OR), heparin sodium salt and FITC (fluorescein isothiocyanate).

2.2) Cell Culture:

a) HEK293 cells:

HEK293 cells were purchased from ATCC (Manassas, VA) and were cultured in Dulbeccos-Modified Eagle's Medium (DMEM) (Thermo Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologics, Lawrenceville, GA), gentamycin sulfate antibiotic (10µg/mL). Cells were incubated at

37°C in a humidified atmosphere with 5% CO_2. HEK 293 cells are very easy to grow and can transfect readily hence widely used in cell biology research.[62]

a) Melanoma cells:

Melanoma cells were purchased from ATCC (Manassas, VA) and were cultured in Dulbeccos-Modified Eagle's Medium (DMEM) (Thermo Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologics, Lawrenceville, GA), gentamycin sulfate antibiotic (10µg/mL). Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

b) HaCaT cells:

HaCaT cells were a generous gift from Texas A&M University and were cultured in Dulbeccos-modified Eagle's Medium (DMEM) (Thermo Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologics, Lawrenceville, GA), L-glutamine (5mM), $MgCl_2$ (0.8112mM), pyruvic acid and gentamycin sulfate (10µg/mL). Cells were incubated at 37 C in an incubator with 5% CO_2.

c) Fibroblast cells:

Fibroblast cells were a gift from Dr. James Lai of Idaho State University. These cells were also cultured in Dulbeccos-Modified Eagle's Medium (DMEM) (Thermo Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologics, Lawrenceville, GA), gentamycin sulfate antibiotic (10µg/mL). Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

2.3) Buffer Solutions:

a. Tris-EDTA buffer (TE 8)

605.7 mg of Tris Base and 146.125 mg of EDTA were dissolved in 450 mL of water and sonicated. The pH was adjusted with 0.3N HCl to 8.0 and the final volume was made up to 500 mL using type 1 water.

b. Tris-Borate EDTA (TBE) 5X

54g of Tris Base, 2.92 g of EDTA and 27.5 g of boric acid were dissolved in 950 mL of water and sonicated. The pH was adjusted with 0.3N HCl to 8.3 and the final volume was made up to 1,000 mL using type 1water.

c. Tris-Acetate EDTA (TAE)

242g of Tris Base, 14.61 g of EDTA and 57.1 mL of glacial acetic acid was dissolved in 950 mL of water and sonicated. The pH was adjusted with 0.3N HCl to 8.0 and the final volume was made up to 1,000 mL using type 1 water.

d. Tris-EDTA sodium phosphate (TES) buffer

1.21g of Tris base (10mM), 292 mg of EDTA (1mM) and 268 mg of $Na_2HPO_4.7H_2O$ (1mM) were dissolved in 950 mL type 1 water and sonicated. The pH was adjusted with 1M CH_3COOH to 7.4 and the final volume was made to 1,000 ml by type 1 water.

e. Phosphate buffer (0.91M)

133.5g of $Na_2HPO_4.7H_2O$ (10mM) and 20.25g of KH_2PO_4 were dissolved in 950 mL of water. pH was adjusted to 7.4 and the final volume was made up to 1000 mL using type 1 water.

f. Tris-sodium phosphate (TS) buffer:

1.21g of Tris base (10mM), and 268 mg of $Na_2HPO_4.7H_2O$ (1mM) were dissolved in 950 mL type 1water and sonicated. pH was adjusted with 1M CH_3COOH to 7.4 and the final volume was made up to 1,000 mL using 1 water.

3) Methods:

3.1) Preparation of Plasmids:

Transformation of *Escherichia Coli*:

a) Stock plasmid solution (1000ng/µL) was diluted to 100pg/µL by sterilized endotoxin free water and placed it on ice.

b) The NEB5-α competent *E.Coli* cells were thawed by keeping the cells on ice for 30 minutes.

c) 100µL of NEB5-α competent *E.Coli* cell solution was taken into a sterile 1.5mL micro-centrifuge tube.

d) Later 1, 5 and 10µL of diluted plasmid solutions (100pg, 500pg and 1000pg) were added to the different tubes of above solution and flicked gently by hand for 5-10 times to mix the solutions.

e) The mixture was then placed on ice for 30 minutes.

f) The micro-centrifuge tube was placed in a 42°C water bath for exactly 45 seconds and immediately transferred to the ice bath again for 5 minutes.

g) 500µL of room temperature SOC (Super Optimal Catabolite repression) medium was then added to the above cooled mixture and spun (700 rpm) at 37°C

for 60 minutes.

h) The tube was then centrifuged at 5000 rpm for 1 minute (4°C) and 400 µL of the supernatant was discarded; the tube was then flicked gently to resuspend the concentrated transformed bacteria into 100 µL of SOC medium.

i) A Kanamycin resistant LB-Agar plate was preheated to 37°C in an incubator for approximately 30 minutes.

j) 100 µL of the concentrated transformed bacteria solution was then spread on the Kanamycin resistant LB-Agar plate using a sterile glass rod.

k) The plate was kept upside down in the incubator for 12 hours for the growth of the colonies.

Amplification & Purification of Plasmids:

Plasmids were amplified by shaking transformed *E. coli* in 10 mL of LB broth at 37°C for 8 hours. After 8 hrs, this 10 ml solution was transferred to a flask which contains 250mL of LB broth and kept it for shaking at 37°C for 16 hours. Qiagen Plasmid Plus Mega kit was used to lyses and purify the plasmids amplified inside the bacteria.

The detailed procedure is as followed:

a) A single colony was picked up from the agar plate using a sterile 20 µL pipet tip and mixed into 10ml of Kanamycin containing LB broth in a polypropylene tube (Falcon

2051). This process was repeated for a total of four tubes at a time.

b) The tubes were then shaken (250 rpm) at 37°C for eight hours using a shaker

(orbit shaker, Labline, Mumbai, Maharashtra, India) to pre-culture the bacteria in the tubes.

c) After eight hours 10 mL of pre-cultured medium was added to 240 ml of Lb-Agar medium and shaken again for 16 hours maintaining the same conditions used for pre- culture.

d) The medium was then centrifuged at 3,000 rpm for 10 minutes at room temperature and the supernatant was discarded.

e) The sediment bacteria were resuspended in 25 mL of buffer P1 by vortexing it gently and then divided into two tubes.

f) Buffer P2 (12.5 mL in quantity) was added to each tube; mixed thoroughly by inverting the tubes several times and then incubated at room temperature.

g) Immediately after five minutes 12.5 mL of buffer S3 was added to each tube and mixed by inverting the tubes several times and then filtered.

h) The clear filtrate was collected and 25mL of binding buffer was added to it.

i) The above solution was then passed through the Qiagen plasmid plus spin column and the column was then washed by 80 ml of buffer ETR and 50 mL of buffer PE subsequently.

j) The column was then placed into a collection tube and was centrifuged at 2,500 rpm (RT600B, Sorvall, Newport Pagnell, Buckinghamshire, England) for 10 minutes to get rid of residual buffer PE.

k) Then the column was transferred to a new centrifuge tube and 1 mL of sterile endotoxin free water was added to the column and kept aside for 10 minutes.

l) The tube containing the column was then centrifuged at 3,000 rpm for 10 minutes at 4°C.

m) The endotoxin free water containing the pure plasmids was collected in a 1.5 mL sterile microcentrifuge tube and stored in a freezer at -20°C.

Determination of Plasmid Concentration by Nanodrop Spectrophotometer: (NanoDrop 2000, Thermo Scientific)

a) The pedestals of the spectrophotometer were cleaned using sterile water prior to use.

b) 2µL of sterile water was placed on the pedestal to measure the baseline absorbance at

260nm and 280nm. Then the spectrophotometer was zeroed for the blank value.

c) The pedestals were then wiped by a tissue paper and 2µl of the sample was placed on the pedestal and the absorbance was measured at 260nm and 280nm.

d) The plasmid concentration was calculated based on the following formula:

Concentration (ng/µL) = Absorbance at 260nm × 50 × dilution factor

e) Sample measurements were done at least three times and the average was determined.

3.2) Preparation of PEI-DNA-PLGA nanoparticles:

PEI-DNA-PLGA nanoparticles were prepared by a modified double emulsion solvent evaporation method. The detailed procedure of this preparation is as follows:

Primary Emulsion	
1.Organic Phase	
Ingredient	Category
PLGA 50:50	Encapsulating Polymer, surfactant for primary emulsion
Dichloromethane	Organic Solvent
2.Aqueous Phase 1	
PEI	Co-Polymer/Entrapment enhancer
3.Aqueous Phase 2	
GFP-Luciferase Plasmid	DNA
Secondary Emulsion (Aqueous phase 2)	
Polyvinyl Alcohol (MW 30,000-70,000)	Surfactant for secondary emulsion
Sterile Purified water	Vehicle

Table 2.1: Formula for preparation of PLGA-PEI nanoparticles

Procedure:

a) Poly (D, L-Lactide-co-glycolide) 50:50 was dissolved in 2ml of different types of organic solvents at room temperature.

b) 100 µl PEI solution of different molecular weights were mixed with 100 µl plasmid DNA and kept aside for 15 minutes.

c) Then 2mL of PLGA solution was added to PEI-DNA complex and sonicated.

d) Then 12 mL of 2% PVA solution was added to the primary emulsion and sonicated again for two minutes to form the W/O/W double emulsion.

e) At this point droplets of samples were taken for measuring the size and polydispersity index.

f) This double emulsion was then stirred at room temperature for various time periods based on the organic solvent used.

g) When evaporation of organic solvent was complete, the nano suspension was subjected to ultracentrifugation by a centrifuge (Avanti® J-26 XPI, Beckman Coulter, Brea, CA) at 4°C to collect nanoparticles.

h) Particles were washed twice with 5 mL of sterile water to get rid of the unentrapped plasmids and residual PVA.

i) Supernatant from original suspension and subsequent washes were collected and stored at 4°C to determine the unentrapped plasmids.

j) Finally the pellets were resuspended in 5mL of sterile water and a few drops were taken to measure the particle size and polydispersity index by a N4 Plus particle size analyzer.

k) 100mg mannitol was added to the suspension as a cryoprotectant.

l) The nanoparticle suspension was frozen by liquid nitrogen and lyophilized for two days by a freeze dryer. (Unitop 400SL, Virtis, Gardiner, NY)

m) Powdered PLGA nanoparticles were stored at 4°C for further use.

Characterization of Nanoparticles:

N4 Plus submicron particle sizer (Coulter Corporation, Miami, FL) was used to determine particle size and polydispersity and the analysis was done by unimodal analysis and size distribution processor (SDP) analysis.

Procedure:

a) Small amount of particles/emulsion was diluted to optimum confluency (50,000-1,000,000 counts per second) using Type 1 particle free water. For the dry particles the suspension was sonicated in a bath sonicator for 2 minutes.

b) The sample was incubated at 25°C for 1, 5 and 10 minutes before the actual measurement was taken.

c) Particle size measurement was done with a light scattering at 90° angle for two minutes.

d) Scanning Electron Microscopy (SEM) analysis:

1) Freeze dried nanoparticles were resuspended in 1mL type 1 water in an eppendorf tube.

2) Centrifugation was done for 10 minutes at 13,000 rpm (4°C) by a bench-top centrifuge (Micromax RF (Thermo Scientific, Waltham, MA)).

3) Then the particles were resuspended again in type 1 water by sonicating in a bath sonicator.

4) The particles were air dried on aluminum stubs.

5) A gold sputter was used to coat the samples by gold under vacuum.

Finally, images were taken by taken by a scanning electron microscope (Quanta FEG 200, FEI, Hillsboro, OR).

3.3) Determination of DNA Entrapment Efficiency:

Encapsulation of plasmid inside the nanoparticles was determined by an indirect method and the efficiency was calculated using the equation below.

$$\% \text{ Encapsultion} = ([DNA]_{total} - [DNA]_{free}) / ([DNA]_{total} \text{ X } 100)$$

Entrapment efficiency could not be calculated by a direct method as presence of polyethylenimine in these formulations resulted in inefficient extraction of DNA from the PLGA nanopartices. Therefore, the DNA lost in the outer phase of double emulsion during nanoparticles preparation and also during the wash steps were determined to calculate the entrapment efficiency.

3.4) *In vitro* Release Study of Nanoparticles:

In vitro dissolution study was done to assess the release profile of plasmids from various types of PLGA-PEI nanoparticles; the study was done at 37°C and the dissolution medium was 0.91M phosphate buffer with pH-7.4. Samples were collected by centrifuging the nanoparticles and picogreen assay was performed on the supernatant to determine the amount of DNA released. The samples were collected at 1st, 3rd, 6th, 12th, 24th, 48th, 96th and 192th hours. Following is the general protocol for the dissolution study:

Dissolution medium	0.91M Phosphate buffer pH-7.4
Dissolution apparatus	Eppendorf-R Thermomixer with attached 1.5mL microcentrifuge tube holder.
Sample volume withdrawn at each time interval	900 µL
Mixing speed	300
Temperature	37±0.5°C
Sampling time intervals	1st, 3rd, 6th, 12th, 24th, 48th, 96th, 192th hours

Table 2.2: Dissolution study protocol

Procedure:

a) Sample and blank PLGA-PEI nanoparticles (3mg including mannitol) were taken into a sterile 1.5 microcentrifuge tube and 1mL phosphate buffer was added to the particles and vortexed briefly to resuspend the particles.

b) Tubes were placed in a thermomixer (Thermomixer R, Eppendorf, NorthAmerica, Hauppauge, NY, USA) and shaken at 300 rpm at 37°C in phosphate buffer.

c) At predetermined time points the tubes were centrifuged using Thermo IEC Micromax RF (Thermo Scientific, Waltham, MA) at 13,000 rpm for 10 minutes (4°C) and 900 µL of supernatant was collected and stored at -20°C.

d) Fresh phosphate buffer (900 µL) was added to each tube and the tubes were subjected to sonication by a bath sonicator for approximately 30 seconds.

e) The tubes were again placed in the thermo-mixer and shaken in the same conditions until the next time point.

f) Picogreen assay was carried out to determine the amount of plasmid release at various time points using the blank particle release medium spiked with several known amount of plasmids as standards.

3.5) Uptake of nanoparticles by cells:

Nanoparticles were prepared with fluorescent labeled polymer PLA-rhodamine with or without the addition of PEI polymer. After preparing nanoparticles, fluorescence microscopy and flow cytometry studies were conducted to determine the uptake of nanoparticles by cells.

Procedure:

a) PLA-Rhodamine (30mg) was dissolved in 2ml of dichloromethane at room temperature.

b) 2kDa PEI-Cholesterol (30mg/mL) was dissolved in about 0.05M acetic acid and pH was adjusted to 4.5.

c) 200µL of water was added to PLA-Rhodamine-dichloromethane solution and sonicated (PLA-Rhodamine nanoparticles).

d) 100µL (3.1mg/ml) of PEI-cholesterol solution was added to the 100µL of water.

e) PLA-Rhodamine-dichloromethane solution was added to ‘d’(PEI-cholesterol) solution and sonicated using probe sonicator at n=7 amplitude in ice bath for 15 sec. (PLA-Rhodamine-PEI-Cholesterol nanoparticles).

f) Then 12 mL of 2% PVA solution was added to the primary emulsion and sonicated again at n=7 amplitude in ice bath for two minutes (10 sec on, 10 sec off) to form W/O/W double emulsion.

g) At this point samples were taken for measuring the size and polydispersity index of

emulsion droplet.

h) The double emulsion was then stirred at room temperature for 4 hrs to evaporate organic solvent.

i) When the evaporation of organic solvent was complete samples were taken again to determine the particle size and polydisepersity index by a N4 Plus particle size analyzer.

j) The nano suspension was subjected to ultracentrifugation by a centrifuge (Avanti® J-26 XPI, Beckman Coulter, Brea, CA) at 4°C to collect nanoparticles.

k) The particles were washed twice again with sterile water to get rid of the residual PVA.

l) Finally the pallets were resuspended in 5mL of sterile water and samples were taken to measure the particle size and polydispersity index again.

m) One hundred (100) mg mannitol was added to the suspension as a cryoprotectant.

n) The nanoparticle suspension was frozen by liquid nitrogen and lyophilized for two days using a freeze dryer. (Unitop 400SL, Virtis, Gardiner, NY)

o) Powdered PLGA nanoparticles were stored at 4°C for further use.

3.6) Cells uptake study using florescence microscopy:

Cells incubation:

a) Sterilized cover slips were placed in each well of a 6 well plate.
b) HEK/HaCaT cells (200,000) were added to each well in 2 mL full media (with FBS).
c) After reaching 70% confluency, media was removed and cells were washed with PBS carefully (HEK cells are easy to detach).
d) 1900 µL of plain (without FBS)/full media was added to each well.

e) 1 mg of nanoparticles were dispersed in 100 µL 5 % mannitol water and sonicated for 2 minutes using sonicater (keeping ice in sonicater).

f) 100 µL nanoparticles suspension was added to each well.

g) After adding nanoparticles, plates were incubated for different time points (6 hours, 24 hours).

Fixing protocol:

a) After reaching a desired time point, media was removed and washed carefully with PBS for twice.

b) Then, added 1 mL of 4% formaldehyde solution to each well and incubated for 5 minutes at room temperature (dark condition).

c) After 5 minutes, formaldehyde was removed and washed again with PBS once.

d) PBS was removed and 1 mL of 0.15M glycerol was added to each well.

e) Now, coverslips were carefully taken off from each well and put on a sterilized slide by flipping so that the cells would be sandwiched between coverslip and slide.

f) Fixing of coverslip was done by applying nail polish on 4 sides of it on slide and excess polish was removed.

g) Immediately slide was placed in a box protected from light.

h) Then, slides were observed under florescence microscope using different lens (10X, 20X, 40X).

3.7) Flow Cytometry Study:

Flow cytometry is a technology that measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream

through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity and relative florescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits florescence.

In the flow cytometer, any suspended particle or cell from 0.2-150 micrometers in size is suitable for analysis[63] When particles pass through the laser intercept, they scatter laser light. Any florescence molecules present on the particle fluoresce. Scattered and florescent light is collected by appropriately positioned lenses. A combination of beam splitters and filters steers the scattered and florescent light to the appropriate detectors. The detectors produce electronic signals proportional to the optical signals striking them.

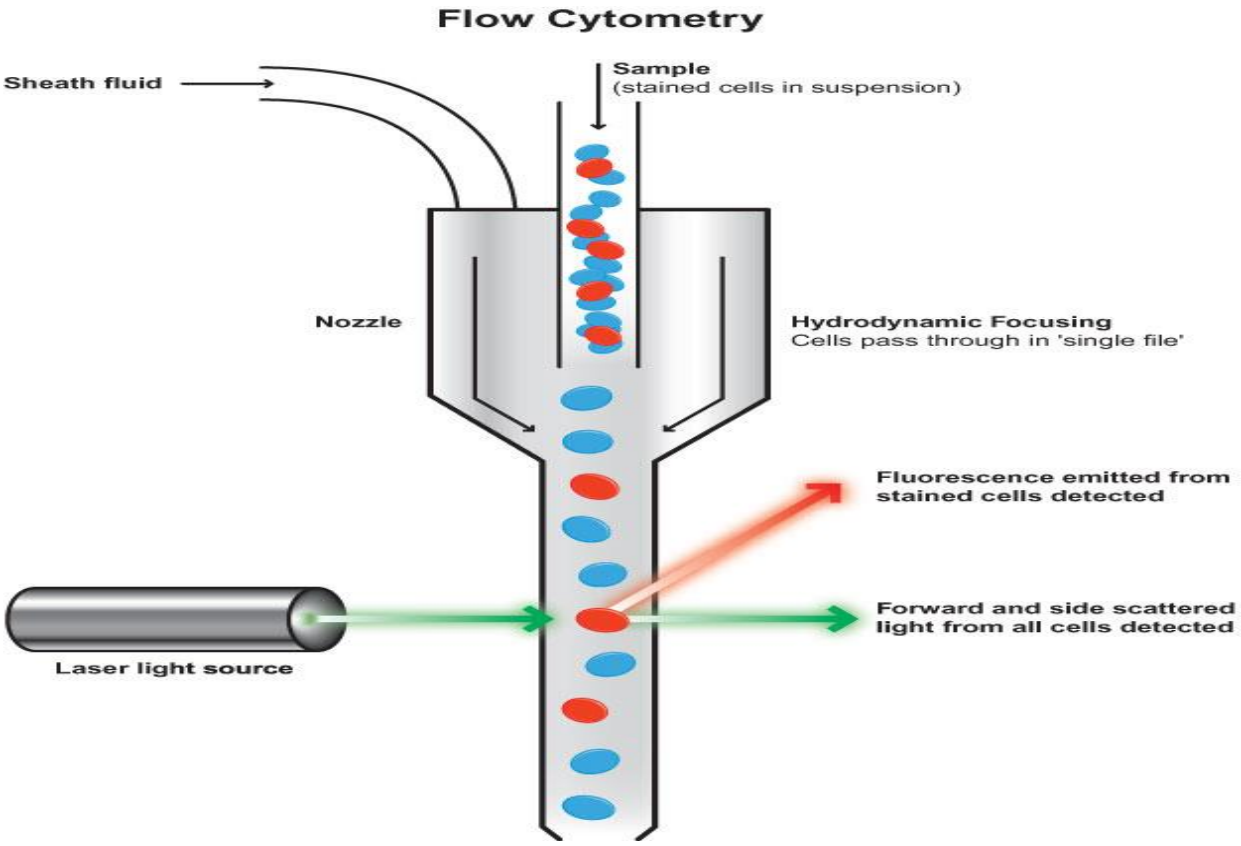

Figure 2.3: Flow Cytometry

Procedure:

Cells incubation:

a) HEK/HaCaT cells (200,000) were added to each well in a 6 well plate. Volume was made up to 2 ml with full media (with FBS).
b) After reaching 70% confluency, media was removed and cells were washed with PBS carefully (HEK cells are easy to detach).
c) 1900 µL of plain (without FBS)/full media was added to each well.
d) 1 mg of PLA-Rhodamine/PLA-Rhodamine-PEI-Cholesterol nanoparticles were dispersed in 100 µL of mannitol (5%) water and sonicated for 2 minutes using a sonicater (keeping ice in sonicater).
e) After sonication, different types of nanoparticle suspensions (100 µL) were added to different wells.
f) After adding nanoparticles, cells were incubated for different time points (6 hours and 24 hours).
g) At appropriate time points, media was removed and washed with PBS once.
h) 500 µL of trypsin was added and incubated the plate for 3-5 minutes.
i) Cell suspension was taken into a micro-centrifuge tube and centrifuged at 1000 rpm for 5 minutes.
j) Supernatant was removed and 500 µL PBS was added and aspirated and analyzed using flow cytometer.

Flow cytometry was performed using a Becton Dickinson Biosciences FACS Calibur flow cytometer, and analyzed using CellQuest software (BD Biosciences, San Jose CA). Dead cells/debris and doublets were gated out of the analysis. A minimum of 10,000

gated (live, singlet) cells were analyzed for particle uptake using Forward and Side Scatter, (as well as FL2 for rhodamine-conjugated particles). Particle uptake by the cells results in vesicular and particle inclusions in the cells, resulting in increased Side Scatter (cite LeClerc L, et al). Data are presented as median fluorescence intensity.

3.8) *In vitro* Transfection Study:

In-vitro transfection study was carried out on HEK 293, HaCaT, Fibroblast cell lines. Transfection studies were carried out on different cells in a 24 well plate. Fugene® HD (Promega, Madison, WI) is a commercially-available liposome commonly used as positive control in some of the *in vitro* transfection studies. It is highly efficient in transferring the genes in *in vitro* settings. Naked plasmids in serum-free medium were used as negative control in all of the studies. Luciferase assay is a sensitive and rapid way to determine the transgene expression inside the cell.[64] The principle behind the assay is oxidation of luciferin by firefly luciferase which is a 62-kDa protein. Transition of luciferin to oxiluciferin produces as flash of light which can be quantified by a conventional luminometer.

Procedure:

Preparation of cells:

a) Cells were split into a 24-well plate a day before transfection with 150,000 cells per well for HaCaT and 180,000 cells per well for HEK293 and fibroblasts cells.

b) Cells were then allowed to grow in 400µL of corresponding full media in a 37°C incubator with 5% CO_2.

c) After 24 hours, when the cell confluency reached to 60-70%, the media was

aspirated, the cells were washed with 300 µL of DPBS and fresh media was added.

d) Positive Control:

   i. 2µg of stock plasmid was mixed with 394 µL of serum free media and gently vortexed.

   ii. 6µL of Fugene HD was added to the plasmid solution, vortexed immediately and incubated in room temperature for 5 minutes.

   iii. 400µL of Fugene® HD-DNA complex serum free medium was added to that well.

e) Nanoparticles:

   i. 360µL of serum-free media was added to each well.

   ii. Depending on plasmid loading, a certain amount of particles were weighed into a sterile 1.5mL micro-centrifuge tube and 5% mannitol in water was added (100µL/1mg) to compensate for the change in tonicity of the medium.

   iii. The tube was gently vortexed followed by a two-minute sonication in a bath sonicator containing ice.

   iv. 40µL of mannitol water-nanoparticles solution containing1µg DNA was added to each well.

f) Negative Control:

   1µg of stock plasmid solution was added to 400 µL of serum-free media, vortexed gently and was added to a well of 24-well plate.

g) The cells were kept in a 37°C incubator with 5 % CO_2.

h) After eight hours the positive control media was replaced with fresh full media. For nanoparticle and negative control containing wells, media was changed at 24 hours.

i) Cells were allowed to grow for 2 days.

j) Right before the luciferase assay, the media was discarded and cells were washed with 300µL of DPBS.

k) 100µL of cell 1x lysis buffer (Promega, Madison, WI) was added to each well and the plate was shaken at 100 rpm (5 minutes) by an orbital shaker SK-330-Pro (Scilogex, Berlin, CT) to cover the cells with 1x lysis buffer.

l) The plate was kept in -80°C freezer for 20 minutes to freeze followed by keeping at room temperature for 10 minutes to thaw. This was done 3 times in succession to achieve a total of three freeze-thaw cycles.

m) The lysed cells in buffer were centrifuged at 12,000g for 2 minutes at 4°C to separate the cell debris.

n) 20µL of supernatant containing luciferase protein was taken into a disposable culture tube (VWR international, West Chester, PA)

o) 100µL of luciferase assay reagent was added to it and mixed properly using a pipette.

p) The luminescence was counted for 12 seconds by Optocomp 1 (MGM instruments, Hamden, CT)

q) The number obtained was adjusted by subtracting the luminescence count from a blank tube.

4) Results and Discussion:

4.1) Characterization of Nanoparticles:

All PEI-DNA-PLGA nanoparticles had shown good particle size. Particle size of these nanoparticles was in the range between 200-300nm. Polydispersity index was also small (less than 0.1-0.2) for all these particles, which means there was narrow distribution of particles.

Figure 2.7 shows the size distribution of one of PLGA-PEI-DNA nanoparticles.

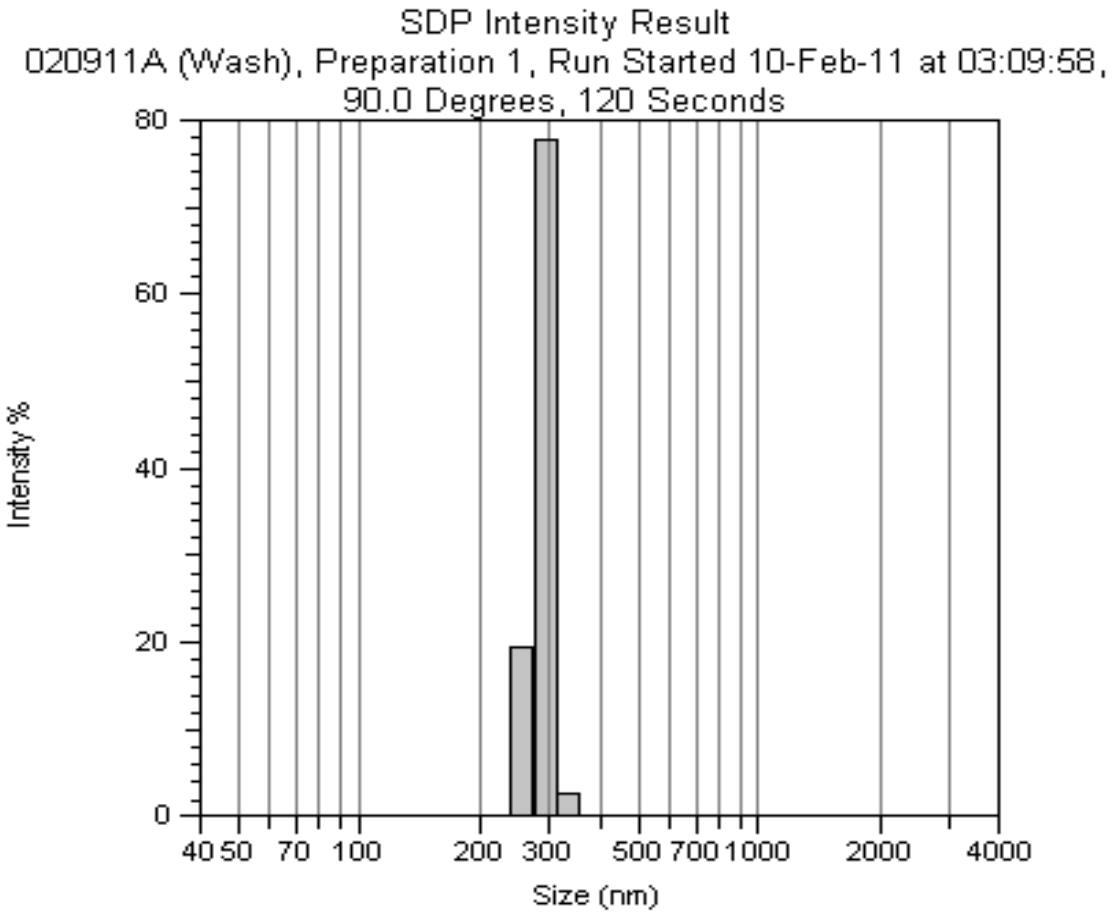

Figure 2.4: Size distribution of PEI-DNA-PGA nanoparticles

It was reported that the shape of the nanoparticles also impacted cellular uptake[65] of the nanoparticles.. All the nanoparticles showed spherical shape, which was confirmed by a scanning electron microscope as shown in figure 2.5.

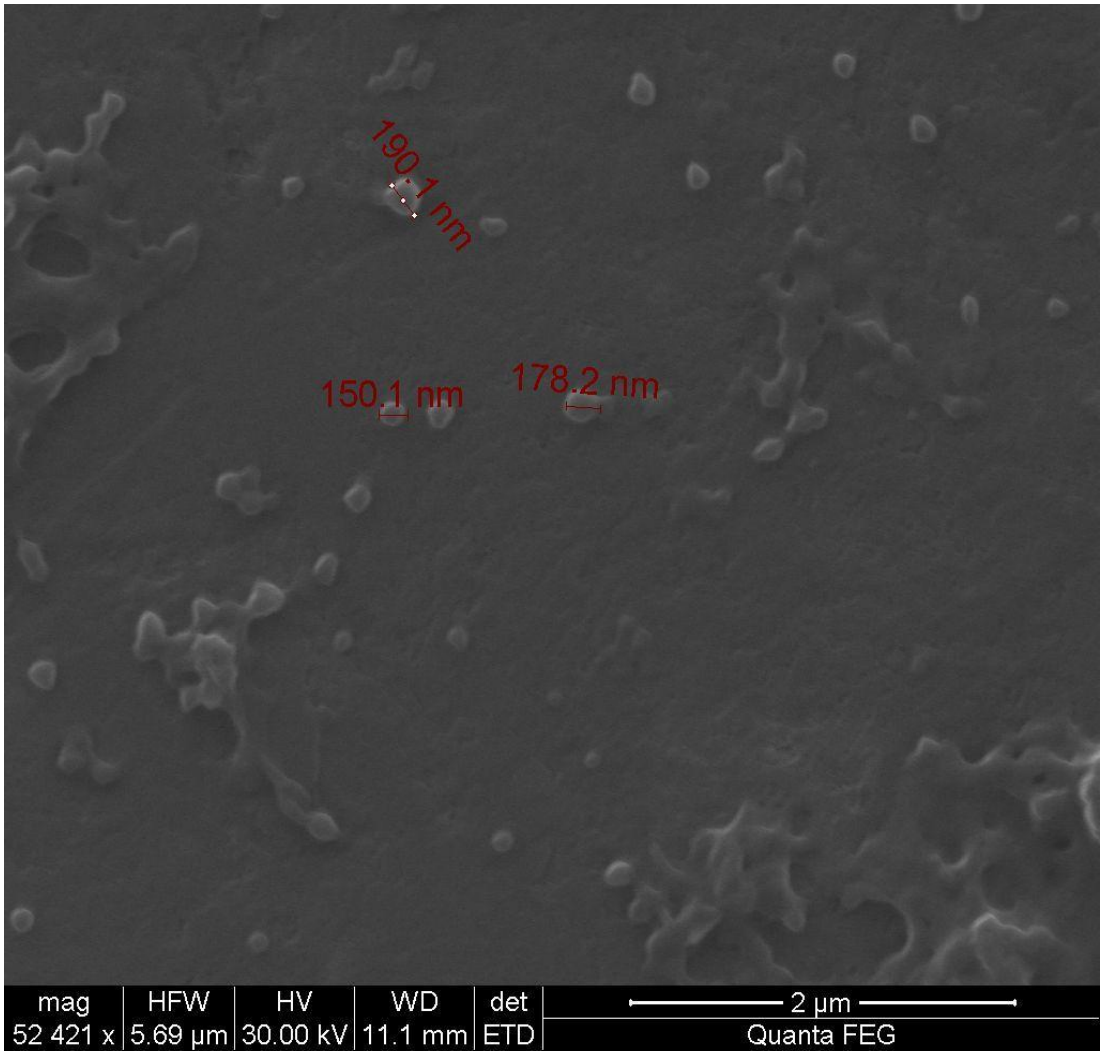

Figure 2.5: Scanning electron microscope image of PEI-DNA-PLGA nanoparticles

4.2) Effect of the method in mixing DNA with PEI:

Two formulations were prepared with different methods of PEI-DNA mixing.

A. 2 mL of PLGA-dichloromethane solution (oil phase) was added to PEI and sonicated. Then DNA (aqueous phase) was added and sonicated again. Lastly, external aqueous phase (2% PVA solution) was added and sonicated.

B. In this method, DNA was added to PEI solution, slowly pipetted 5 times and the mixture was kept aside for 15 minutes. Then oil phase was added and sonicated. Later, external aqueous phase was added and sonicated.

Type	Median Particle Size in nm	Polydispersity index	Entrapment Efiiciency
A(**110712C**)	247.2	0.072	88%
B(**110712A**)	224.4	0.048	97%

Table2.3: Effect of way of mixing of PEI-DNA

Although both particles showed same particle size, particles from method A showed less entrapment compared to particles from method B. The reason could be that when DNA was mixed with PEI first in the method B, PEI-DNA polyplex was formed and it would not come out easily from complex compared to that from method A. *In vitro* dissolution study of method A particles showed 30% of DNA release in 8 days with 20% of burst release in first 6 hours. Whereas, Method B particles showed 43% of DNA release in 8 days with 45% burst release in initial 6 hours. The reason could be that PEI-DNA complexes were retained on the surface of the PLGA nanoparticles. When PEI and DNA were mixed first in method B, more PEI-DNA complex were formed and retained on the surface and thus dissolved immediately. When they were mixed later (method A

particles), less amount of PEI-DNA was complexed, and thus less amount of DNA burst released compared to the particles from method B.

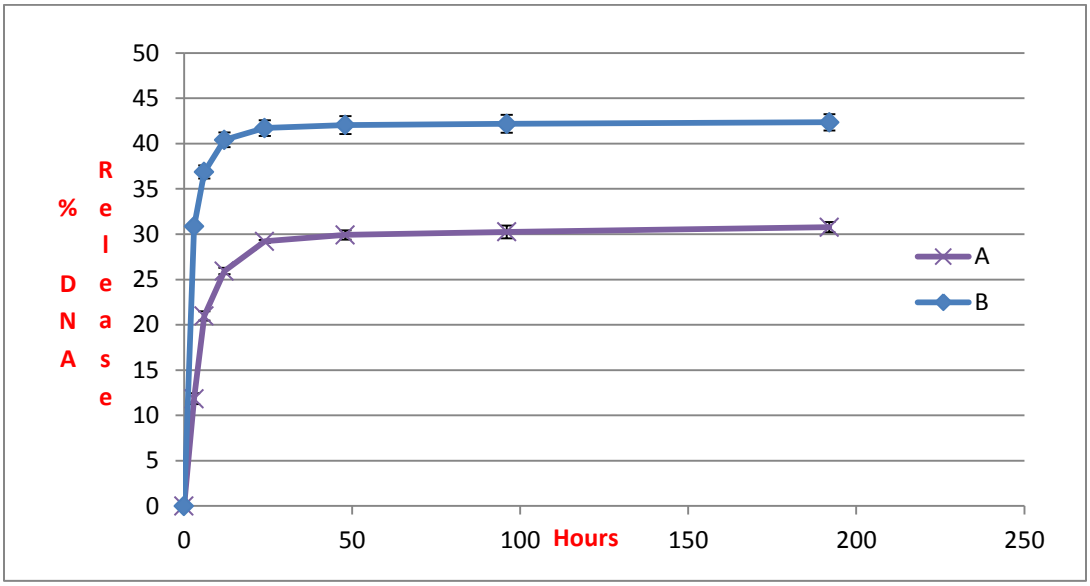

Figure 2.6: *In vitro* dissolution study of PEI-DNA-PLGA nanoparticles made with different way of mixing

4.3 N/P ratio of PEI-DNA complex:

PEI is a highly water soluble synthetic polymer. Structure of linear PEI has secondary amino and branched PEI has primary, secondary and tertiary amino groups. Every third atom of PEI is a nitrogen atom that can be protonated. Approximately 20% of the nitrogens of PEI are protonated under physiological conditions. PEI is a positively charged polycation because of these amino groups. The phosphodiester backbone of DNA is negatively charged; therefore, positively charged both branched and linear PEI forms complex with DNA. These electrostatic interactions are responsible for PEI mediated gene delivery. DNA condensation is dependent a cation to anion ratio or more specifically the nitrogen of PEI to the phosphate of DNA ratio (N/P ratio). This

condensation protects DNA from nuclease degradation in the cell. These closely packed particles can easily be taken up by cells through endocytosis or phagocytosis. Complexation and condensation is dependent on various factors such as polymer molecular weight and ratio of polymer to DNA.

Figures 2.5 shows gel electrophoresis study of the PEI-DNA complex with different N/P ratios. When the ratio was 1:1 by weight, the complexation was partial as there are 2 to 3 distinct bands in the gel. When the ratio was above 2, good condensation was achieved.

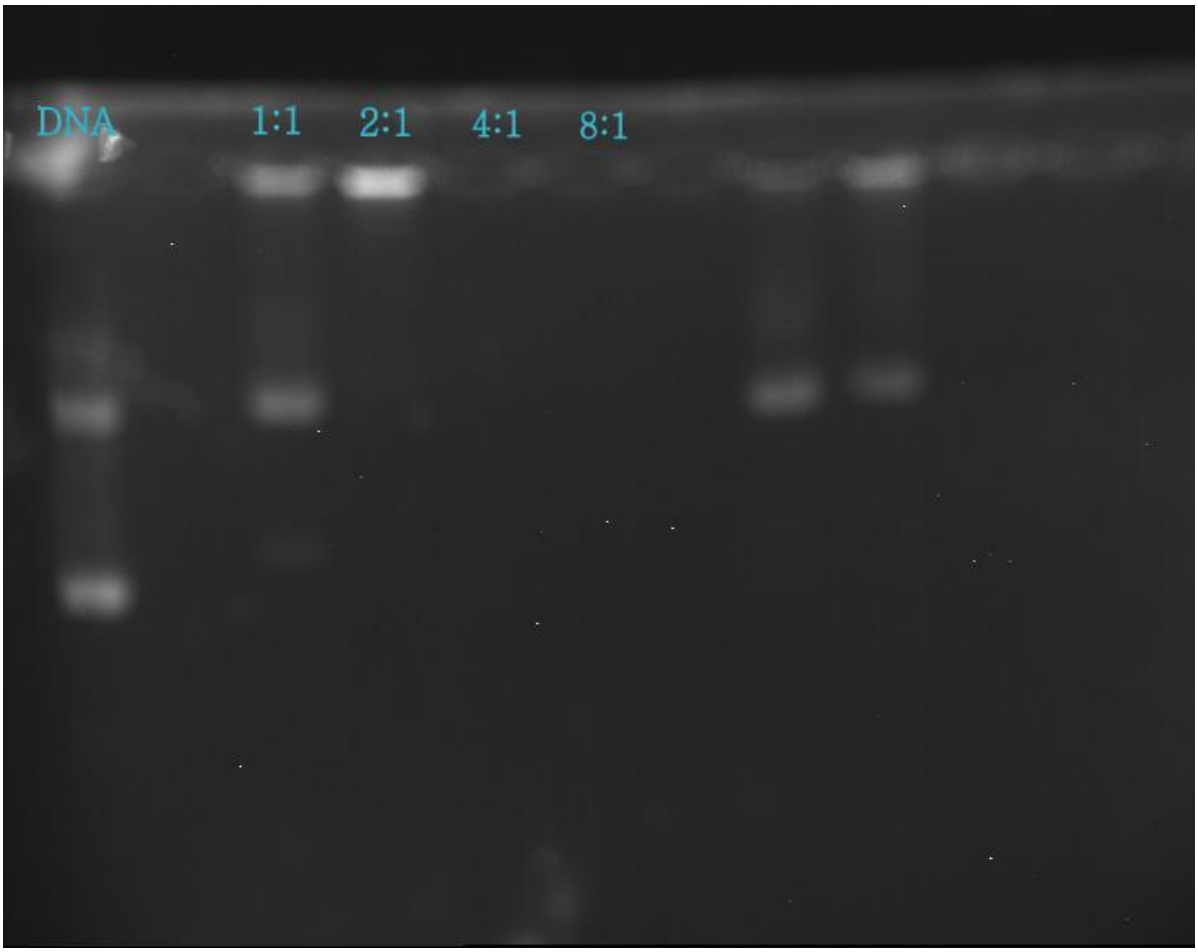

Figure 2.7: Gel electrophoresis of 25 kDa PEI-DNA complexes with different N/P ratios

Based on this, to study the effect of N/P ratio of PEI-DNA complex in PLGA nanoparticles, different types of formulations were prepared (using B method) with N/P ratio ranging from 2.5 to 10 with 25kDa PEI. All the formulation showed good condensation as the N/P ratio was above 2 in all of them.

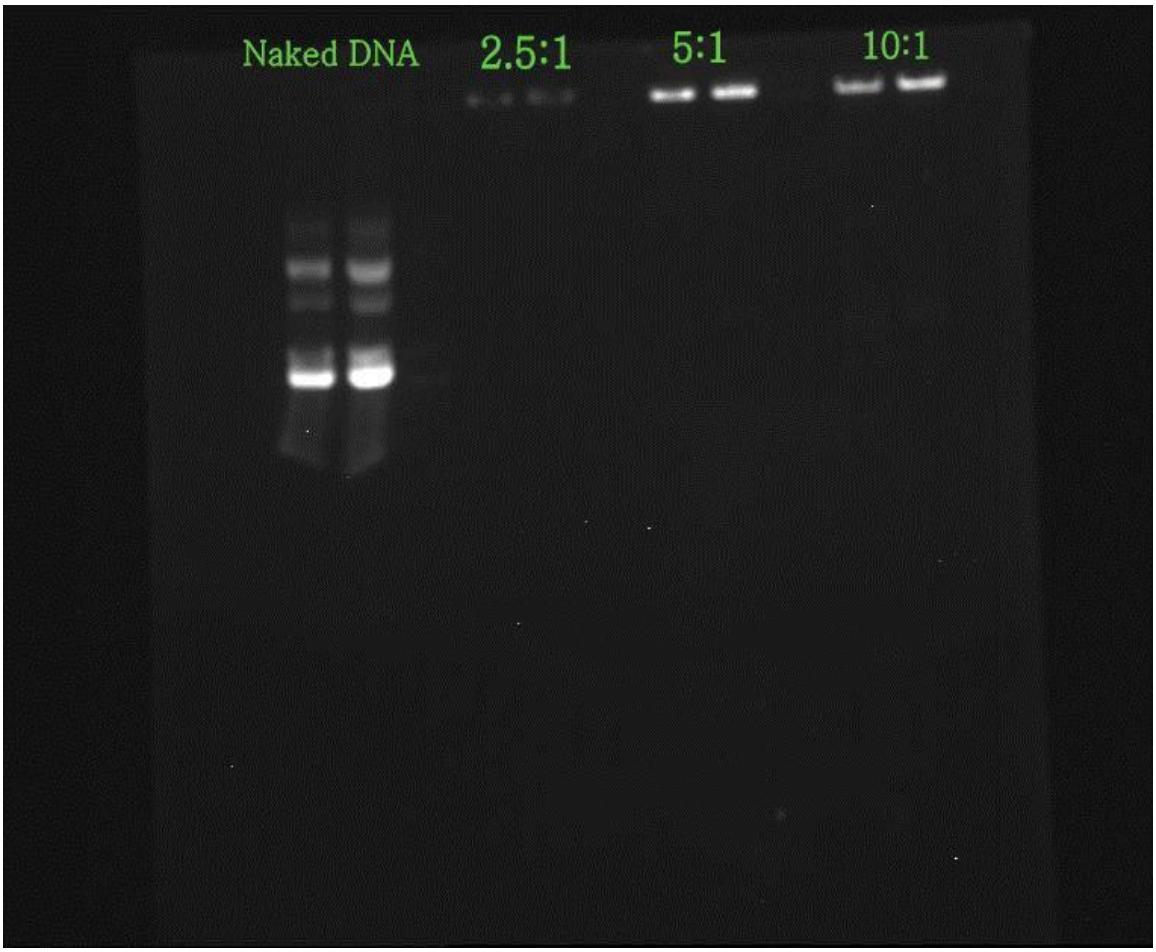

Figure 2.8: Gel Electrophoresis with different N/P ratio (25kDa PEI-DNA complex in PLGA nanoparticles)

To evaluate the effect of N/P ratio on DNA release in the cell, 25kDa PEI-DNA-PLGA nanaoparticles prepared with different N/P ratios were used.

PEI-DNA (N/P ratio)	Median Particle Size in nm	Polydispersity index	Entrapment Efiiciency
2.5 (**103112C**)	237	0.035	95%
5 (**110712A**)	224.4	0.048	97%
10 (**111512A**)	250	0.125	95%
20 (**111512C**)	257.2	0.185	78%

Table 2.4: Effect of N/P ratio

Nanoparticles with N/P ratio =2.5 had median particle size of 237 nm with a polydispersity index of 0.035, which means narrow distribution. The entrapment efficiency was 95%. When N/P ratio =5 nanoparticles were prepared, the median particle size was 225nm with 97% entrapment efficiency and 0.048 polydispersity. N/P ratio =10 nanoparticles did not show much difference compared to those from N/P ratio of 2.5 or 5. These particles had median particle size of 250nm and 95% entrapment efficiency. But, 20:1 N/P ratio formulation showed a little difference with 78% entrapment efficiency, though particle size and polydispersity has not changed much (257nm).

In vitro dissolution study of 2.5:1 N/P ratio particles showed 38% of DNA release in 8 days with 28% of burst release in first three hours. 5:1 ratio particles showed 45% of DNA release in 8 days with 35% burst release in initial 6 hours. 10:1 particles showed 60% DNA release in 8 days with 50% burst release in first 6 hours. As the ratio of N/P increases, release of DNA was also increased. The reason is, with the increase of PEI amount, amount of DNA to be complexed with PEI was also increased. So, N/P ratio of

10 showed more DNA release compared to 5 and N/P of 5 showed high DNA release compared to 2.5. But 20:1 particles showed less release compared to 10:1, which could be attributed to the stronger interaction between DNA and the higher amount of PEI. These particles showed 45% DNA release in eight days with 40% burst release in six hours. Though amount of released DNA was varied with different N/P ratios, all the particles showed burst release of DNA in initial 3-6 hours. This probably could be due to that significant amount of PEI-DNA complex was only entrapped on the surface of the PLGA nanoparticles.

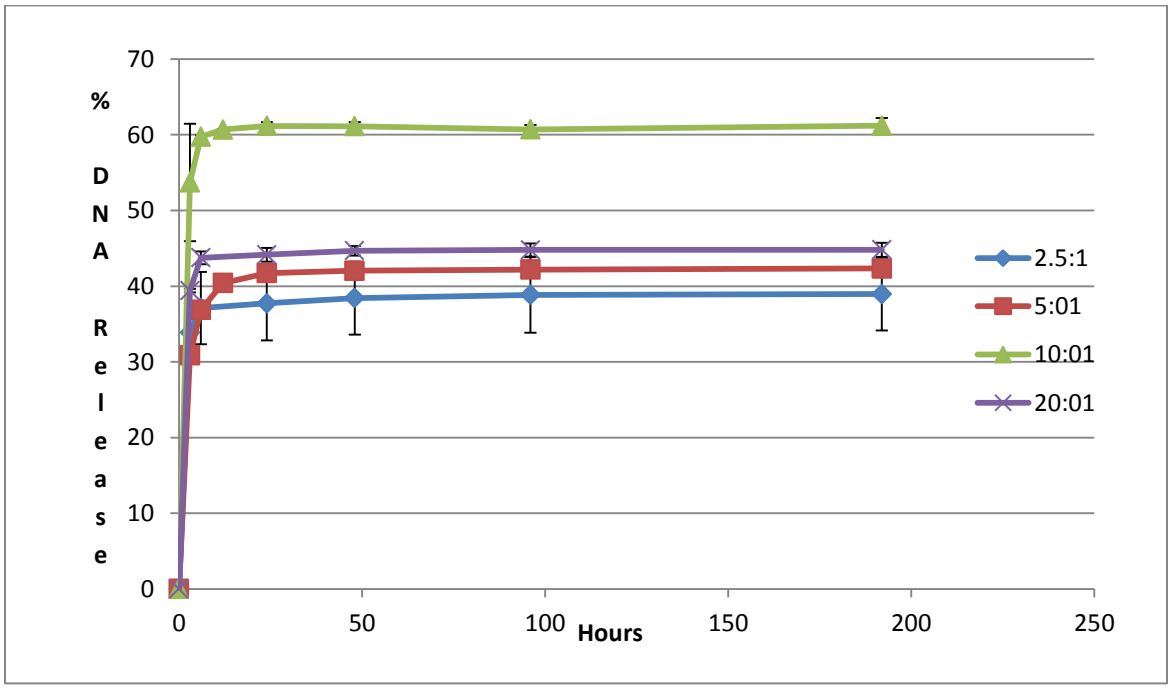

Figure 2.9: *In vitro* dissolution study of 25kDaPEI-DNA-PLGA nanoparticles made with 2.5:1, 5:1, 10:1, 20:1 N/P ratio

4.4) Effect of grade of PLGA:

To determine the effect of molecular weight of PLGA on PEI-DNA-PLGA nanoparticles, formulations with 0.24-0.54 dL/g and 0.95-1.2 dL/g PLGA containing

10:1 ratio of 25kDa PEI and plasmid DNA using method B, were prepared. 20 mg of PLGA in 2ml of dichloromethane was used for both formulations.

PLGA Grade	Median Particle Size in nm	Polydispersity index	Entrapment Efficiency
0.25-0.54dL/g **(LMW) (112012A)**	215.7	0.121±0.069	83%
0.95-1.2dL/g **(HMW) (111512A)**	250.2	0.125 ± 0.018	95%

Table 2.5: Effect of different grades of PLGA

Low molecular weight PLGA nanoparticles showed 215.7nm particle size and 83% entrapment efficiency. High molecular weight nanoparticles showed 250.2 nm particles size with high entrapment efficiency (95%). This is attributed to the high viscosity of the HMW PLGA. As outer oil layer of double emulsion is more viscous, that will limit the escape of PEI-DNA from the oil phase (see figure 2.10). *In vitro* dissolution test of particles made with LMW PLGA showed 10% more DNA release compared to HMW PLGA. The reason is, lower grade PLGA undergoes faster dissolution compared to high grade, thus more release of DNA was observed. When 0.25-0.54 dL/g PLGA (LMW) was used there was a burst release of about 60% of the DNA in first 6 hours and no more release later on. 0.95-1.2 dL/g PLGA (HMW) nanoparticles showed a 50% of burst release in 6 hours and less than 2% release afterwards. However, none of the formulations showed a good sustained release after the burst release. The reason is most of the released DNA was entrapped in the surface of the PLGA nanoparticles. PLGA polymer takes at least 30 days or more to degrade completely. As we performed

dissolution study just for 8 days, it can be understandable that burst release was because of the outside DNA and remaining DNA was still inside the PLGA polymer.

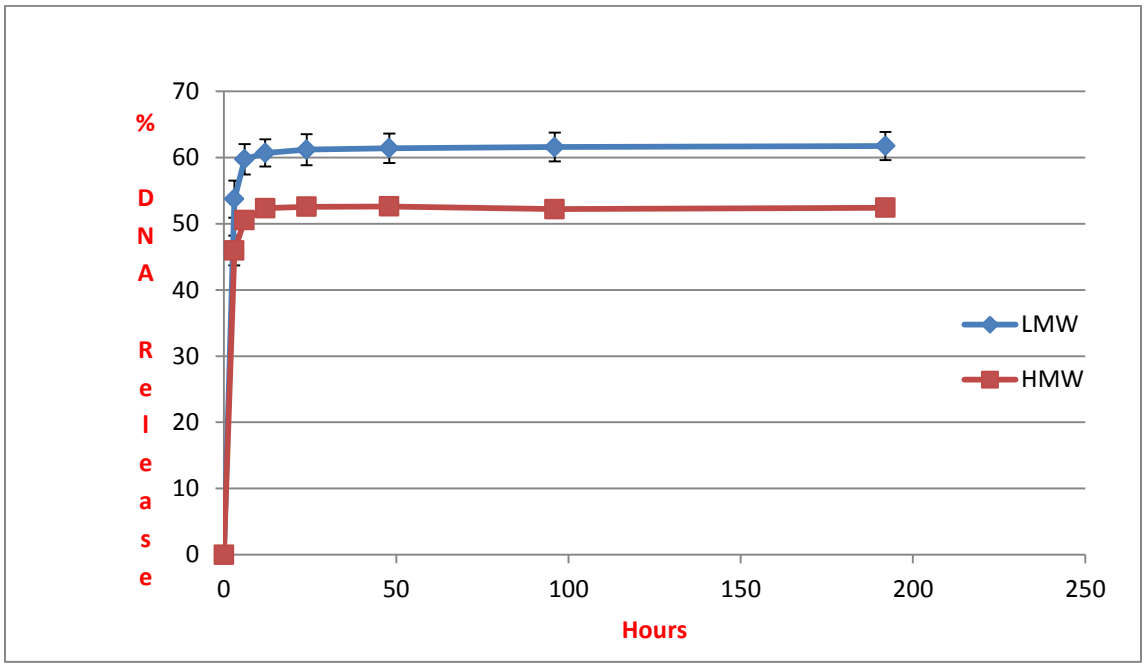

Figure 2.10: *In vitro* release of PEI-DNA-PLGA nanoparticles made with different grades of PLGA

4.5) Nanoparticles cell up taken study:

Florescence study has been done to check whether nanoparticles are being up taken by HEK293 cells.

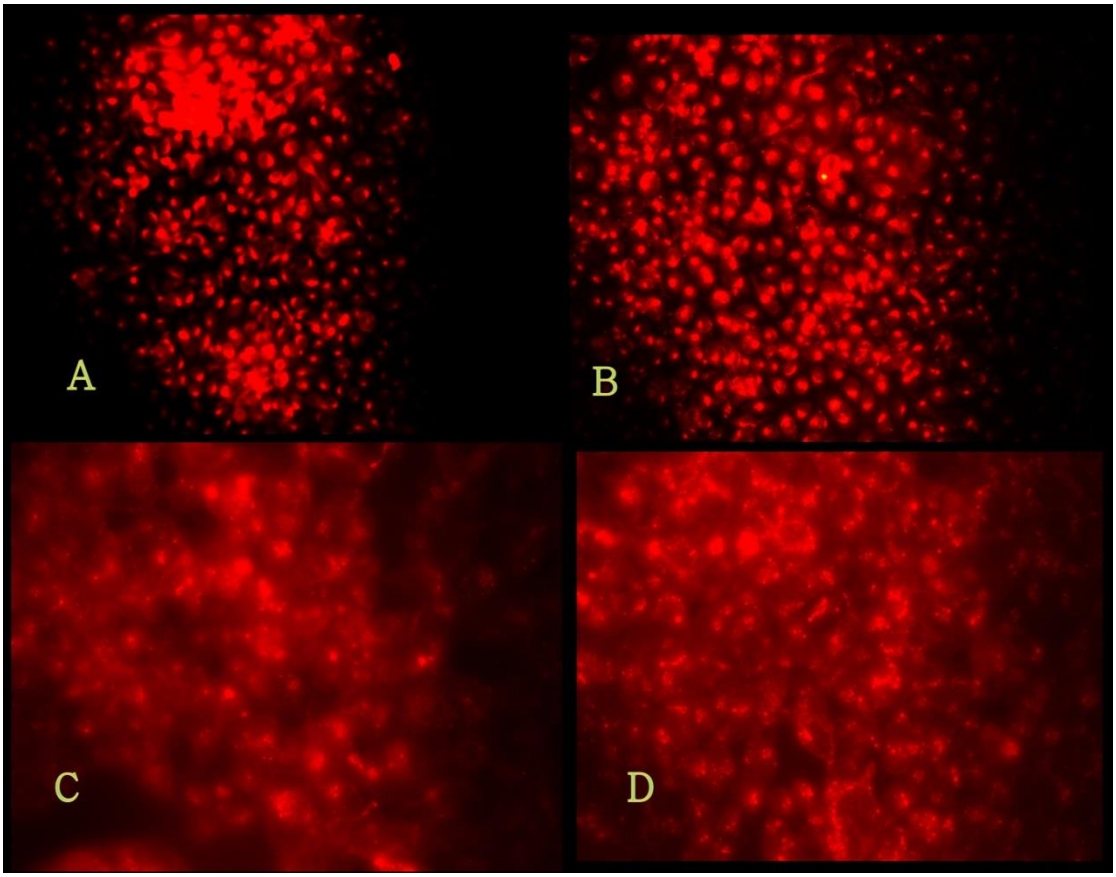

Figure 2.11: Florescence of PLA-Rhodamine and PLA-PEI-Cholesterol-Rhodamine nanoparticles in HEK293 cells at 6 hour and 24 hours

A. **HEK293 cells showing florescence of PLA-Rhodamine nanoparticles at 6 hours (20X lens)**
B. **HEK293 cells showing florescence of PLA-Rhodamine-PEI-Cholesterol at 6 hours (20X lens)**
C. **HEK293 cells showing florescence of PLA-Rhodamine nanoparticles at 24 hours (40X lens)**
D. **HEK293 cells showing florescence of PLA-Rhodamine-PEI-Cholesterol nanoparticles at 24 hours (40X lens)**

HEK293 cells under florescence microscope can be seen in figure 2.11. These cells showed florescence, when florescent light was on which means PLA-Rhodamine

nanoparticles prepared with PEI-cholesterol were being up taken by HEK293 cells. It can be observed in above figure.

After confirmation of the nanoparticles up taking, experiments were carried to check the up taking of nanoparticles at different incubation time using HEK293 cells.

When the confluency of plated cells reached 70%, nanoparticles were added to the cell media and incubated for 6 or 24 hours and then the florescence was observed under microscope.

From figure 2.11, it can inferred that more florescence was seen at 24 hours compared to 6 hours, which means more particles were up taken at 24 hours compared to 6 hours. The reason for this depends on many factors such as size,[66,67] shape[68] and importantly cell cycle.[69] Also, PLA-Rhodamine-PEI-Cholesterol nanoparticles showed more florescence compared to PLA-Rhodamine particles. The reason is, positively charged PEI easily crosses negatively charged cell membrane barrier.

Cell cycle is a process of cell division and replication. It consists four phases. G1, S, G2 and M phase. Each phase depends on the completion of previous phase.[70] Cell cycle starts with G1 phase, during which the cell increases its size. Cell synthesizes DNA in S phase and synthesizes proteins other materials in G2 phase to prepare for cell division. Finally, cell division takes place in M phase in which two daughter cells form and enter G1 phase. Cellular processes vary during each stage which means up taking of foreign materials/particles vary from phase to phase.[71] Endocytosis rate, which is essential for particles up take, increases during mitosis (after 23 hours).[72]

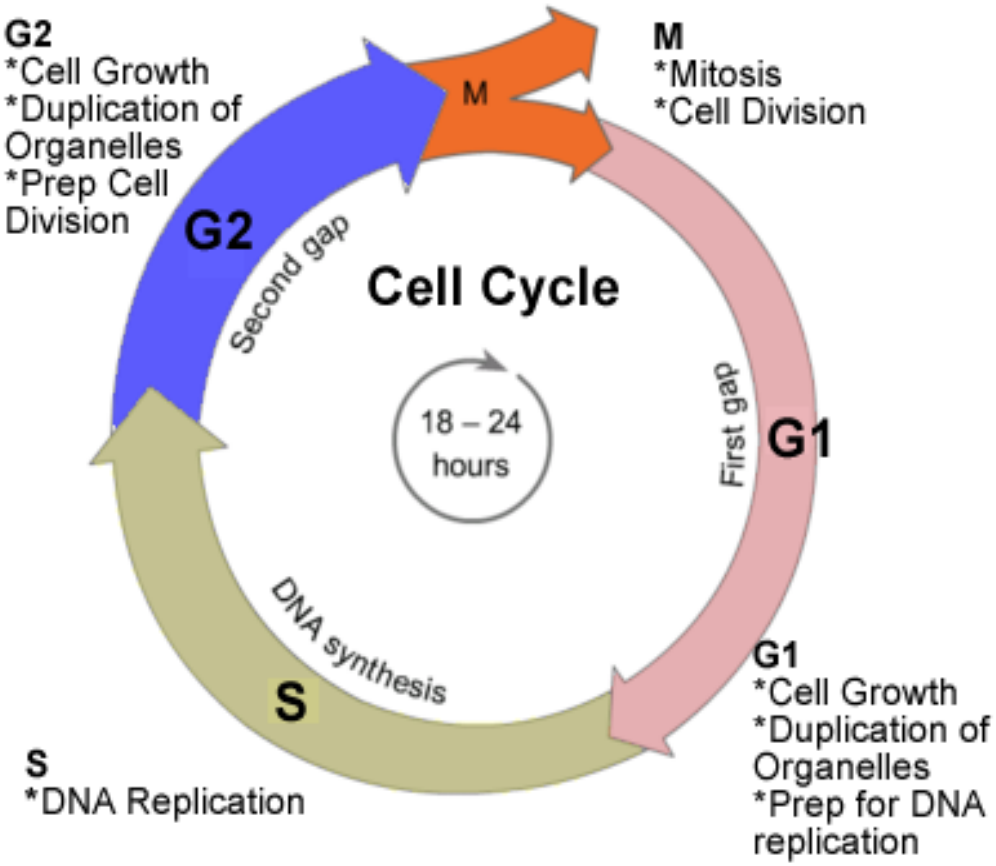

Figure 2.12: Cell cycle

Because of this rate increase of endocytosis, more particles had been up taken, which was the reason for more florescence at 24 hour time point.

We observed similar behaviors from other cell lines (HaCaT and Fibroblast) as well.

HaCaT cells:

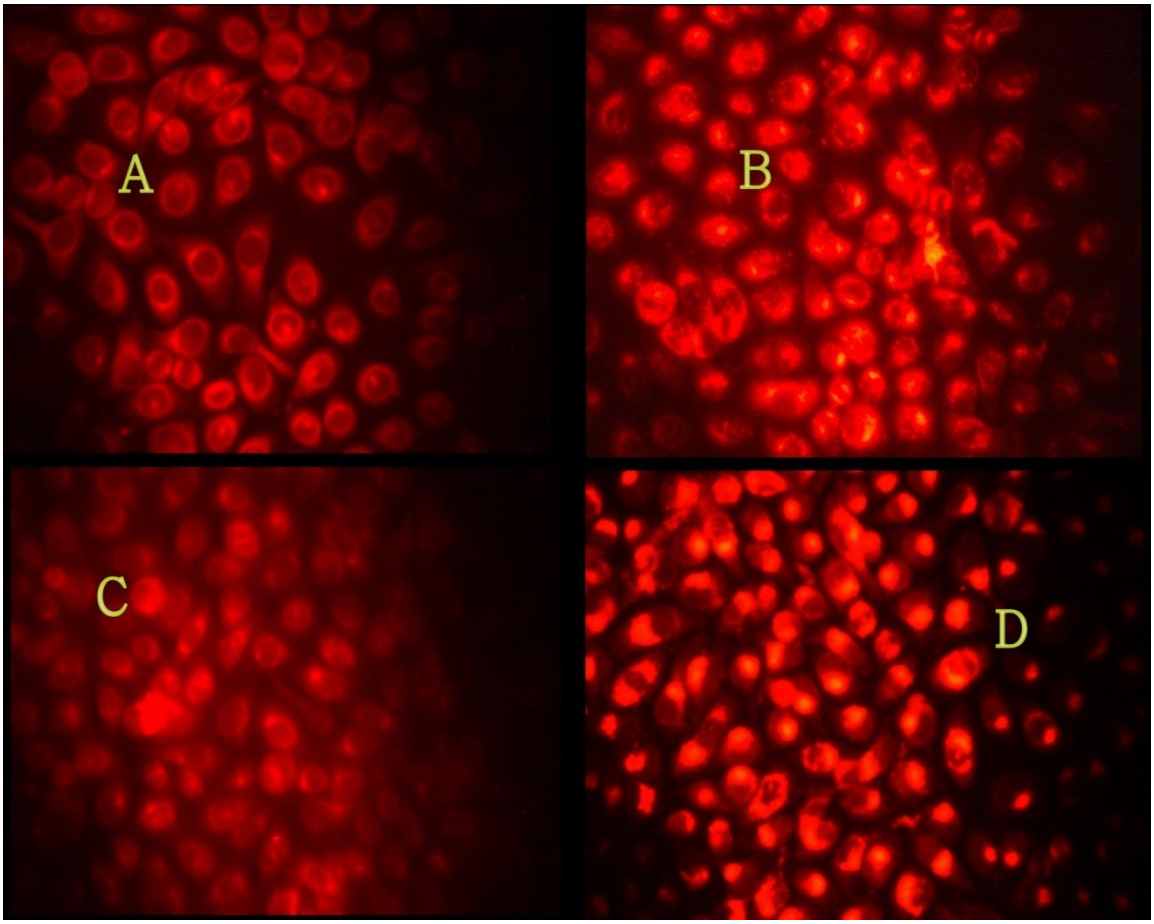

Figure 2.13: Florescence of PLA-Rhodamine and PLA-PEI-Cholesterol-Rhodamine in HaCaT cells at 6 hour and 24 hours

A. **HaCaT cells showing florescence of PLA-Rhodamine nanoparticles at 6 hours (40X lens)**
B. **HaCaT cells showing florescence of PLA-PEI-Cholesterol-Rhodamine nanoparticles at 6 hours (40X lens)**
C. **HaCaT cells showing florescence of PLA-Rhodamine nanoparticles at 24 hours (40X lens)**
D. **HaCaT cells showing florescence of PLA-PEI-Cholesterol-Rhodamine nanoparticles at 24 hours (40X lens)**

Fibroblast cells:

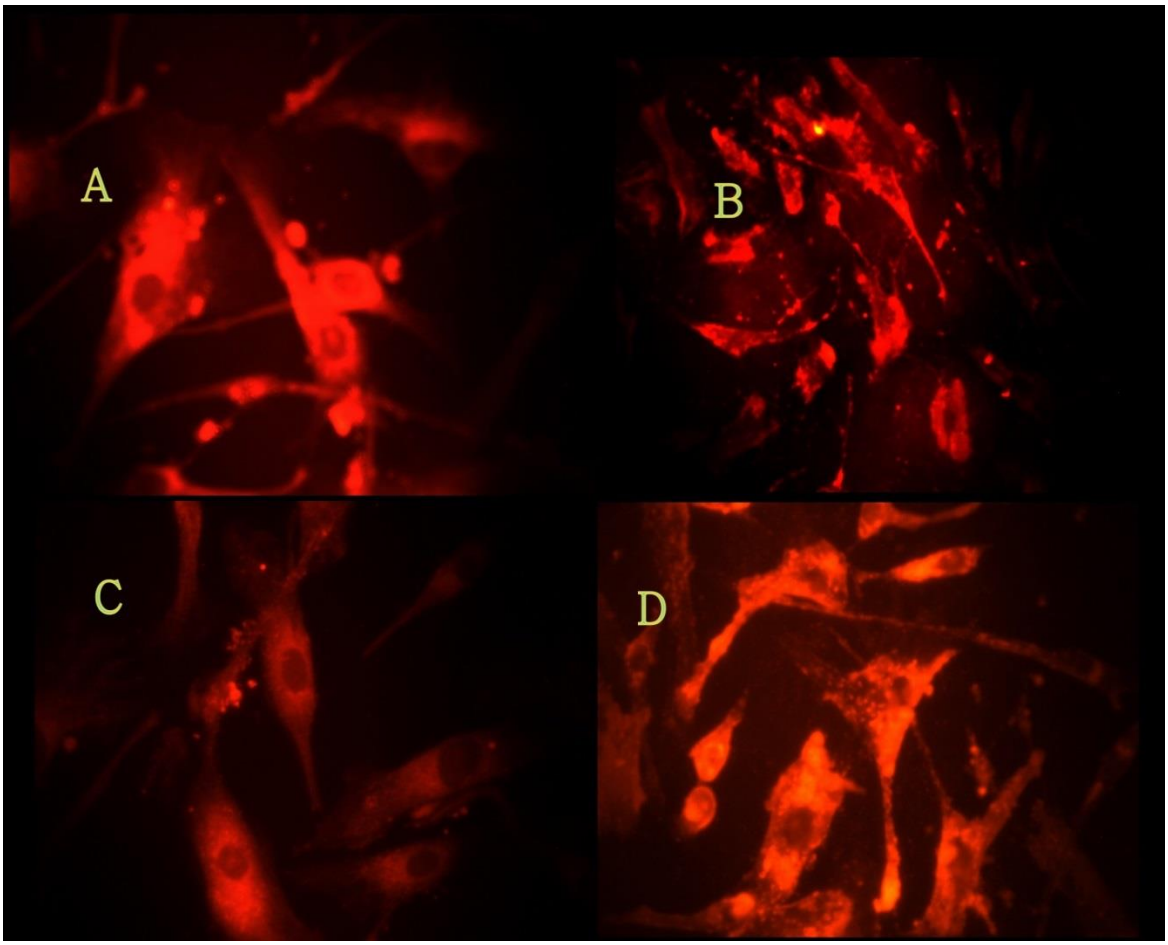

Figure 2.14: Florescence of PLA-Rhodamine and PLA-PEI-Cholesterol-Rhodamine in Fibroblast cells at 6 hour and 24 hours

A. **Fibroblast cells showing florescence of PLA-Rhodamine nanoparticles at 6 hours (40X lens)**
B. **Fibroblast cells showing florescence of PLA-PEI-Cholesterol-Rhodamine nanoparticles at 6 hours (40X lens)**
C. **Fibroblast cells showing florescence of PLA-Rhodamine nanoparticles at 24 hours (40X lens)**
D. **Fibroblast cells showing florescence of PLA-PEI-Cholesterol-Rhodamine nanoparticles at 24 hours (40X lens)**

Based on the above pictures, it can be concluded that all the cell lines had up taken nanoparticles and all of them had taken more particles at 24 hours compared to 6 hours.

The same was confirmed with flow cytometry studies also.

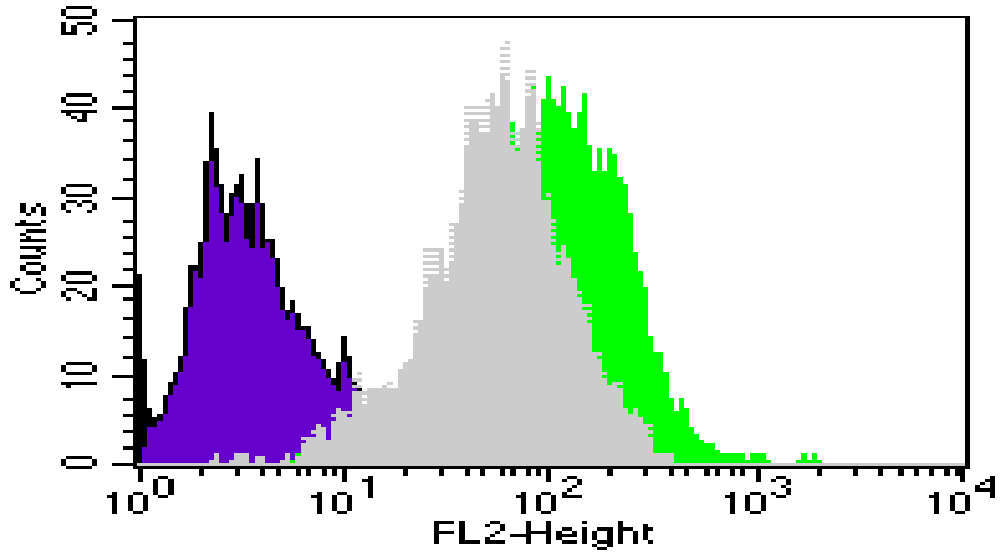

Figure 2.15: HEK293 live cells showing florescence with different particles at 6 hours

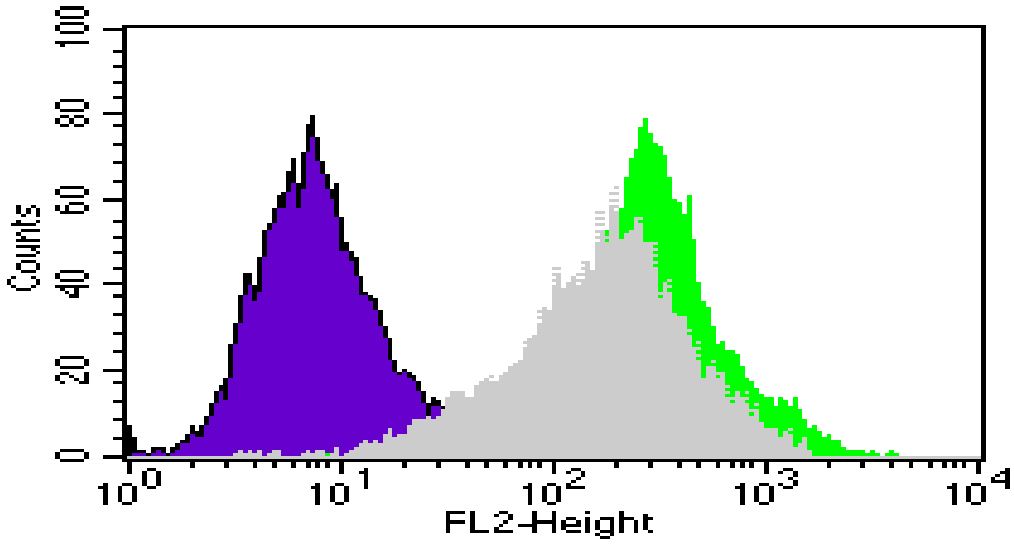

Figure 2.16: HEK293 live cells showing florescence with different particles at 24 hours

Blue- no particles (control), Grey- PLA-Rhodamine nanoparticles, Green- PLA-Rhodamine-PEI-Cholesterol nanoparticles

Figure 2.15 and 2.16 shows; there was no florescence when particles were not added.

When the study was carried out at different time points (6 hours and 24 hours) by adding PLA-Rhodamine and PLA-Rhodamine-PEI-Cholesterol nanoparticles, florescence was observed.

It can be seen in above pictures.

As explained earlier, the reason for more florescence at 24 hour time point is more up taking of nanoparticles by cells. Up take of particles depends on shape, size and cell cycle. As endocytosis rate is high at the end of the cell cycle which usually lasts for 24 hours, more up taking of nanoparticles at 24 hour time point can be understandable. Because of the more up taking, more florescence was shown by which was measured using flow cytometry. Also, PLA-Rhodamine-PEI-Cholesterol nanoparticles showed more florescence compared to PLA-Rhodamine particles. The reason is, positively charged PEI easily crosses negatively charged cell membrane barrier.

4.6) Transfection:

In vitro transfection study was carried out by PEI-DNA-PLGA nanoparticles. Luciferase assay was performed to get relative luminescence units by the amount of luciferase gene expressed.

Polyethylenimine is being widely investigated as an ideal carrier for gene delivery. As PEI has protonated amine, it has a positive charge which effectively condenses plasmids and protects them from degradation. Moreover, due to its positive charge, the probability of endocytosis is more. Transfection study by the PEI-DNA complex was done in various N/P ratios with different types of PEI with different

molecular weight. All the formulations showed better transfection than negative. Most of them, however, did not achieve transfection levels as compared to positive. 25kDa PEI-DNA (1µg) complex (N/P ratio of 10:1) was used as positive in all transfections. The reason why I used 25kDa PEI as positive control was because; it showed almost equal levels of transfection with Fugene® HD. Fugene® HD is a positively charged liposome which shows very high transfection in serum-free media. The main disadvantage with Fugene® HD is, in presence of serum the liposomes complex with serum and fail to transfect the cells, making it them ineffective *in vivo*. To overcome this problem, we used 25kDa PEI-DNA complex (N/P ratio of 10:1) as positive control, which could be effective *in vivo* also.

25 kDa branched PEI has become a remarkable polymer compared to other because of the following reasons:

1) High gene delivery efficiency[32, 73]
2) Cost effective and readily available.

After cellular uptake of nanoparticles by endocytosis, endosomal release is a critical barrier which affects the gene transfer efficiency as most of the DNA is retained in the endosomes and eventually degraded by lysosomal enzymes. Many methods have been developed to increase endosomal release. One amongst those is, using high buffer capacity systems known as "proton sponge" which is believed to reduce acidification of the endosome that results in swelling and rupture of membrane.[74]

Many transfection experiments were done to assess the effect of molecular weight of PEI, way of mixing and also the effect of two different grades of PLGA using different cell lines.

With the increase of molecular weight of PEI polymer, transfection efficiency has also been increased. It can be clearly observed from the below figure where 25kDa PEI-DNA complex showed higher transfection levels compared to 1.3kDa and 2kDa. This is mainly because; condensation ability increases with an increase in the molecular weight of the polymer which forms smaller, compact and much stable PEI/DNA polyplexes. These stable PEI/DNA polyplexes contribute to higher transfection efficiency of PEI 25kDa.[75]

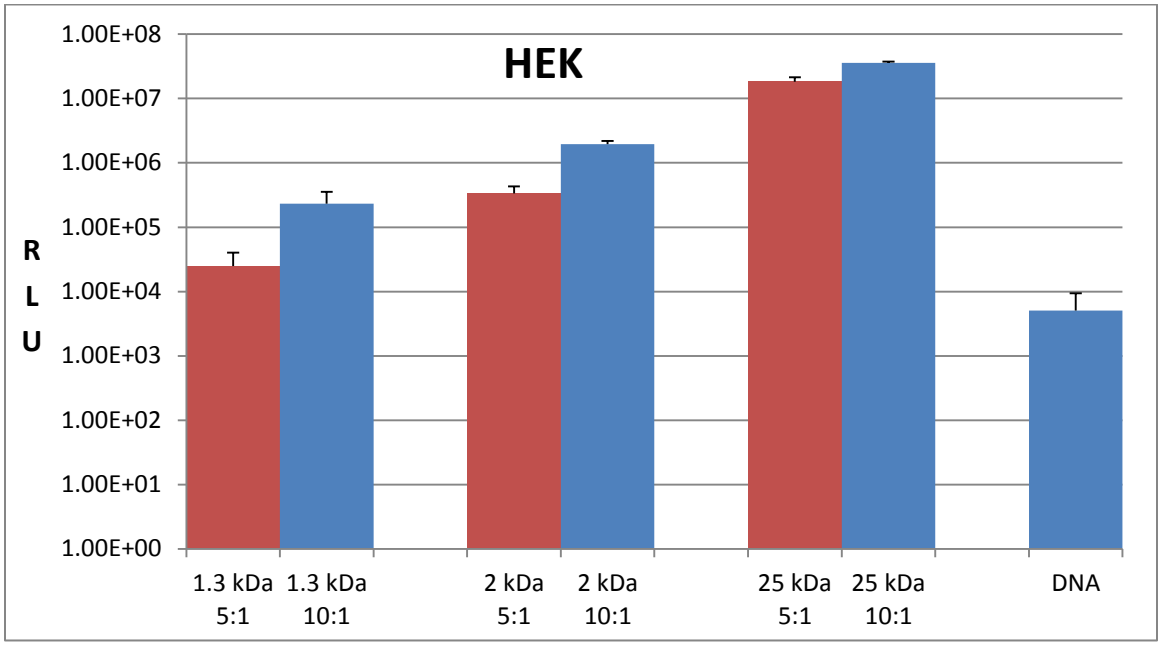

Figure 2.17: Comparison of transfection levels of different molecular weights of PEI-DNA complex with different N/P ratio in HEK 293 cells

All three molecular weights of PEI-DNA complexes showed similar transfection results with 5:1 and 10:1 N/P ratio.

25kDa PEI-DNA-PLGA nanoparticles prepared with method B showed higher transfection levels with 10:1 N/P ratio compared to 5:1. However, both of them showed less efficiency compared to positive control PEI-DNA complex.

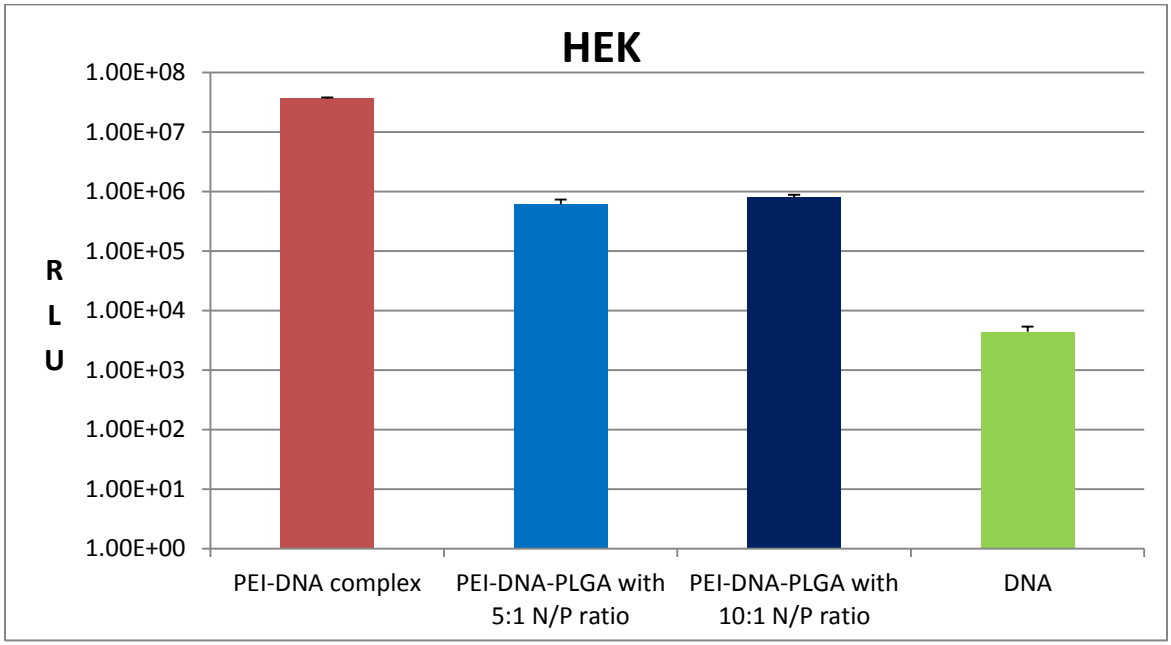

Figure 2.18: Comparison of transfection levels of 25kDaPEI-DNA-PLGA nanoparticles with different N/P ratio in HEK293 cells

Although, lower level of transfection was observed by HaCaT cells compared to HEK cells, they showed a similar pattern to HEK cells when transfection levels were compared with different molecular weights of PEI/DNA complex along with transfection levels of PEI-DNA-PLGA nanoparticles with different N/P ratio.

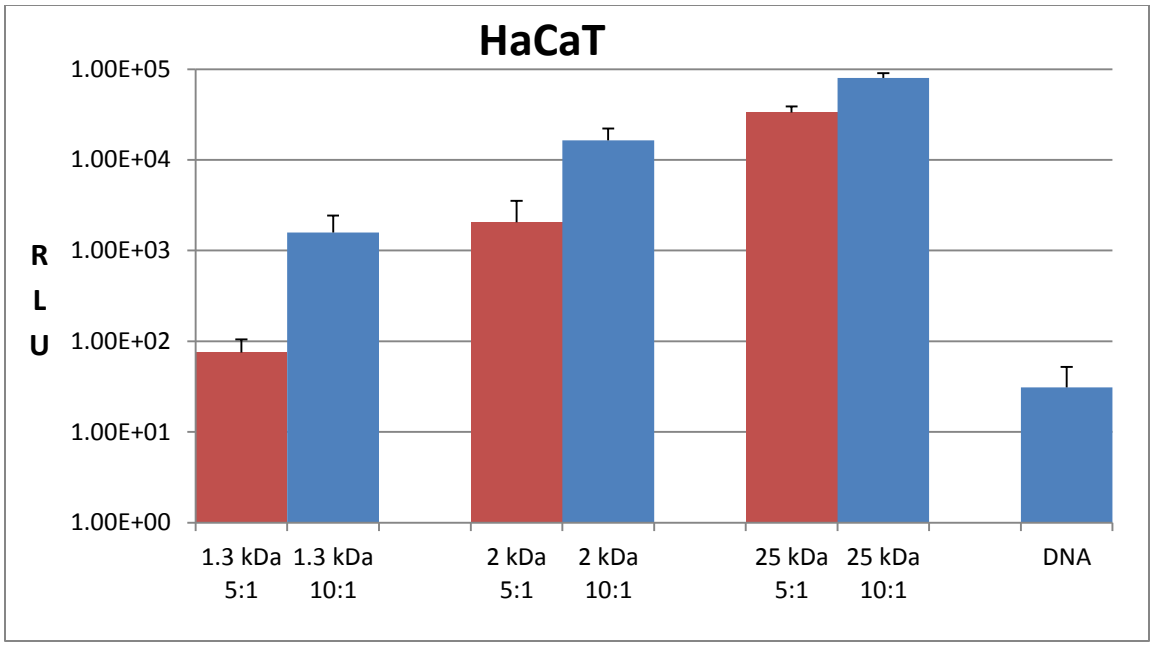

Figure 2.19: Comparison of transfection levels of different molecular weights of PEI with different N/P ratio in HaCaT cells

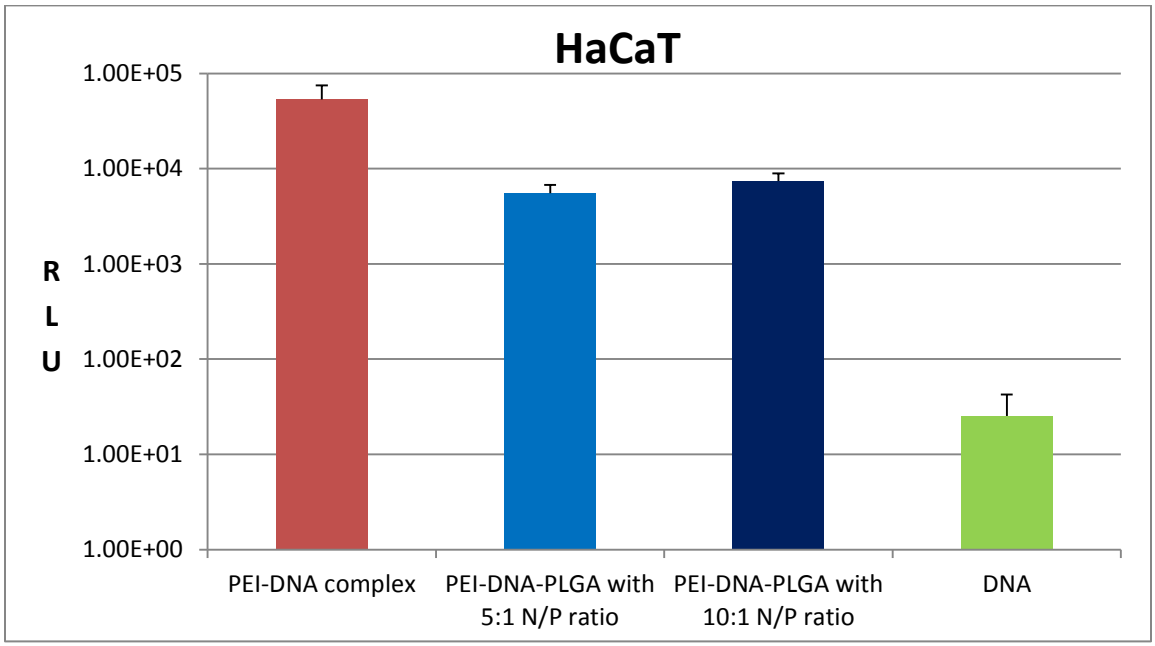

Figure 2.20: Comparison of transfection levels of 25kDaPEI-DNA-PLGA nanoparticles with different N/P ratio in HEK293 cells

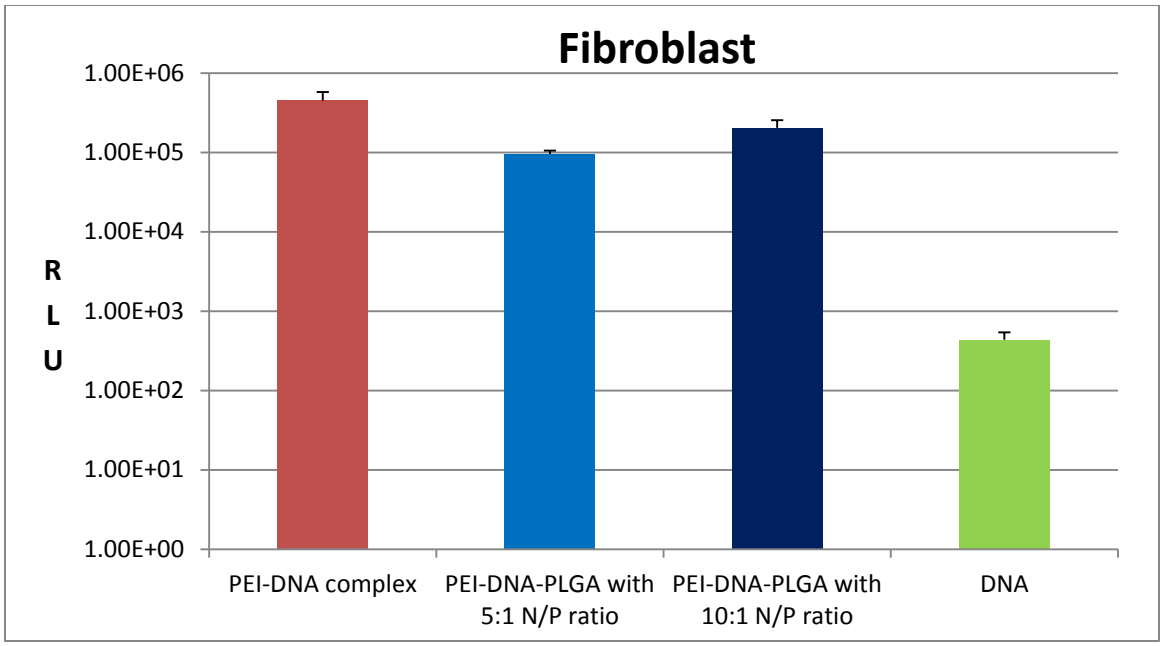

Figure 2.21: Comparison of transfection levels of 25kDaPEI-DNA-PLGA nanoparticles with different N/P ratio in Fibroblast cells

Along with HEK 293 cells, HaCaT and fibroblast cells also showed similar results of transfection. N/P ≥10 showed higher transfection levels with all molecular weights of PEI I used. When comparing transfection efficiency of all molecular weights, 25 kDa showed better gene delivery efficiency than 1.3 and 2 kDa PEI without causing any cell death.

As discussed earlier, condensation ability increases with an increase in the molecular weight of the polymer which forms smaller, compact and much stable PEI/DNA complexes. These stable PEI/DNA complexes contribute to higher transfection efficiency of PEI 25kDa in all cell lines.

Though HEK293, fibroblast cells showed good transfection results, HaCaT cells showed lower levels of transfection. The reason is, HEK293 and fibroblast cells are very easy to transfect. On the other hand, it is very difficult to transfect to HaCaT.

5. Conclusions:

The objective of this study was to develop a PLGA-PEI-DNA formulation with good transfection levels which will eventually help for *in vivo* gene delivery. The following conclusions were made after the completion of the study.

1. When plasmid DNA and PEI is mixed together, entrapment efficiency and DNA release are better compared to not mixed particles.
2. Good condensation can be achieved when N/P ratio of PEI-DNA is ≥2.
3. Entrapment efficiency and dissolution of PLGA nanoparticles or release of DNA is dependent on the molecular weight/viscosity grade of PLGA. High molecular weight PLGA shows high entrapment along with slow dissolution of nanoparticles compared to low grade.
4. Nanoparticles are up taken by different cell lines. More particles are up taken at 24 hour time point compared to 6 hours.
5. PEI-DNA complex with 10:1 N/P ratio shows good transfection results which are on par with other positive controls such as Fugene® HD.
6. HEK293, HaCaT, Fibroblast cells show good transfection with PEI-DNA-PLGA nanoparticles. However, HEK293 cells show higher transfection levels compared to other two cell lines.

Chapter 3

Evaluation of CaP-PEI-DNA-PLGA nanoparticles

1) Introduction:

Although good gene expression levels were observed with PEI-DNA-PLGA nanoparticles in the previous chapter, they were far lower than that from the PEI-DNA complex. The PEI-DNA complexes showed 100 fold higher gene expression levels compared to all PEI-DNA-PLGA formulations. The purpose of the study in this chapter was to exploring a new method for improving the transfection efficiency of the PEI-DNA-PLGA nanoparticles. In this method, calcium ions from calcium chloride salt react with phosphate ions of sodium phosphate and form calcium phosphate precipitate, which entraps the DNA molecules in the precipitation.[76] Concentration of calcium, phosphate, DNA play important role in forming these particles.[77]

Calcium phosphate precipitation is a good transfection agent [78] that was initially investigated by Graham.[79] At the time, he used it with adeno virus DNA. Laterly, Wigler found that exogenous DNA can be integrated into mammalian chromosomes using calcium phosphate. [80] Gradually, many methods have been developed for gene delivery using calcium phosphate precipitate. The formation of calcium phosphate precipitation from calcium chloride salt and sodium phosphate dibasic salt will also generate a hydrochloride acid. The generated acid can be used to protonate the PEI for condensation of the DNA molecules during the encapsulation into the PLGA nanoparticles.

2) Methods:

2.1) Preparation of CaP-PEI-DNA-PLGA nanoparticles:

CaP-PEI-DNA-PLGA nanoparticles were prepared by a modified double emulsion solvent evaporation method. The detailed procedure of this preparation is as follows:

Primary Emulsion	
1.Organic Phase	
Ingredient	Category
HMW PLGA 50:50	Encapsulating Polymer, surfactant for primary emulsion
Dichloromethane	Organic Solvent
2.Aqueous Phase 1	
PEI+$Na_2HPO_4.7H_2O$	
3.Aqueous Phase 2	
GFP-Luciferase Plasmid+ $CaCl_2$	
Secondary Emulsion (Aqueous phase 2)	
Polyvinyl Alcohol (MW 30,000-70,000)	Surfactant for secondary emulsion

Table 3.1: Formula for preparation PEI-DNA-PLGA-Calcium phosphate nanoparticles

Procedure:

a) HMW Poly (D, L-Lactide-co-glycolide) 50:50 was dissolved in 2ml of dichloro methane at room temperature.

b) 100µL of 25kDa PEI (7.81 mg/ml) solution was mixed with 100 µL of 0.66M $Na_2HPO_4.7H_2O$ and kept aside for 15 minutes.

c) 150µL of plasmid DNA (2.4 mg/ml) was mixed with 50µL of 2M $CaCl_2$ and kept aside for 15 minutes.

d) Then 1ml of PLGA-DCM solution was added to b and c complexes and sonicated.

e) Then these two solutions were mixed and sonicated again.

f) Lastly, 12 ml of 2% PVA solution was added to the primary emulsion and sonicated again for two minutes to form the W/O/W double emulsion.

g) At this point samples were taken for measuring the size and polydispersity index of the emulsion droplet.

h) The double emulsion was then stirred at room temperature for 4 hours.

i)When the evaporation of organic solvent was complete, the nano suspension was subjected to ultracentrifugation by a centrifuge (Avanti® J-26 XPI, Beckman Coulter, Brea, CA) at 4°C to collect the nanoparticles.

j) The particles were washed two times with 5mL of sterile water to get rid of the unentrapped plasmids and residual PVA.

k) The supernatant of original suspension and subsequent washes was collected and stored at 4°C to determine the unentrapped plasmids.

l) Finally the pallets were resuspended in 5ml of sterile water and samples were

taken to measure the particle size and polydispersity index by a N4 Plus particle size analyzer.

m) 100mg of mannitol was added to the suspension as a cryoprotectant.

n) The nanoparticle suspension was frozen by liquid nitrogen and lyophilized for two days by a freeze dryer. (Unitop 400SL, Virtis, Gardiner, NY)

o) The powdered PLGA nanoparticles were stored at 4°C until further use.

2.2) Characterization of Nanoparticles:

N4 Plus submicron particle sizer (Coulter Corporation, Miami, FL) was used to determine the particle size and polydispersity and the analysis was done by unimodal analysis and size distribution processor (SDP) analysis.

Procedure:

a) Small amount of particles/emulsion was diluted to optimum confluency (50,000-1,000,000 counts per second) using Type 1 particle free water. For the dry particles the suspension was sonicated in a bath sonicator for 2 minutes.

b) The sample was incubated at 25°C for 1, 5 and 10 minutes before the actual measurement was taken.

c) Particle size measurement was done with a light scattering at 90° angle for two minutes.

d) Scanning Electron Microscopy (SEM) analysis:

1) Freeze dried nanoparticles were resuspended in 1mL type 1 water in an eppendorf tube.

2) Centrifugation was done for 10 minutes at 13,000 rpm (4°C) by a bench-top centrifuge (Micromax RF (Thermo Scientific, Waltham, MA)).

3) Then the particles were resuspended again in type 1 water by sonicating in a bath sonicator.

4) Then the particles were air dried on aluminum stubs.

5) A gold sputter was used to coat the samples by gold under vacuum.

Finally, images were taken by taken by a scanning electron microscope (Quanta FEG

200, FEI, Hillsboro, OR).

2.3) Determination of DNA Entrapment Efficiency

Encapsulation of plasmid inside the nanoparticles was determined by an indirect method. Encapsulation efficiency was determined by the following equation:

$$\% \text{ Encapsultion} = ([DNA]_{total} - [DNA]_{free}) / ([DNA]_{total} \times 100)$$

Entrapment efficiency was not determined by a direct method as presence of polyethylenimine in these formulations resulted in inefficient extraction of DNA from the PLGA nanopartices. Therefore, the DNA lost in the outer phase of double emulsion during the nanoparticles preparation and also in the wash steps were determined to calculate the entrapment efficiency.

2.4) *In vitro* Release Study of Nanoparticles:

In vitro dissolution study was done to assess the release profile of plasmids from various types of PLGA-PEI nanoparticles; the study was done at 37°C and the dissolution medium was 1 ml Tris-EDTA and sodium phosphate buffer pH-7.4. Samples were collected by centrifuging down the nanoparticles and the picogreen assay was done on the supernatant to determine the amount of DNA released. The

samples were collected at 1st, 3rd, 6th, 12th, 24th, 48th, 96th and 192th hours. Following is the general protocol for the dissolution study:

Dissolution medium	Tris-EDTA Sodium Phosphate buffer pH-7.4
Dissolution apparatus	Eppendorf-R Thermomixer with attached 1.5ml microcentrifuge tube holder.
Sample volume withdrawn at each time interval	900 µL
Mixing speed	300
Temperature	37°C
Sampling time intervals	1st, 3rd, 6th, 12th, 24th, 48th, 96th, 192th hours

Table 3.2: Dissolution study protocol

Procedure:

a) Sample and blank PLGA-PEI nanoparticles (3mg including mannitol) were taken into a sterile 1.5mL micro-centrifuge tube and 1mL buffer solution was added to the particles and vortexed briefly to resuspend the particles.

b) Tubes were placed in a thermomixer (Thermomixer R, Eppendorf, NorthAmerica, Hauppauge, NY, USA) and shaken at 300 rpm at 37°C in phosphate buffer.

c) At predetermined time points the tubes were centrifuged down by Thermo IEC Micromax RF (Thermo Scientific, Waltham, MA) at 13,000 rpm for 10 minutes (4°C) and 900 µL of supernatant was collected and stored at -20°C.

d) Fresh buffer (900µL) was added to each tube and the tubes were subjected to sonication by a bath sonicator for approximately 30 seconds.

e) The tubes were again placed in the thermo-mixer and shaken in the same conditions until the next time point.

A picogreen assay was carried out to determine the amount of plasmid release at various time points using the blank particle release medium spiked with several known amount of plasmids as standards.

2.5) *In vitro* Transfection Study:

In-vitro transfection study was carried out on HEK 293, HaCaT and Melanoma cell lines. Transfection studies were carried out on these cells in a 24 well plate. Transfection was carried out for three days. 25 kDa PEI with 1µg DNA was used positive control. Naked plasmid in full medium was used as negative control in all of the studies. Luciferase assay is a sensitive and rapid way to determine the transgene expression inside the cell. The principle behind the assay is the oxidation of luciferin by firefly luciferase which is a 62-KDa protein. Transition of luciferin to oxiluciferin produces as flash of light which can be quantified by a conventional luminometer.

Procedure:

Preparation of cells:

a) Cells were split into a 24-well plate one day before the transfection with 150,000 cells per well for HaCaT and 180,000 cells per well for HEK293 and melanoma cells.

b) The cells were then allowed to grow in 400µl of corresponding full medium in a 37°C incubator with 5% CO_2.

c) After 24 hours, when the cell confluency was reached to 60-70%, the medium was aspirated, and cells were washed with 300µL of DPBS and then full medium was added.

d) Positive Control:

i. 25µL of plasmid DNA (40µg/mL) was complexed with 25µL of 25 kDa PEI (52µg/mL) (N/P ratio of 20:1) and kept aside for 15 minutes.

ii. After 15 minutes, 50µL of this complex was added to 350µL of full medium in a well.

e) Nanoparticles:

i. 360µL of full medium was added to each well.

ii. Depending on the plasmid loading, certain amount of particles were weighed and taken in a sterile 1.5mL micro-centrifuge tube and 5% of mannitol water was added (100µL/1mg) to compensate for the change in tonicity of the medium.

iii. The tube was vortxed gently followed by a two-minute sonication in a bath sonicator containing ice.

iv. 40µLof mannitol water-nanoparticles (1µg DNA) solution was added to each well.

f) Negative Control:

1µg of stock plasmid solution was added to 400µL of full medium and vortexed gently and added to a well of 24-well plate.

g) Cells were then kept in the 37°C incubator with 5 % CO_2.

h) After 24 hours, medium was replaced with 400 µL of fresh medium.

i) Cells were allowed to grow for two more days.

j) Before the luciferase assay, the medium was discarded and cells were washed with 300µL of DPBS.

k) 100µL of cell 1x lysis buffer (Promega, Madison, WI) was added to each well and the plate was shaken at 100 rpm (5 minutes) by an orbital shaker SK-330-Pro (Scilogex, Berlin, CT) to cover the cells with 1x lysis buffer.

l) The plate was then kept in -80°C freezer for 20 minutes to freeze and then kept at room temperature for 10 minutes to thaw. This was done three times to achieve a total of three freeze-thaw cycles.

m) The lysed cells in the buffer were centrifuged at 12,000g for two minutes at 4°C to separate the cell debris.

n) 20µL of supernatant containing the luciferase protein was taken in a disposable culture tube (VWR international, West Chester, PA)

o) 100µL of luciferase assay reagent was added to it and mixed properly by a pipette.

p) The luminescence was counted for 12 seconds by Optocomp 1 (MGM instruments, Hamden, CT)

q) The number obtained was adjusted by subtracting the luminescence count for the blank tube.

2.6) Cytotoxicity study:

Cytotoxicity study was carried using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay to check the toxic effects of PEI-DNA complex and CaP-PEI-DNA-PLGA nanoparticles.

MTT assay:

HEK293 cells were seeded at a density of 7000 cells/well in 96-well plates after harvesting cells from cultures and the cells were allowed to attach to the bottom of the plates. After 24 hours of incubation, cells were treated with PEI-DNA complexes and specified solutions of CaP-PEI-DNA-PLGA nanoparticles prepared in medium supplemented with serum. Untreated cells were used as control. Following the desired incubation time, 20 μL of solution of MTT (prepared in 1X PBS at a concentration of 5 mg/mL) was added and incubated for 3 hours in the incubator at 37^0C. Following that incubation, the medium with the MTT dye was aspirated and 100 μL of DMSO was added to each well to solubilize the formazan crystals and the resultant absorbance was measured at 570 nm using a plate reader.

Cell viability (%) = (absorption test/ absorption control) * 100

3) Results and Discussion:

3.1) Effect of Heparin on release of DNA from PEI:

Two formulations were prepared using plasmid DNA, calcium chloride and sodium dibasic phosphate. PEI was added to one formulation and the other was made without PEI.

Type	Median Particle Size in nm	Polydispersity index	Entrapment Efiiciency
A. With PEI (**061513A**)	1150	0.432	98%
B. Without PEI (**062513A**)	950	0.350	96%

Table 3.3 Particle size of nanoparticles prepared with calcium phosphate

Both formulations showed similar entrapment efficiency although particle size was varied. The reason for increase in particle size was due to aggregation of amorphous calcium phosphate precipitate in aqueous phase. Because of this aggregation, particles became bigger with 900-1100 nm size compared to PLGA-PEI-DNA smaller particles. Polydispersity was also increased which means particle distribution was broad.

In vitro dissolution study of method A particles showed 30% of DNA release in 8 days with 20% of burst release in first 12 hours. Method B particles showed 43% of DNA release in 8 days with 45% burst release in initial 12 hours.

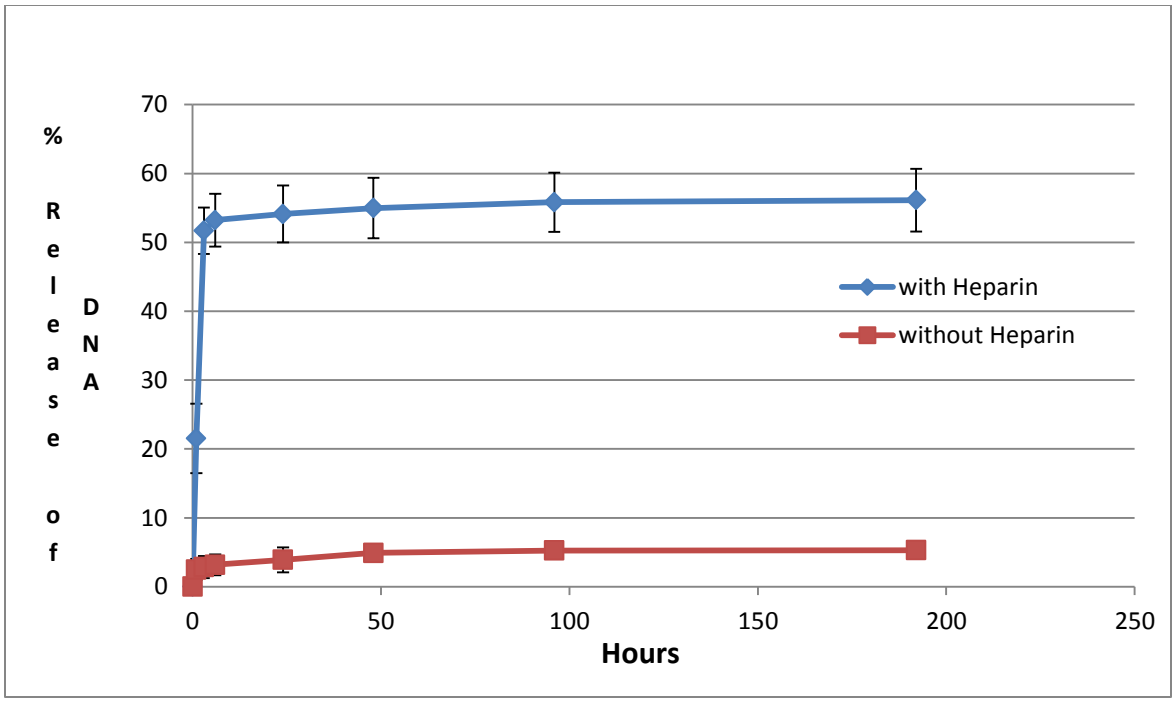

Figure 3.1: Release of DNA from CaP-PEI-DNA-PLGA nanoparticles

Dissolution test was done with Tris-sodium phosphate buffer (pH 7.4). Nanoparticles, which had PEI in their formulation, showed less than 5% DNA release. But some polyanions are able to release DNA from cation complexes.[81] Heparin is one of those polyanions.

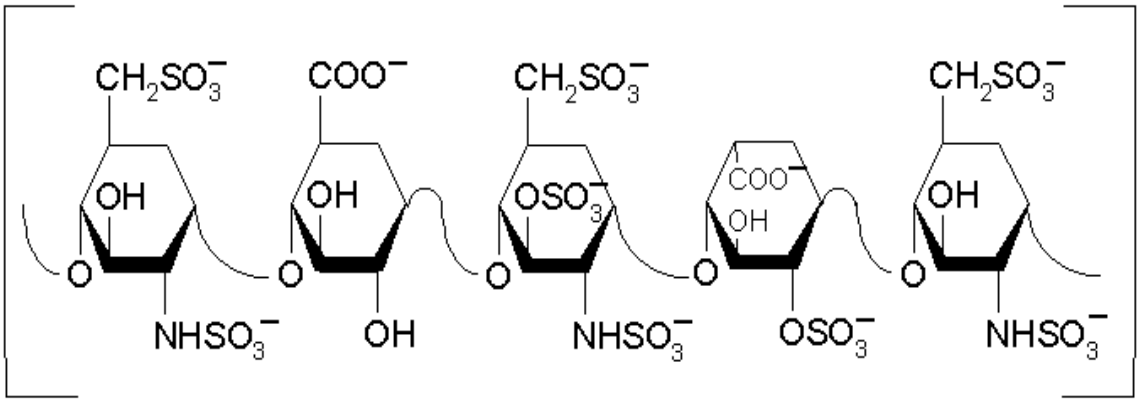

Figure 3.2: Heparin structure

At pH 7.4, which is alkaline medium, secondary and tertiary ammoniums of PEI are easily deprotonated and then disassociate from DNA. At the same time, heparin, a polyanion, competes with DNA and forms complex with PEI which will eventually help in DNA release.[82] Thus, heparin helps in DNA release from PEI-DNA complex. Same was proved when DNA release test was carried out with and without addition of heparin.

PLGA-PEI-DNA-calcium phosphate nanoparticles showed less than 5% DNA release when heparin was not added. But, 50 % of DNA was released when heparin included.

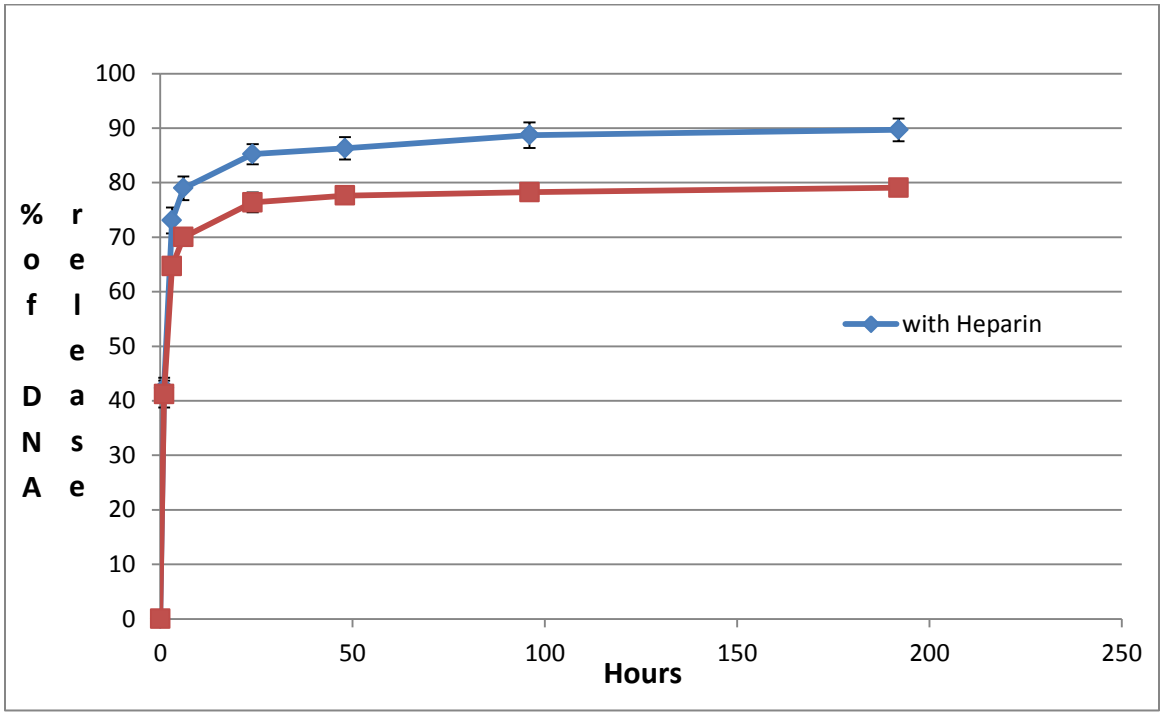

Figure 3.3: Release of nanoparticles (CaP-DNA-PLGA) prepared without PEI

Nanoparticles which were prepared without PEI shown almost similar amounts of DNA release irrespective of heparin effect.

These results conclude, DNA was conjugated with PEI and when heparin was not added, the conjugate did not break and the DNA was not released. With the addition of heparin in dissolution medium, it helps in freeing of DNA from PEI-DNA complex.

3.2) Particle size reduction:

Although good DNA release profile was seen with addition of calcium phosphate into PEI-DNA formulation, size of the particles was a big concern. Most of the particles showed wide distribution with half of the each formulation showing more than 1000nm size. I tested on more sonication time, but it did not help in reducing the size of the particles. Another method was to filter out the big particles using 8µm filter membrane. Results of this procedure are as followed:

Type	Median Particle Size in nm	Polydispersity index
A. With PEI	195.7	0.055
B. Without PEI	195.7	0.187

Table 3.4: Particle size of formulations prepared with filtration method

Particle size was reduced successfully with filtration method. All the particles showed 200nm with narrow distribution. The reason could be that all the big calcium phosphate particles (500-1000nm) were stopped by 8µm filter membrane.

Dissolution test was done using Tris-EDTA-Sodium phosphate (pH 7.4). The DNA content in the filtered particles was still based on the original DNA content in the particles without filtering.

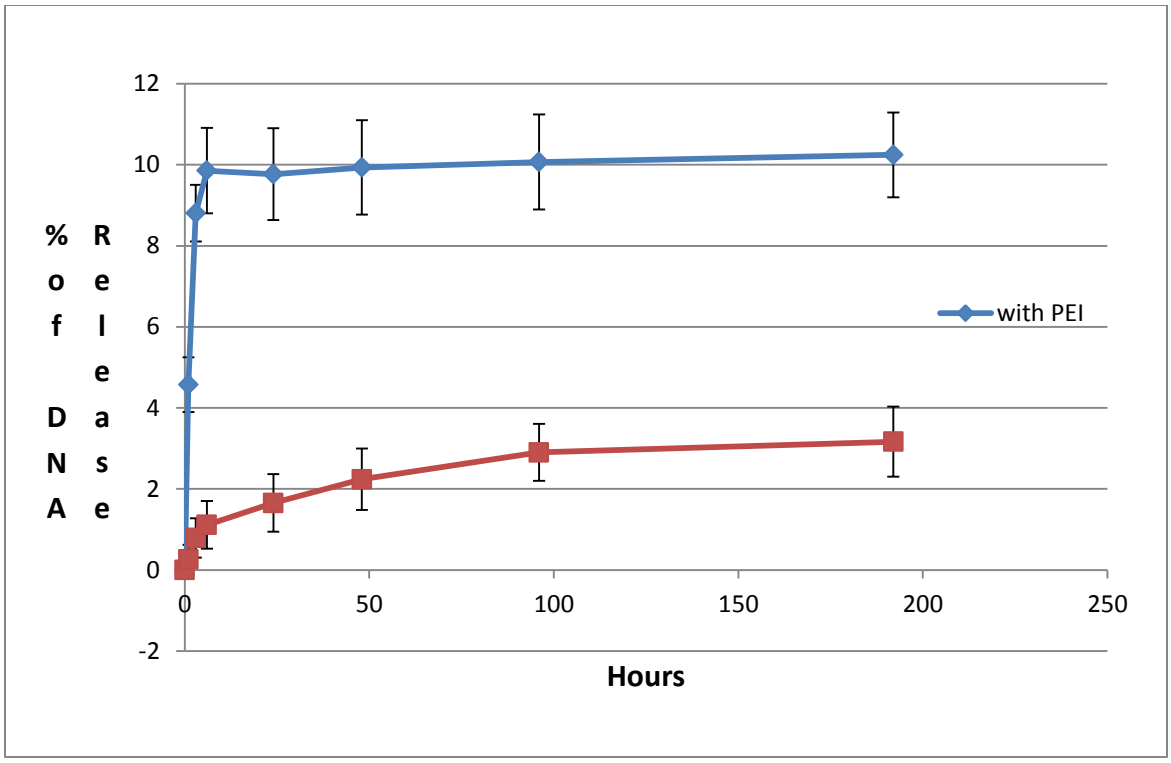

Figure 3.4: Release of particles prepared with filtration method

Although filtered formulations shown good particle size, none of the formulations showed much DNA release form those particles. It could be attributed to the loss of DNA with big calcium phosphate particles during filtration step or blockage of DNA in filter membrane. However, we tried to dissolve the CaP particles out of the filter membrane with diluted HCl and collected the solution. But we did not retrieve much DNA from that.

The large CaP particles could be due to the release of Ca^{2+} and PO_4^{3-} ions out of the oil phase during the second emulsion step before they have a chance to form precipitation in the oil phase. To ensure the CaP form precipitation in the oil phase, the primary emulsion was kept for shaking for 10-30 minutes. This method also showed very good particle size.

Type	Median Particle Size in nm	Polydispersity index	Entrapment Efficiency
A. CaP-PEI-DNA-PLGA, 10 minutes shaking, 10 mg PLGA (**101113A**)	224.7	0.095	98%
B. CaP-DNA-PLGA (**102213D**)	195.7	0.145	99%
C. CaP-PEI-DNA-PLGA, 30 minutes shaking (**102213B**)	257.2	0.165	97%
D. CaP-PEI-DNA-PLGA, 40 mg PLGA, 10 minutes shaking (**102213A**)	294	0.125	89%

Table 3.5: Particle size of formulations prepared with shaking method

All the formulations showed good particles with good polydispersity and very good entrapment efficiency without loss of calcium phosphate. All of them showed good size and distribution along with entrapment efficiency. The reason for small particle size is, shaking of emulsion for 10-30 minutes. This made calcium phosphate to form nano precipitation instead of aggregation of big particles. These small nanoparticles then got encapsulated in PLGA which made them reduce their size to 200-300nm.

Dissolution test of these formulations was done using Tris-Sodium phosphate buffer (pH 7.4). Although it was burst release, all of these formulations showed good DNA release profile. DNA might be still on the out phase of the PLGA particles, which could be the possible reason for burst release.

DNA release comparison of formulations prepared with shaking method can be seen in below figures. (Figure 3.5A and B).

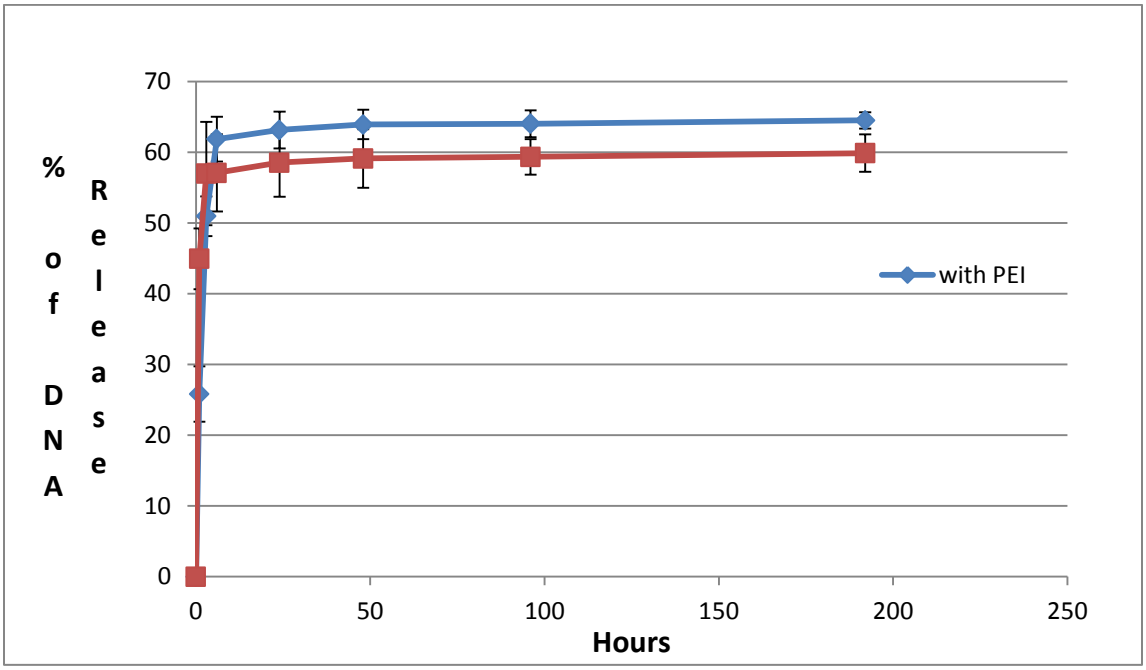

Figure 3.5 A

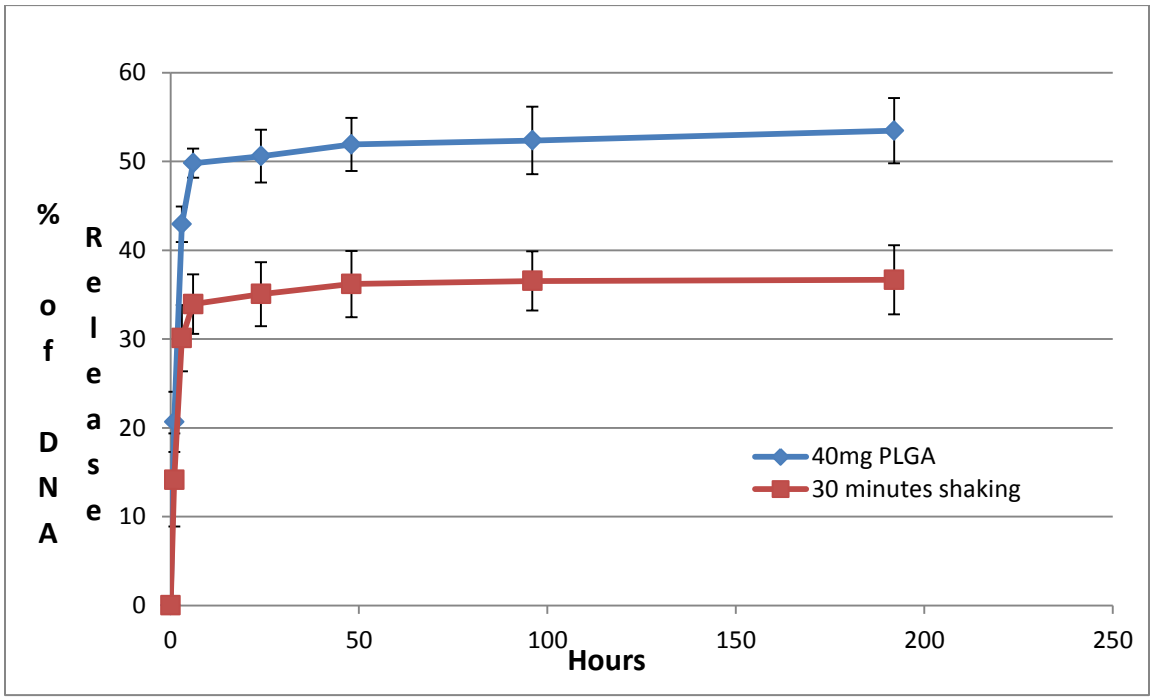

Figure 3.5 B

3.3) Transfection:

In vitro transfection study was carried out by the PEI-DNA-Calcium phosphate-PLGA nanoparticles. BCA protein assay was done to normalize the relative luminescence units by the amount of cell protein. The protein concentration was determined by a standard curve.

Many transfection experiments were done to assess the effect of calcium phosphate, PEI and calcium phosphate and PEI combination and high amount of PLGA using different cell lines. Also, effect of plain and full DMEM media was studied.

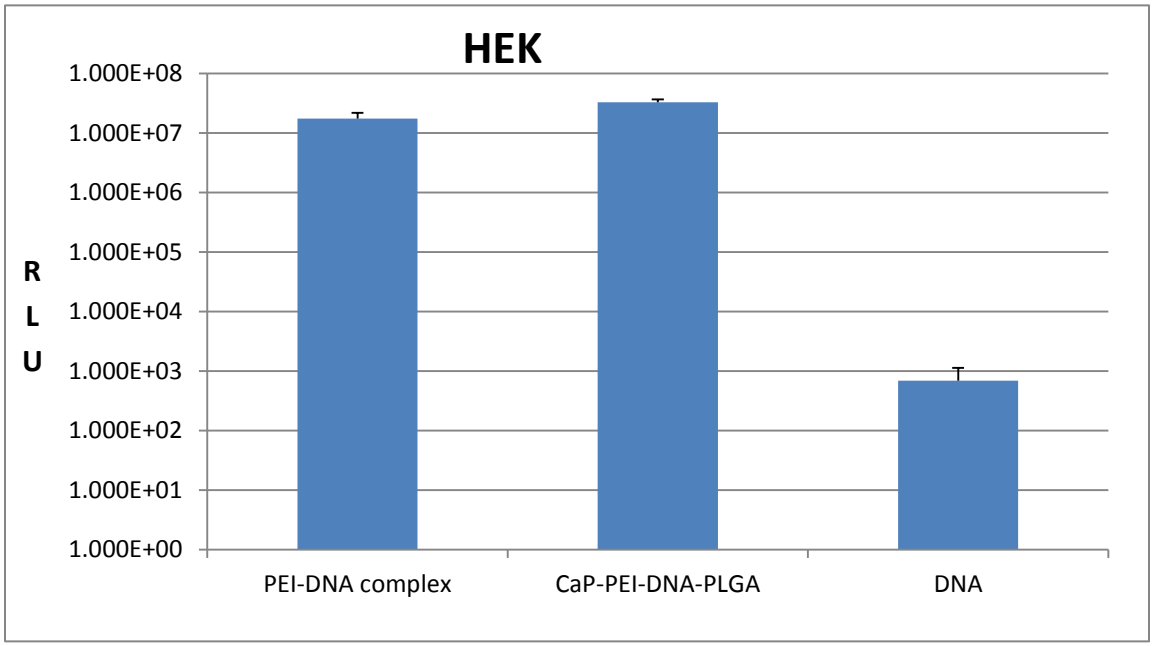

Figure 3.6: Transfection with PEI-calcium phosphate particles using HEK 293 cells

Formulations, which were prepared with PEI and calcium phosphate, showed equal levels of transfection with positive control. This was not achieved when cells were transfected with PEI particles alone (without calcium phosphate). The reason for this is formation of calcium phosphate while preparing these nanoparticles. Calcium phosphate helped in encapsulating DNA. Once nanoparticles were taken up by cells, calcium

phosphate dissolved in acidic environment of cell and released encapsulated DNA which helped in high levels of transfection.

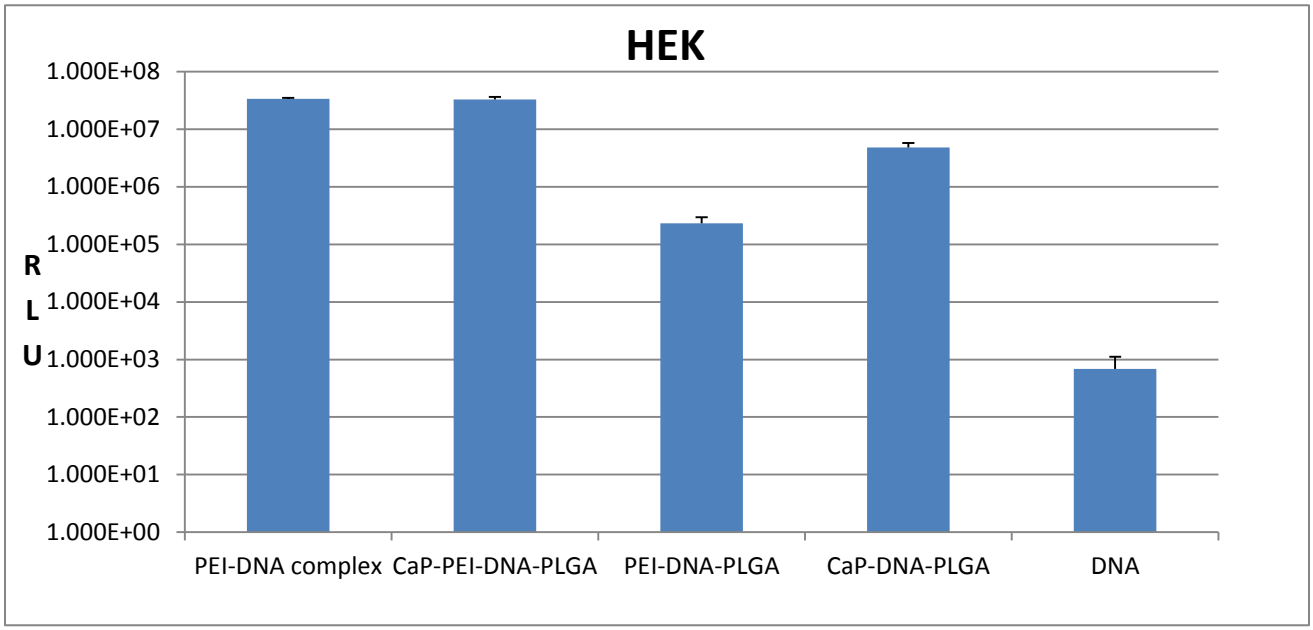

Figure 3.7: Comparison of transfection levels of PEI-DNA-PLGA, CaP-DNA-PLGA and PEI-CaP-DNA-PLGA nanoparticles using HEK293 cells

As from above results, it can be inferred that calcium phosphate plays major role in achieving transfection levels equal to positive control. Formulations were prepared using CaP-PEI-DNA-PLGA and CaP-DNA-PLGA and also PEI-DNA-PLGA complexes. PEI-DNA-PLGA particles never showed very high transfection levels. To achieve high transfection efficiency, insertion of calcium phosphate method was developed and has been improved. Formulations which had PEI-calcium phosphate-DNA complex showed higher transfection, which were comparable to positive. Effect of calcium phosphate was proved, when CaP-DNA complex particles (without PEI) showed more transfection compared to PEI-DNA complex particles (without calcium phosphate). The reason for this is, PEI alone was not effective in entrapping DNA. But formed calcium phosphate along with PEI was entrapped DNA efficiently and showed high transfection levels.

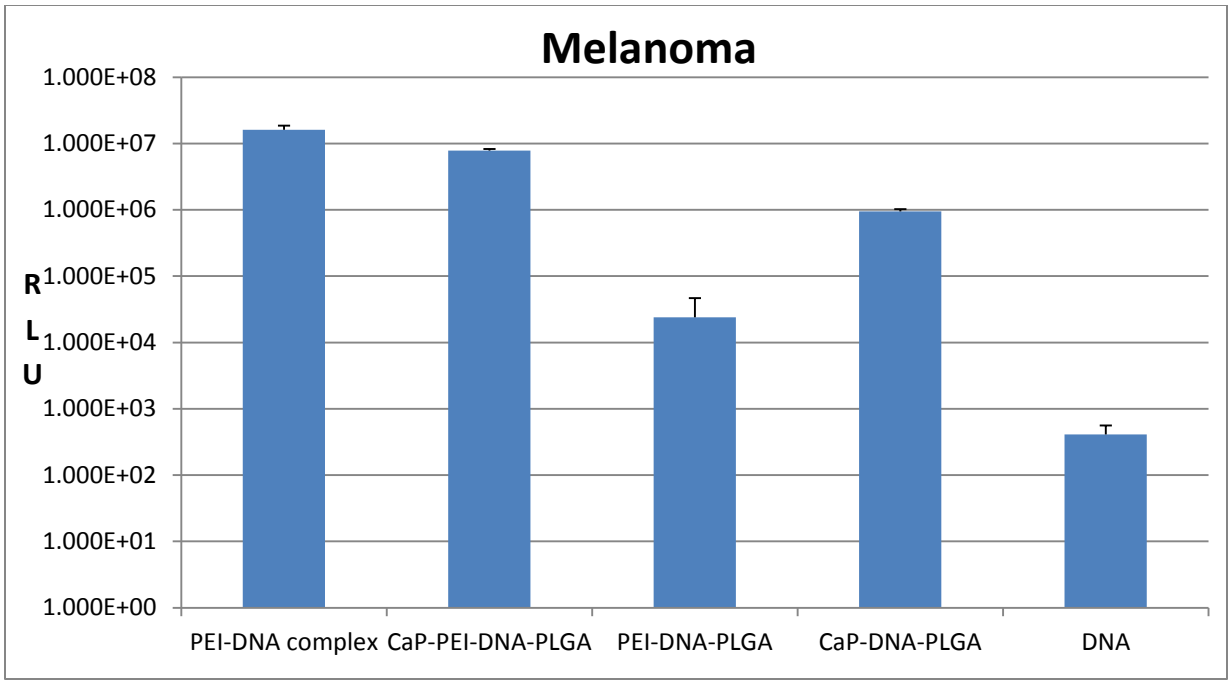

Figure 3.7: Comparison of transfection levels of PEI-DNA-PLGA, CaP-DNA-PLGA and PEI-CaP-DNA-PLGA nanoparticles using Melanoma cells

Transfection with melanoma cells showed similar results to HEK 293 cells. PEI-CaP-DNA-PLGA formulation showed more transfection efficiency compared to PEI-DNA-PLGA and CaP-DNA-PLGA formulations.

Although good transfection efficiency was achieved with PEI-CaP particles, particle size was not good in any of those formulations. So developments were done to improve particle size as it is necessary for *in vivo* gene delivery. Filtration of big calcium phosphate particles resulted in good particle size.

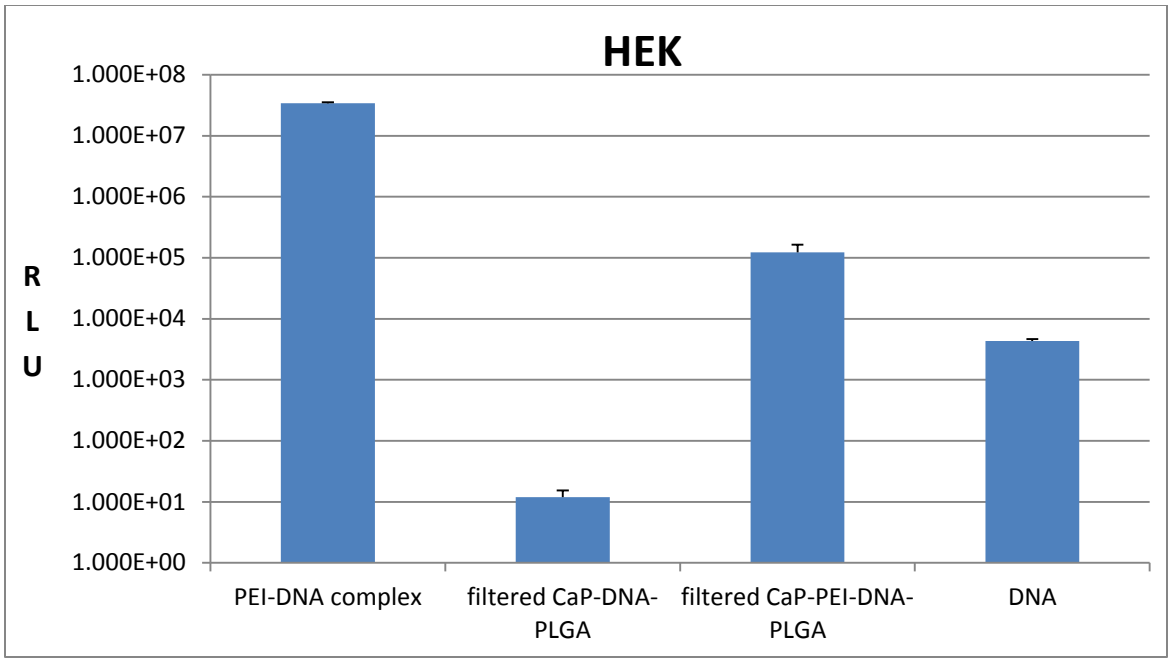

Figure 3.9: Transfection with filtered nanoparticles using HEK 293 cells

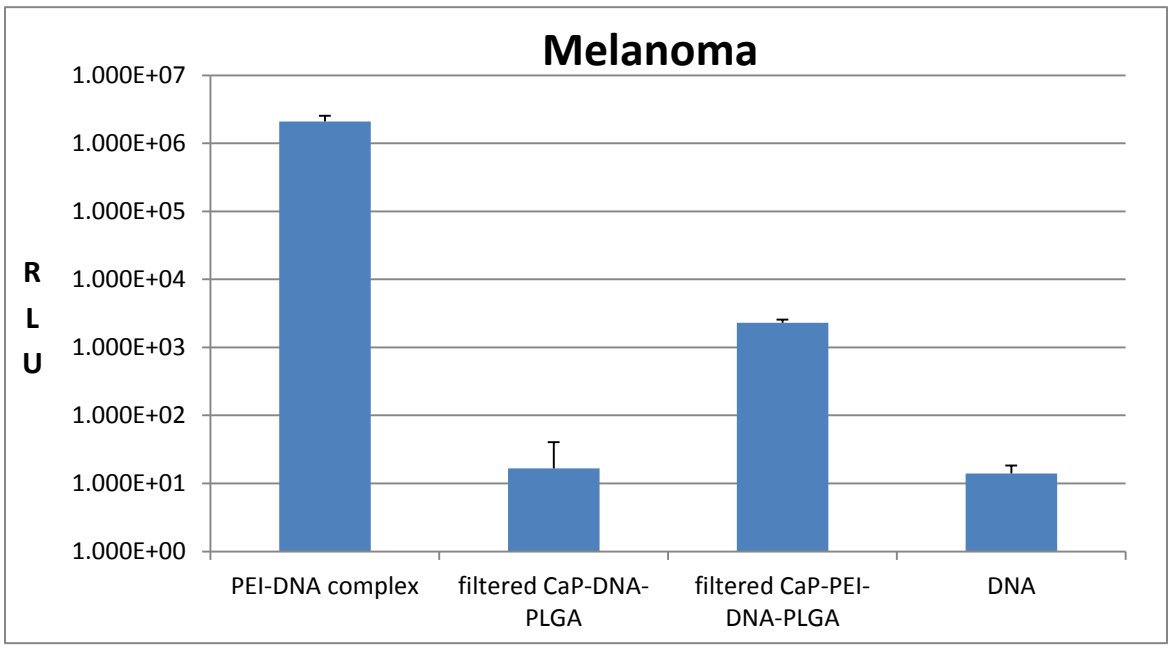

Figure 3.10: Transfection with filtered nanoparticles with melanoma cells

Even though desired particle size was achieved with this preparation method, none of the cell lines showed good transfection these particles. Reasons could be:

1. Plasmid DNA might have lost with big calcium phosphate particles while filtration.
2. DNA might have stuck in filter paper.

To achieve good transfection along with good particle size, primary emulsion with longer shaking method was evaluated. With this, aggregation of calcium phosphate particles could be reduced while protecting DNA from loss.

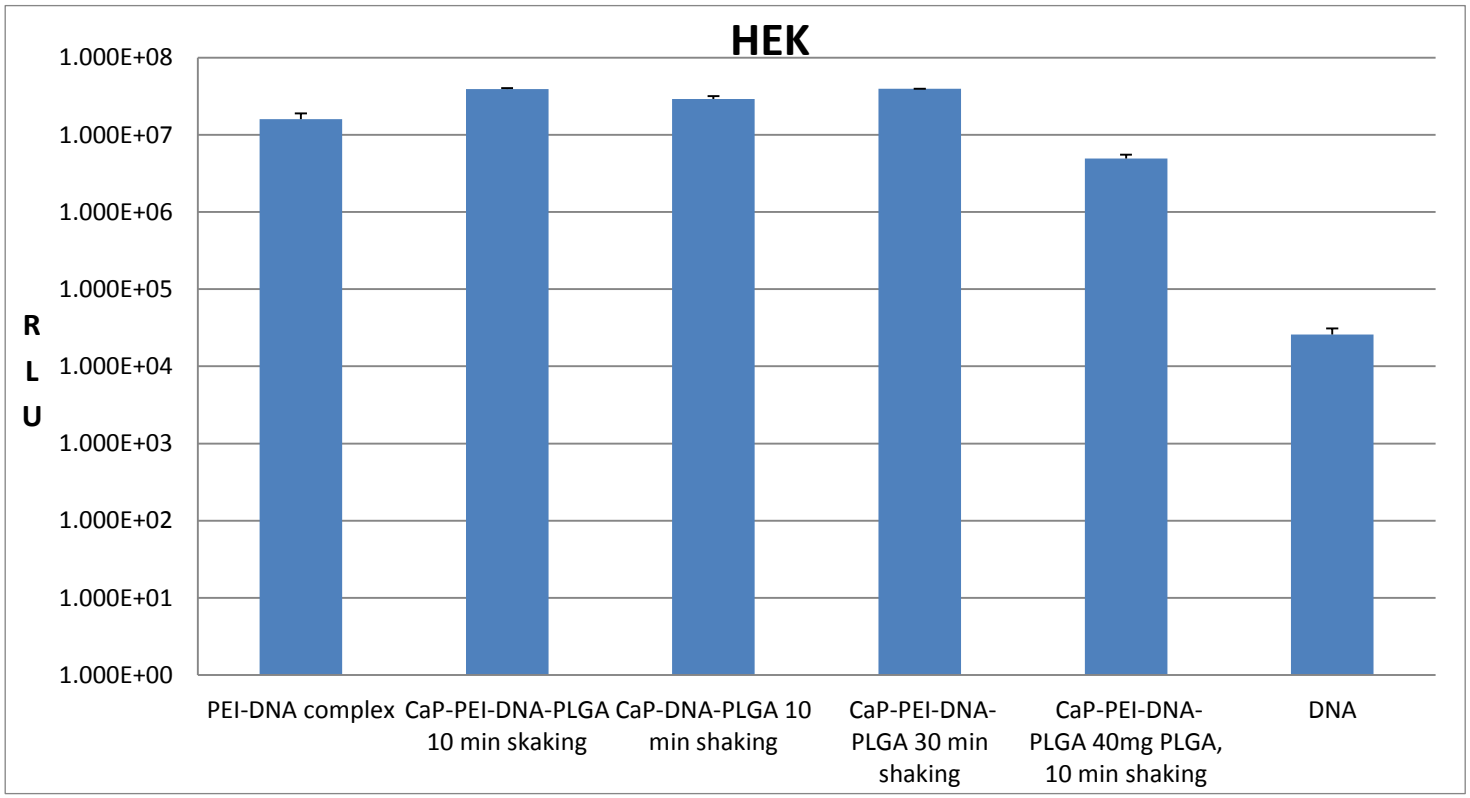

Figure 3.11: Transfection with shaking method nanoparticles using HEK 293 cells

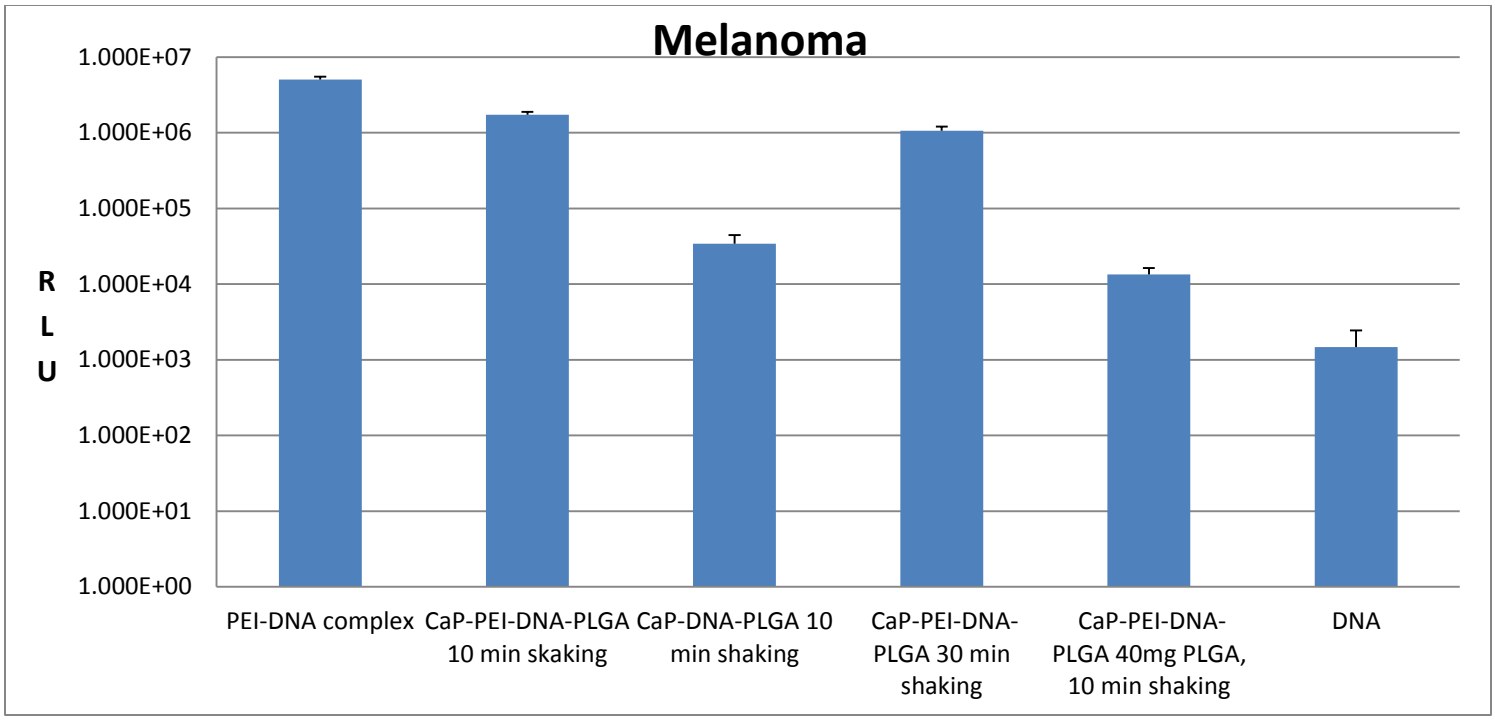

Figure 3.12: Transfection with shaking method nanoparticles using melanoma cells

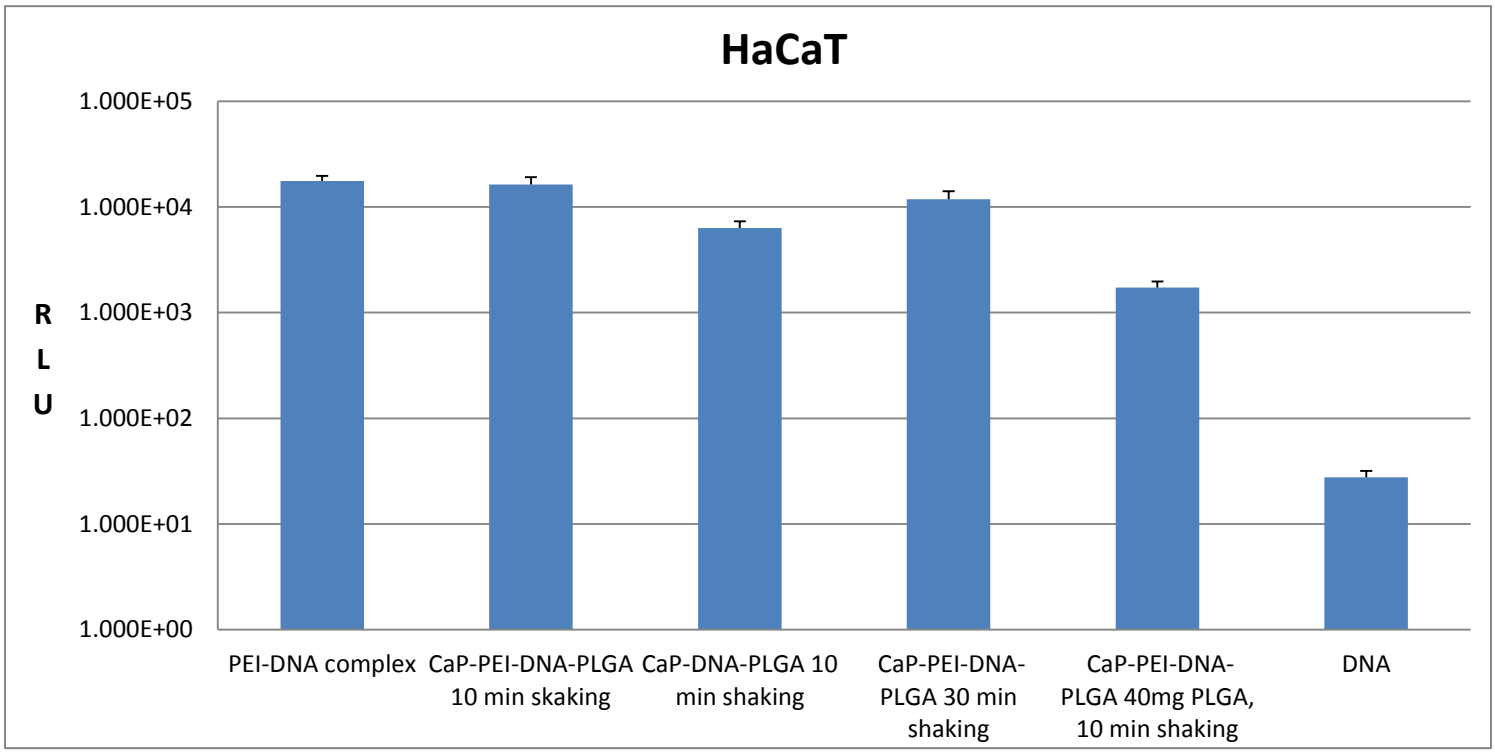

Figure 3.13: Transfection with shaking method nanoparticles using HaCaT cells

These particles showed high transfection efficiency while maintaining good particle size. The reason is calcium phosphate precipitate was retained in the formulation unlike filtration method, where whole calcium phosphate precipitate was blocked to filter membrane. Calcium phosphate is essential to entrap DNA and deliver it to cells which will eventually show good transfection levels.

3.4) Cytotoxicity (MTT assay):

MTT assay was carried using PEI-DNA complexes and CaP-PEI-DNA-PLGA nanoparticles with the increasing concentration of DNA while maintaining N/P ratio at 20:1

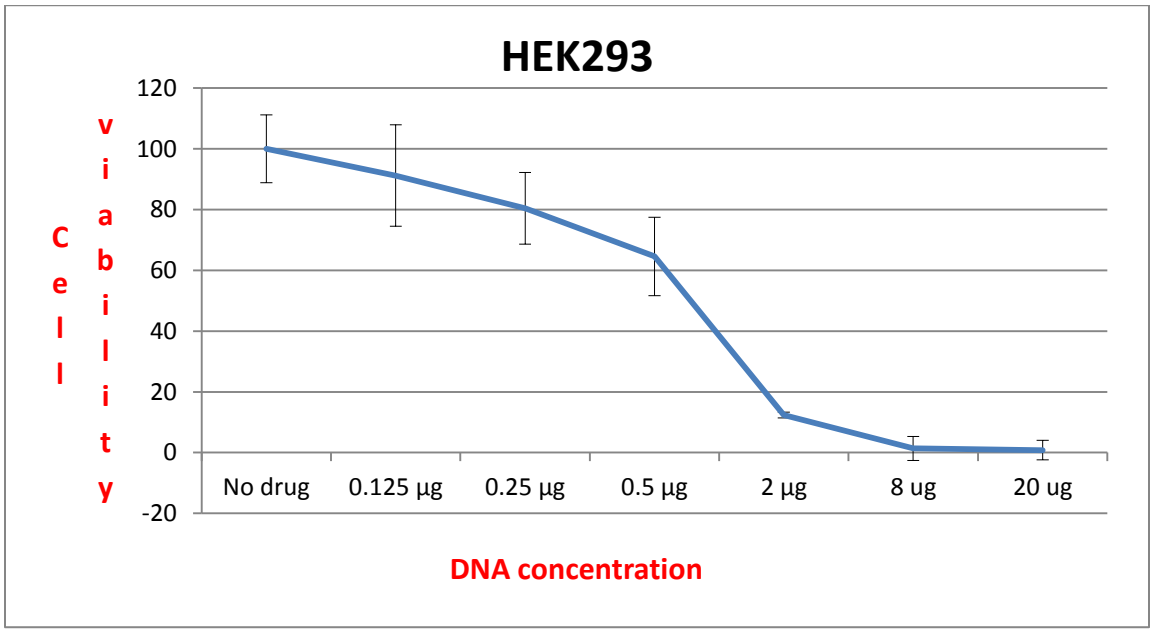

Figure 3.14: MTT assay of HEK293 cells using various 25 kDa PEI-DNA complexes

Figure 3.14 shows, toxic levels of PEI-DNA complex alone. At low concentrations of DNA (0.125 µg to 0.5 µg) (20:1 N/P ratio was used for all the complexes), at least half of the cells were able to survive. But, when concentration was increased to 2, 8 and 20µg (which eventually increases the concentration of 25 kDa PEI) all the cells were died. The

reason is, PEI is very toxic at higher concentration levels when it is given alone. But, when it was modified to CaP-PEI-DNA-PLGA nanoparticles, they showed less toxicity compared to PEI-DNA complexes at same concentration levels.

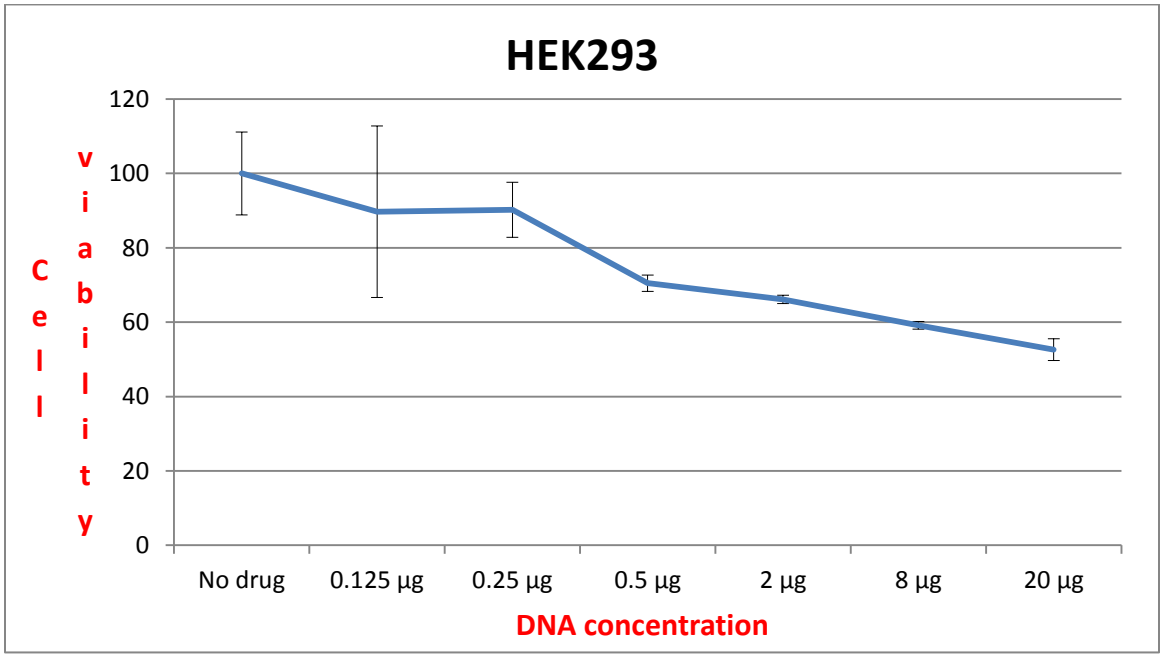

Figure 3.15: MTT assay of HEK293 cells using CaP-PEI-DNA-PLGA nanoparticles

Reasons could be probably due to the decrease in charge of PEI in nanoparticles with decrease in primary amine. [83] Also, when PEI-DNA complexes modified to CaP-PEI-DNA-PLGA nanoparticles, cytotoxicity might decreased due to decrease in free PEI polymer concentration in cells[84] and biocompatible PLGA polymer.

Similar cytotoxicity results were observed by melanoma cells as well.

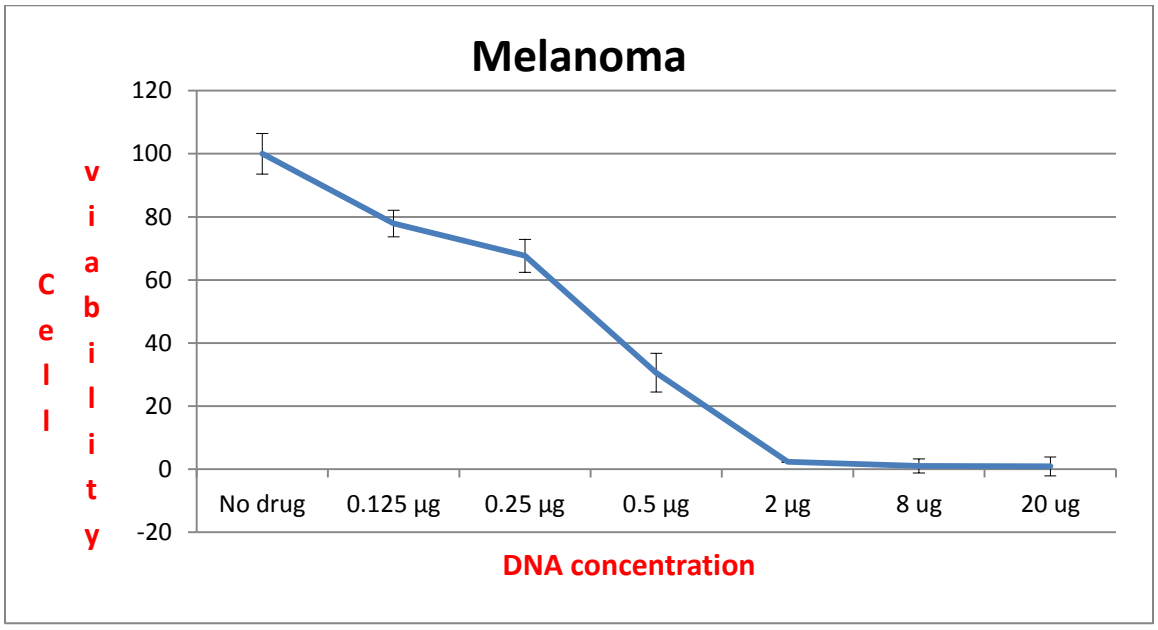

Figure 3.16: MTT assay of Melanoma cells using various 25 kDa PEI-DNA complexes

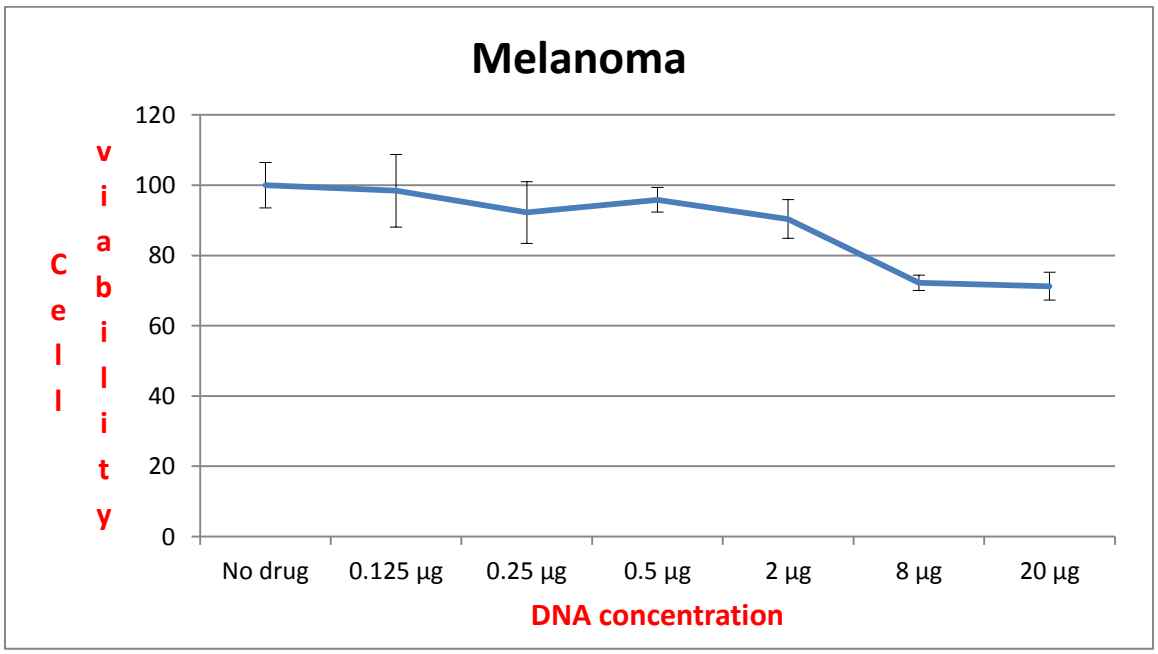

Figure 3.17: MTT assay of HEK293 cells using CaP-PEI-DNA-PLGA nanoparticles

4) Conclusions:

The intention of the study was to develop a novel vehicle for the delivery of gene inside the cells. PLGA-PEI-DNA-CaP formulation was used to achieve higher transfection levels compared to PLGA-PEI-DNA nanoparticles. The following conclusions were made after the completion of the study.

1. Heparin reacts with PEI and helps in releasing DNA from PEI-DNA complex.
2. Higher transfection levels are shown by HEK293, HaCaT and melanoma cells using CaP-PEI-DNA-PLGA nanoparticles which are equal to positive control. But the particle size is big.
3. CaP-PEI-DNA-PLGA nanoparticles size can be reduced using 8µm filter membrane. But all the calcium phosphate precipitation is blocked on filter membrane which reduces the transfection levels.
4. Emulsion shaking method has shown good particle size along with retention of calcium phosphate nano precipitate in the formulation. These particles have shown higher transfection levels too.
5. The cytotoxicity and *in vitro* transfection studies indicated that CaP-PEI-DNA-PLGA nanoparticles are a good novel gene delivery system with lower cytotoxicity and good transfection levels compared to PEI-DNA complex.

5. Future Directions:

The study was carried out to develop a novel non-viral safe carrier of gene to achieve good transfection efficiency. Both PLGA-PEI and PLGA-PEI-CaP nanoparticles can transfect the cells *in vitro.* However, the transfection efficiency of PLGA-PEI was not excellent and far lower than the positive control (PEI-DNA complex with 10:1 N/P ratio). But PLGA-PEI-CaP nanoparticles were developed and showed good transfection efficiency compared to positive. As a continuation of this study, experiments can be done to check how PEI and calcium phosphate entrapping DNA. Dissolution studies also need to be done to improve the DNA release with sustained profile.

Confocal microscopic studies need to be carried out to check the nanoparticles movement across the cell and also to monitor the endocytosis. It is also necessary to determine the cytotoxicity of the prepared nanoparticles.

Finally, animal studies should be carried out to determine the effectiveness of these nanoparticles *in vivo*. As our particles do not contain any targeting moiety, therefore, it would be more appropriate for injection into the site of action and check the protein development.

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