DEVELOPMENT OF A GENETIC MATERIAL TRANSFER APPROACH FOR GENE THERAPY

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Approval of the Graduate School of Natural and Applied Sciences.

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ABSTRACT

DEVELOPMENT OF
A GENETIC MATERIAL TRANSFER APPROACH
FOR GENE THERAPY

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This thesis is focused on the development of a gene delivery system, especially for the purpose of DNA vaccination. DNA expression vectors have the potential to be useful therapeutics for a wide variety of applications. A carrier system was designed to realize the delivery of genes to cells and the promotion of controlled adequate expression in the target cells. The low gene delivery efficiency observed with systems composed of polyplexes is mainly due to low stability of polycation e.g polyethylenimine-DNA complexes and inability of most of the complexes to the reach nucleus after entering the cells. The encapsulation of polyethylenimine-DNA complexes inside the alginate microspheres was expected to provide protection from nuclease-based attack, thereby, increasing the stability of the complex and also to achieve controlled release of the complex at the target tissue.

In this study, controlled release of complexes from alginate microspheres was studied with DNA staining. In Tris-HCl buffer, the release of PEI-DNA complexes were completed in 48 h, however in cell culture medium (DMEM) 18 % of complexes were released in 48 h because of presence of Ca^{+2} ions in DMEM. Also, in order to provide mucosal gene delivery for mucosal immunization polyethylene glycol (PEG) was introduced into the composition of microspheres and the two systems were compared in terms of release kinetics of the complexes. In the presence of PEG, release of PEI-DNA complexes from alginate microspheres in the cell culture medium (DMEM) were enhanced and 50 % of PEI-DNA were released from the microspheres in 48 h. To understand the effect of the PEG on the surface of microspheres zeta potential analysis and microscopic examination were carried out. By increasing percentage of PEG (0, 15, 30, 50) in microspheres, less negative zeta potential value were measured. Mucoadhesion of alginate and PEG-alginate microspheres were evaluated by using modified microbalance method, and in the presence of PEG enhancement of mucoadhesion was observed. In this way a gene delivery system with a possible route through mucosa of tissues was prepared.

Keywords: DNA vaccine, gene delivery, polyethylenimine (PEI) alginate, mucoadhesion.

ÖZ

GEN TERAPİSİ AMACIYLA GENETİK MATERYAL TRANSFER YAKLAŞIMI GELİŞTİRİLMESİ

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Yüksek Lisans; Biyoteknoloji Bölümü
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Ocak 2004, 64 sayfa

Bu tez çalışmasında, özellikle DNA aşılarında kullanılabilecek bir gen taşıyıcı sistemin geliştirilmesi üzerine yoğunlaşılmıştır. DNA ekspresyon vektörleri çeşitli uygulamalar için terapötik ajan olma potansiyeli taşırlar. Taşıyıcı sistem, genlerin hücrelere aktarılmasını hedef hücrede kontrollü olarak ekspresyonun sağlanması başarmak için geliştirildi. Kompleksler aracılığıyla gen aktarım veriminin düşük olmasına neden olan başlıca etkenler; polikatyon (polietilenimin)-DNA komplekslerinin dayanıklılığının az olması, ve hücre içine alınan komplekslerin bir çoğunun çekirdeğe ulaşamamasıdır. PEI/DNA kompleksinin aljinat mikroküreler içine hapsedilmesiyle, komplekslerin dış etkenlerden korunarak dayanıklılıklarının artması ve kompleksin hedef bölgeye kontrollü olarak salımı sağlanması hedeflenmiştir.

Bu çalışmada komplekslerin mikroküre lerden salımı DNA boyaması ile incelenmiştir. PEI-DNA komplekslerinin aljinat mikroküre lerinden salımı Tris-HCL tampon çözeltisinde 48 saatte tamamlanmıştır. Fakat hücre kültürü ortamı olan DMEM Ca^{+2} iyonları içerdiğinden, hücre kültürü ortamında 48 satte yalnızca komplekslerin % 18'i salınmıştır. Aljinat mikroküre lerine ek olarak, PEG-aljinat mikroküreleri yapılarak, iki farklı mikroküre sisteminin dayanıklılığı ve salım profilleri incelenmiştir. PEG'in varlığında PEI-DNA komplekslerinin aljinat mikroküre lerinden salımını hücre kültürü ortamında artmıştır ve 48 saatte komplekslerin % 50'si ortama çıkmıştır. arttır. PEG'in aljinat mikroküre lerde varlığını göstermek ve mikroküre lerin yüzeyine etkisini incelemek amacıyla zeta potansiyel analizi yapılmıştır. PEG'in yüzdesi arttıkça (0, 15, 30, 50) daha az negatif yüzey potansiyeli ölçülmüştür. Aljinat yüzeyinde bulunan PEG'in asıl işlevi mikroküre lerin mukosal tutunmasını sağlayarak bu yoldan gen transferini sağlamaktır. Böylelikle mukosal katmanlara sahip dokular için etkin bir gen aktarım sistemi geliştirilmiştir.

Anahtar Kelimeler: DNA aşıları, gen aktarımı, polietilenimin (PEI), aljinat, mukozal tutunma.

To My Family

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ABREVIATIONS

DMEM: Dulbecco's Modified Eagle Medium

FBS: Fetal bovine serum

PEI: Polyethylenimine

PEG: Polyethylene glycol

CHAPTER 1

INTRODUCTION

1.1 Gene Delivery

Gene transfer into animals and humans has become a popular research tool since the basics of molecular genetics and gene transfer in bacteria were established in the 1960s. Achievements in recombinant DNA techniques and human genome project gave rise to developments in new approaches of gene delivery for treating and controlling diseases.

Gene delivery involves transferring nucleic acids usually in the form of DNA to the cells, which results in a therapeutic effect by correcting genetic defects or by expressing therapeutically useful proteins. The rate limiting step of gene delivery is the transfer of genetic material to appropriate tissues or organs [1].

There are two important steps for a successful gene delivery; first one is the transferring of DNA molecules into the cell and nucleus, second one is the expression of DNA. Although functioning of transferred genes against many diseases was accomplished, gene therapy is still an unsolved problem because of lack of effective and safe gene transfer methods.

Delivery of DNA is limited because of extracellular factors like clearance or degradation of DNA by nucleases and also because of the physicochemical properties of the DNA. Since a cell membrane is negatively charged and is not permeable to the negatively charged macromolecules like DNA, gene delivery requires DNA delivery vehicles called vectors. Generally, two different approaches have been utilized for the delivery of nucleic acids in gene therapy, viral vectors and non-viral delivery systems (mainly cationic polymers or lipids) [2-3].

1.1.1 Viral Vectors

Viruses are small intracellular parasites that contain either DNA or RNA as genetic material. The structure of virion is composed of DNA or RNA, a capsid, and sometimes an envelope. Viral vectors including retroviruses, adenoviruses and adeno-associated viruses have impressively high efficiencies in introducing their genetic material into host cells. In nature, viruses transfect cells; thus, enter the cells and achieve expression of their genetic material in the cell. Non-replicating viral vectors created in the laboratory through gene deletions are devoid of pathogenicity but retain their high efficiency of gene transfer [3-4].

1.1.1.1 Retroviral Vectors

These viruses carry RNA as genetic material and contain reverse transcriptase, which is essential for reverse transcription, the production of a molecule of DNA from a RNA template. Retroviral vectors have the advantage of stable transforming cells; viral DNA is integrated into the host genome and cells clonally expanded. However, this could be a disadvantage because integration of foreign DNA into host may cause activation of oncogenes. Retroviruses requires cell division for infection thus; this type of vectors can be used for dividing cells.

1.1.1.2 Adenoviral Vectors

They are an alternative to retroviruses because their life cycle does not require target cell division. Moreover, foreign DNA is not integrated into the host genome; for this reason cells are transduced for only 1-2 weeks. It can be used for the cases where a transient transfection like vaccination is required. Adenoviruses seem very well suited for in vivo gene transfer, but they carry the risk of immunogenicity.

1.1.1.3 Adeno-associated Virus Vectors

Adeno-associated viruses are single stranded DNA viruses, they require helper viruses for infection. They can infect non-dividing cells also, integration of foreign DNA in the host occurs.

1.1.1.4 Herpes Simplex Virus Vectors

Herpes simplex viruses are very promising in gene delivery since they have the ability of infecting non-dividing cells and have large DNA loading capacity. However, they are cytotoxic and immunogenic. Through some genetic alterations toxicity and antigenicity of the viruses were decreased to some extent. Their transgene expression is very low and for these reasons their use is limited.

1.1.2 Non Viral Vectors

Although viral vectors show excellent transfection efficiencies but in the recent years their usage is being reconsidered owing to serious safety risks because of the their potential oncogenicity due to insertional mutagenesis. Besides, they develop a high immunogenicity after repeated administration since the mammalian immune system has strategies developed to eliminate viral invaders.

Other problems associated with viral vectors are the limited size of DNA that can be carried and limited number of cell types that could be transfected by viruses. Furthermore, the inclusion of a targeting moiety in order to transfect specific cell types or tissues is problematic. Because of these concerns non-viral vectors are emerging as a viable alternative [6-8].

Non-viral delivery systems are limited in their low gene transfer efficiency but still are very attractive because of their non- pathogenicity, limitless foreign gene capacity and ease of production. The ideal nonviral DNA delivery system should be nontoxic, should protect the DNA from degradation, have enhanced cellular uptake and provide controlled expression [8-10].

1.1.2.1 Materials for Gene Delivery

1.1.2.1.1 Liposomal Gene Delivery (Cationic Lipids)

Negatively charged (anionic) classical liposomes were initially evaluated for gene delivery applications, however, they have low loading and low gene transfer efficiency. Cationic lipids that interact with the negatively charged DNA through

electrostatic attractions were used as liposomal carriers or as lipid aggregates. All cationic lipids possess an amine group in addition to hydrophobic group. The amino group binds DNA electrostatically, while the hydrophobic groups facilitate the assembly of cationic lipids into bi-layer vesicles. For internalization cationic lipids like 1.2-dioleoyl phosphatidylethanolamine (DOPE) are necessary. After internalization they provide endosomal escape of liposome/DNA complexes by affecting the endosome [7-8].

1.1.2.1.2 Cationic Polymers

Cationic polymers generally bear amines that give high density positive charge through protonated amines. The cationic polymers help the condensation of negatively charged DNA into small particles and provide an overall positive charge to the complexes that facilitate their uptake into the cells via electrostatic interactions with anionic cell membrane groups such as proteoglycans (Figure 1.1). Polymers possess many advantages as vectors for gene delivery. They can be modified easily in accordance with requirements of application and can be produced in large amounts with the same properties. After identifying a suitable polymer structure a scale-up to the large quantities is rather simple as well. [9-12]

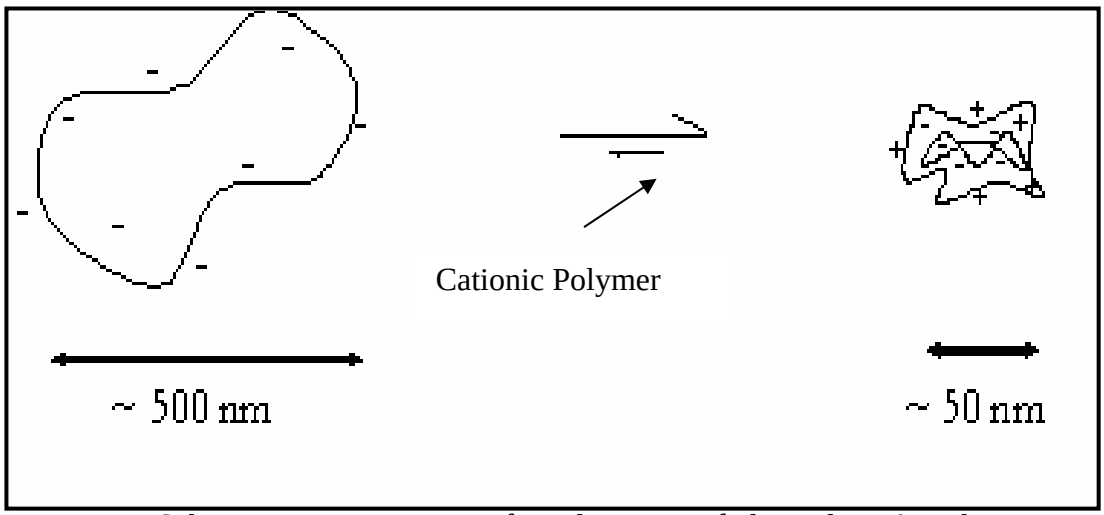


Figure 1.1: Schematic representation of condensation of plasmid DNA with cationic polymers.

1.1.2.1.2.1 Poly(L-lysine)

Poly(L-lysine), PLL, was one of the first polymers to be used in non-viral gene delivery and a large variety of polymers with different molecular weights have been utilized in experiments. Due to its polypeptide structure poly(L-lysine) is biodegradable, a property that makes it especially suitable for in vivo use. However, it exhibits modest-to-high toxicity. Transfection efficiency is lower than that of Polyethylenimine (PEI), because PLL-DNA complexes are lysed in the endosomal pH. The inclusion of targeting moieties or joint application of endosomolytic agents like chloroquine improves reporter gene expression. This indicates that the main reason of lower transfection with PLL is the degradation of the complexes in the endosome [3, 9-12].

1.1.2.1.2.2 Polyethylenimine

PEI polymers with different molecular weights and degrees of branching have been synthesized and evaluated in vitro as well as in vivo. PEI polymers are able to effectively complex even with large DNA molecules and are capable of transfecting cells efficiently in vitro as well as in vivo. They offer a significantly more efficient protection against nuclease degradation than other polycations because of their higher charge density and more efficient complexation [3, 10-14]. Every third atom is an amino nitrogen with the capacity to be protonated. High charge density of PEI provides a unique property to these complexes; these complexes escape from the endosomes (Figure 1.2). Complexes enter the cell via endocytosis, and endocytosed particles are directed to the lysosome for degradation [15-16]. Protonation of PEI in acidic environment provides destabilization of endosomes. There are two explanations; it may be because of high buffering capacity of the PEI, where buffering of environment results in ion accumulation and osmotic swelling and in an acidic environment the size of PEI-DNA complexes increases and thus result in swelling of endosomes. Although it is the most successful nonviral delivery vector, its transfection efficiency is substantially lower than viral vectors, and in addition it is toxic in high concentrations. There are a large number of studies aimed at improving PEI transfection efficiency and to improve the toxicity of the polymer.

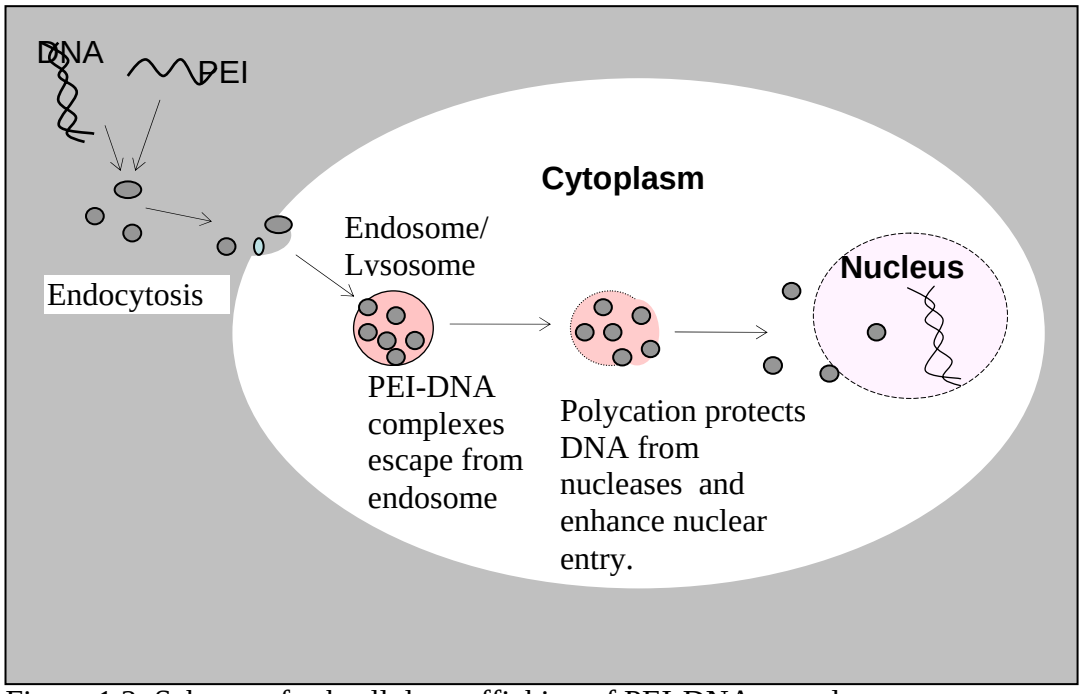


Figure 1.2: Scheme of subcellular trafficking of PEI-DNA complexes.

1.1.2.1.3 Sustained DNA delivery of plasmids with polymers.

Polymeric delivery represents an alternative approach that can increase residence time within the tissue and protect against degradation. Since DNA is a macromolecule, transport of the DNA through tissues is very limited. Encapsulation of DNA promotes localized delivery. For sustained delivery of plasmid DNA collagen based materials, hydroxyapatite-based hydrogels, poly-l-glycolic acid (PLG), ethylene vinylacetate (EVAc) polymers were used. DNA-releasing polymers administered to multiple sites in vivo have demonstrated the capacity to transfect cells locally and promote sustained protein production. Nanospheres loaded with plasmid DNA fabricated from PLG, gelatin, chitosan, alginate provided transfection. [17-21]

1.1.2.1.4 Sustained delivery of DNA complexes

An alternative gene delivery is encapsulation of DNA after complexation with cationic polymers or lipids. This system combines the properties of the cationic polymers and lipids. In addition, release of DNA complexes from the carrier system may enhance or localize gene transfer in vivo and in vitro. They protect the DNA against degradation and can also facilitate intracellular trafficking, which includes

endosomal escape, cytoplasmic transport, and nuclear targeting. Porous PLG or collagen scaffolds with encapsulated polyplexes or lipoplexes achieved substantial transfection in vitro and in vivo. Also sustained delivery from carrier systems decreased toxicity of complexes [17, 21-26]

1.1.2.1.5 Physical methods

Electroporation, a physical gene delivery method, is the transport of the gene of interest into target cell by means of an electric field. This procedure is simple, but it provides short-term transient expression of therapeutic gene. This technique has not been used for any clinical applications. Though another physical method called microinjection provides very efficient gene delivery, it is also transient, and is very labor-intensive because each individual cell must be injected one by one [27].

1.2 Gene Delivery Approaches

Gene delivery has potential not only in the treatment of genetic disorders but also in their prevention. Gene delivery to the animal cells is also very useful tool in molecular biology studies. Since in vitro gene transfection studies using viruses are not convenient and constitute a safety risk, nonviral vectors are mostly preferred.

1.2.1 Gene Therapy

With advances in the molecular biology, the understanding of genetic basis of both inherited and acquired illnesses provide new tools for gene therapy against diseases, disorders and infections. There are a large number of studies for gene therapy of cancer and AIDS. In clinical gene therapy applications, approaches involving viral vectors are more frequently used. Because of occurrence of tumor formation after viral gene therapy studies the clinical applications of gene delivery was prevented by FDA. Among the nonviral gene therapy applications, liposomes are the most studied ones. There are a few nonviral commercial products like the derivatives of PEI (Exgen 500, JetPEI) used in vitro and in vivo gene delivery but they were found to be unsuitable for clinical studies.

1.2.2 DNA Vaccine

Vaccines are one of the oldest and most effective ways to fight against disease, and DNA vaccines represent one of the most significant, fundamental additions to the technology in recent years. DNA vaccines represent one of the latest advances in vaccinology, based upon a bacterial plasmid encoding the antigen of interest, which is generally under the control of a strong viral promoter [28-30]. This in turn leads to induction of antigen-specific immune responses. First, the expression of secreted protein antigens may allow the production of subunit vaccines that stimulate both arms of the immune system. Second, intracellular expression of a non-secreted antigen should specifically induce or stimulate cellular immunity (Figure 1.3).

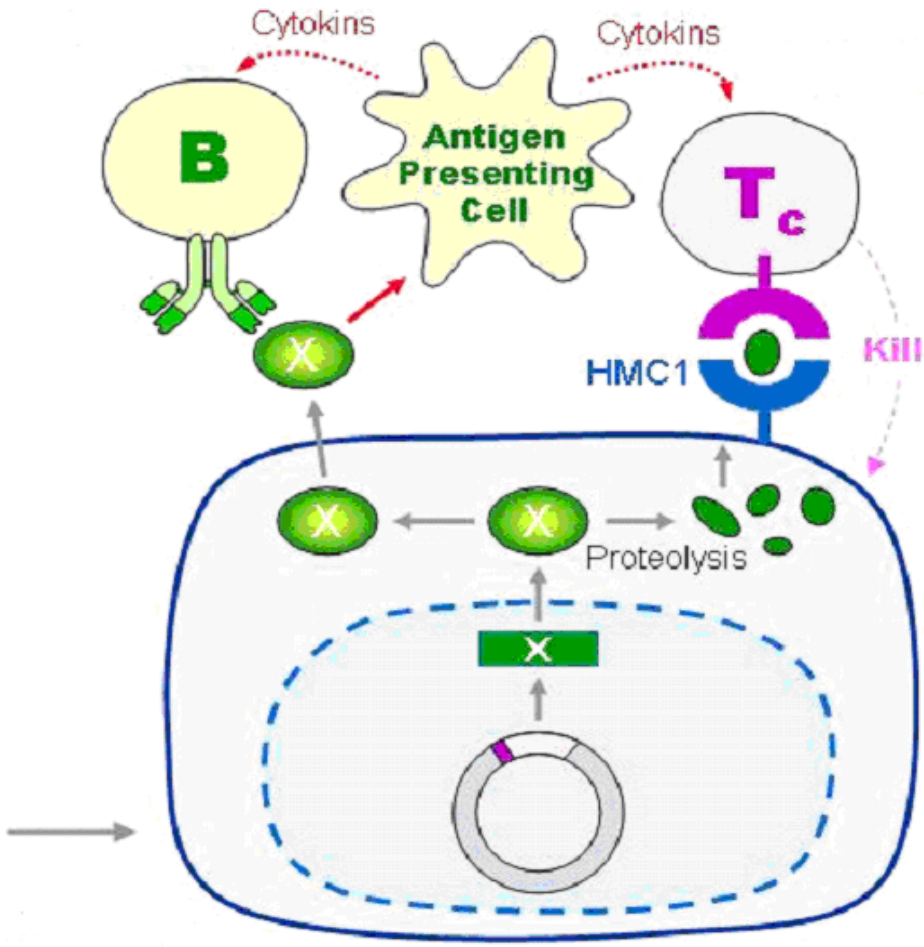


Figure 1.3: Induction of humoral and systemic immunity via DNA vaccination. Tc: T cytotoxic cell.

Compared to current vaccines DNA vaccines offer a number of potential advantages because they are economical, easy to produce, and do not require special storage or handling. DNA vaccines allow repeated inoculations in the same patient. Without using no infectious agents, DNA vaccination can induce both humoral and cellular responses. Because of prolonged antigen synthesis, DNA vaccination provides long-lasting immunity, therefore, there is no need for several applications. Also DNA vaccination method possesses adjuvant properties because of immunostimulatory CpG sequences (unmethylated bacterial DNA sequences) [30].

For a successful vaccination, the design of DNA vector, carrier system and administration route are crucial. DNA vaccine vectors should include: a bacterial origin of replication (ori), a prokaryotic selectable marker gene such as an antibiotic resistance, antigen-encoding sequences, B-cell and/or T-cell epitopes, eukaryotic transcription regulatory elements such as promoter and enhancer sequences that are most often viral in origin, but can also be tissue specific, a transcription termination element, such as that derived from the bovine growth hormone gene, to ensure appropriate termination of the expressed mRNA and polyadenylation. Although naked plasmid DNA can be used in immunization, they are not so effective, and for efficient immunization several administrations are required. Like gene therapy, the success of DNA vaccine depends on the carrier systems. The carrier system is also critical for the selection of the route of administration [30-35].

The most important target of vaccines are mucosal surfaces since the vast majority of human and veterinary pathogens are transmitted across the epithelia of the respiratory, gastrointestinal, genital, or ocular tissues [36]. Systemic immunity can not prevent entry of pathogenic organisms at mucosal surfaces. Mucosal immune responses could block entry of pathogen, neutralize pathogen or virus that has invaded epithelial cells intracellularly and neutralize by activating specific Ig-A that are unique to mucosal tissues. IgA (S-IgA) are primarily secretory and function to prevent entry of the pathogen into the body via the mucosal surfaces. Thus, the development of an effective local immune response is essential for the prevention of most infectious diseases. Furthermore, mucosal immunization is highly desirable

for mass vaccination, since it is fast and easy to administer, requires minimal trained personnel, and carries no risk of needle stick injury or cross-contamination [37]. DNA vaccines have recently been shown in several studies to induce both local and distal mucosal and systemic responses following administration by mucosal routes [33]. For improved and needle free mucosal immunity, delivery of antigens or DNA is problematic because of the properties of mucosal tissues. In delivery of therapeutics through the mucosa the most common method is the use of mucoadhesive carrier systems to increase availability of drugs, antigens etc. Diverse classes of polymers have been investigated for their potential use as mucoadhesives. These include synthetic polymers such as poly(acrylic acid) (PAA), hydroxypropyl methylcellulose and poly(methylacrylate) derivatives, as well as naturally occurring polymers such as hyaluronic acid, chitosan and alginate [38-39]

1.2 Aim of the Study

In this study, the aim was to design and prepare a local, controlled, mucosal gene delivery system. DNA was complexed with PEI and loaded into alginate microspheres (Figure 1.4). Controlled release of PEI-DNA complexes from the microsphere at the target site was expected to increase the availability of the complex and thus transfection efficiency to be increased. This approach would also eliminate the toxicity of the system.

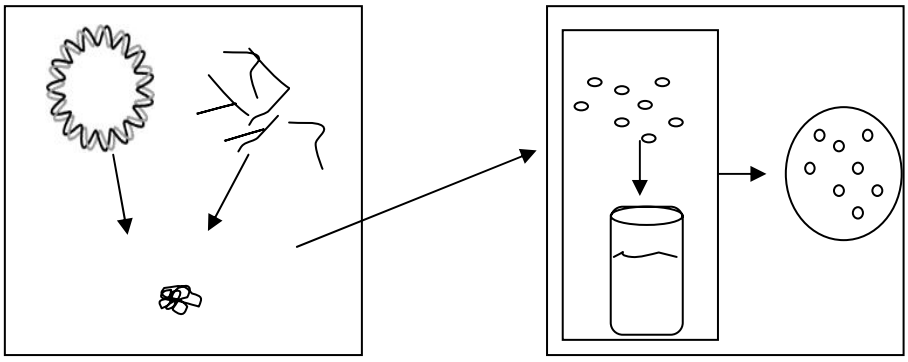


Figure 1.4: Schematic representation of the aim of the study.

DNA vaccine is a very promising alternative to the traditional vaccines to have immunity safely. Systemic administrations of antigens or antigen coding genes provides systemic immunity, in prevention of most of the infectious diseases mucosal immunity provides first defense mechanism. To provide mucosal immunity in this study oral mucosal surfaces were chosen as target. It is known that alginate is resistant to pH of the stomach, thus it can deliver its content to the intestinal mucosa. Mucoadhesion of the microspheres will be studied and to improve mucoadhesion PEG will be used.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Polyethylenimine (branched, 25000 Dalton) was purchased from Aldrich Chemical Company, Inc. (USA). Fetal bovine serum (FBS) was obtained from Biochrome KG (Germany). Dulbecco's Modified Eagle Medium (DMEM) was obtained from Gibco Invitrogen Corporation (New Zealand). MTS kit was purchased from Promega Corporation (USA). Picogreen was purchased from Molecular Probes Inc. (The Netherlands). Heparin was obtained from pharmaceutical company Mustafa Nevzat A.Ş. (Turkey) under the trade Nevparin. Restriction enzymes (Sal I, Pst I, mlu I and Hpa I) and DNA ladders (PhiX/HaeIII, Lambda/Hind III) were purchased from Fermentas (Lithuania). Alginic acid, trypsin-EDTA (0.25 %), Hepes, CaCl2, LB broth, agarose, agar, ethidium bromide, IPTG, X-gal and chemical components of buffers were supplied by Sigma Chemical Corporation (USA). Acridine Orange was obtained from BDH Chemicals Ltd. (UK). The RPE cell line D407 was a kind gift of Dr. R. Hunt (Department of Ophthalmology, University of South Carolina Medical School, and Columbia, S.C., USA). Plasmid, pCMVβ encoding β-galactosidase under the control of cytomegalovirus promoter and *E.coli DH5α* were a gift from Prof. A. Özkul (Department of Veterinary, Ankara University, Ankara, TURKEY), purchased from (Clonetech).

GenElute HP Plasmid Maxiprep Kit and bichincronic acid protein assay kit, β galactosidase expression assay kits were purchased from Sigma Chemical Corporation (USA). Giga plasmid isolation kit was obtained from Qiagen Corporation (Germany)

2.2. Methods

2.2.1. Preparation of Plasmids

2.2.1.1. Plasmids

The reporter gene plasmid pCMVβ encoding β-galactosidase under the control of cytomegalovirus promoter was used. The genetic map of the plasmid is available in Appendix A.

2.2.1.2. Transformation of Bacteria

To amplify large quantities of plasmid DNA, plasmid vector has to be transformed into a bacterial host. To perform transformation host bacteria should be made competent to incorporate plasmid DNA in a stable fashion.

2.2.1.2.1. Preparation of Competent Hosts

For the transformation of pCMVβ (Clonetech, BID, USA), *E.coli* DH 5α which was kindly provided by Prof. A. Özkul was used. Bacteria from frozen stock of *E.coli* DH 5α was inoculated with an inoculation loop into a 2.5 ml LB media and growth by using shaking platform overnight at 37 ºC . In order to prepare fresh culture, growth bacteria was diluted 200 times, 25 µl were taken and inoculated into 5 ml of LB media and incubated additional 2 hours. Cells were collected by centrifugation at 2500 rpm for 10 minutes (Heraus Christ Minifuge GL, Germany) and supernatant was decant and obtained pellet was resuspended in 0.5 ml transfection storage medium (TSS) (LB containing 10% PEG 8000, 20mM $MgSO_4$ and 5% DMSO at pH: 6.5) and mixed by using vortex. Suspension was transferred pre-chilled 1.5 ml eppendorf tube and incubated for 1 h on ice.

2.2.1.2.2. Transformation of Chemically Competent *E.coli DH 5α* with Plasmid DNA by Heat Shock

Plasmid pCMVβ was thawed and 2 µl of plasmid was diluted 100 times by using transfection culture medium (TCM) composed of 11 µl 1M Tris-HCl pH 7, 11 µl 1 M $MgCl_2$, 11 µl 1 M $CaCl_2$, 967 µl sterile distilled water and allowed to cool on ice. 200 µl of plasmid suspension was gently mixed with 200 µl of competent *E.coli DH 5α*. Cell and plasmid mixture was incubated on ice for 1 h and then heat-shocked in a water bath at 45 ºC for 2 min. After heat shock, the cell suspension was returned to ice and incubated for 5 min. 600 µl of SOC solution was added to mixture and left for incubation for 30 min at 37 ºC. In order to prepare sterile SOC solution first, 20 g bacto-tryptone, 5 g bacto-yeast extract and 0.58 g NaCl were dissolved in distilled water after adding 2.5 ml 1 M KCl solution. After completing to 1 L it was autoclaved. Into this solution sterile 10 ml 1 M $MgCl_2$ and 20 ml 1M glucose were added.

The medium was spread on the selective media containing ampicillin, IPTG and Xgal. In order to prepare the selective media, LB and agar were dissolved in nanopure water at a concentration of 2 % and 1.5 %, respectively and autoclaved for 10 min at 121 ºC. After cooling to 50 ºC, 100 mg/ml ampicillin in a final concentration of 100 µg/ml was added to media. X-gal was prepared in DMF (dimethylformamide) 20mg/ml and added to LB agar to a yield a final concentration of 240 µg/ml. 240 µg/ml IPTG was also added. IPTG stock solution was prepared at a concentration of 200 mg/ml and sterilized by filtering through a 0.22 micron filter. Prepared LB agar was introduced to sterile petri plate.

After solidification of the medium, 100 µl of bacteria suspension was inoculated by spreading the bacteria over the surface under aseptic conditions. Plates were incubated overnight at 37 ºC in an inverted position and stored at 4 ºC to allow full development blue colour. Blue and white colonies were observed and 10 blue colonies were picked separately and inoculated into 2.5 ml LB medium containing 100 µg/ml ampicillin.

2.2.1.3. Plasmid Isolation

2.2.1.3.1 Mini Prep Isolation

Small scale plasmid isolation was performed by alkaline lysis method according to the protocol of Sambrook et. al. [40]. In order to harvest cells, 1 ml of each culture was transferred to eppendorf tubes and was centrifuged in microfuge (micro Hettich Zentrifugen, Germany) 30 seconds for 12 000 rpm. The supernatant was discarded and bacterial pellet was resuspended in 100 µl of ice cold Solution I (50 mM glucose, 25mM Tris-HCl pH 8.0, 10 mM EDTA) by vortexing. After resuspension, to lyse the bacterial cells, freshly prepared 200 µl of Solution II (0.2 N NaOH, 1% SDS) was added and gently mixed by inverting the tube. For neutralization 150 µl of ice cold Solution III (3 M potassium acetate 60 ml, glacial acetic acid 11.5 ml, and H_2O 28.5 ml) was added and mixed by vortexing for 10 s. After 5 min storage on ice, the viscous cell lysate was centrifuged for 3 min at 4 ºC by using microfuge at 12 000 rpm. The supernatant was transferred to a new tube and 1 ml of absolute ethanol was added at room temperature to precipitate the DNA. Solution was centrifuged for 3 minutes in microfuge at 12 000 rpm. Supernatant was discarded by aspiration and pellet was washed by 70 % ethanol. After aspiration pellet was air dried for 10 min. Dry pellet was dissolved in 40 µl of TE buffer containing RNase at a final concentration of about 25 µg/ml.

The positive result of the miniprep was guaranteed by analytical digestion with Sal I, Pst I, Mlu I and Hpa I. 4 µl DNA sample, 3.5 µl of distilled water, 1 µl of enzyme buffer and 1.5 µl enzyme were added to an eppendorf tube and incubated for 1 h. After preparation of 1.5% agarose gel (molecular biology grade, Sigma), restricted plasmids and DNA ladder PhiX/HaeIII, Lambda/Hind III were loaded to the gel. After electrophoresis the DNA bands were visualized.

The bacteria strain that gave positive result, in plasmid production was amplified in ampicillin containing LB at 37 Cº and a glycerol stock of bacteria was made for further

use. Briefly, glycerol was sterilized by autoclaving for 20 min at 121 ºC and 0.15 ml of glycerol was added to the 0.85 ml of bacterial culture. The culture was vortexed and transferred to a cryogenic vial and tubes were transferred to -70 ºC.

2.2.1.3.2 Maxi Prep Plasmid Isolation

The plasmid "maxiprep" method is useful for preparing partially purified plasmid DNA in large quantities. GenElute HP Plasmid Maxiprep Kit (Sigma Chemical Corporation, USA) was used according to supplemented procedure. In order to obtain growth of large volume of transformed bacteria, 2.5 ml of LB media at a concentration of 20 g/L was prepared and sterilized by autoclaving at 121 ºC for 15 min. After cooling to 50 ºC, 1 µl/ml ampicillin was added (50 mg/ml ampicillin stock solution). By using inoculation loop, bacteria from frozen stock of pCMVβ transformed *E.coli DH 5α* was transferred and incubated at 37 ºC 15-16 h. For maxiprep plasmid isolation, grown pCMVβ transformed *E.coli* DH 5α inoculated into 250 ml LB containing ampicillin and incubated overnight. Cells were harvested from 250 ml culture by centrifugation at 5,000 X g (Sorvall RC-5B, USA) for 10 min and the supernatant was discarded. Pellet was resuspend by using 12 ml of resuspension solution by vortexing. The resuspended cells were lysed by adding 12 ml of the lysis solution and mixed by inverting gently. After obtaining a clear and viscous mixture by 5 min incubation, the lysate was neutralized by adding 12 ml of chilled neutralization solution and gently mixed. To provide binding of plasmids to the silica membrane 9 ml of binding solution was added and poured into the filter syringe that did not allow cell debris and protein lipid aggregates to go through. Binding column of kit including silica membrane was placed into a 50 ml collection tube and 12 ml of the column preparation solution was added to the column and centrifuged at the settings of 3 for 2 min (bench top centrifuge). The eluate was discarded and cell lysate was filtered by filter syringe into the binding column that was placed into the collection tube and centrifuged at the setting of 3 for 2 min and eluate was discarded. After passing all the suspension through silica membrane, the column was washed by 9 ml washing solution. Bound plasmids were

eluted from the membrane by using elution buffer (10 mM Tris-HCl, pH 8.5) into a new collection tube by the help of centrifugation at the settings of 5 for 5 min.

2.2.1.3.3 Giga Plasmid Isolation

To obtain large amounts of plasmid DNA, Qiafilter Plasmid Giga Kit (Qiagen, Germany) was used. In order to obtain growth of 2.5 L of transformed bacteria, 5 ml of LB media at a concentration of 20 g/L was prepared and sterilized by autoclaving at 121 ºC for 15 min. After cooling to 50 ºC, 1 µl/ml ampicillin was added (50mg/ml ampicillin stock solution). After inoculation of pCMVβ transformed *E.coli DH 5α* from frozen stock, bacteria were incubated at 37 ºC for 8 h with vigorous shaking (200 rpm). 2.5 L of LB broth was prepared and distributed to culture flasks. After sterilization of cultures, 1 µl/ml ampicillin was added. Starter culture was seeded to flasks with 800 fold dilution. For amplification, bacteria were incubated at 37 °C for 16 h with vigorous shaking (200 rpm). The bacterial cells were harvested by centrifugation at 4000 g for 20 min at 4°C in a Sorvall centrifuge. After removal of the supernatant, pellet was resuspended with P1 buffer and cell suspension was collected in a 1L bottle. 125 ml P2 buffer (lysis buffer) was added and lysate was neutralized with prechilled P3 buffer. After screwing the QIAfilter Giga Cartridge onto a 45 mm-neck glass bottle, the fluffy lysate was applied and filtering of lysate was provided by vacuum. The filtrate was applied to the precalibrated column of the kit and passed through it. After washing, plasmid DNA was eluted from column by 75 ml of elution buffer. To concentrate the plasmid DNA eluate, it was centrifuged at 18 000 g for 30 min (Sigma 30K, USA). After removal of supernatant, 10 ml of 70 % ethanol was added and centrifuged for 12 min. Supernatant was removed and the pellet was dried. To resuspend the pellet 6 ml of Tris-HCl buffer pH 8.5 was used.

2.2.1.4. Nucleic Acid Quantification

The most commonly used method of DNA quantification is the UV spectroscopy. Nucleic acids absorb UV light at 260nm and an OD of 1 corresponds to approximately

50 µg/ml of double-stranded DNA and 40 µg/ml of single-stranded DNA and RNA. As a blank Tris-HCl buffer was used, and 10 µl of plasmid stock was diluted to 800 µl with Tris-HCl buffer. Absorbance of diluted plasmid was measured by using spectrophotometer (Shimadzu UV 1601, Japan). Absorbance at 280 nm was also measured to evaluate the purity of the plasmid DNA. Absorbances were used to calculate the concentration of DNA as described below.

C µg/ml = A_{260} x 50 µg/ml x DF DF: Dilution Factor (in the above case DF is 80)

2.2.1.5. Agarose Gel Electrophoresis

In order to observe isolated plasmids, agarose gel electrophoresis was made by using the set up composed of electrophoresis tank and power supply. As the electrophoresis buffer to fill the tank and prepare the gel, 1X Tris-Borate-EDTA (TBE) was used, TBE was prepared as 10X stock. Powder agarose was added into 150 ml of 1X TBE buffer in a flask to a final concentration of 0.6 % (w/v) and the slurry was heated in a microwave oven (Arçelik, Turkey) (5 min) to dissolve agarose.

After cooling to 50 ºC, ethidium bromide 7.5 µl (0.5 µg/ml) was added and mixed thoroughly. In the electrophoresis tank tray positioned vertically so that a mold was obtained and the comb (12 well) was positioned to produce wells after solidification of gel. Warm agarose gel was poured to mold and allowed the gel to settle down by cooling to room temperature in 30-40 min. Comb was removed carefully and the tray was positioned horizontally. Gel tank was filled completely with electrophoresis buffer. Isolated plasmids were mixed with 6X loading dye, 8 µl plasmid DNA and 2 µl of loading dye (0.25 % bromophenol blue, 0.25 % xylene cyanol, 0.40 % sucrose in water) and mixture was loaded into the wells. The lid of gel tank was closed, electrical leads were attached and 60 mV was applied. After one h, power was turned off and the gel was examined by ultraviolet light and photographed.

2.2.2 PEI-DNA Complexes

2.2.2.1. PEI-DNA Complex Formation

A stock solution of Polyethylenimine (PEI) (1 mg/ml) was prepared in ultra pure water. The PEI solution was slowly added to the diluted DNA solution in appropriate concentrations and vortexed for 30 s. The mixture was allowed to stand at room temperature for 15 min. The ratio of PEI and DNA in complexes was determined according to the number of the charged groups (PEI nitrogen and DNA phosphate) N/P ratio was calculated by taking into account that $3.3.10^8$ μg DNA has 1 mole of phosphate and $4.31.10^7$ μg PEI has 1 mole of amine nitrogen.

$3.3.10^8$ μg DNA	1 mole of Phosphate
1 μg DNA	X mole Phosphate

$$X = 3.03.10^{-9} \text{ mole Phosphate}$$

For N/P=1 $\Longrightarrow$ $3.03.10^{-9}$ mole amine is required

1mole of amine	$4.31.10^7$ μg PEI
$3.03x10^{-9}$ mole amine	Y μg PEI

$$Y = 0.13 \text{ μg PEI}$$

2.2.2.2. Characterization of Polyplexes

2.2.2.2.1. Gel Retardation Assay

Binding of polycations to plasmid DNA results in the neutralization of negative charges on the phosphate backbone of DNA, and, in turn, in the formation of large neutralized complexes unable to migrate toward the anode in the agarose gel. Complexes for this

assay were formed at nitrogen to phosphate ratios of 0, 0.25, 0.5, 1 and 4. PEI solution was prepared at a concentration of 100 µg/ml. In each case; an appropriate amount of the PEI was diluted with distilled water in an epppendorf tube. Plasmid DNA 1 µg was added into the tubes, and were incubated at 37 °C for 20 min, and 10 µl of solution was mixed with 2 µl of the loading dye solution, and loaded onto ethidium bromide containing agarose gel (0.8% agarose in Tris-borate EDTA buffer). Electrophoresis was carried out at 100 V for 1 h, and DNA bands were visualized.

2.2.2.2.2. Spectrofluorometric Analysis

In order to observe complexation quantitatively, PEI-DNA complexes in different ratios of N/P were stained with ethidium bromide (EtBr). DNA (30 µg) were complexed with appropriate amount of PEI. After addition of 2.5 µl (1mg/ml) EtBr, fluorescence was measured with spectrofluorimeter (Shimadzu RF 5301, Japan) at λex: 535 nm, λem: 590 nm.

Light scattering of DNA and PEI was examined to observe complex formation. Intensities of light scattering were measured by spectrofluorimeter with a quartz cuvette (1×3 cm). The light scattering intensity was measured at 300 nm with slit a width of 5.0 nm for both the excitation and emission according to the protocol Zhou et.al [41] DNA (2.13 µg) was complexed with PEI (1.11 µg) in 2 ml of 0.05 mol l^{-1} Tris–HCl to yield a N/P of 4. Complexes and their 2, 4 and 8 fold dilutions were prepared and light scattering was measured. The effect of pH on light scattering by PEI-DNA complexes was also studied. Light scattering of PEI, DNA and PEI-DNA were measured in Tris-HCl buffer with different pH (2, 3, 4, 5, 6 and 7).

2.2.2.3. Quantification of PEI-DNA Complexes

Quantification of DNA is essential for the calculation of PEI-DNA loading in microspheres and release of PEI-DNA from microspheres. In order to quantify the

complexes, PEI-DNA complexes were dissociated and then quantified by using fluorescent staining.

2.2.2.3.1. DNA Quantification by Using Fluorescent Staining

In the quantification of free DNA after the dissociation of the complexes, different fluorescence stains were evaluated. To evaluate suitability of Hoechst bisbenzimidine, fluorescence of PEI (5 μg), DNA (2 μg) and complexes of PEI-DNA were measured in the presence of 2 μg Hoechst in a 2 ml by using spectrofluorimeter (λex: 345 nm, λem: 470 nm). PEI-DNA complexes were also examined by using Picogreen DNA dye. PEI (0.05 μg), DNA (0.1 μg) and complexes of PEI-DNA (N/P: 4) prepared in 1 ml of Tris-HCl buffer (10 mM Tris-HCl, pH 7.5). After 200 fold dilution of Picogreen with Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), 1 ml of Picogreen reagent was added to the sample and incubated 15 min at room temperature. The level of fluorescence was measured by spectrofluorimeter (λex: 480 nm, λem: 520 nm) (10 nm slit width). Since in loading and release studies two different buffers were used (5 M phosphate buffer for loading, 10 mM Tris-HCl for release), the effect of these buffers were studied.

2.2.2.3.2. Dissociation of PEI-DNA Complexes

Since DNA can not be stained and quantified correctly when in the complex, “heparin” was used to dissociate PEI from DNA. To determine appropriate amount of heparin 100 μl of DNA (1 μg DNA) and 100 μl of PEI-DNA (0.52 μg DNA with N/P: 4) treated with different concentrations of heparin for 10 min and DNA was quantified by using fluorescent staining with Picogreen as described earlier. The optimum concentration of heparin was determined.

2.2.2.3.3. Preparation of a Calibration Curve in the Presence of Heparin

Two different calibration curve were prepared for use in loading and release assay, For loading, 5 M phosphate buffer (PB, pH 7.5), and for release studies Tris-HCl (T-HCl, 10mM Tris-HCl, pH 7.5) were used. The calibration curves were prepared in duplicate and in the presence of 1400 U/ml of heparin.

DNA (0.2, 0.4, 0.8 and 1.2 µg) were prepared in Eppendorf tubes by using 4 µg/ml DNA stock and the volumes of samples were completed to 720 µl with assay buffer (T-HCl or PB). After addition of 280 µl of heparin (1400 U) to the each tube, samples were vortexed for 3 s and incubated for 10 min at room temperature. The fluorescence levels of DNA samples were determined with Picogreen assay. A calibration curve of fluorescence versus DNA concentration was prepared.

2.2.3. Microencapsulation

2.2.3.1 Alginate Microsphere Preparation

2.2.3.1.1 Water-In-Oil Emulsification Method

Medium viscosity alginic acid was dissolved in 14 ml of distilled water to yield a final concentration of 3 % and 3.5 ml of buffer was added instead of complexes and mixed. Alginate solution, 70 ml of canola oil and 1.6 ml Sorbitan monooleate (Span 80) emulsifier was added to a 3-necked flask as schematized in figure 2.1. The suspension was mixed by using an overhead stirrer for 15 min at level 3, 17.5 ml $CaCl_2$ was added during mixing and suspension was mixed for an additional 12 min.

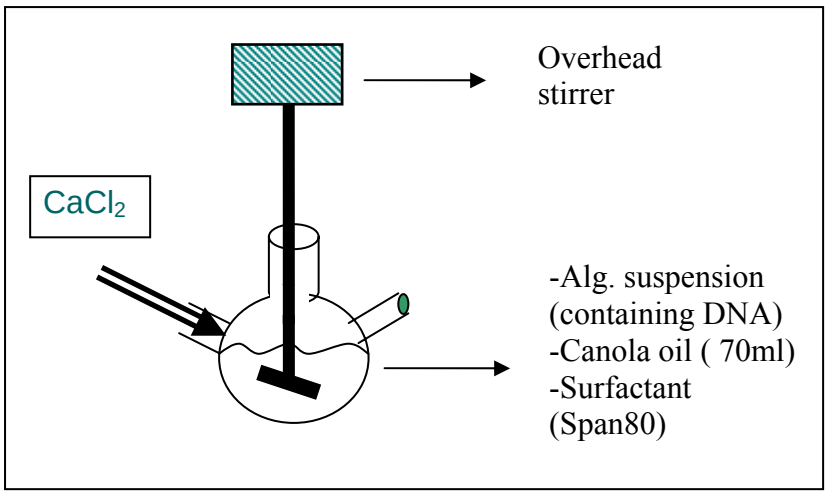


Figure 2.1: Schematic representation of alginate microsphere preparation by water-in-oil emulsification method.

To collect the microspheres, the suspension was dispersed into 50 ml falcon tubes and centrifuged for 15 min at level 5 by using bench top centrifuge. Pellet was resuspended in ethanol and centrifuged for 15 min at level 5 and supernatant was removed carefully to remove the oil. Pellet was resuspended in ethanol and transferred to a teflon sheet to prevent adhesion of spheres to the surface and left for air drying.

2.2.3.1.2 Ionic Gelation

Alginic acid was dissolved in distilled water to yield a concentration of 4.5 % by using magnetic stirrer. To prepare PEG modified alginate microspheres, PEG was added to the alginate solution during the dissolution stage. After adding appropriate amounts of PEI-DNA solution, alginate was diluted to the final concentration of 3%. The suspension was transferred to a home-made applicator set up and introduced into magnetically stirred 100 ml 1 % $CaCl_2$ as shown in figure 2.2. To obtain small size microspheres oxygen gas was applied. Microspheres were collected by centrifuging in a bench top centrifuge. To remove the remainder $CaCl_2$ and microspheres were washed with 70 % aqueous ethanol and were transferred into a teflon container. Microspheres were dried in laminar hood.

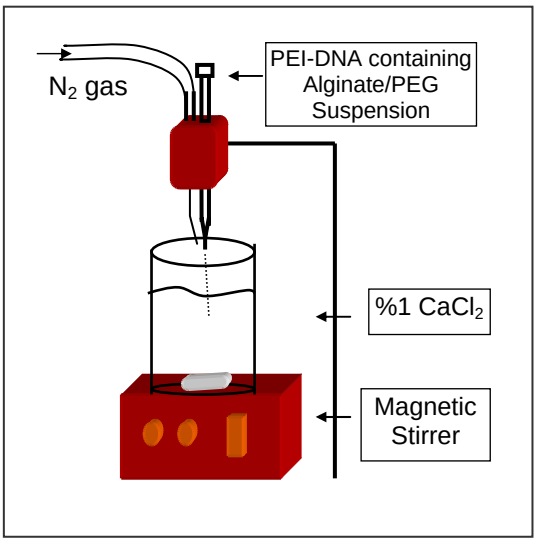


Figure 2.2: Summary of alginate microsphere preparation by ionic gelation method.

2.2.3.2. In Situ Characterization of Alginate Microsphere Preparation Methods

2.2.3.2.1. Morphology and Size Determination of Alginate Microspheres

Polyplexes (N/P: 4) were encapsulated into microspheres in a polyplexes/ microsphere ratio of 1/100. The morphology of alginate and alginate-PEG microspheres was studied using scanning electron microscopy (SEM) (JSM 6400, JEOL, Japan). Dry microspheres were attached on a double sided tape and stuck onto stub before coating with gold. The size distribution of microspheres was analyzed by stereo microscopy (Nikon SZM 1500, Japan). Also alginate microspheres were immersed into 1 mg/ml acridine orange dye solution and incubated for 2 h. After washing, microspheres were examined by using fluorescence microscope (Olympus IX 70, Japan).

2.2.3.2.2. Zeta Potential Measurement of Microspheres

Zeta potentials of alginate and PEG-alginate microspheres were measured by using a Zeta Meter 3.0+ (USA) at TAGEM, Afyon Kocatepe University (Figure 2.3).

Figure 2.3: Zeta Meter 3.0+ (USA)(TAGEM, Afyon Kocatepe University).

Alginate microspheres were prepared by ionic gelation method with different PEG concentration (0, 15, 30, 50 %) and then equilibrated in distilled water (pH 6.5) in a concentration of 1 mg/ml. The pH of the suspensions was adjusted to pH 7 by using 0.1 N NaOH. After transferring the samples to the electrophoresis tank of the Zeta Sizer, electrodes were placed and a 20 mV voltage was applied. The mobility of the particles was tracked with a microscope and the surface potential of microparticles was calculated.

2.2.3.2.3 Determination of Encapsulation Efficiency

The amount of microencapsulated PEI-DNA complex was quantified by extraction method. PEI-DNA loaded microspheres (5-10 μg) were introduced to 5 ml 5 M phosphate buffer of pH 7.5 and left on orbitary shaker for 12 h. The sample was then centrifuged at 2000 rpm for 10 min and the aqueous layer removed. PEI-DNA

complexes were released into the PB buffer and to determine amount of complex, PEI and DNA was dissociated by heparin and DNA was quantified.

2.2.3.2.4. In Vitro Release of PEI-DNA Complexes from Microspheres

The cumulative release of PEI-DNA complexes from microspheres was determined by using an in vitro release system. Microspheres (76 mg and 88 mg) were suspended in 2 ml of 10 mM T-HCl buffer and maintained at 37 °C in a shaking water bath. At 12, 24, and 48 h, suspension of the microspheres were replaced with 2 ml of T-HCl buffer using a syringe. Each time, the volume of collected suspension was measured and used in corrections of calculations. The amount of DNA in each sample was determined using a Picogreen assay after dissociation of PEI-DNA complexes. 720 µl of the supernatant was collected and 280 µl (1400 U) heparin was added and incubated for 10 min incubation. A Picogreen assay as described earlier (section 2.2.2.3.1) was then performed. The amount of DNA in the sample was calculated from the calibration curve.

2.2.4. Mucoadhesion

2.2.4.1 Determination of Bioadhesion Strength of Alginate and PEG-Alginate To Intestinal Mucosa

In order to stimulate the surface of a microsphere, sintered glass was coated with alginate. Sintered glass was cut into rectangular shapes and the dimensions were measured by using micrometer. In order to hang the sintered glass in the microbalance a piece of tape was rolled on the sintered glass and a hole was introduced in tape. Sintered glass was immersed into 3 % of alginate which 0, 15, and 30 % of PEG. After complete embedding of alginate, the sintered glass was transferred into 1 % $CaCl_2$ for 30 s and left to air dry (Figure 2.4).

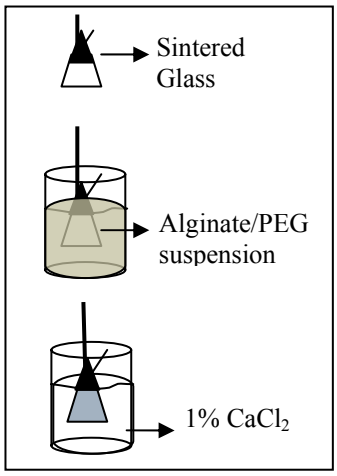


Figure 2.4: Preparation of alginate w/o PEG coating for mucoadhesion studies.

Jejunum intestine was obtained from newly scarificed calf. Part of jejunum (20-30 cm in length) was taken, after closing of two ends of intestinal segment, the segment was immersed into ice cold Dulbecco's phosphate buffered saline (DPBS) (pH: 7.5). Mucosal tissue was washed 3 times by using DPBS buffer, and then it was transferred into 1000 U/ml Penicillin containing DPBS for storage.

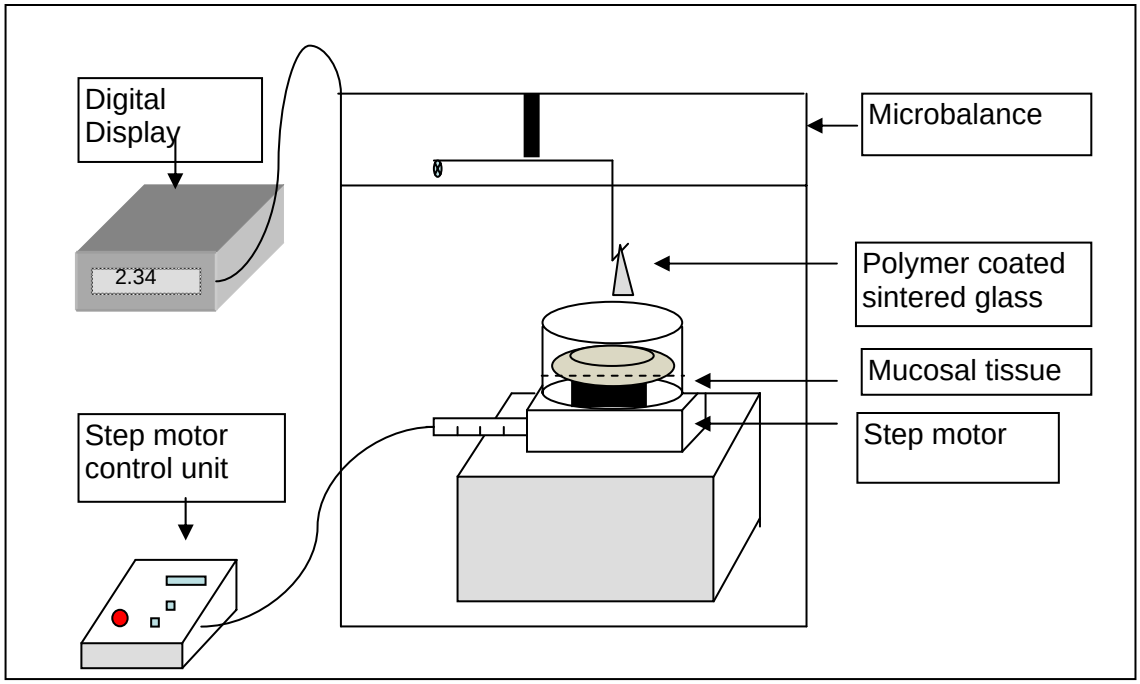


Figure 2.5: Schematic demonstration of the electro balance method for mucoadhesion test.

Mucosal tissue was stretched on a stage by using rubber. To prevent drying of the tissue, overhanging part of tissue was immersed into saline (0.87 % $NaCl_2$) in a beaker. The container was placed on the elevator of microbalance. The sintered glass was hanged up hook of microbalance (Figure 2.5). By using elevator, the contact of tissue and the alginate-coated sintered glass was achieved and the force that was required to separate them was measured in milligrams. By using the dimensions of contact surface area force was converted to adhesion strength (dyne/cm^2).

2.2.5. In Vitro Studies

2.2.5.1. Cell Culture of D407

D407 cell line (retinal pigment epithelial) passage number (PN: 10-18) was used in these experiments. The cells were grown in 75-cm^2 flasks for 7 days in DMEM supplemented with 5 % FCS, 10 mM Hepes and 50 µg/mL penicillin at 37 °C in a 90 % humidified incubator and 5% CO_2. The cell culture medium was changed every 2 days. After 80% of confluency was reached, cells were incubated in 3 ml of 0.1 % Trypsin-EDTA for 5 min, and the cell suspension was transferred to a conical tube containing 5 ml of medium including FCS. Cell suspension was centrifuged at 6000 rpm for 5 min. Cell pellet was resuspended in the medium and cells were seeded to new flasks 6 or 24-well plates. Number of cells was determined by using Nucleo Counter. Cell suspension was transferred (150 µl) into a sterile Eppendorf tube and 150 µl of solution A and solution B each was added to achieve the lysis of the cells. Lysate (50 µl) was loaded to the cassette of the Nucleo Counter.

2.2.5.2. Transfection

2.2.5.2.1 Transfection of D407 Cells With DNA/PEI-DNA

D407 cells were seeded at a seeding density of 1 x 10^5 cells/well on 6-well plates and grown in DMEM supplemented with 5 % FCS, 100 units/ml penicillin and 100 units/ml streptomycin at 37 °C in a 90 % humidified 5 % CO_2 incubator (MCO-17AIC, Sanyo Electric Co. Ltd., Japan) for 2 days. Prior to starting the transfection experiment, the cells were rinsed twice with warm phosphate-buffered saline (DPBS, pH 7.4), and every well was supplied with 1350 µL DMEM including 5 % FCS, 10 mM Hepes and antibiotics. Exactly 150 µL of the sample (DNA, PEI-DNA and PEI-DNA encapsulated alginate microspheres) were added to each well. The negative control group consisted of naked DNA in DMEM. The final pDNA concentration was 1.5 µg/well. The cells were transfected for 4 h and rinsed with warm DPBS, supplied with 1.5 ml culture medium, and allowed 48 hours for β-galactosidase protein expression. After 48 h, β-galactosidase expression was quantified.

In the transfection studies naked DNA (Sample I) and naked PEI-DNA (Sample II) complexes and PEI-DNA loaded alginate microspheres (Sample III) with the same DNA content were used.

Sample I:	15 µl DNA (100µg/ml) +	120 µl 1% Hepes +	15 µl ddH2O
Sample II:	15 µl DNA (100µg/ml) +	120 µl 1% Hepes +	15 µl PEI (0.052µg/µl)
Sample III:	8.6 mg PEI-DNA loaded alginate microsphere +		120 µl DMEM

2.2.5.2.2 β-Galactosidase Enzyme Assay

β-galactosidase expression was quantified by using β-gal enzyme assay kit. Briefly the cells were rinsed with DPBS and incubated in 250 µL cold lysis buffer (250 mM HEPES, pH 7.5, 25 mM CHAPS) for 15 min at room temperature. The cellular debris was pelleted by cold centrifugation at 14 000 rpm for 5 min, and 50 µl of supernatant and 50 µl of lysis buffer as blank were transferred to 96 well plate and 50 µl of assay buffer (200 mM sodium phosphate buffer, pH 7.3, 2 mM $MgCl^2$, 100 mM β-

mercaptoethanol, 1.33 mg/ml o-nitrophenyl β-D-galactopyranoside (ONPG)), was added to each well. When the yellow colour developed, 150 µl of stop solution was added and absorbance was measured at 405 nm by using a microplate reader (Vmax, Molecular Devices, USA).

In the calculations of the units of β-galactosidase (β-gal), the final assay volume Vf, the lysate volume and required time for colour development (t_{min}) were taken into consideration and the absorbance of 1 µmol/ml (1 mM) of enzyme was accepted as 4.6 for an optical path of 1 cm according to manufacturer's protocol.

$$\text{Units/Sample} = \frac{\text{OD} \times V_f}{4.6 \times \text{tmin}} \times \frac{\text{Lysis Volume}}{\text{Sample Volume}} \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

2.2.5.2.3 Determination of Total Protein

The amount of protein was standardized by the total amount of protein present in the cell lysate that was measured with the bicinchoninic acid (BCA) assay (Sigma) kit. In the assay, initially, BCA working reagent was prepared by mixing 50 parts of Reagent A (bicinchoninic acid, sodium carbonate, sodium tartrate, and sodium bicarbonate in 0.1 N NaOH pH 11.25) with 1 part of Reagent B (4 % (w/v) copper (II) sulphate pentahydrate). In each assay, to prepare a calibration curve, 0, 40, 60 and 80 µg/ml of BSA was prepared by diluting a 1 mg/ml standard BSA with lysis buffer. Each BSA (0.1 ml) protein standard, lysis buffer (as blank), and cell lysate were transferred into test tubes and 2 ml of the BCA Working Reagent were added and vortexed. After 30 min incubation at 37 ºC, absorbances of the solutions were measured at 562 nm with spectrophotometer. By using net absorbances and BSA standard concentrations, a calibration curve was created and the total protein content of the cell was calculated.

2.2.5.3. Measurement of Cytotoxicity of the Gene Delivery System

A cytotoxicity assay based on cell proliferation was made by using MTS assay (Nonradioactive cell proliferation assay, Köse et al., 2003). A calibration curve was constructed using the predetermined cell numbers (counted with NucleoCounter) of D407 cells. $2.5.10^4$, $5.0.10^4$, $1.0.10^5$, $2.5.10^5$, $5.0.10^5$ and $1.0.10^6$ cells were seeded on 24 well plates in triplicate and incubated for 4 h. After removal of the medium wells were washed with DPBS. MTS solution (500 µl, 10 % PMS and MTS in DMEM low glucose medium) was added. After 2 h of incubation at 37 ^{0}C, 150 µl of this solution was transferred to a 96-well plate. The optical density (OD) at 490 nm was determined with the microplate reader (Maxline Vmax®, Molecular Devices, USA).

For the evaluation of cytotoxicity of the PEI-DNA loaded alginate microspheres and naked PEI-DNA complexes, after counting cells with Nucleo Counter, cells were seeded to 24 well plates at a density of $5.0.10^4$ cell/well. After 24 h PEI-DNA loaded alginate microspheres (1 mg and 5 mg), PEI-DNA complexes (1 µg and 5 µg) were suspended in 100 µl T-HCl buffer and added to 400 µl medium containing wells. MTS assay was carried on 48 h later.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Transformation of Bacteria and Plasmid Isolation

Plasmids pCMV β were transferred to the bacterial cell and transformed *E.Coli DH5α* colonies were selected by blue white screening. The plasmid includes Ampicillin resistance gene and Lac Z, in the presence of IPTG, inducer of lac operon, bacterial cells that have pCMV β plasmids produce blue colonies after utilization of substrate of X-gal

After isolation, plasmids obtained from different clones were examined on the gel and the clones that produced the highest concentration of plasmids were amplified.

3.1.1. Restriction Analysis of the Plasmid

The positive result of the mini prep was guaranteed by restriction enzyme analysis. Four different restriction enzymes were chosen by using restriction map of plasmid that was provided by the manufacturer. Sal I has one restriction site and it was used for the linearization of plasmid, Pst I has two, Mlu I has 3 and Hpa I has 4 restriction sites in the pCMVβ (Appendix B). According to restriction map of pCMVβ, expected fragment sizes were;

Sal I: 7164 b
Pst I: 2741 b, 4423 b
Mlu I: 425, 780 b, 5949 b
Hpa I: 624 b, 716 b, 2431 b, 3393 b

To demonstrate DNA fragments and their size distribution, the restricted plasmids were run on the agarose gel in the presence of DNA ladder (PhiX HaeIII, Lambda Hind III). Figure 3.1 shows the restriction analysis of the isolated plasmid; the fragments and sizes are (seen below) to be consistent with results expected from manufacturer's specifications.

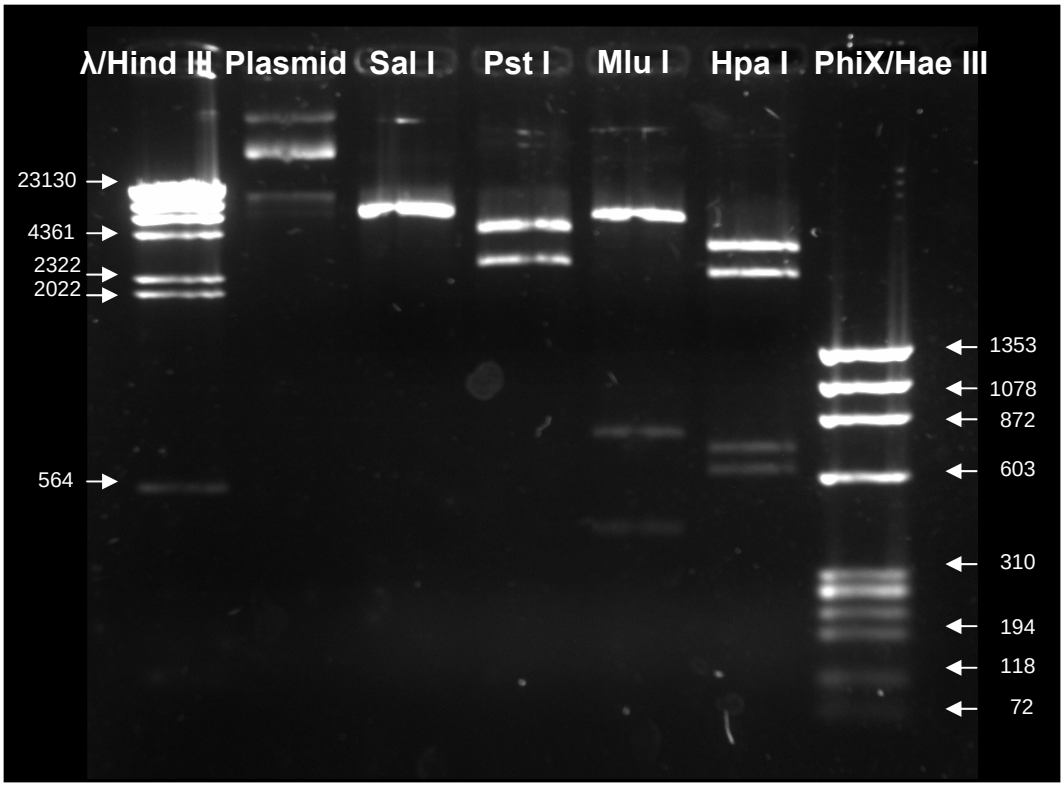


Figure 3.1: Restriction analysis of plasmid DNA

3.1.2 Plasmid Isolation in Larger Quantity

Large amount of plasmids needed for the tests were obtained by using Maxi and Giga plasmid isolation kits. The total quantity of plasmid obtained by Maxi prep and Giga prep was 300 µg and 6 mg respectively. After each isolation the purity of the sample was examined by checking the ratio of absorbances at 260 nm and 280 nm. The ratio was around 1.8.

3.2 PEI-DNA Complexes

3.2.1. Gel Retardation Assay

To demonstrate the interaction between the positively charged PEI and the negatively charged plasmid DNA, a gel retardation assay was performed using electrophoresis. Figure 3.2 shows the electrophoretic mobility shift assay for different N/P (ratio of NH_2 of PEI to phosphate of DNA) ratios. There was a decrease in the electrophoretic mobility of the plasmid with increasing polymer concentration. The N/P ratio for a given amount of plasmid, with no DNA mobility was observed with complexes prepared at the N/P ratio of 4 and in agreement with previously reported data. N/P is an important factor in gene transfer because it determines the net charge and the size of the complexes. It was earlier reported that slightly positive charge was very effective in transfection [42].

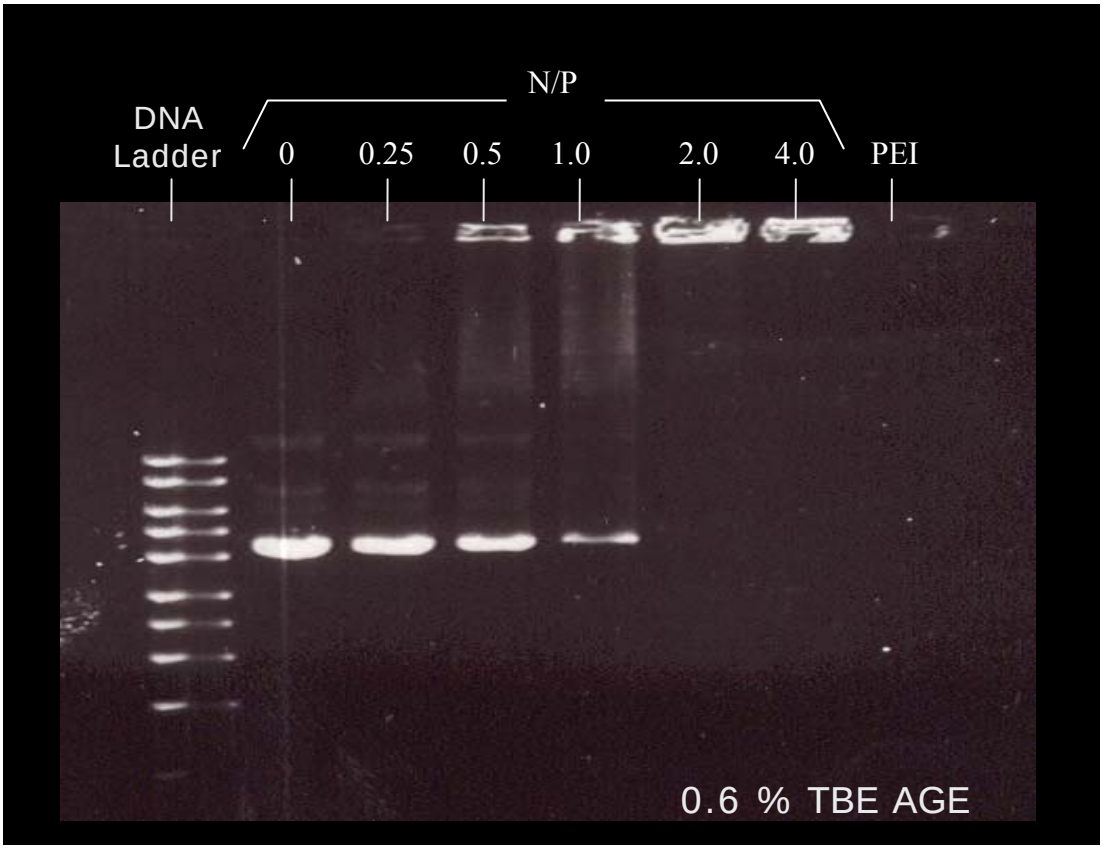


Figure 3.2: Examination of complexes by agarose gel electrophoresis. N/P ratio is the ratio of NH_2 of PEI to phosphate of DNA.

3.2.2. Spectrofluorometric Analysis of Complexes

To observe the interaction between PEI and DNA, dye exclusion method was used. Ethidium bromide is a polycyclic fluorescent dye that binds to double-stranded DNA molecules by intercalating a planar group between the stacked base pairs of the nucleic acid. Complexation prevents binding of EtBr to the DNA and leads to a decrease in the level of fluorescence. Fluorescence of DNA was measured for different N/P ratio (Figure 3.3). By increasing the PEI concentration, the fluorescence level was decreased. For N/P ratios of 2 and 4, fluorescence levels were nearly zero. Gel retardation assay and spectrofluometric analysis of complexes gave the same result.

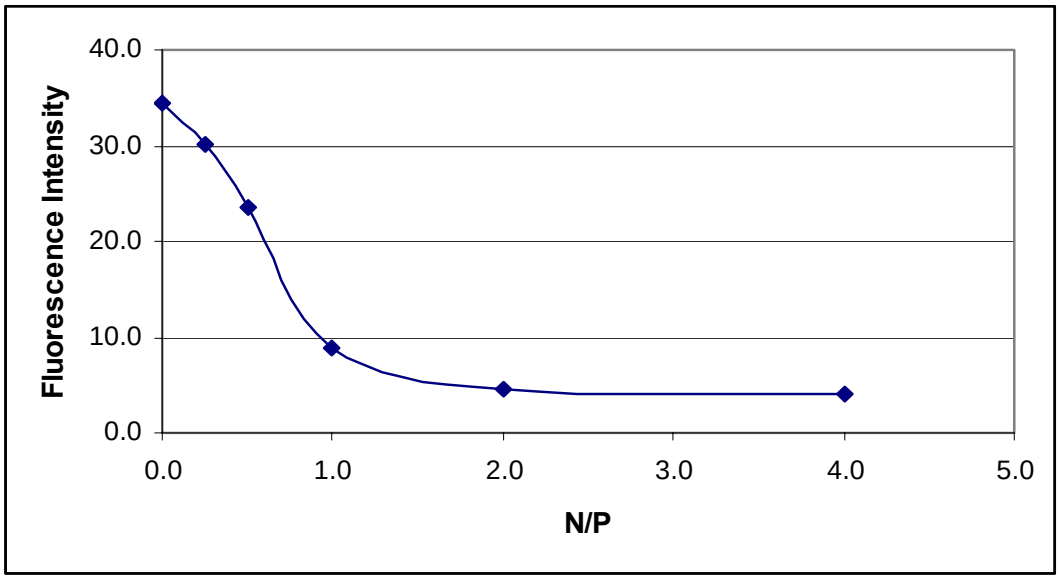


Figure 3.3: Examination of complexes with dye exclusion method.

3.2.3. Observation of the Complexation of PEI and DNA by Fluorescence Spectroscopy

The principle of light scattering was based on measurement of scattered fluorescence light from particulates, thus it depends on particle formation. It has been widely used to determine nucleic acids based on an interaction between DNA and other reagents. Light scattering by complexes is a very sensitive method in the quantification of PEI and DNA. It was observed that enhancement in light

scattering, when PEI-DNA complexes formed [41]. To evaluate sensitivity of the light scattering method, PEI-DNA complexes with 1 µg DNA content were prepared and light scattering of the complex as it is and after dilutions were measured by using spectrofluorimeter. The linear results are presented in Figure 3.4. However, this method could not be used in the determination of encapsulation efficiency and release of PEI-DNA complexes. It was reported that light scattering was affected by ionic strength, pH and presence of other chemicals. Also, it was possible that the amount of PEI in the complex could change during release studies and during loading because of the reversibility of complexation of PEI-DNA.

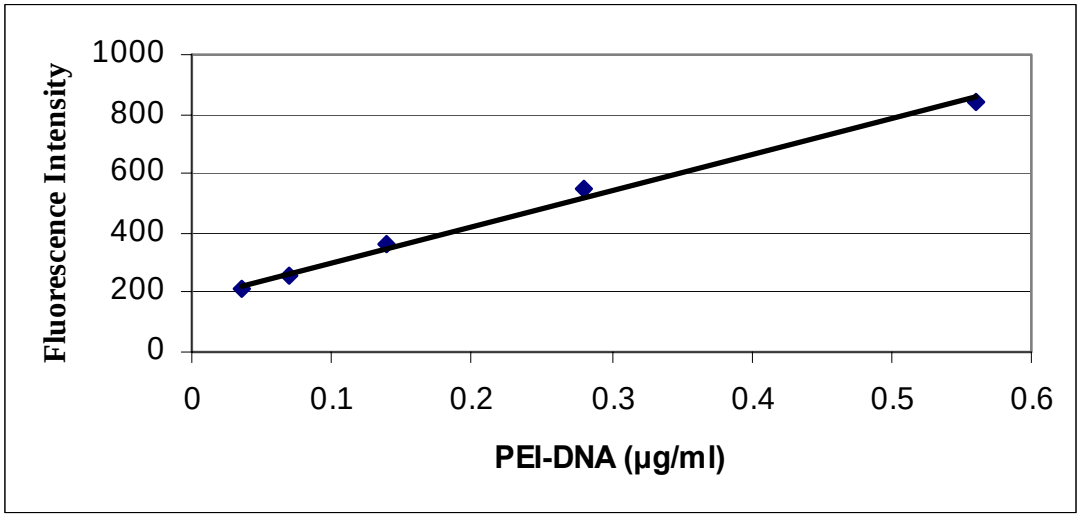


Figure 3.4: Analysis of sensitivity of light scattering to the concentration of complexes.

In order to understand the behaviour of PEI-DNA complexes at different pHs, light scattering was used and results are presented (Figure 3.5). At around pH 4, light scattering of PEI and PEI-DNA complex was enhanced, thus indicating that particle sizes were increased. It was reported that, at low pH, higher protonation of PEI occurs and this induces a stronger repulsion of intramolecular amino groups causing enlargement in the PEI [15]. Also, higher protonation of PEI increased complexation of PEI and DNA. However, this effect was not observed at pH lower 4. In the study of Zhou et. al (2004) the effect of pH (2, 3, 4, 5, 6 and 7) was also discussed; they could not observe any changes in the light scattering of PEI. They used PEI with molecular weight of 50 000-60 000 (Acros Organics), in this study

PEI with MW 25 000 was used. Differences in branching of the two PEI and MW used could be responsible for the differences in the results.

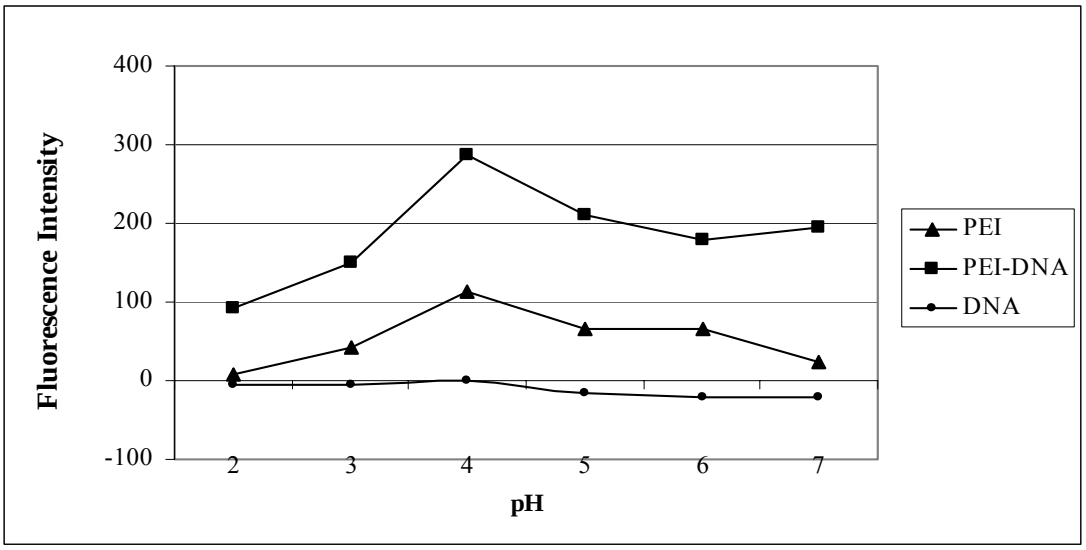


Figure 3.5: The influence of the pH on the interaction of PEI and DNA by spectrofluorimetery.

In order to quantify DNA, Hoechst 33258 (bisbenzimide) was evaluated. As shown in figure 3.6, Hoechst 33258 interacted with PEI and a nonproportional fluorescence signal was obtained. Therefore, another fluorescent dye, PicoGreen, with higher sensitivity range for DNA quantitation was tested.

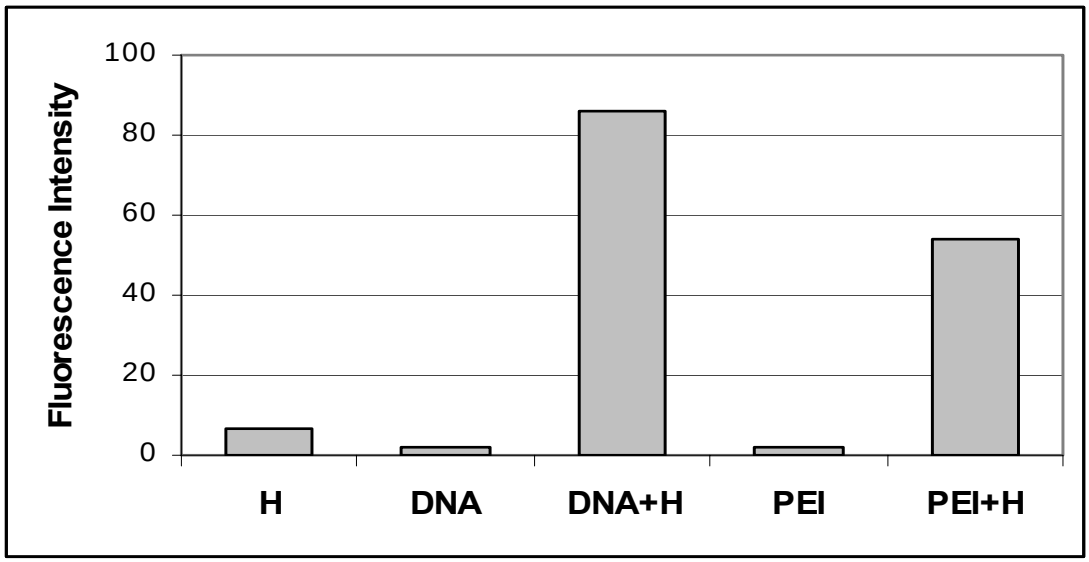


Figure 3.6: The evaluation of Hoechst 33258 (bisbenzimide) as fluorescent dye in quantification of PEI and DNA. H: Hoechst 33258

Interactions of PicoGreen with PEI, DNA, and PEI-DNA complexes were evaluated in two different buffers (Figure 3.7). Since a fluorescence signal was observed only in the presence of DNA, and since fluorescence intensity was high even at low concentrations of DNA, PicoGreen was used as indicator dye in the rest of the experiments.

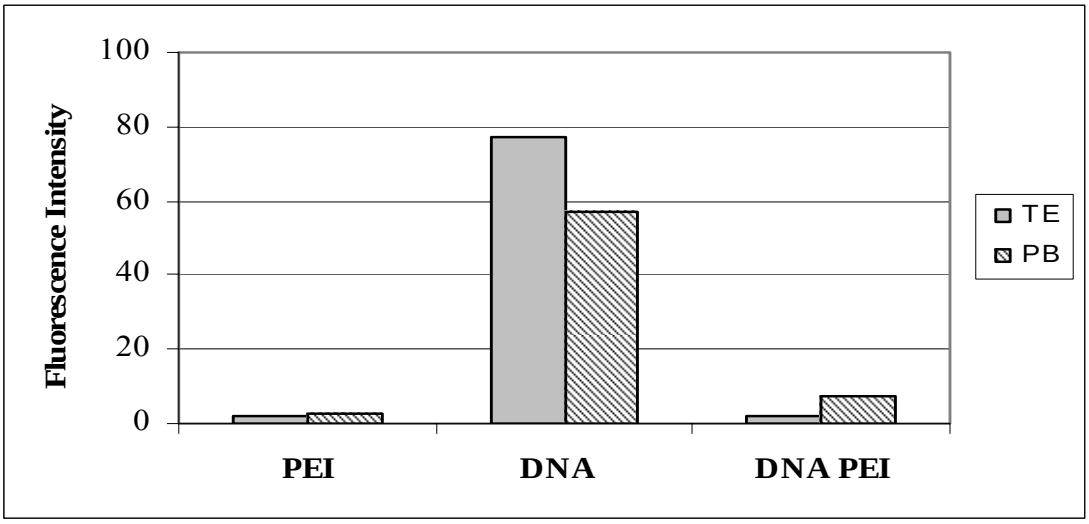


Figure 3.7: PicoGreen quantitation of PEI, DNA and PEI-DNA in two different buffers 10 mM T-HCl and 5 M PB at pH 7.5 (DNA: 0.1µg, PEI: 0.05)

Fluorescence intensity upon DNA staining in 5 M PB was lower than in 10 mM T-HCl buffer at pH 7.5. It was observed that 5 M PB probably affects DNA conformation and causes coiling of DNA and decrease accessing of dye to the DNA. Since staining in these buffers had different fluorescence intensity, two different calibration curves were prepared. The calibration curves of plasmid concentration versus fluorescence intensities with PicoGreen in different buffers 5 M PB and 10 mM T-HCl are presented in Appendix C and Appendix D respectively.

3.2.4. Dissociation of PEI-DNA complexes

In order to determine DNA, PEI-DNA complexes had to be dissociated and for this heparin was used. Heparin is a negatively charged polymer, and produces complexes with PEI causing the release of DNA. The fluorescence intensities of DNA and PEI-DNA were compared in the presence of different concentrations of

heparin. As shown in Figure 3.8, by increasing the concentration of heparin, the fluorescence of DNA decreased and with 1400 U/ml heparin, PEI-DNA complexes had the same fluorescence intensity as naked DNA. It meant that, 1400 U/ml heparin could remove all the DNA from the PEI-DNA complex.

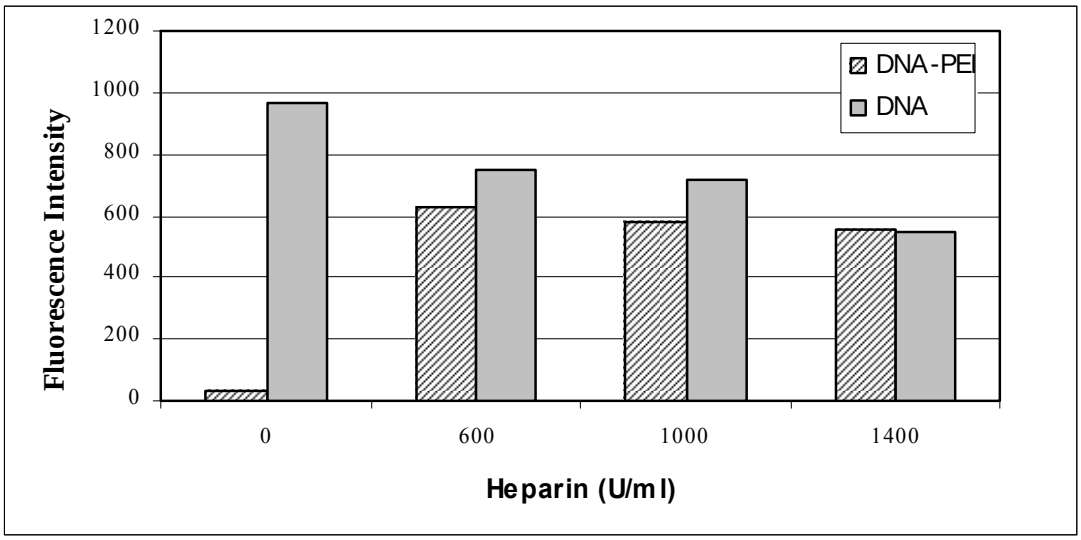


Figure 3.8: The influence of amount of heparin concentration on fluorescence intensities of DNA and PEI-DNA complexes with PicoGreen.

3.3. Characterization of Alginate Microspheres

3.3.1. Properties of Microspheres Prepared by Emulsification

By using water-in-oil emulsification method, alginate microspheres with 5-10 μm size range were obtained. To examine the microspheres with fluorescence microscopy, they were stained with acridine orange (Figure 3.9).

After microsphere preparation several solvents including ethanol, acetone and chloroform were used to remove the oil and the number of washing steps was also increased. However, neither of them removed all the oil. Also, in order to dry, the microspheres, air drying, vacuum drying and freeze drying were tested. In all cases alginate microspheres were fused.

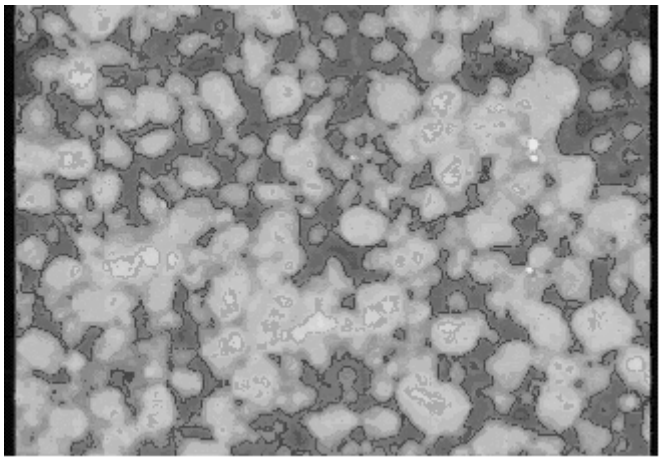


Figure 3.9: Fluorescence microscopy of alginate microspheres prepared by emulsification method after staining with Acridine Orange (X20)

Alginate microspheres prepared by emulsification method were reported in number of applications including encapsulation of antigen, DNA and viruses for vaccine applications [19-20, 43-44] where alginate microspheres were either dried by spray drying were used while wet. In those reports there was no mention of the remaining oil. Unsuccessful drying and remained oil prevented the calculation of doses that is used in transfection.

3.3.2. Characterization of Microspheres Prepared By Ionic Gelation

Microspheres were prepared by gelation of alginate droplets in $CaCl_2$ solution. It is simple and does not include chemicals like surfactants and oil. The size of microspheres obtained were larger than those obtained with the emulsification method, was around 90 μm (Figure 3.10 and 3.11)

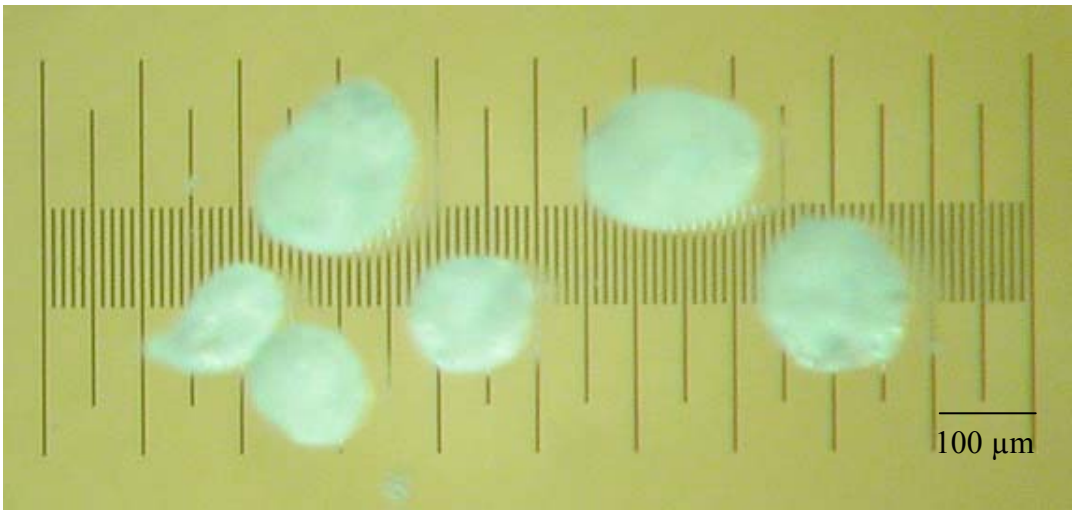


Figure 3.10. Stereomicrographs of alginate microspheres

PEG contaning microspheres were prepared with ionic gelation method and the effect of PEG on the morphology of the alginate microspheres was examined by scanning electron microscopy. As shown in Figure 3.12, increasing PEG concentration was reflected on the surface of the microspheres as increased smoothness. Drying of highly swollen alginate causes the shrink and the surface appears rough. In the presence of PEG, surface roughness of the spheres was decreased, this could be explained by the difference in water loss from microspheres. This observation is an indirect proof of the presence of PEG on the surface, and of formation of semi-interpenetrating network of PEG-alginate.

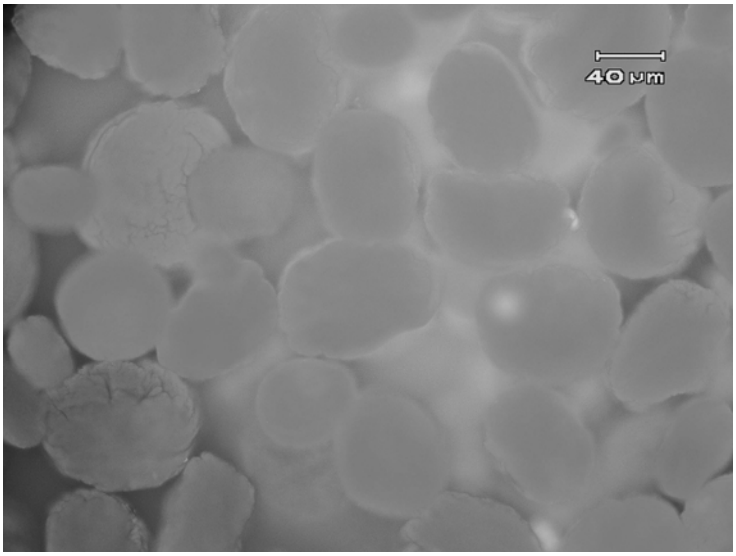
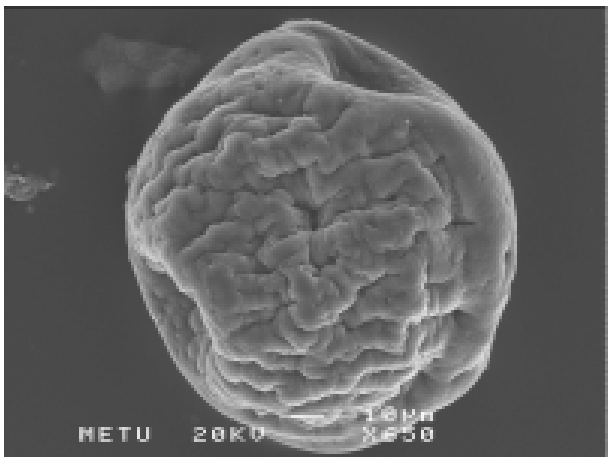


Figure 3.11: Fluorescence microscopy of alginate microspheres prepared with ionic gelation after staining with acridine orange (X20)



A)

B)

C)

Figure 3.12: Scanning electron micrographs of microspheres. A) Alginate microsphere (X650), B) PEG-Alginate (15:85) microsphere (X450), C) PEG-Alginate (30:70) microsphere (X350)

3.3.3. Surface Charge Analysis by Measurement of Zeta Potential of Microspheres

In order to study the surface charge of the prepared microspheres and the effect of presence of PEG on the surface of microspheres, zeta potentials were measured. It is known that Ca-alginate microspheres are negatively charged and PEG has no net charge. Alginate microspheres were measured to have a surface charge of -24 mV. By increasing the PEG content of the alginate microspheres, the negative zeta potentials of the particles decreased, since PEG has no charge (Figure 3.13). In PEG-Alginate 50:50, microspheres were in disc shape instead of sphere, because with higher PEG concentration, the quantity of alginate was insufficient to entrap PEG and alginate could not be effectively crosslinked with Ca^{+2}.

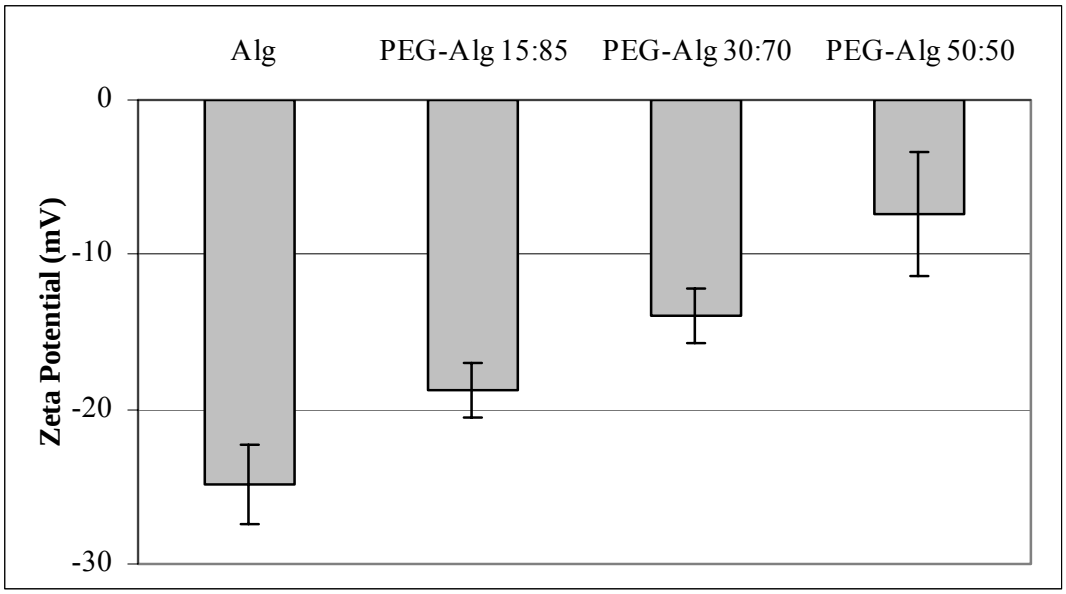


Figure 3.13: The influence of PEG content of alginate microspheres on their Zeta potential.

3.3.4. Calculation of Loading of PEI-DNA Complexes to Microspheres

For each batch of microspheres loading of PEI-DNA into them was calculated after lysis of microspheres by concentrated 5 M phosphate buffer at pH 7.5. In the presence of phosphate, calcium interact with them, since the solubility of the resultant calcium phosphate is low, nearly all of the calcium ions precipitate.

Centrifugation of lysate provides a clear supernatant consisting of PEI-DNA complexes. After dissociation of complexes with heparin and PicoGreen assay, the concentration of DNA was calculated by using calibration curve. Input DNA content of microspheres was 1 %, thus 100 mg alginate microspheres contained 1 mg of DNA and enough PEI to yield in N/P 4. Loading and loading efficiency were calculated as;

PEI-DNA loading % = Amount of DNA (mg)/ Formulation weight (mg) x 100 (2)

Loading efficiency % = Amount of DNA(mg)/ Input amount of DNA (mg) x 100 (3)

PEI-DNA loading and loading efficiency of the microspheres were found to be as 0.59 ± 0.03 % and 59 ± 3 % respectively. Alginate microspheres prepared with ionic gelation method are known to have high encapsulation efficiencies especially for macromolecular drugs e.g. 95.0% for BSA-fluorescein isothiocyanate; 80.0% for blue dextran. Encapsulation efficiencies were generally lower for low molecular weight drugs, e.g. 4.0% for nitrofurantoin, 32% for indomethacin [45, 46]. Encapsulated poly-L-ysine and oligonucleotide complexes in alginate microsphere with 49.45 % efficiency [26]. The main reason for differences in loading efficiency was explained as the high porosity of alginate microspheres. It is also known the complexes of polycation and DNA (70-120 μm) can leak out from alginate microspheres during washing. By optimizing the concentration of alginate, $CaCl_2$ and PEI-DNA complexes, loading efficiency of alginate microspheres can be improved. Also, the net charge of PEI-DNA complexes affects the loading efficiency and the release rate because of ionic interactions between alginate and complexes. If the N/P ratio is increased to 4 or more, complexes become positively in charge [24] and the loading of positively charged molecules into alginate is higher than neutral and negative ones [47]. Moreover, the encapsulation efficiencies of water soluble drugs are in general lower than those of slightly soluble or insoluble drugs, a property which can also be modified with by optimizing the N/P ratio.

Polycation-DNA complexes were encapsulated in PLGA microspheres other than alginate. The encapsulation efficiency of PLGA microspheres were observed to be 13-17 %. This low encapsulation efficiency was explained by the remaining of the complexes at the interphase between solvent and aqueous phases during microsphere preparation [24-25].

3.3.5 Release of PEI-DNA Complexes from Microspheres

Since alginate microspheres were disintegrated in phosphate buffer easily, PEI-DNA release experiments were carried out in Tris-HCl buffer (10 mM, pH 7.5) in a shaking waterbath at 37 ºC. At predetermined time points, 2 ml release buffer was removed and replaced with 2 ml fresh Tris-HCl. Sample (720 µl) was treated with heparin and DNA released was quantitated with PicoGreen assay using the calibration curve (Appendix D). DNA concentration of the total sample and DNA concentration in 1 mg of alginate microsphere were calculated and cumulative release of complexes from alginate microspheres were determined.

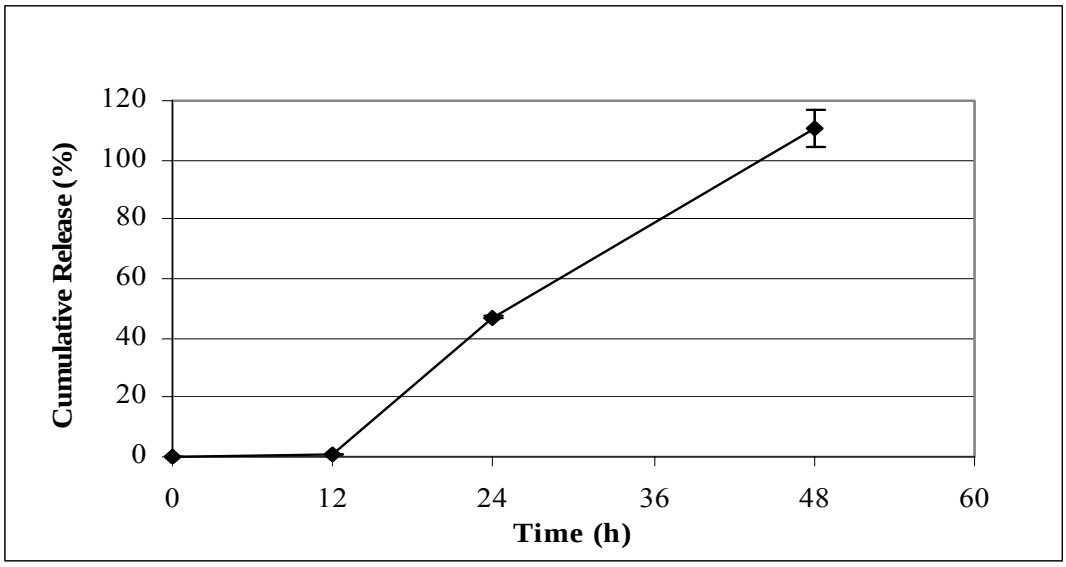


Figure 3.14: Release of PEI-DNA complexes from alginate microspheres in Tris-HCl buffer (10 mM, pH 7.5)

The release profile of PEI-DNA complexes are shown in Figure 3.14. There is an initial lag phase for at least 12 h followed by a significant burst. The lag in the release of complexes could be explained with the effect of alcohol washing and

over drying preparation of microspheres. During the lag the microsphere swells its pores open. The release of drugs or particles from alginate microspheres depends on diffusion and erosion [45-46]. Diffusion of molecules occurs via the water phase that fills the matrix of the microspheres. Erosion of the matrix is the result of the reversion of the Ca^{+2} crosslinking procedure. Depending on the release medium one of these routes becomes predominant. In PBS buffer or EDTA containing buffers release is controlled by erosion; in other cases diffusion is the main mechanism of release. Although there was a lag phase, release of complexes was completed in 48 h, and at the end microspheres were disintegrated because of swelling.

The final aim of this study was in vivo application of the developed system, release of complexes in vitro cell culture was studied. Release of complexes in the DMEM were decreased with respect to those obtained in Tris-HCl medium (Figure 3.15) It is suspected that the Ca^{+2} ions in the DMEM stabilize the alginate microsphere preventing swelling due to loosing gel and initiation of release.

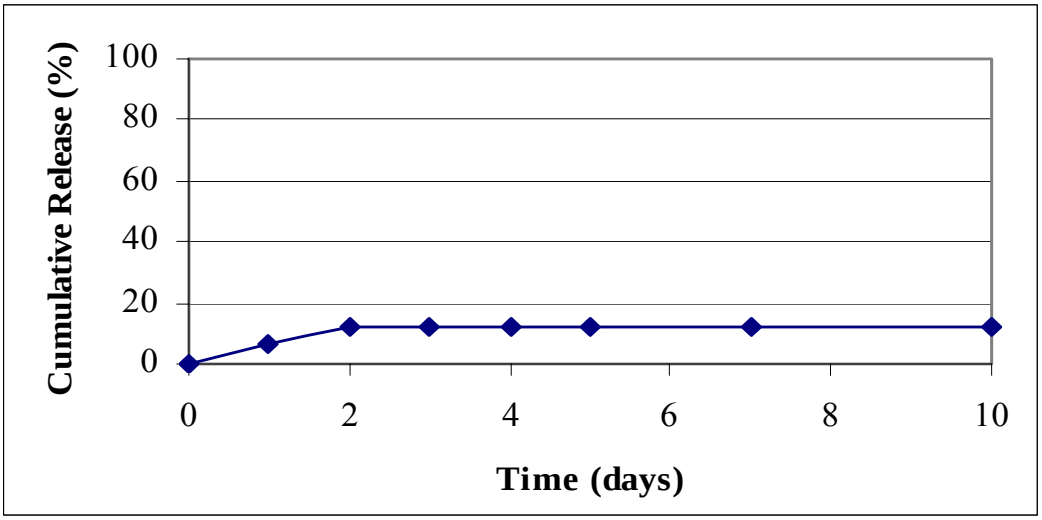


Figure 3.15: Release profile of PEI-DNA complexes from alginate microspheres in DMEM.

For comparison, release of complexes from PEG-alginate (30:70) was also studied. In the DMEM, the release of complexes was enhanced in comparision to results represented in the Figure 3.15. Most probably, incorporation of PEG into alginate leads to an increase in pore sizes, thus looser microspheres were formed (Figure 3.16).

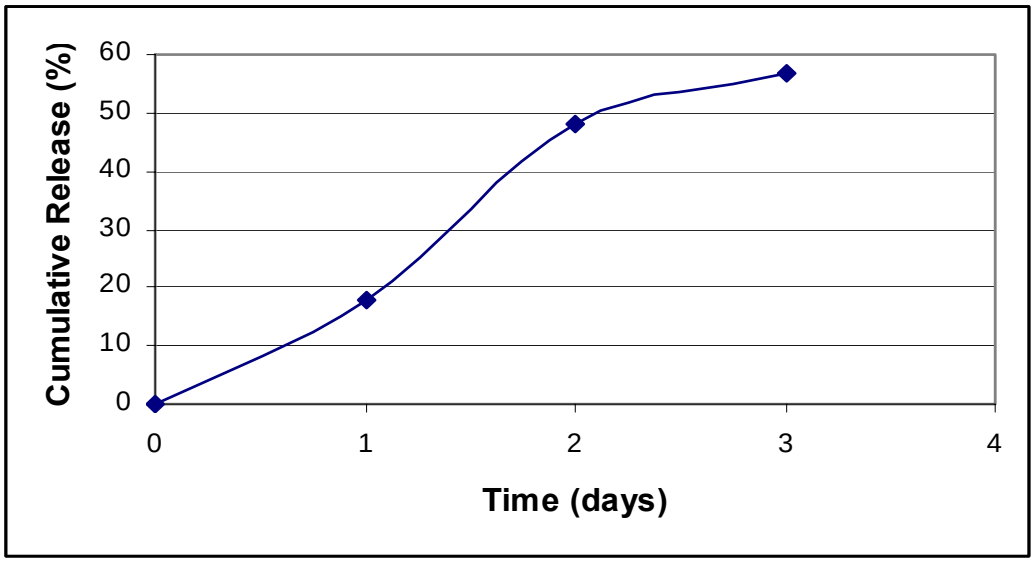


Figure 3.16: Release profile of PEI-DNA complexes from PEG-alginate (30:70) microspheres in DMEM.

Release of complexes from the carrier is crucial for transfection. Up to now different carrier systems like alginate, chitosan, PLGA etc. have been used for complex delivery, but there is no systematic study on this subject to optimize delivery [23-26]. Alginate is suitable for modifications; in order to slow down release from alginate microspheres can be coated with poly(l-lysine), chitosan etc. or to increase release hydrophilic components like PEG can be used. Since the system was developed as potential DNA vaccine delivery system, for improved gene delivery and vaccination, release profile of the system in simulated in vivo systems should be optimized.

3.4. Mucoadhesion

It is known that alginate possesses mucoadhesive properties, which could increase the contact time between alginate microspheres and the absorptive mucosa and therefore, could enhance the uptake of encapsulated complexes that are released from microspheres [46,49]. In this system the alginate microsphere was used as a reservoir for the PEI-DNA complexes. To study the adhesion of alginate to mucosa and the effect of PEG on adhesion a method based on microbalance was used.

Adhesion force between alginate and intestinal tract mucosa was measured as 3184±8 dyne/cm^2. This indicated that alginate could be considered as an excellent bioadhesive. Alginate is known to have mucoadhesive properties because of its negative charge, hydrophilicity, high swelling capacity and interpenetration of glycoprotein network of mucus and alginate [50].

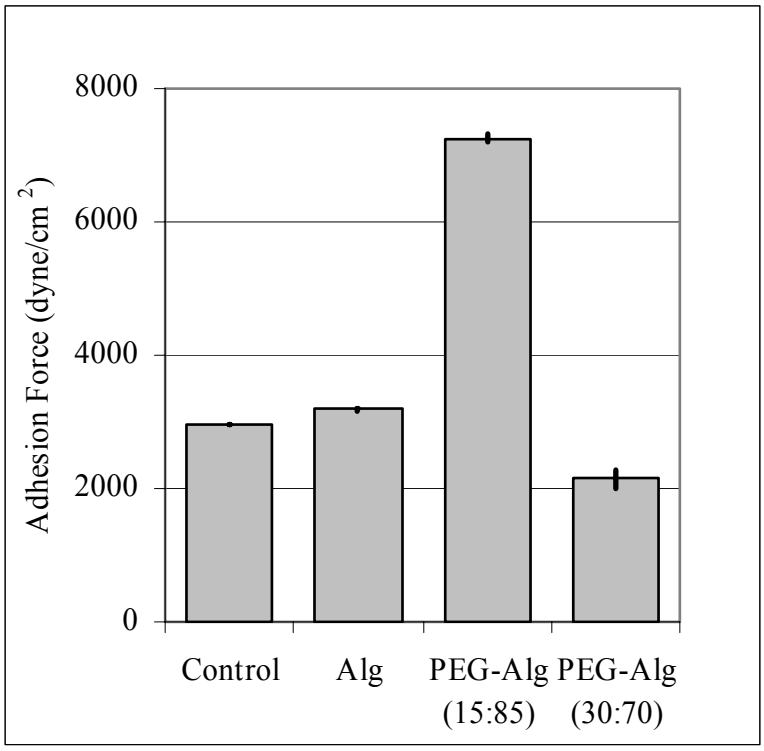


Figure 3.17: Adhesion force of alginate and PEG-Alginate to intestinal mucosa by using an microbalance based method. Control was uncoated sintered glass.

Although incorporation of PEG to alginate resulted in decreased negative charge and density of alginate molecules on the surface of microspheres, it enhanced its mucoadhesive properties (Figure 3.17). The adhesion force was measured as 7256 ± 43 dyne/cm² for the PEG:alginate ratio of 15:85. PEG is known as a mucoadhesion enhancer increased potential for hydrogen bond formation, since the lone pair electrons of oxygen in the repeat unit (CH CH O) of PEG would serve as hydrogen bond acceptors [51]. Also, free PEG chains incorporated in the microspheres enhanced mucoadhesion because of free PEG chains penetrating the mucin network and interacting easily as schematized in Figure 3.18.

However, in the case of PEG-alginate (30:70), mucoadhesion was lower (2147 ± 151 dyne/cm²) than pure alginate and PEG-alginate (15:85). This could be the result unefficient coating of sintered glass.

It was shown that in this study mucoadhesion of the alginate could be measured by modified electrobalance method. Adhesive properties of the alginate could be increased by incorporating optimum amount of PEG.

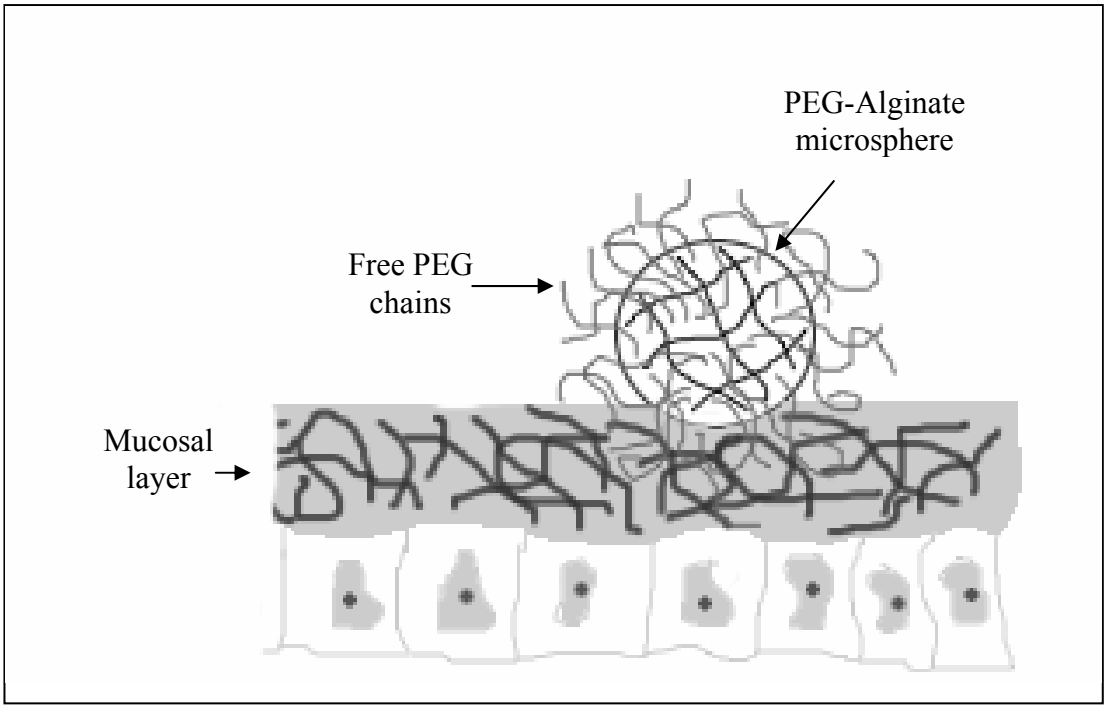


Figure 3.18: Schematic representation of PEG chains and mucus layer [50].

3.5. In Vitro Studies

3.5.1. Gene Transfection Efficiency of DNA and PEI-DNA

The transfection efficiencies in D407 cells with DNA and polyplexes based on PEI and pCMV β complexes were quantified by β-galactosidase enzyme assay. In order to normalize results total protein was determined with BCA assay and the standard curve of the BCA assay is represented in Appendix E. The results were represented in Table 3.1.

Table 3.1: The results of BCA assay and β-galactosidase enzyme assay

Sample	**BCA Assay** Total protein (μg/ml)	**β-galactosidase Activity** (unit/ml)	**β-galactosidase Activity** (Unit/ μg cellular protein)
Control	995.85	0	0
DNA	823.54	3.53×10^{-4}	4.28×10^{-7}
DNA-PEI	829.69	23.7×10^{-4}	28.56×10^{-7}

PEI-DNA complexes had a significantly higher transfection efficiency compared to DNA as expected (Table 3.19 and Figure 3.20). the optimum N/P ratio for Cos-7 cell line was determined as 4 [42]. In determining optimum N/P ratio, transfection efficiency and cellular toxicity were taken into consideration. For 3 μg/ml DNA complexed with appropriate amount of PEI (25 K) to yield N/P: 4, they showed that transfection was the maximum with no obvious cytotoxicity.

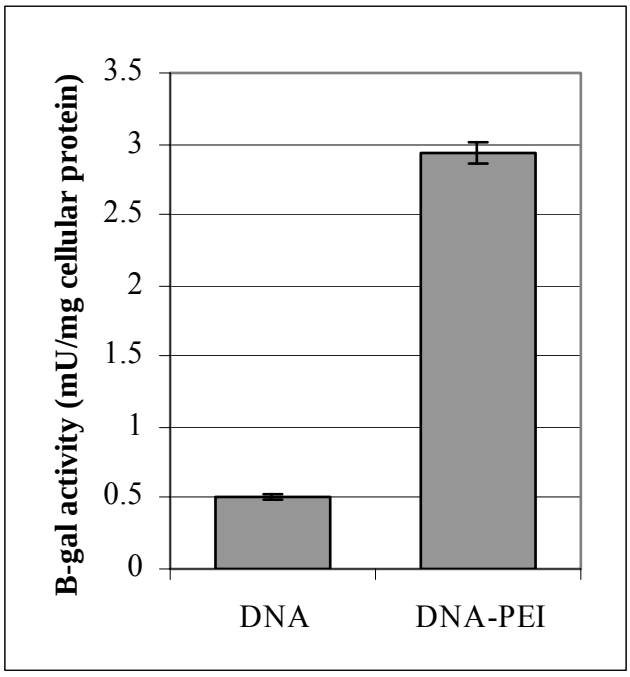


Figure 3.20: Transfection efficiencies of DNA and PEI-DNA complexes (1.5 μg DNA to each well for 6 well plate)

PEI-DNA loaded alginate microspheres were however ineffective in transfection because there was not sufficient PEI-DNA release from the microspheres. This is because of the cell culture medium contained enough calcium to prevent swelling of microspheres and the release of PEI-DNA.

3.5.2. Cellular Toxicity of the PEI-DNA and Alginate

Cytotoxicity of the PEI-DNA loaded alginate microspheres (1 mg and 5 mg) and PEI-DNA complexes (1 µg and 5 µg) with the same DNA concentration were examined by using MTS test which measures cellular activity. The plot of viability of cells in a variety of cases are given in Figure 3.21. It was shown that at high concentration of PEI-DNA, there was 50 % cell death. For effective gene delivery, high concentrations of PEI-DNA is required; this could also achieved by sustained delivery of the PEI-DNA delivery and this could reduce cellular toxicity. PEI-DNA loaded alginate microspheres did not cause toxicity however it could be result of insufficient release of the PEI-DNA complexes in cell culture media during the incubation period.

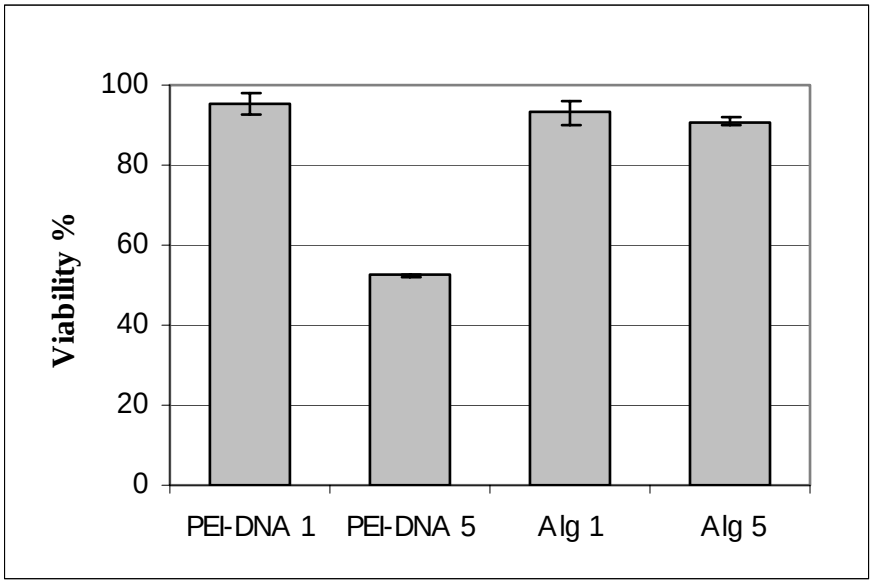


Figure 3.21: Cytotoxicity of PEI-DNA complexes and PEI-DNA loaded alginate microspheres on D407 cells after 48 h of incubation

CHAPTER 4

CONCLUSIONS

In this thesis, pCMV β plasmids were transformed to E.coli DH5 α and the plasmid was amplified in the bacteria. After PEI was complexed with DNA the effect of the pH on this process were examined by using electrophoretically and fluorescence spectroscopy.

Two different alginate microsphere preparations, water-in-oil emulsification and ionic gelation methods were carried out and the resultant alginate microspheres were compared. Smaller microspheres were obtained with water-in-oil emulsification system but because of difficulties in drying and removal of oil and surfactant, this method was abondened and ionic gelation method was used in the rest of study. PEG modified alginate microspheres also were prepared by the same method. After zeta potential measurements and SE Micrography of PEG-alginate microspheres, the presence of PEG on the surface of the alginate microspheres was proven. Zeta potential of alginate microspheres became less negative by increasing PEG concentration on the surface of because of the decrease in charge density of alginate microspheres.

Mucoadhesion of alginate and PEG-alginate microspheres were analysed by using microbalance. Presence of 15% PEG greatly increased cell adhesion, possibly as a result of interaction of free PEG chains with the mucous layer. This also indicated that by using ionic gelation alginate, PEG could be incorporated into the microspheres with some PEG chains staying on the surface. Upon increase of this PEG concentration the effect was reversal.

PEI-DNA complexes effectively were loaded into alginate microspherese with an efficiency of 59 ± 3 %. Release of polyplexes from alginate microspheres were studied in Tris-HCl buffer (10 mM, pH 7.5) and in the cell culture medium (DMEM). The results showed that in vitro cell conditions are not suitable for this system because in DMEM the release of complexes were prevented. The preliminary results showed that presence of PEG in the gel significantly affected the release from the microsphere. In calcium containing medium (DMEM) release of PEI-DNA complexes were not observed. By using PEG in the alginate microsphere preparation a faster release was obtained. This result can be used in order to understand the optimal release rate on transfection efficiency.

Transfection ability of PEI-DNA complexes were evaluated in the cell culture and compared with naked DNA. Complexation of DNA enhanced gene delivery at least 6 times. To evaluate gene delivery efficiency and immunogenic response level studies should be carried out in vivo. Before in vivo studies, release studies could be carried out in a simulated in vivo system and the relationship of release rate of polyplexes and gene transfer efficiency has to be studied by using PEG modified alginate microspheres.

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APPENDIX A

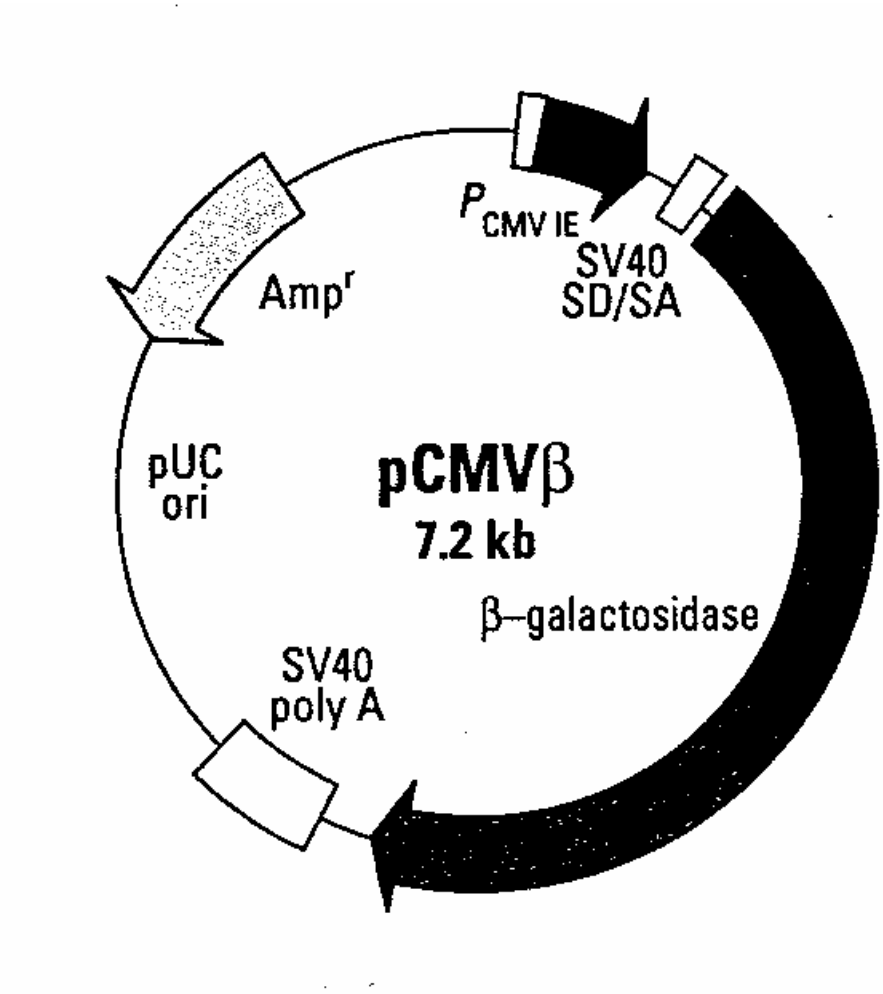

Figure A-1: Genetic Map of Plasmid pCMVβ

APPENDIX B

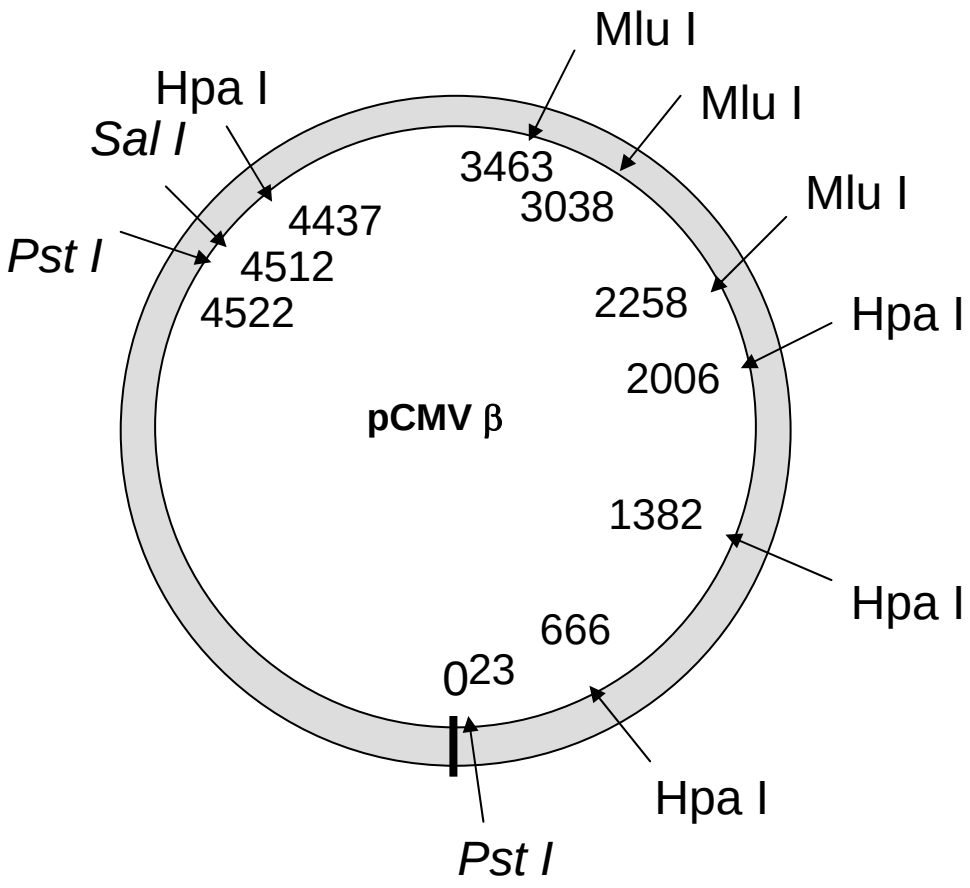

Figure A-2: Restriction Map of the Plasmid pCMVβ

APPENDIX C

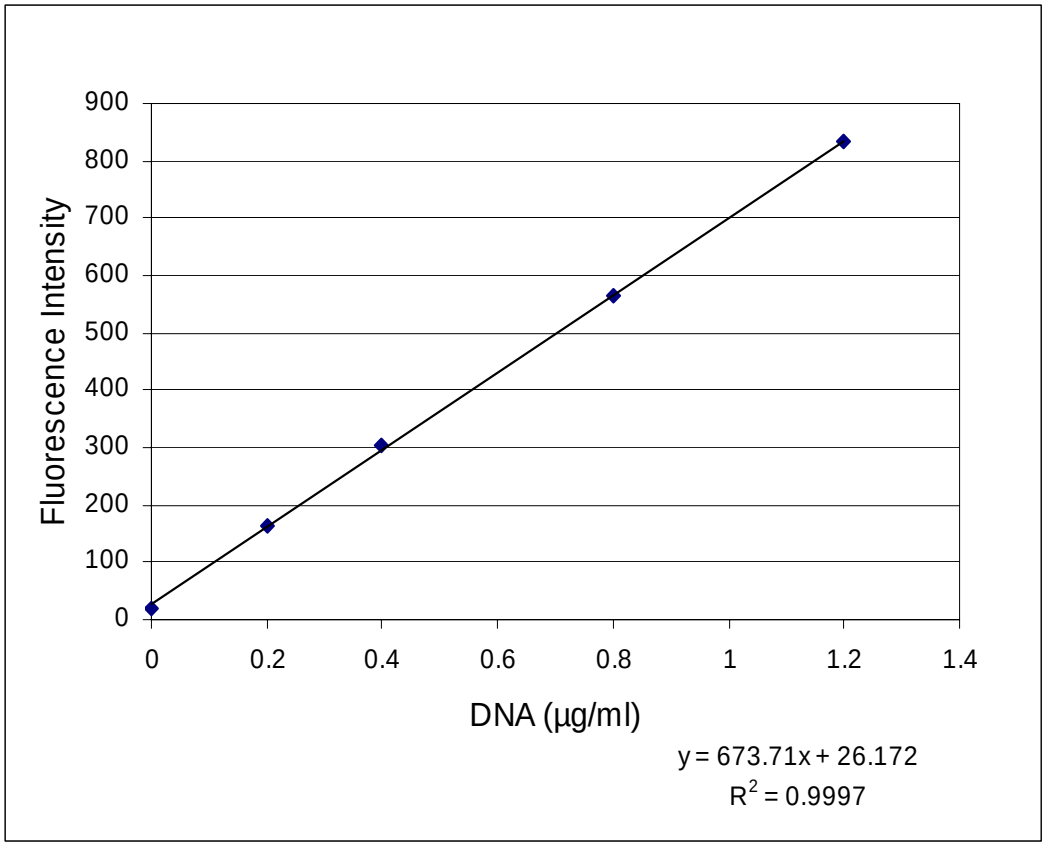

Figure A-3: Calibration Curve for PicoGreen Staining of DNA in Phosphate Buffer (5 M, pH 7.5)

APPENDIX D

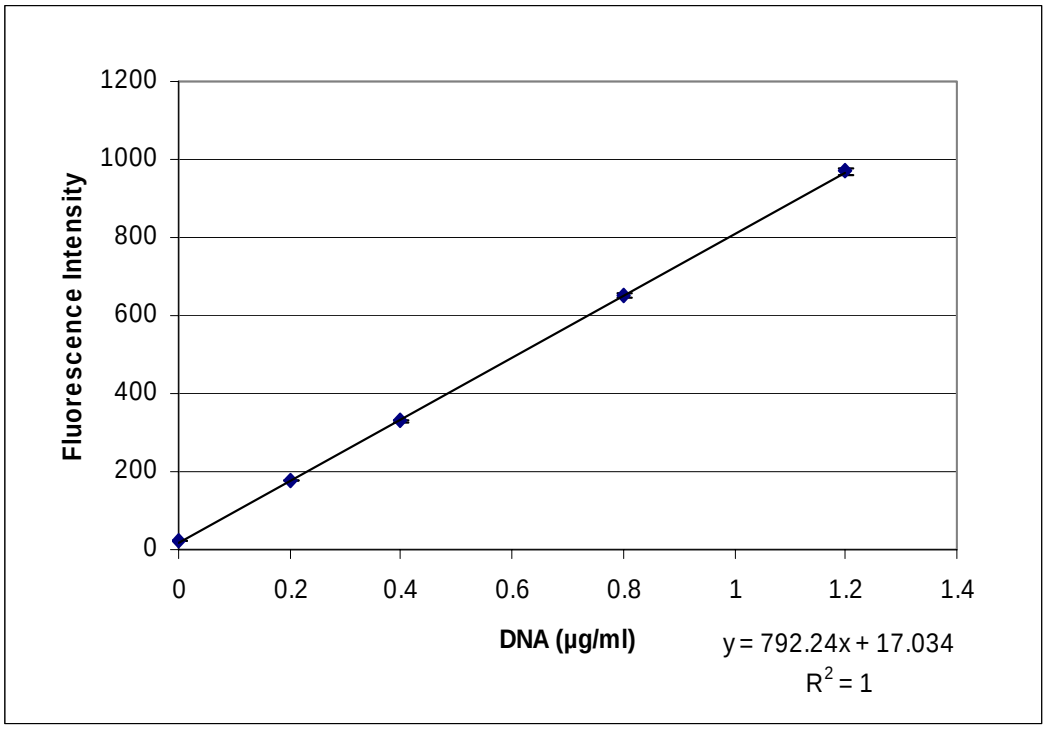

Figure A-4: Calibration Curve for PicoGreen Staining of DNA in Tris-HCl Buffer (10 mM, pH 7.5)

APPENDIX E

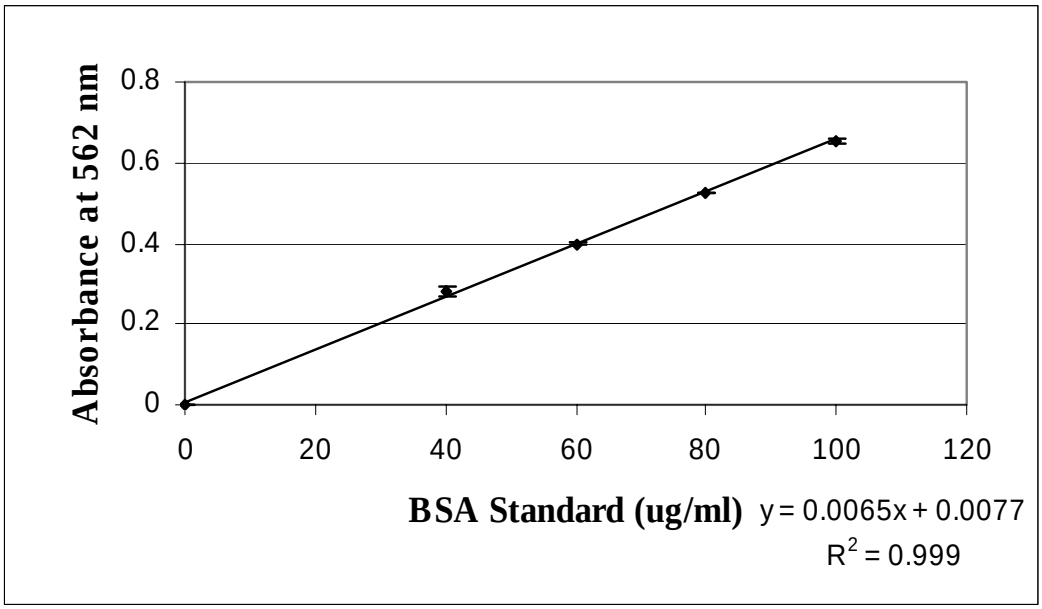

Figure A-5: Calibration Curve for Protein Quantification with BCA Assay