DEVELOPMENT OF GENE CARRIERS USING LIPOSOMES WITH SURFACTANTS COATED WITH CATIONIC POLYMERS

By
Kingkan Subsantisuk

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree
MASTER OF PHARMACY
Program of Pharmaceutical Technology
Graduate School
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การพัฒนาตัวพายีนโดยใช้ลิโพโซมร่วมกับสารลดแรงตึงผิวเคลือบด้วยพอลิเมอร์ประจุบวก

โดย

นางสาวกิ่งกาญจน์ สืบสันติสุข

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต

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In the present study, two types of liposomes; cationic polymers-coated anionic liposomes and cationic liposomes were prepared by sonication method and studied for their use as gene carriers. The anionic liposomes were composed of egg yolk phosphatidylcholine (EPC) and anionic surfactants i.e. sodium oleate (NaO) or sodium taurocholate (NaT) or zwitterionic surfactant 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) at the molar ratio of 10:1, 10:1.5 and 10:2. Subsequently, these liposomes were coated with cationic polymer chitosan lactate (CSL), methylated *N*-(4-pyridylmethyl) chitosan (TM_{69}-Py_{62}-CS) or polyethylenimine (PEI). In the preparation of cationic liposomes, EPC, cholesterol (Chol) and various cationic surfactants i.e. stearylamine (SA), dodecyl trimethylammonium bromide (DTAB) and cetylpyridinium chloride (CPC) at the molar ratio of 10:2:1, 10:2:2, 10:2:3 and 10:2:4 were used. In the gene delivery study, the liposomes were used to form complexes with plasmid DNA encoding green fluorescent protein (pEGFP-C2) and investigated for their ability to transfect human hepatoma cell lines (Huh-7 cells) compared with PEI and Lipofectamine2000^{TM}. The results revealed that all liposomal formulations were able to form complex with DNA depending on the carrier to DNA weight ratios. The liposomes coated with PEI showed higher transfection efficiency than that coated with TM_{69}-Py_{62}-CS or CSL. While the transfection efficiency of PEI-coated anionic liposomes was significantly higher than that of the PEI/DNA complexes, and the liposomes coated with TM_{69}-Py_{62}-CS or CSL showed lower transfection efficiency than the cationic polymer/DNA complexes. Among PEI-coated liposomes, the formulations which were prepared by using NaO as surfactant showed higher transfection efficiency than those using NaT or CHAPS. The complexes of PEI coated-liposomes were EPC:NaO (10:2) liposomes at the weight ratio of 0.5 > EPC:CHAPS (10:2) liposomes at the weight ratio of 0.75 > EPC:NaT (10:1.5) liposomes at the weight ratio of 0.75. In case of cationic liposomes, only EPC:Chol:SA liposomes/DNA complexes showed gene expression and the highest transfection efficiency was thus obtained at the molar ratio of 10:2:4. All liposomal formulations had lower cytotoxicity than PEI. PEI coated NaO-liposomes had the highest transfection and low cytotoxicity, suggesting that these formulations have the potential to be used as effective and safe gene delivery carrier.

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ในการศึกษาครั้งนี้เตรียมลิโพโซมสองชนิดคือลิโพโซมประจุลบซึ่งเคลือบด้วยพอลิเมอร์ประจุบวกและลิโพโซมประจุบวกเตรียมโดยวิธีลดขนาดด้วยคลื่นเสียงความถี่สูงและศึกษาการเป็นตัวนำส่งยีนของลิโพโซม ลิโพโซมประจุลบประกอบด้วยฟอสฟาทิดิลโคลลีนจากไข่แดง (EPC) และสารลดแรงตึงผิวประจุลบ ได้แก่ โซเดียมโอลีเอต (NaO) หรือโซเดียมทอโรโคลเลต (NaT) หรือสารลดแรงตึงผิวที่มีทั้งประจุบวกและลบ ได้แก่ 3-[(3-คอลลามิโดโพรพิล)-ไดเมทธิลแอมโมนิโอ]-1-โพรเพนซัลโฟเนต (CHAPS) ที่อัตราส่วนโมลาร์ เท่ากับ 10:1, 10:1.5 และ 10:2 นำลิโพโซมประจุลบเหล่านี้มาเคลือบด้วยพอลิเมอร์ประจุบวก คือ ไคโตซานแลคเตต (CSL), เมธิเลตเตต เอ็น-(-4-ไพริดิลเมทธิล) ไคโตซาน (TM_{69}-Py_{62}-CS) หรือพอลิเอทิลีนอิมมีน (PEI) ในการเตรียมลิโพโซมประจุบวกประกอบด้วย EPC, คอเลสเตอรอล (Chol) และสารลดแรงตึงผิวประจุบวกชนิดต่างๆ ได้แก่ สเตียริลเอมีน (SA), โดเดคซิลไตรเมธิลแอมโมเนียมโบรไมด์ (DTAB) และ เซทิลไพริดิเนียมคลอไรด์ (CPC) ที่อัตราส่วนโมลาร์เท่ากับ 10:2:1, 10:2:2, 10:2:3 และ 10:2:4 ในการศึกษาการนำส่งยีนนั้นทำโดยนำลิโพโซมมาทำให้เกิดสารประกอบเชิงซ้อนกับดีเอ็นเอที่สามารถแปลรหัสได้เป็น green fluorescent protein (pEGFP-C2) และศึกษาประสิทธิภาพการถ่ายโอนยีนเข้าสู่เซลล์เพาะเลี้ยงที่ใช้คือ human hepatoma cell lines (Huh7 cells) เปรียบเทียบกับ PEI และไลโปเฟคตามีน ผลการศึกษาพบว่าลิโพโซมทุกตำรับสามารถเกิดสารประกอบเชิงซ้อนกับดีเอ็นเอได้ขึ้นกับอัตราส่วนโดยน้ำหนักของตัวพาต่อดีเอ็นเอ ลิโพโซมซึ่งเคลือบด้วย PEI จะให้ประสิทธิภาพในการถ่ายโอนยีนสูงกว่าการเคลือบด้วย TM_{69}-Py_{62}-CS หรือ CSL ประสิทธิภาพการถ่ายโอนยีนของลิโพโซมซึ่งเคลือบด้วย PEI สูงกว่าสารประกอบเชิงซ้อนของ PEI กับดีเอ็นเออย่างมีนัยสำคัญ ในขณะที่ลิโพโซมซึ่งเคลือบด้วย TM_{69}-Py_{62}-CS หรือ CSL จะให้ประสิทธิภาพการถ่ายโอนยีนต่ำกว่าสารประกอบเชิงซ้อนของพอลิเมอร์ประจุบวกกับดีเอ็นเอ ในกลุ่มลิโพโซมซึ่งเคลือบด้วย PEI ตำรับที่เตรียมโดยใช้ NaO เป็นสารลดแรงตึงผิวจะให้ประสิทธิภาพในการถ่ายโอนยีนสูงกว่าตำรับที่เตรียมจาก NaT หรือ CHAPS สารประกอบเชิงซ้อนของลิโพโซมซึ่งเคลือบด้วย PEI ซึ่งเตรียมจาก EPC:NaO = 10:2 ที่อัตราส่วนโดยน้ำหนัก 0.5 > EPC:CHAPS = 10:2 ที่อัตราส่วนโดยน้ำหนัก 0.75 > EPC:NaT = 10:1.5 ที่อัตราส่วนโดยน้ำหนัก 0.75 ในกรณีของลิโพโซมประจุบวกพบว่าสารประกอบเชิงซ้อนของ EPC:Chol:SA ลิโพโซมกับดีเอ็นเอเท่านั้นที่เกิดการแสดงออกของยีนและให้ประสิทธิภาพการถ่ายโอนยีนสูงสุดที่อัตราส่วนโมลาร์เท่ากับ 10:2:4 ลิโพโซมทุกตำรับมีความเป็นพิษต่อเซลล์ต่ำกว่า PEI โดยที่ NaO ลิโพโซมซึ่งเคลือบด้วย PEI จะให้ประสิทธิภาพการถ่ายโอนยีนสูงสุดและมีความเป็นพิษต่อเซลล์ต่ำ แสดงให้เห็นว่าลิโพโซมตำรับนี้เป็นตัวพายีนที่มีประสิทธิภาพและมีความปลอดภัย

สาขาวิชาเทคโนโลยีเภสัชกรรม บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร ปีการศึกษา 2551

ลายมือชื่อนักศึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ 1. ..... 2. .....

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CHAPTER I

INTRODUCTION

1. Statement and significance of the research problem

The success of gene therapy is predicated on the development of gene transfer vectors that are safe and efficacious. The application of viral vectors in clinical therapy is limited because they suffer from numerous problems such as immunogenicity, oncogenic effects, feasibilities of endogenous virus recombination (Byrnes et al. 1995 : 1015). Considering these limitations of viral vectors, non-viral vectors such as cationic liposomes and cationic polymers formed complexes with DNA that facilitate cellular uptake and delivery (Pedroso de Lima et al. 2001 : 278). Therefore, non-viral vector has received significant attention because they are simple to prepare, rather stable, easy to modify and relatively safe compared to viral vectors. However, non-viral vector has a significant limitation, owing to its low transfection efficiency and short duration of gene expression. Nevertheless non-viral vectors should be ideal vectors for gene therapy. Non-viral vectors are commonly cationic in nature. Cationic polymers and cationic liposomes are non-viral vectors that are widely used to deliver nucleic acids, including plasmid DNA, antisense oligonucleotide and siRNA. In addition to their lack of immunogenicity, liposomes can improve the biological stability of plasmid DNA by entrapping and shielding them from nuclease degradation (Patil, Rhodes and Burgess 2004 : 1). However, cytotoxicity and inactivation in the presence of serum have restricted the potential applications of some cationic liposomal vectors.

Chitosan (CS), a cationic polymer is produced by alkaline deacetylation of chitin. It is a copolymer of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN), which are linked by β(1,4) glycosidic bond. CS is a weak base with a pK_a value of about 6.2-7.0; therefore, it is insoluble in water, but soluble in acidic medium such as acetic acid, citric acid, hydrochloric acid and lactic acid, etc. CS has been used as a vector for gene delivery. In addition, CS is biocompatible, biodegradable and non-toxic; therefore, it has been proposed as a safer alternative to other non-viral vectors such as cationic lipids and cationic polymers (Borchard 2001 : 146). At acidic pH, below pK_a, the primary amines in the CS backbone become positively charged. These protonated amines enable CS to bind to negatively charged DNA and condense DNA into particles. Moreover, CS has shown capability to protect DNA from DNase I&II degradation and has been successful used as *in vitro* and *in vivo* gene delivery (MacLaughlin et al. 1998 : 259). Furthermore, incubation of cells with CS demonstrated low cytotoxic activity (Carreno-Gomez and Duncan 1997 : 234). These results suggest that CS has comparable efficacy without the associated toxicity of other synthetic vectors and can, therefore, be an effective gene-delivery vehicle *in vivo*.

The drawback of chitosan is insoluble in water and low transfection efficiency. Many researchers synthesized CS derivatives in order to improve solubility of CS in physiological pH. Lee et al. designed thiolated chitosan, which was prepared by reaction between 33 kDa chitosan (90% degree of deacetylation) and thioglycolic acid. The thiolated chitosan with plasmid DNA encoding green fluorescent protein (GFP) has been evaluated for their transfection efficiency in various cell lines. These results demonstrated that thiolated chitosan (CSH-360) with plasmid DNA complexes induced higher GFP expression in HEK293, MDCK and HepG-2 cell lines than unmodified chitosan. Complexes of disulfide-crosslinked CSH360/DNA showed sustained DNA release and continuous expression in cultured cells lasting up to 60 hours post transfection (Lee et al. 2007 : 157). Kim et al. prepared water-soluble chitosan by coupling with urocanic acid bearing imidazole ring which can play the crucial role in endosomal rupture through proton sponge effect. The urocanic acid-modified chitosan (UAC) was formed complexes with DNA. It showed good DNA binding ability, high protection of DNA from nuclease attack, and low cytotoxicity. The transfection efficiency of chitosan into 293T cells was much enhanced after coupling with urocanic acid (UA) and increased with increasing of UA contents in the UAC (Kim et al. 2003 : 392-400). Thanou et al. synthesized CS derivative *N*-trimethylated chitosan (TMC) from oligomeric chitosan. These oligomers (40 and 50% degree of quaternization) were examined for their potency as DNA carrier systems in two cell lines, COS-1 (monkey kidney) and Caco-2 (human colon carcinoma). *N*-trimethylated chitosan oligomers (TMOs) were superior to oligomeric CS in transfecting COS-1 cells; however, none of the used CS-DNA and lipofectin-DNA complexes was able to increase transfection efficiency in differentiated Caco-2 cells (Thanou 2002 : 156-157). Opanasopit et al. have successfully synthesized methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitoan (TM-Bz-CS) (Opanasopit et al. 2009 : 147) and methylated N-(4-pyridinylmethyl) chitosan (TM-Py-CS) (Opanasopit et al. 2008 : 132). Both TM-Bz-CS and TM-Py-CS were soluble in pH 7.4 and expressed transfection efficiency higher than unmodified CS.

Polyethylenimine (PEI) is a cationic polymer that has been successfully used in gene delivery. It has been widely used to transfer DNA both *in vitro* and *in vivo* (Boussif et al. 1995 : 7297). Ko et al. applied technology of PEGylated liposomes for delivery of PEI polyplex of oligodeoxynucleotides (ODN), based on encapsulation of the PEI/ODN polyplexes into PEGylated liposomes. The PEI/ODN polyplex was prepared with a low-branched PEI with MW 2.7 kDa and 20-mer double stranded ODN and was then entrapped into PEGylated liposomes. The PEG-stabilized liposome (PSL) entrapping PEI/ODN polyplexes remained stable in the presence of serum as compared to the naked PEI/ODN complex (Ko, Bhattacharya and Bickel 2008 : 1). In addition, surfactant has been applied for component of liposomes, to modify the surface charge of liposomes, such as stearylamine (SA) which has prepared positively charge liposomes. Wang et al. studied stearylamine liposome for DNA transfection of eukaryotic cells. The transfection efficiency was evaluated and compared with that of the Lipofectin reagent and the calcium phosphate transfection method. Results demonstrated that stearylamine liposome had advantages over the Lipofectin reagent and calcium phosphate precipitation method. The transfection efficiency in 12 cell lines were significantly higher than that of the calcium phosphate method and in 8 cell lines were significantly higher than that of the Lipofectin reagent method (Wang, Jing and Lin 1996 : 453).

In this study, the gene carriers using liposomes have been developed with surfactants : anionic surfactants (sodium taurocholate; NaT and sodium oleate; NaO); cationic surfactants (SA and dodecyl trimethylammoniumbromide (DTAB)); zwitterionic surfactants (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; CHAPS) and cationic polymers such as chitosan salts, chitosan derivatives (TM_{69}-Py_{62}-CS) and PEI. *In vitro* transfection efficiency and cytotoxicity were evaluated. A number of variables that influenced transfection efficiency such as weight ratio, particle size, zeta-potential, pH of culture medium, were determined.

2. Objective of this research

2.1 To develop liposomes with surfactants coated with cationic polymers as gene carrier.

2.2 To investigate the influence of formulation factors such as type of surfactants, type of cationic polymers and the carrier/DNA weight ratio on physicochemical properties (particle size, surface charge, DNA condense) and transfection efficiency.

2.3 To examine the cytotoxicity of cationic polymer-coated anionic liposomes and cationic polymer-coated anionic liposomes/DNA complexes.

3. The research hypothesis

3.1 Liposomes with surfactants coated with cationic polymers can be used as gene carrier.

3.2 Formulation factors such as type of surfactants, type of cationic polymers and carrier/DNA weight ratio significantly influence on physicochemical properties (particle size, surface charge, DNA condense), transfection efficiency and cytotoxicity.

CHAPTER II

LITERATURE REVIEW

1. Gene therapy

Gene therapy, the transfer of therapeutic genes to the target cells, is the use of specific genes for the "treatment or cure of genetic diseases in human" (Huang and Tseng 1998 : 206). Many genetic diseases are caused by lack of production of a single gene production or are due to the production of a mutated gene product incapable of carrying out its natural function. This approach is based on the principle of correcting the basis of diseases by delivery and subsequent expression of exogenous DNA, which encodes for a missing or defective gene product (Walsh 2003 : 463). Some of the genetic conditions for which the defective gene has been purposed are summarized in Table 1.

Gene therapy starts with the introduction of an appropriate vector (viral, non-viral or cell based) into the body either locally (direct tissue injection) or into the blood stream (systemic delivery). The vector needs to find its target tissue, enter the target cells and traffic through the cytoplasm to reach and enter the nucleus. Once there, the therapeutic (trans) gene needs to be transcribed and the mRNA formed needs to be appropriately translated into the therapeutic protein. The protein then acts on its receptor(s) either on the cell which produced it (intracrine or autocrine mechanism), on neighboring cells (paracrine mechanism) or at distant sites after entering the blood circulation (endocrine mechanism, e.g., erythropoietin, coagulation factors, growth hormone, etc.). Finally, after interacting with its receptor, the protein needs to induce an appropriate biological effect which results in therapeutic benefits.

Table 1 Some examples of genetic diseases for which the defective gene responsible has been identified.

Disease	Defective genes protein product
Hemophilia A	Factor VIII
Hemophilia B	Factor IX
Thalassemia	β-Globin
Sickle cell anemia	β-Globin
Familial hypercholesterolemia	Low-density protein receptor
Severe combined immunodeficiency	Adenosine deaminase, purine nucleoside phosphorylase
Niemann-Pick disease	Sphingomyelinase
Gaucher's disease	Glucocerebrosidase
Cystic fibrosis	Cystic fibrosis transmembrane regulatory
Emphysema	α_1-Antitrypsin
Leukocyte adhesion deficiency	CD18
Hyperammonemia	Ornithine transcarbamylase
Citrullinemia	Arginosuccinate synthetase

Disease	Defective genes protein product
Phenylketonuria	Phenylalanine hydroxylase
Maple syrup disease	Branched chain α-keto acid dehydrogenase
Tyrosinemia Type 1	Fumarylacetoacetate hydrolase
Glycogen storage deficiency type 1A	Glucose-6-phosphatase
Fucosidosis	α-L-Fucosidase
Mucopolysaccharidosis type VII	β-Glucuronidase
Mucopolysaccharidosis type I	α-L-Iduronidase
Galactosemia	Galactose-1-phosphate uridyl transferase

Source : Garry Walsh, Biopharmaceuticals : Biochemistry and Biotechnology, 2nd ed. (Britain : Wiley, 2003), 483.

2. Gene therapy vector

Carriers for gene therapy, which have successfully demonstrated the delivery of exogenous genes *in vivo*, can be divided into viral and non-viral systems. Viral vectors, such as retroviruses, adenoviruses are still the most popular vectors in lab studies and clinical trials. They are very high transfection efficiency; however, there are major limitations associated with toxicitiy and oncogenic side effects of viral vectors. On the contrary, non-viral vectors do not have such serious effects but the efficiency of non-viral vectors is not satisfactory (Irvine et al., quoted in Gregoriadis 2007 : 294).

2.1 Viral vector systems

Among the viral vectors, retrovirus and adenovirus are the two of the most commonly used for gene therapy vectors for human clinical trials. However, other viral vectors developed in the laboratory have shown promise in preclinical studies. (Table 2)

2.1.1 Retrovirus

Retrovirus is a RNA virus which contains an enzyme called reverse transcriptase which is an RNA-dependent DNA polymerase allowing the formation of proviral DNA which becomes integrated into the infected host cell chromosome. Retroviruses preferentially infect dividing cells, thereby targeting actively replicating cells while sparing nondividing cells. Retrovirus has an insert-size limit of 8 kbp. Several tissue specific promoters work reasonably well in retrovirus but re-transfection is proved to be more difficult to achieve than with adenovirus. A major disadvantage of using a retrovirus as a vector is the propensity of the transferred gene to integrate randomly into the chromosomes of the recipient cells. Other limitations include difficulty in large-scale production of the virus, as well as the inability to infect non-dividing cells (Walsh 2003 : 468).

2.1.2 Adenovirus (Ad)

Adenoviruses are large, housing double-stranded DNA-containing viruses, which are pathogenic in human causing endemic and epidemic respiratory and intestinal infections. Adenoviruses can infect both dividing and non-dividing cells and use in both laboratory and clinic. They are replication incompetent, therefore, viral replication is prevented in patients who receive this vector system. The advantages of using adenoviral vectors are several including easy to produce in large quantities and high levels of gene expression. Adenovirus shares with other viral vectors in the problem that first generation vector preparations are contaminated with replication-competent virus (RCV). However, a new combination of adenovirus vector and producer cells can eliminate RCV and is suitable for large-scale manufacture (Keegan et al. 1998 : 1909). In addition, significant problems still exist. The major problem of adenovirus vectors stems from their tendency to elicit strong immune and (at high doses) inflammatory responses such as adenovirus gene transfer lead to an inflammatory response in the brain (Byrnes et al. 1995 : 1015).

2.1.3 Adeno-associated virus (AAV)

AAV, a single strand of DNA encapsulated with a protein shell belongs to a member of the parvovirus group. It is non pathogenic in human and appears to integrate into the host cell chromosome. However, AAV has its problem including difficulties with large-scale production, loss of ability of the AAV vector to integrate into the host genome, and limitations in insert size (Xiao et al., quoted in Lu and Qie 2004 : 81). Different serotypes of AAV vectors have shown high transduction specificity for cell types. For example, AAV1 and AAV5 mostly infect neurons in the central nervous system, and are able to diffuse into the brain parenchyma for several millimetres, while AAV4 infects ependymal cells (Ballana et al. 2008 : 134).

2.1.4 Herpes simplex virus (HSV)

HSV is a neurotrophic virus and large double-stranded DNA virus. HSV-1 is one of the human herpes viruses; the other members of DNA virus group include HSV-2, VZV (varicella zoster virus), CMV (cytomegalovirus), EBV (Epstein–Barr virus). HSV is commonly present in more than 90% of the human population. However, it remains latent in its activity. When active, it is capable of infecting neuronal tissue. Due to its ability to infect neurons, HSV is being proved as a vector for the treatment of neuronal disorders, including cancer of neuronal origin (Kennedy et al. 1997 : 1250-1251).

2.1.5 Vacinia virus (VV) or Poxvirus

These viruses were used as vaccines, which eradicate smallpox virus worldwide. VV is a large double-stranded DNA virus and is the only vaccine to have damaged a human infectious disease globally (El-Aneed 2004 : 1). Poxvirus is considered to be one of the most promising vaccine vectors against a variety of infectious pathogens including HIV, malaria, and tuberculosis, as well as for gene therapy and treatment of human cancers. Numerous recombinant VVs (rVVs) have been engineered and shown to induce potent cellular and humoral responses.

However, using the replication-competent VV as vaccine vector is prohibited by the high rates of post-vaccination medical complications, such as post-encephalitis and dermatologic disease. Therefore, many approaches have been proposed to enhance the safety of poxvirus, such as attenuated and host range-restricted poxviruses that undergo limited replication in human and other mammalian cell lines. Although these poxvirus vectors have been shown to be relatively safe, they do not replicate efficiently *in vivo*, and reduce the expression of target antigen; hence, they impaired immunogenic potency both in preclinical and clinical trials (Dai et al. 2008 : 5062).

Table 2 Advantages and disadvantages of gene-transfer vector.

Vector	Advantages	Disadvantages
Adenovirus	- Very high transfection efficiency *ex vivo* and *in vivo* - Transfect proliferating and non-proliferating cells - Substantial clinical expierence acquired - Efficient retargeted transfection acquired	- Repeat dosing ineffective owing to strong immune responses - Insert-size limit of 7.5 kbp - Manufacture, storage and QC are moderately difficult - Short duration of expression
Retrovirus	- Fairly prolonged expression - High transfection efficiency *ex vivo* - Substantial clinical experience *ex vivo* - Low immunogenicity	- Low transfection efficiency *in vivo* - Insert-size limit of 8 kbp - Transfect only proliferating cells - Risk of insertional mutagenesis - Manufacture, storage and QC are extremely difficult
Lentivirus	- Transfects proliferating and non-proliferating cells *in vivo* - Transfects haematopoietic stem cells	- Manufacture, storage and QC are extremely difficult - Insert-size limit of 8 kbp - No clinical experience
AAV	- Efficiently transfect a wide variety of cell *in vivo* - Very prolonged expression *in vivo* - Low immunogenicity	- Insert-size limit of 4.5 kbp - Manufacture, storage and QC are very difficult - Little clinical experience - Risk of insertional mutagenesis - Repeat dosing affected by neutralizing antibody responses
Naked DNA	- Manufacture storage and QC are simple and cheap - Very low immunogenicity - Clinical efficacy demonstrated in critical limb ischemia - Very good safety profile	- Very short duration of expression in most tissues - Very inefficient transfection *ex vivo* and *in vivo* - Retargeting transfection very difficult
Cationic lipids	- Inefficient transfection *in vivo*, efficient transfection *ex vivo*	- Very short duration of expression

Vector	Advantages	Disadvantages
Condensed DNA particles	- Low immunogenicity - Good safety profile - Relatively simple manufacture, storage and QC - Efficient transfection *ex vivo* - Low immunogenicity - Good safety profile - Retargeted transfection demonstrated	- Little clinical experience - Retargeting transfection difficult - Inefficient transfection *in vivo* - Very short duration of expression - No clinical experience

Source : J. Navarro et al., "Gene Therapy and Intracytoplasmatic Sperm Injection (ICSI)," Placenta 29 (2008) : S194.

2.2 Non-viral vector systems

Viral vectors can induce immune response and activate oncogenes, therefore non-viral vectors have been introduced and tested for their potential to be safer. Non-viral vectors usually do not elicit an immune response and they are relatively less toxic to the target cells, which is a great advantage for drug target validation over viral vectors. Among the non-viral vectors, cationic liposomes and cationic polymers have gained increasing attention because both of them are positively charged and can form complexes with negatively charged of DNA, and also, mediate transfection via condensing DNA into nanoparticles, protecting DNA from enzymatic degradation, and facilitating the cell uptake and endolysosomal escape.

2.2.1 Cationic liposomes

Cationic liposomes, which are positively charged, are the most commonly used for gene therapy for non-viral vectors. They made from positively charged lipid formulations such as 3β-[*N*-(*N′*,*N′*-dimethylaminoethane)-carbamoyl] cholesterol hydrochloride (DC-Chol), dioctadecylamidoglycylspermine (DOGS) (Transfectam), dioctadecyldimethylammonium bromide (DDAB)/ dioleoylphosphatidylethanolamine (DOPE) (LipofectinACE), 1,3-dioleoyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA)/DOPE (LipofectAMINE) and *N*-(2-hydroxethyl)-*N*,*N*-dimethyl-2-3-bis(tetradecyloxy)-1-propana-minium bromide (DMRIE) (Figure 1). Usually, cationic liposomes are mainly composed of binary mixtures of cationic lipids and a zwitterionic or neutral colipids such as DOPE or cholesterol, respectively to form liposomes. Inclusion of a colipid is not always essential. For instance, the cationic lipid, 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) is active in the absence of a co-lipid in a variety of cells *in vitro*. Most cationic lipids used as transfection reagents consist of three parts : (i) a hydrophobic lipid anchor group; (ii) a linker group, such as an ester, amide or carbamate; and (iii) a positively charged head-group, which interacts with pDNA, leading to its condensation (Figure 2). The hydrophobic lipid anchors group can be either a cholesterol group or fatty chains. Lipid anchor helps in forming liposomes and determines the physical properties of a lipid bilayer.

The linker group is an important part, which determines the chemical stability and biodegradability of a cationic lipid. The head groups of cationic lipid appear to be critical for transfection and cytotoxicity of corresponding liposome formulations (Mahato 2005 : 700-701).

Cationic liposomes popularity is only due to their ability to deliver nucleic acids into cells both *in vitro* and *in vivo* for gene therapy of diseases such as cancer (Campbell et al. 2002 : 6831). However, some limitations with lipid vectors, including their lower efficiency than viral vectors in gene transfer and their transient gene expressions (Mhashilkar et al. 2001 : 279). In 1987, Felgner et al. developed and synthesized the first cationic lipid formulation, Lipofectin used as a transfecting agent. Lipofectin is composed of the cationic lipid, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*-trimethylammonium chloride (DOTMA) and DOPE, which became commercially available as a transfection reagent and provided evidence that this positively charged lipid formed with DNA a stable complex allowing delivery and expression of the gene material into the cell (Felgner et al. 1987 : 7413). Cationic lipids interact with the negatively charged DNA through electrostatic interactions and promoting the condensation of DNA into a more compact structure (Dalkara, Chandrashekhar and Zuber 2006 : 353).

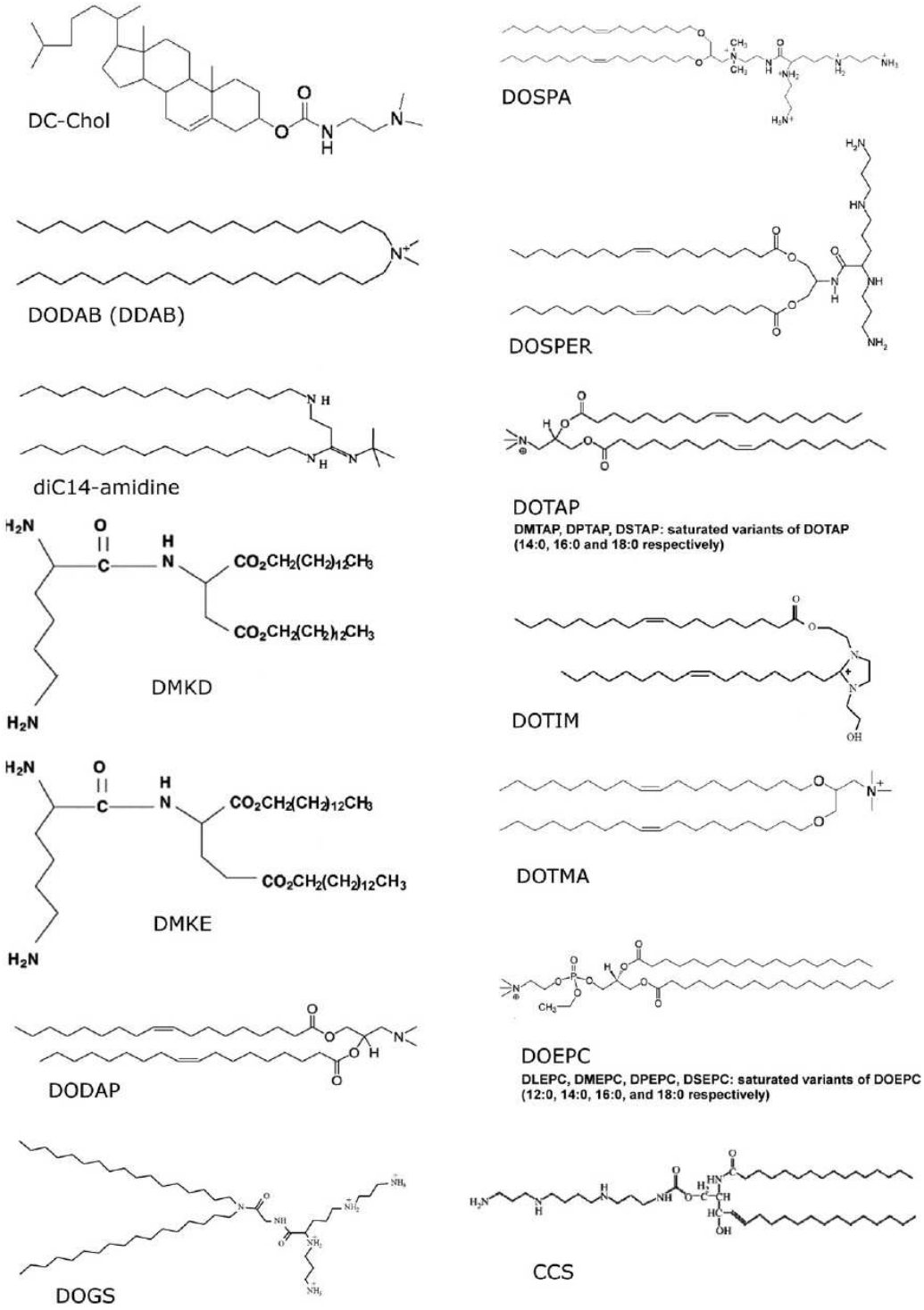

Figure 1 Chemical structures of some cationic lipids commonly used for gene delivery.

Source : Caroline Lonez, Michel Vandenbranden, and Jean-Marie Ruysschaert, "Cationic Liposomal Lipids : from Gene Carriers to Cell Signaling," Progress in Lipid Research 47 (2008) : 342.

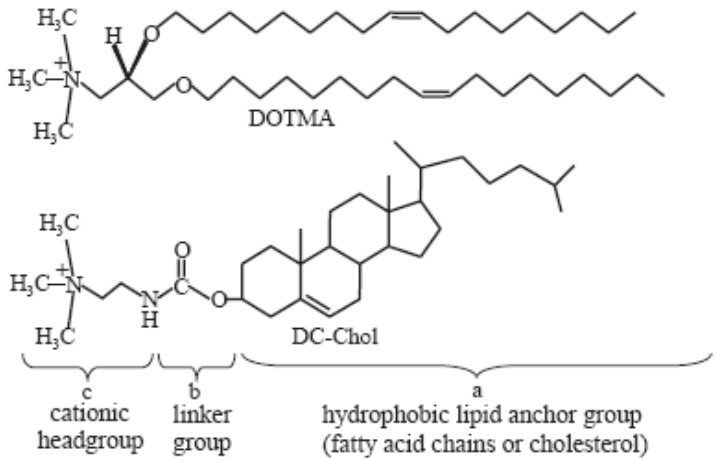

Figure 2 Basic components of cationic lipids (a) Hydrophobic lipid group; (b) linker group; (c) cationic head group.

Source : Ram I. Mahato, "Water Insoluble and Soluble Lipids for Gene Delivery," Advanced Drug Delivery Reviews 57 (2005) : 701.

Ito et al. studied a group of tumor suppressor genes on human chromosome 3p21.3 that are frequently deleted in human lung and breast cancers such as *FUS1* gene. Intratumoral administration of *FUS1* gene complexed to DOTAP:Cholesterol (DOTAP:Chol) liposome into subcutaneous H1299 and A549 lung tumor xenograft resulted in significant inhibition of tumor growth. Furthermore, intravenous injections of DOTAP:Chol–*FUS1* complex into mice bearing experimental A549 lung metastasis demonstrated significant decrease in the number of metastatic tumor nodules. Finally, lung tumor-bearing animals when treated with DOTAP:Chol–*FUS1* complex demonstrated prolonged survival compared to control animals (Ito et al. 2004 : 773). Ramesh et al. tested ability of extruded DOTAP:Cholesterol cationic liposome. Cationic liposome efficiently delivers the therapeutic tumor suppressor genes *p53* and fragile histidine triad (*FHIT),* which are frequently altered in lung cancer, to primary and disseminated experimental metastatic murine and human lung tumors compared with conventional liposomes. These results demonstrated that extruded DOTAP:Chol cationic liposome-mediated gene delivery effectively transfected both primary and disseminated murine tumors and human lung tumor xenografts, and this was associated with suppressed tumor growth and prolonged animal survival with minimal toxicity (Ramesh et al. 2001 : 337). Maitani et al. prepared cationic liposomes composed of DC-Chol and DOPE, molar ratio, 1:1 or 3:2 prepared by the dry-film method. A more efficient transfection in medium with serum was achieved using DC-Chol/DOPE liposomes (molar ratio, 1:2) than those (3:2), and preparation method by a modified ethanol injection than the dry-film. The most efficient DC-Chol/DOPE liposome for gene transfer was molar ratio (1:2) and prepared by a modified ethanol injection method. The enhanced transfection might be

related to an increase in the release of DNA in the cytoplasm by the large lipoplex during incubation in optiMEM, not to an increased cellular association with the lipoplex. The use of a modified ethanol injection method might enhance the role of DOPE that is aid in destabilization of the plasma membrane and/or endosome (Maitani et al. 2007 : 33).

To combine advantages of lipid and polycation, Chen et al. prepared polycationicliposomes (PCLs). PEI, MW 800-cholesterol, firstly designed to modify the surface of liposomes, was synthesized. Polycation liposomes composed of soybean phospholipids, cholesterol and PEI 800 – Chol were prepared using film hydration method. Compared to Lipofectamine ^{TM} 2000, PCLs have high transfection efficiency with significantly low cytotoxicity as well as the protection ability from serum (Chen et al. 2007 : 255). A synergistic effect on the transfection efficiency by the combined use of polycations and cationic liposomes was found by Ko et al. A synergism between PEGylated liposome for delivery of PEI polyplex of oligodeoxynucleotides (ODN), is based on encapsulation of the PEI/ODN polyplexes into PEGylated liposomes. The PEI/ODN polyplex was prepared with a low-branched PEI with MW 2.7 kDa and 20-mer double stranded ODN and was then entrapped into PEGylated liposomes. The PEG-stabilized liposome (PSL) entrapping PEI/ODN polyplexes remained stable in the presence of serum as compared to the naked PEI/ODN complex (Ko et al. 2008 : 1).

2.2.2 Cationic polymers

Cationic polymer are the most probable alternative to viral delivery systems; the possibility of their application for gene delivery. Cationic polymer can be combined with DNA to form a particulate complex, polyplex capable of gene transfer into the targeted cells.

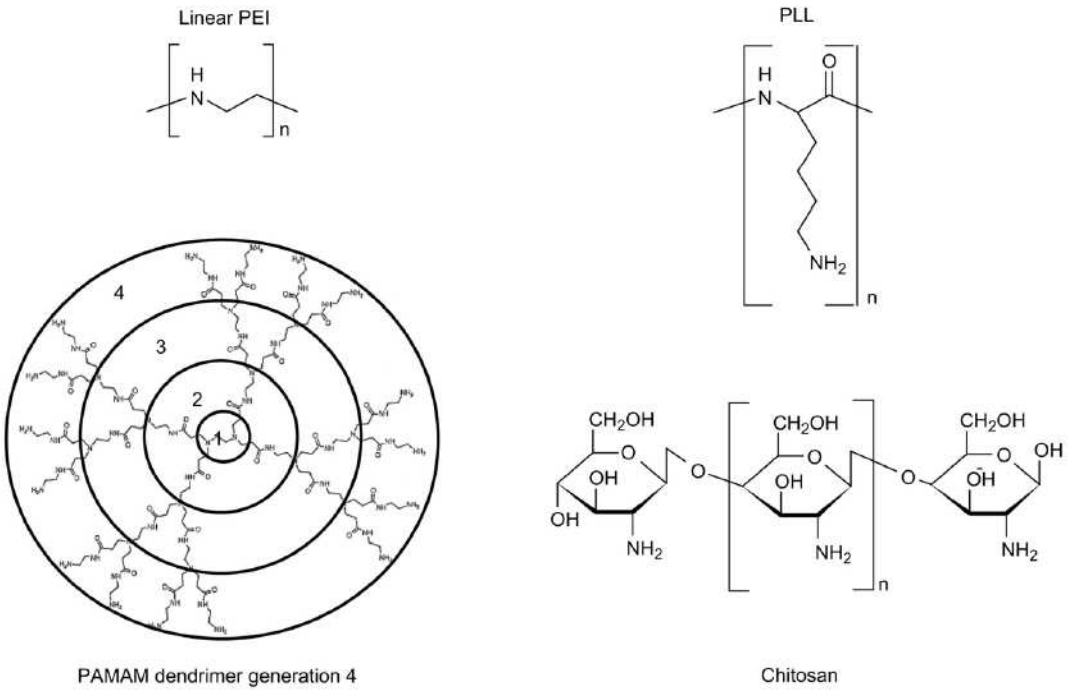

Figure 3 Structures of cationic polymers used in gene therapy.

Source : Marie Morille et al., "Progress in Developing Cationic Vectors for Non-Viral Systemic Gene Therapy Against Cancer," Biomaterials 29 (2008) : 3478.

2.2.2.1. Poly(amino acid)s

Cationic polymers that have been used in gene delivery are polyornithine, polyarginine and poly-L-lysine(PLL)s. PLL has been popularly used as a DNA delivery vehicle due to its excellent ability to condense DNA (Laemmli 1975 : 4289). PLL is synthesized by polymerization of the *N*-carboxy-anhydride of lysine. They are linear polypeptides with the amino acid lysine as the repeat unit; thus, they possess a biodegradable nature. PLL remains under extensive investigation due to the versatility of molecular weight and the ease with which other functionalities can be incorporated, by conjugation to the ε-amino groups of the molecule (targeting agent or ligand) (Figure 3). PLL with high molecular weights usually elicit significant cell cytotoxity (Choi et al. 1998 : 45). Nevertheless, PLL/DNA complexes are rapidly bound to plasma proteins and cleared from the circulation (Ward, Read and Seymour 2001 : 2221). In addition, successful transfection requires co-application of endosomolytic agent such as chloroquine, which reduces the lysosomal degradation of lipoplexes. In fact, levels of transfection achieved by DNA complexes produced solely with PLL are poor (Pouton et al. 1998 : 289). The condensation pathways and condensation efficiencies are strongly dependent on the charge ratios, the length of PLL and DNA concentration. DNA condensation is monomolecular at low DNA

concentrations and involves multi-molecular condensation also at higher DNA concentration. PLL of the smallest length (containing on an average 19 amino acid residues) was found to be the most efficient in condensing DNA at low DNA concentrations (Mann, Richa and Ganguli 2008 : 252).

2.2.2.2 Polyethylenimine (PEI)

PEI exists in either a branched or linear morphological structure depending on the linkage of the repeating ethylenimine units (Figure 3) (Lungwitz et al. 2005 : 248). PEI is usually branched with every third amino nitrogen atom being primary, secondary and tertiary amines. The property of PEI confers an extraordinary buffering capacity over a wide range of pH, which gives PEI-carrying nucleic acid an opportunity to escape from the acidic endolysomal compartment via a hypothetical "proton sponge" effect. After PEI entered into the cell by endocytosis, proton and chloride ions entered into the endosome with protonation of PEI. As the pH value of endosome increased, water molecules were imported into the endosome by osmosis, and finally, endosome has disrupted. PEI has different molecular weights associated with different transfection efficiencies. *In vitro* cytotoxicity was only apparent at concentrations that were much greater than those affording optimal transfection (Boussif et al. 1995 : 7297). Nevertheless, lower, more acceptable levels of cytotoxicity were seen with lower molecular weight PEI (11.9 kDa).

Ahn et al. studied the delivery of DNA/PEI nano-particles into rat bone marrow stem cells (rBMSCs). Their results indicated that DNA was sufficiently condensed by branched PEI to give DNA/PEI nano-particles and the optimal packing was achieved at nitrogen-to-DNA phosphate (N/P) ratio of 4. The DNA/PEI nano-particles were transfected into rBMSCs. After gene transfection, naked DNA showed no enhanced green fluorescence protein (EGFP) expression, whereas delivery of DNA/PEI nano-particles to rBMSCs showed EGFP expression and resulted in 2–10% transfection (Ahn et al. 2008 : 116). Huh et al. determined the optimal transfection conditions of a 25-kDa linear polyethylenimine (25 kDa L-PEI) and examined whether it has comparable transfection efficiency with other commercially available reagents, ExGen 500, LipofectAMINE 2000, and Effectene by using EGFP expression vector in different cell lines. With the increase of N/P ratio and DNA amounts, transfection efficiency increased with a slight variation in cell types. The optimal amounts of 25 kDa L-PEI were determined at N/P ratio 40 and DNA concentration varied among the cell types. In addition, 25 kDa L-PEI worked efficiently and was less toxic than other reagents. However, the efficiency and toxicity of all these reagents varied according to cell types as well as the ratio of DNA to reagents and the amounts of DNA (Huh et al. 2007 : 165).

2.2.2.3 Poly (2-dimethylamino) ethyl methacrylate (pDMAEMA)

pDMAEMA is a water-soluble cationic polymer which can electrostaticlly interact with DNA to form stable polyelectrolyte complexes with a size of ca. 200 nm at a ratio greater than 2/1 (polymer/DNA, w/w) (Van de Wetering et al. 1998 : 145). Transfection efficiency of pDMAEMA increases as the molecular weight

of the polymer increases. Transfection efficiency was also studied as a function of p(DMAEMA) molecular weight. Polymers with molecular weight over than 300 kDa were better transfection agents in both COS-7 and OVCAR-3 cells than low molecular weight polymers. Dynamic light scattering measurements showed that high molecular weight polymers were able to condense DNA effectively resulting in particles of 0.17-0.21 μm. In contrast, when plasmid was incubated with low molecular weight p(DMAEMA), large complexes were formed (size of approximately 1.0 μm) (Van de Wetering et al. 1997 : 59). Verbaan studied the *in vivo* fate of pDMAEMA-based polyplexes after intravenous administration into mice. In positively charged polyplexes at a dose of 30 μg DNA, most of the radioactivity was found in the lungs and the liver 60 minutes after injection. In the case of pDMAEMA/DNA polyplexes with a negative charge, uptake occurred mainly by the liver. Administration of positively charged complexes at a 30 μg DNA dose resulted in reporter gene expression primarily in the lungs. Injection of negatively charged complexes and naked plasmid did not result in luciferase expression in any of the organs examined (Verbann et al. 2003 : 419).

2.2.2.4 Dendrimers

Dendrimers (orginate from Greek words *dendron* : tree, and *meros* : part) consist of a central core molecule which acts as the root from which a number of highly branched, tree-like arms orighinate in an ordered and symmetric fashion (Figure 4). Dendrimer are usually synthesized by stepwise synthesis based on either the divergent method, in which branch chains grow from the multifunctional core, or the convergent method, in which the growth of branches starts from the end groups and terminates with the final attachment of the core (Tomalia et al. 1985 : 117). Among the broad range of dendrimers, cationic dendrimers, including commercially available poly(amidoamine) (PAMAM) have been used for gene delivery. First dendrimers were discovered in 1980 by Doctor Donald A. Tomalia (The Dow Chemical company). They are characterized by their distinct architectural feature, in which several hyper-branched arms emanate from a central core to the surface in an ordered manner. The first report of the use of Starburst ^{TM} PAMAM dendrimers as trasfection agents demonstrated that these agents could efficiently induce expression of reporter genes in adherent and suspension cell cultures with the G6 (NH_3) dendrimer having optimum efficiency (Haensler and Szoka 1993 : 372).

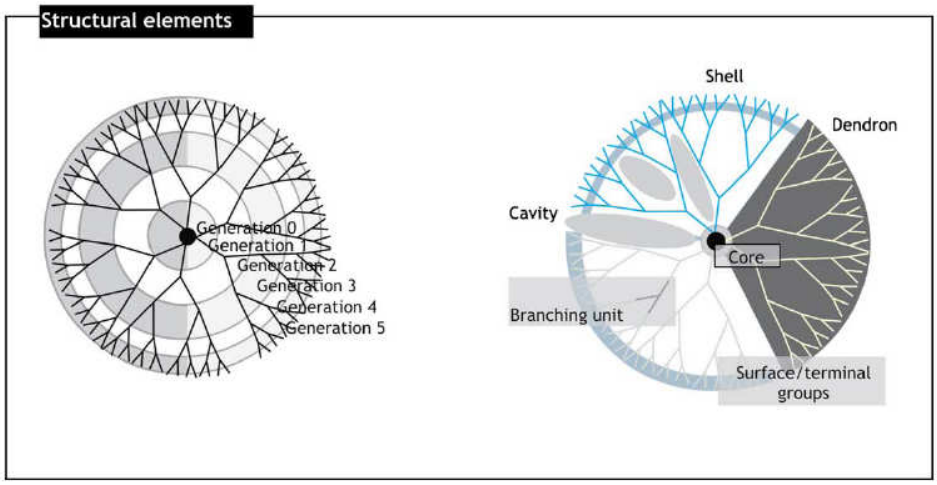

Figure 4 Dendrimer structure.

Source : Christine Dufes, Ijeoma F. Uchegbu, and Andreas G. Schatzlein, "Dendrimer in Gene Delivery," Advanced Drug Delivery Reviews 57 (2005) : 2181.

Nam et al. synthesized biodegradable PAMAM esters that contain arginines or lysines at the peripheral ends of PAMAM with hydroxyl group dendrimer through ester bond linkages. The PAMAM esters were readily degradable under physiological conditions (pH 7.4, 37°C), with more than 50% of the grafted amino acids hydrolyzed within 5 hours. Moreover, these amino-acid-modified polymers showed excellent buffering capacities between pH 5.1 and 7.4, facilitating endosomal escape of polyplexes. While the lysine-grafted PAMAM ester did not display significant improvement in transfection efficiency, the arginine conjugated PAMAM ester-mediated transfection of a luciferase gene showed better transfection efficiency than the branched 25 kDa PEI and PAMAM conjugated with arginine (peptide bond), and lower cytotoxicity, especially with primary cells such as human umbilical vein endothelial cells and primary rat aorta vascular smooth muscle cells (Nam et al. 2009 : 665).

Another polylysine 'branch' structure was studied by Yamagata et al., which compared 2 types of poly(L-lysine) (PLL) as carriers, that is, dendritic poly(L-lysine) (KG6) and linear PLL. KG6 formed a neutral DNA complex, and its DNA compaction level was weaker than that of PLL. The amount of DNA binding and uptake into cells mediated by PLL was 4-fold higher than that with KG6. However, KG6-mediated gene expression was 100-fold higher than that by PLL. Even though large DNA amounts were internalized into cells, most of the DNA would not take part in gene expression systems in the nucleus. Amount of induced cytokine production after intravenous injection of DNA complexes with KG6 and PLL was low, and was similar to the case when DNA was injected alone. Therefore, no significant difference in effects on cytokine production was observed between KG6 and PLL (Yamagata et al. 2007 : 526).

2.2.2.5 Chitosan and its derivatives

Chitosan is the natural cationic polysaccharide and is produced by alkaline deacetylation of chitin (Figure 3). Chitin is the second most abundant natural polysaccharide which is present in crustacea, insects and yeasts. Studies on chitin and chitosan have increased since the 1990's to find value-added uses of these polysaccharides that show excellent biological properties such as biocompatibility, biodegradability, lack of toxicity (Borchard et al. 2001 : 146). The unique properties of chitosan arise from its amino groups that carry positive charges at pH values below 6.5, enabling it to bind to negatively charged materials such as enzymes, cells, polysaccharides, nucleic acids. Chitosan is insoluble at alkaline and neutral pH but soluble in inorganic and organic acids such as hydrochloric acid, acetic acid, lactic acid and glutamic acid. The drawback of chitosan is insoluble in water and low transfection efficiency. Many researchers synthesized CS derivatives in order to improve solubility of CS in physiological pH. For example, Lee et al. designed thiolated chitosan, which was prepared by reaction between 33 kDa chitosan (90% degree of deacetylation) and thioglycolic acid. The thiolated chitosan with plasmid GFP has been evaluated for their transfection efficiency in various cell lines. These results demonstrated that thiolated chitosan (CSH-360) with plasmid DNA complexes induced higher GFP expression in HEK293, MDCK and HepG-2 cell lines than unmodified chitosan. Complexes of disulfide-crosslinked CSH360/DNA showed sustained DNA release and continuous expression in cultured cells lasting up to 60 hours post transfection (Lee et al. 2007 : 157). Kim et al. prepared water-soluble chitosan by coupling with urocanic acid bearing imidazole ring which can play the crucial role in endosomal rupture through proton sponge effect. The urocanic acid-modified chitosan (UAC) was formed complexes with DNA. It showed good DNA binding ability, high protection of DNA from nuclease attack, and low cytotoxicity. The transfection efficiency of chitosan into 293T cells was much enhanced after coupling with urocanic acid (UA) and increased with increasing of UA contents in the UAC (Kim et al. 2003 : 392-400). Thanou et al. synthesized CS derivative *N*-trimethylated chitosan (TMC) from oligomeric chitosan. These oligomers (40 and 50% degree of quaternization) were examined for their potency as DNA carrier systems in two cell lines, COS-1 (monkey kidney) and Caco-2 (human colon carcinoma). *N*-trimethylated chitosan oligomers (TMOs) were superior to oligomeric CS in transfecting COS-1 cells; however, none of the used CS-DNA and lipofectin-DNA complexes was able to increase transfection efficiency in differentiated Caco-2 cells (Thanou 2002 : 156-157). Opanasopit et al. have successfully synthesized methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitoan (TM-Bz-CS) (Opanasopit et al. 2009: 147) and methylated *N*-(4-pyridinylmethyl) chitosan (TM-Py-CS) (Opanasopit et al. 2008 : 132). Both TM-Bz-CS and TM-Py-CS were soluble in pH 7.4 and expressed transfection efficiency higher than unmodified CS.

To combine two cationic polymers (chitosan and PEI), Zhao et al. designed chitosan/DNA complex combined with PEI. The combination of PEI with the chitosan/DNA complex enhanced the gene expression of Hela cells to 1000-fold of that induced by chitosan alone. Cytotoxicity of PEI was decreased upon combination with the chitosan/DNA complex. Furthermore, the PEI/chitosan/DNA

could maintain the gene expression efficiency in the presence of serum (Zhao et al. 2008 : 65).

2.2.2.6 Collagen

Collagen is a major extracellular matrix protein and accounts for 20–30% of the total body protein. It is bioabsorbable and non-toxic, and has low antigenicity. It was used as a gene carrier. In the form of hydrogel or pellet matrix, collagen has been shown to deliver DNA to cells. There are various types of collagens that have different structures, properties, and tissue distributions, and their applications have been studied by various approaches. During the last decade, the analysis of mechanisms for delivering properties of type I collagen triple helices in gene delivery systems has attracted huge interest. Honma et al. reported that atelocollagen, the telopeptides at the ends of the collagen molecules are removed, could be utilized in the delivery systems for genes. They developed an efficient technique for high-throughput gene transfer and expression screening in mammalian cells in microarrays by precoating a microplate with an atelocollagen complexed with DNA to which cells are then seeded. The complexes with a nanoparticle form were efficiently transduced into cells without use of any additional transfection reagent, and they allowed for long-term gene expression without apparent chromosomal integration (Honma et al. 2001 : 1075). Furthermore, detailed analysis of the mechanisms of interaction between collagen triple helix and double helix of nucleic acids leads to a better understanding of the mechanisms of gene delivery systems, which is crucial for gene therapy. (Svintradze and Mrevlishvili 2005 : 283). Collagen has pH of 5.8 and carries net negative charge at neutral pH, therefore, they cannot form complexes with DNA at neutral pH. Although it could form complexes with DNA at low pH, these complexes aggregate rapidly at neutral pH and do not confer significant protection to DNA due to its poor stability in serum.

Wang et al. prepared DNA complexes with methylated collagen (MC) and unmodified native collagen (NC) to deliver genes into cells. MC was prepared by methylation of the carboxyl groups of collagen, rendering the collagen net positively charged at neutral pH. NC/DNA complexes were prepared at pH ~ 3, but aggregated rapidly at neutral pH. These methyl ester groups can be hydrolyzed *in vivo* releasing collagen. This positively charged methylated collagen (MC) can form complexes with DNA at neutral pH. These complexes did not confer significant protection to DNA due to its poor stability in serum. MC carried a positive charge at neutral pH and formed complexes with DNA in PBS; therefore MC improved DNA binding ability and the stability of the complexes at physiological conditions. MC/DNA complexes were smaller and more stable than NC/DNA complexes in PBS, and sustained released of DNA from MC/DNA complexes was observed for up to 3 weeks in PBS at 37°C. In contrast, NC/DNA complexes released almost all the DNA within 6 hours under the same condition. *In vitro* gene transfection experiments revealed that MC mediated a higher gene expression than NC, although the level of gene expression was still much lower than that achieved with PEI/DNA complexes. In contrast to *in vitro* results, NC/DNA complexes yielded a 3.8-fold higher gene expression than naked DNA and MC/DNA complexes ($P < 0.05$) at week 2 following intramuscular injection at a DNA dose of 3 μg per muscle and a weight ratio of 1.

Higher weight ratios resulted in significant decrease of transfection efficiency, particularly for MC/DNA complexes (Wang et al. 2004 : 115).

3. Barriers to DNA delivery

Naked DNA must overcome serum nucleases, conserved immune receptors, nonspecific clearance, cellular membrane barriers, endosomal degradation, and intracellular trafficking to ensure optimal localization and expression (Figure 5).

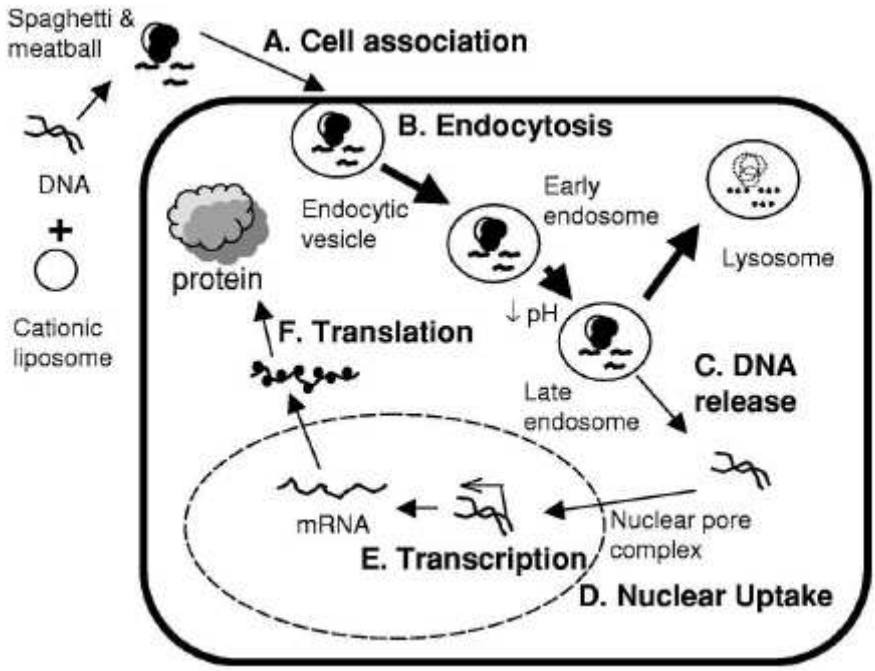

Figure 5 Mechanism of cellular transfection by cationic lipid-based gene transfer vectors.

Source : Feng Liu and Leaf Huang, "Development of Non-Viral Vectors for Systemic Gene Delivery," Journal of Controlled Release 78 (2002) : 261.

3.1 Physiological barriers

3.1.1 DNA protection

In order to avoid the rapid degradation of DNA, the DNA must be protected from nuclease attack. Non-viral gene delivery systems, including cationic lipids and polymers, have been shown to protect DNA effectively. These complexes are formed by the electrostatic interaction between the positively charged of cationic lipid/polymer and the negatively charged phosphate molecules on the DNA backbone. This electrostatic interaction produces an uncontrolled aggregation and subsequent particle formation. The resulting lipid/DNA complexes or polymer/DNA complexes must meet stringent size criteria, such as the cationic liposomes used are typically

small (~ 100 nm) before adding to DNA; however, complexes formed with DNA exhibit diameters that range from as small as 200 nm to structures as large as 2 μm (Wasan, Reimer and Bally 1996 : 427). The aggregation problem was depending on : (i) the cationic lipid used, (ii) the type and quantity of additional lipids (e.g. DOPE, 1,2-di-oleoyl-sn-glycero-3-phosphocholine (DOPC) or cholesterol) used to prepared the cationic liposome, (iii) the composition of the diluent and the buffers used, (iv) the method and rate of mixing, (v) the temperature of mixing, (vi) DNA purity and (vii) the length of time after complex formation. Therefore, in addition to shielding, the DNA packaging process must include DNA compaction. Liposome system containing spermidine-condensed DNA and negative cone-forming lipids improved gene delivery and gene expression. It combines the high compaction of polyplexes and the facilitated endosomal escape of the lipoplexes. In addition, they provide more protection to DNA (Ibanez et al. 1996 : 633).

3.1.2 Biodistribution (delivery to target cells)

Biodistribution of plasmid to either extracellular or intracellular targets is dependent on the structure of capillary walls, (patho) physiological conditions, the rate of blood and lymph supply, the physicochemical properties of plasmid and its carrier molecules. An accumulation of cationic vectors in the lung can be acheived by changing the zeta-potential of DNA/cationic (lipid or polymer) complex from negative to positive values. This accumulation could be explained by the electrostatic interaction of cationic system with negatively charged erythrocyte membranes. Targeted gene delivery into subcutaneously growing tumors after systemic application was achieved using electroneutral adenovirus-enhanced transferrinfection (AVET) complexes and sterically stabilized PEGylated transferrin-polyethylenimine (Tf-PEI)/DNA complexes, whereas application of positively charged polycation/DNA complexes resulted in predominant gene expression in the lungs (Kirchesis et al. 1999 : 111). Polymeric nano-particles coating with polyethylene glycol and water-soluble chitosan inhibited the macrophage uptake and extended the circulation time (Sheng et al. 2009 : 2340). Tumors are known to have defined, leaky vasculature that allows particles to extravasate into the tumor tissue, where they are retained owing to a lack of effective lymphatic drainage i.e., enhanced permeation and retention effect (the EPR effect). The use could be made of this effect in gene delivery from the systemic circulation, but penetration of the tumor tissue may be restricted by the high hydrostatic pressures which build up due to the lack of lymphatic drainage (Thrope and Burrows 1995 : 237).

3.2 Cellular barriers

After vectors cellular internalization, intracellular barriers (endosomal escape, cytoplasm trafficking, nucleus entry) are additional hurdles, in which each of the listed steps can be a major drawback for the efficiency of such a gene delivery system.

3.2.1 Cell Entry (Cellular internalization)

There are a multitude of endocytic pathways that can be processed by the vector systems : clathrin-coated pit formation, caveolea-mediated (Kirkham and

Parton 2005 : 273). Some cell types are capable of internalizing extracellular fluid via macropinocytosis and large particles via phagocytosis. The predominant way of entry gene delivery systems seems to be by non-specific adsorptive endocytosis followed by the clathrin-coated pit mechanism, because negatively charged, proteoglycans present on the cell membrane, are able to interact with the positively charged systems (Mounkes et al. 1998 : 26164). Using specific inhibitors of different endocytosis pathways, DOTAP/DNA lipoplex uptake proceeded only by clathrin-mediated endocytosis, while PEI/DNA polyplexes can be taken up by two mechanisms, one involving caveolae and the other clathrin-coated pits (Rejman, Bragonzi and Conese 2005 : 468). However, the internalization pathway depended on the system used and the cells to transfect. Therefore, factors such as cell membrane composition or surface charge and the size of complexes particles may influence the balance in favour of either one or the other pathway. The size itself of (ligand devoid) particles can determine the pathway of entry. The clathrin-mediated pathway of endocytosis shows an upper size limit for internalization of approx. 200 nm, and kinetic parameters may determine the almost exclusive internalization of such particles along this pathway rather than via caveolae (Rejman et al. 2004 : 159).

3.2.2 Endosomal release

If the gene delivery system enters the cells via an endocytosis pathway, escape from endosomes of the vector is essential for efficient transfection. Furthermore, an escape of DNA from the endocytic compartments is one of the major barriers to efficient gene delivery. The mechanisms involved in endosomal release of DNA by cationic polymer-based vectors are uncertain. Two hypothesis have been suggested to explain this escape. The first one is based on the idea that a physical disruption of the negatively charged endosomal membrane occurs on direct interaction with the cationic polymer. Such a mechanism has been suggested for both PAMAM dendrimers and PLL (Zhang and Smith 2000 : 811). The second hypothesis involves the use of DNA delivery systems that possesses a high buffering capacity in the endosomal compartment, has been termed the ‘‘proton-sponge’’ hypothesis (Figure 6). This hypothesis suggests that polymers such as PEI containing a large number of secondary and tertiary amines, is led to buffering inside endosomes. The additional pumping of protons into the endosome, along with an influx of chloride ions to maintain charge neutrality, increase ionic strength inside the endosome. This is then though to cause osmotic swelling and rupture of the endosome, resulting in the escape of the vector from the degradative lysosomal trafficking pathway (Boussif et al. 1995 : 7297). In the case of cationic lipid-based vectors, another model has been proposed for local endosomal membrane destabilization, after electrostatic interaction of cationic lipoplexes with the endosomal membrane induce the replacement of anionic lipids from the cytoplasm-facing lipid monolayer of the endosomal membrane are proposed to flip-flop mechanism. Anionic lipids diffuse into the complex and form a charge neutral ion pair with cationic lipids. The DNA dissociates from the complex and enters the cytoplasm (Figure 6). Interestingly, it has been suggested that transfection with bis-guanidinium-trencholesterol (BGTC) containing a tertiary amine with a low pK_a could be able to escape from the endosome to a similar process to that

proposed earlier for cationic polymers, PEI : the ‘‘proton-sponge’’ mechanism (Vigneron et al. 1996 : 9684).

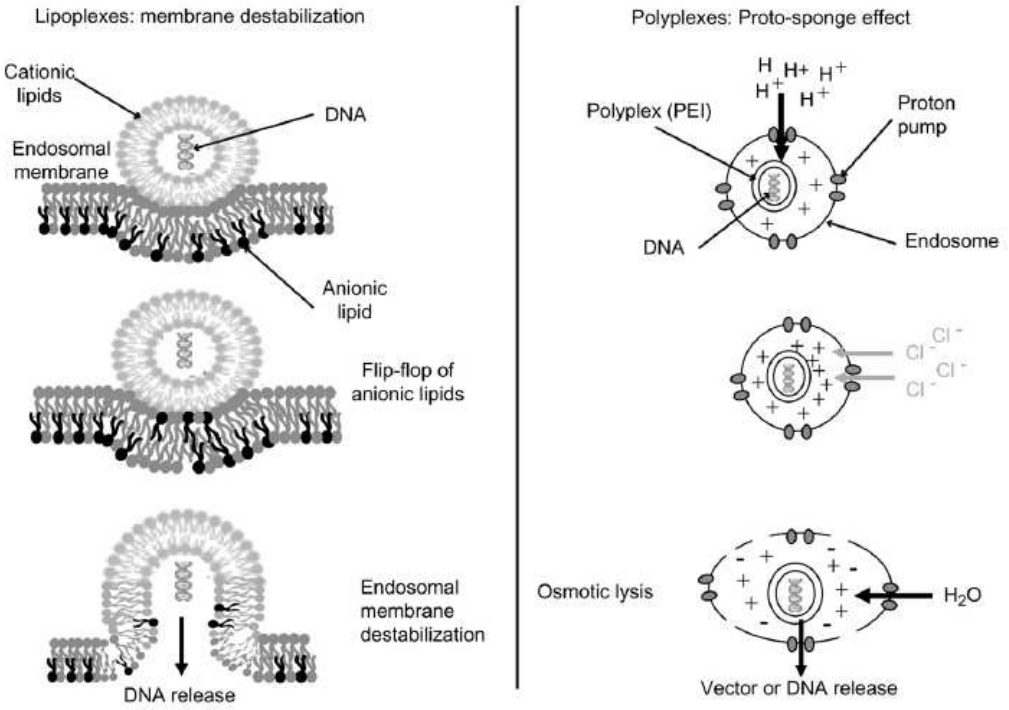

Figure 6 Hypothesis of endosomal escape of lipoplexes and polyplexes gene delivery systems.

Source : Marie Morille et al., “Progress in Developing Cationic Vectors for Non-Viral Systemic Gene Therapy Against Cancer,” Biomaterials 29 (2008) : 3483.

A number of approaches have been used to overcome the problem of endocytic entrapment of DNA. Some of them are summarized in Table 3. For example, the use of endolytic pathway with lysosomotropic agent such as chloroquine was found to enhance the gene expression and protect DNA from degradation (Yang et al. 2009 : 1). In addition, pH sensitive liposomes which are formulated to undergo rapid destabilization in the acidic environments of endosomes. Hiraka et al. concluded that the pH-sensitive liposome retaining the Fe-porphyrin could be delivered by the pH-sensitive liposome from the endosome to the cytosol (Hiraka et al. 2008 : 57).

Table 3 Endolytic strategies for non-viral delivery.

Agent	Mechanism
pH-sensitive liposomes	Destabilization of membrane at low pH
Fusogenic peptides	Membrane lytic activity (eg., HA2 from influenza virus)
DMI-2	Specific inhibitor of DNase I, inhibits lysosomal nucleases
Chloroquine	Lysosomotropic agent
Glycerol, PVP, sucrose, PEI	Proton sponge, osmotic swelling

Source : Mansoor M. Amiji, Polymeric Gene Delivery : Principles and Applications (USA : CRC Press, 2005), 46.

3.2.3 Nuclear localization

Following endosomal escape, transport of DNA to the nucleus is required for gene expression. In eukaryotic cells, the nucleus is separated from the cytoplasm by a double lipid bilayered membrane system called the nuclear envelope. The nuclear membrane separates the nucleoplasm from the cytoplasm. Nuclear pore complexes (NPCs) are the sites of exchange of macromolecules between cytoplasm and nucleoplasm. However, nuclear trafficking of DNA is the next hurdle to efficient transfection. The mechanism of nuclear translocation is depended on the type of delivery system. There are possible routes for DNA entry into the nucleus to have any therapeutic effect : moving through the nuclear pores; entering during mitosis when the nuclear envelope breaks down; and moving physically across the nuclear membrane. Some viruses, such as adenovirus, simian virus 40 (SV40), HIV, and herpes virus are know to induce rapid migration of their genomes to the nucleus even in nondividing cells. In SV40, viral capsid proteins contain nuclear localization signal (NLS) that trigger translocation of the virion to the nucleus and disassembly of the virion within the nucleus. The NLS concept has been used to improve the uptake of plasmid DNA into the nucleus. Several groups demonstrated that the covalent attachment of an SV40 nuclear localization sequence (SV40NLS) either directly to DNA or to polymers that form complexes with DNA led to an increase of nuclear import resulting in enhanced protein expression (Eguchi et al. 2005 : 507). Furthermore, a PNA (peptide nucleic acid) molecule linked to an SV40 NLS peptide increased the nuclear uptake of oligonucleotides and enhanced the transfection efficacy of plasmids (Branden, Mohamed and Smith 1999 : 784). Talsma et al. designed recombinant reovirus type 3 σ1 attachment protein modified with a nuclear localization sequence (σ1-NLS) as a targeting ligand. Purified σ1-NLS was covalently conjugated to the PEI using a carboxyl-reactive cross-linking agent and complexed with plasmid DNA. This allowed these carriers to specifically bind to plasma membrane cell surface receptors when located outside the cell and engage the nuclear import machinery for enhanced nuclear translocation after uptake into cells. The results demonstrated that delivery vehicle resulted in substantially greater levels of in vitro gene expression (Talsma et al. 2006 : 271). Moffatt et al. newly synthesized a multi-functional PEIbased polyplex for systemic p53-mediated gene therapy. This PEGylated vector attached with a CNGRC peptide for CD13 targeting in tumors also

carries two systems targeting the nucleus: a Simian Virus (SV) 40 peptide (nuclear localization signal) and an oligonucleotide based nuclear signal (DNA nuclear targeting signal). This promising vector exposed a significant tumor regression and 95% animal survival after 60 days (Moffat, Wiehle and Cristiano 2006 : 1512).

3.2.4 Gene expression

After conquering all the hurdles described earlier, the DNA finally reaches the nucleus where the transgene can be expressed. Lee et al. showed that upon synergistic effect of PEI and cationic liposome, the transfection efficiency is dependent on the polymer to lipid to DNA ratio. At ratios where the highest expression level was achieved by using the combination of PEI 25 kDa (0.65 μg/μg of DNA, N/P ratio = 4.5) with 10 nmole of DOTAP–cholesterol (DOTAP–Chol, 1:1 w/w). This DNA complex formulation dramatically increased the luciferase expression 10- to 100-fold, which was much higher than those of other polycations alone, cationic liposomes alone or the combination (Lee et al. 2003 : 55). Colonna et al. proposed that upon type of cationic lipid, the powders kept a good transfection efficiency as compared to the fresh colloidal formulations. Lipid/polycation/DNA (LPD) complexes were prepared by an optimized liposomes/protamine/pDNA mass ratio (w/w/w) of 3:2:1 with liposomes obtained from cationic lipids or 5:2:1 with chitosan loaded liposomes : these conditions led to pDNA complexation efficiencies approaching 100%. LPD complexes prepared effectively condensed pDNA, protecting it from enzymatic degradation (Colonna et al. 2008 : 108-118).

3.3 Immunological barriers

A significant barrier to *in vivo* gene delivery is immune system, as this response to the vector can reduce the biological activity of the secreted protein eliminate transfected cells, or prevent repeated dosing. Gene delivery can up-regulate inflammatory cytokines, such TNF-α, IL-1, IL-6, IL-12, and IFN-γ, which can inhibit promoter activity and attenuate gene transcription. Positively charged particles can be opsonized with plasma protein such as immunoglobulin M, complement C3 and proteins of the coagulation cascade leading to their rapid clearance resulting from phagocytic cells of mononuclear phagocyte system (MPS) in the liver, spleen, lungs and bone marrow. The clearance rate of these vectors from the circulatory system in fact depends on their physicochemical surface characteristics. To be undetectable by macrophage, vectors have to be as small and neutral as possible (Vonarbourg et al. 2006 : 4356-4362). Additionally, systemic administration of cationic lipid/DNA complexes can display potent antitumor activity depending on both NK cells and interferon-γ (IFN-γ). Moreover, lipoplex vaccination can elicit large numbers of functionally active and tumor-specific infiltrating $CD8^+$ T cells (Dow et al. 1999 : 1552). The molecular weight of PLL/DNA vectors has a significant effect on the circulation of PLL/DNA complexes in mice, with PLL 211 kDa/DNA complexes displaying up to 20 times greater levels in the blood after 30 minutes compared with PLL 20 kDa/DNA complexes. It is shown that PLL 20 kDa/DNA complexes fix mouse complement C3 *in vitro*, independent on immunoglobulin binding; are less soluble in the blood *in vivo*; bind erythrocytes; are rapidly removed by the liver, where

they associate predominantly with Kupffer cells; and result in a rapid increase in hepatic leukocytes expressing high levels of complement receptor 3 (CR3). Whereas, PLL 211 kDa/DNA complexes bind erythrocytes and associate with Kupffer cells but, in contrast, do not fix mouse complement *in vitro* (Ward et al. 2001 : 2221). Another example of vector in gene delivery are viral vectors such as Ad vectors, which elicit strong antibody response in mice and primates against both transgene product and the vector. Intramuscular delivery of AAV vectors in mice and humans elicits antibodies against both the transgene product and the vector, but in generally only AAV capsid-specific antibodies are induced when the vectors are injected into the liver (Jooss and Chirmule 2003 : 956-958). Coaggulation factor IX (F.IX) delivered to hepatocytes by a lentivirus vector was shown to result in off-target cell expression in hematopoietic cells of the spleen, which in turn triggered an F.IX transfecr. Incorporation of a microRNA sequence specific spleen and, most importantly, resulted in sustained expression of F.IX (Brown et al. 2007 : 4144).

4. Techniques for evaluation of complex formation between DNA and cationic vectors

4.1 Physicochemical evaluation techniques

4.1.1 Gel electrophoresis retardation

The binding of the cationic vectors to DNA was studied by analysis of the electrophoretic mobility of the DNA within an agarose gel, the so-called electrophoretic mobility shift assay. DNA, electrophoresed on agarose gels and visualized by ethidium bromide fluorescence, usually gives clear mobility and an obvious signal. However, following complex formation, the ethidium bromide signal often disappears and the complex remains at the origin, where it can be visualized by post-electrophoretic incubation of the gel in cationic glycolipids/pDNA complexes, restoring ethidium bromide fluorescence. The gel pattern revealed that the glycolipids were capable completely inhibiting electroresis mobility of plasmid DNA when lipoplexes were prepared at a high lipid : DNA charge ratios 8:1 and 4:1,whereas the lower lipid : DNA charge ratios (2:1 and 1:1) exhibited strong binding interactions with plasmid DNA thereby inhibiting the electrophoretic mobility of DNA lipoplexes formulations (Mukthavaram et al. 2009 : 2380). This technique is often used for comparison of complexes formed at different charge ratios, and a definite “gel shift’ appear to occur at about the charge ratio where particles form of DNA migration during electrophoresis of the cationically modified cationic vectors.

4.1.2 Particle size and charge analysis

Particle size and charge analysis is a size and surface charge measurement of the complexes in the form of colloidal particles which are smaller than micrometer scale. The high sensitivity of tool, which popular method for determination of particle sizes is dynamic light scattering (DLS), or photon correlation spectroscopy (PCS), is a rapid method for provide information on the sizes and uniformity of particles in a sample, via the zeta-average diameter and polydispersity

index (PI) reading. This method can be applied for investigations into the colloidal stability of particles (Gaumet et al. 2008 : 2).

Surface charge analysis is a technique that provide on the principle of electrophoresis, an external electric field applied for measure the zeta-potential of particles will induce them to move, with the mobility of the particles being releated to the charge magnitude. Experimentally, not actually the potential at the particle surface that is measured. The potential just outside the bound later of counterions from the medium (the stern layer), known as the zeta-potential is, generally marginally lower than the surface charge. Surface charge highly dependent on the ionic strength of the medium, whereas ionic strength increases, the zeta-potential falls. Thus, to perform experiments in low ionic strength buffers is the best method. Peng et al. studied that the size distribution and zeta-potential of the prepared nanoparticles (NPs) in aqueous environment could be investigated by dynamic light scattering (DLS). An increase in the amount of γ-polyglutamic acid (γ-PGA) incorporated, the size of NPs increased appreciably while their PI and zeta-potential value decreased noticeably. The diameters of NPs measured by DLS were relatively larger than those observed by transmission electron microscopy (TEM). This is because the diameters of NPs obtained by DLS reflected their hydrodynamic diameters swelled in aqueous solution, while those observed by TEM were the diameters of dried NPs (Peng et al. 2009 : 1799).

4.1.3 Microscopic investigation

4.1.3.1 Transmission electron microscopy (TEM)

TEM is a microscopy technique whereby a beam of electrons is transmitted through an ultra thin specimen, interacting with the specimen as they pass through. An image is formed from the interaction of the electrons transmitted through the specimen, which is magnified and focused onto an imaging device, such as a fluorescent screen, as is common in most TEMs, on a layer of photographic film, or to be detected by a sensor such as a CCD camera. TEM was used to examine the morphology, size, shape and arrangement of the particles which make up the specimen as well as their relationship to each other on the scale of atomic diameters. Zou. et al. studied the morphology of bioadhesive poly(D,L-lactide-co-glycolide) (PLGA) nanoparticles, that was examined by TEM. The result was shown that the obtained nanoparticles stabilized with different stabilizers appeared similar spherical in shape and separated from each other (Zou. et al. 2009 : 188). Serikawa et al. used electron microscopy and found that the typical spherical structure of several cationic liposomes was often lost in serum-containing medium. Vesicular structures of cationic liposome containing a cationic lipid, O, O'-ditetradecanoyl-N-(α-trimethylammonioacetyl) diethanolamine (DC-6-14) dispersed in serum-free medium. When this liposome was mixed with the GFP plasmid in serum-free medium at the concentrations used for transfection, large aggregates in spherical and granular form. In 5% serum-containing medium as used for transfection, this cationic liposome/DNA complex formed rather smaller and uniform vesicular structures with a diameter of 100-200 nm, and these vesicular structures were stably maintained for 1 hour after the formation of complex and were seen for at least 6 hours. In contrast, the spherical structure of

Lipofectamine seen in serum-free Dulbecco's modified Eagle's medium (DMEM) was completely lost in the serum containing medium (Serikawa et al. 2000 : 425-426).

4.1.3.2 Atomic force microscopy (AFM)

AFM, has been reported as a useful microscopy to visualize not only submicron-size particles but also DNA, protein or living cells at very high resolution without drying the samples (Kawaura et al. 1998 : 69). AFM offers a distinct advantage over other methods for investigating the DNA condensates. One of the greatest advantages is AFM's ability to view the structure of the delivery vehicle in its hydrated state, as it would appear in use. In addition, with AFM's nanometer-scale resolution, researchers can easily image the DNA strands and see how they react and condense with a particular polymer or liposome (Nakano et al. 2008 : 203). Ahn et al. used AFM to examine the structure of the PEI/DNA complexes formed at N/P charge ratios of 1, 2, and 4. At a N/P charge ratio of 1, the complex was the shape of a large strand. The size decreased as the N/P charge increased. At an N/P charge ratio of 4, most of the PEI/DNA complexes were spherical, with few nonspherical forms. The diameters of the PEI/DNA complexes determined by AFM agreed well with the values determined from DLS (Ahn et al. 2008 : 2418). Yang et al. studied AFM images of naked DNA and cationic polyrotaxane/DNA complexes at N/P ratio of 2 and 10. Results from the AFM study showed that the complexation of DNA by polyrotaxane led to the formation of compact nanoparticle. The loose, supercoiled structure of pDNA could be found when the pDNA was not condensed by polymer. At N/P ratio of 2, supercoiled plasmid DNA could still be identified under AFM while some of the pDNA was condensed to nanoparticles by polyrotaxane. Compared to this partial condensation at N/P ratio of 2, the same amount of pDNA could be tightly packed and formed pDNA complexes at N/P ratio of 10 completely (Yang et al. 2009 : 1386).

4.2 Biological evaluation techniques

4.2.1 *In vitro* cell culture for cytotoxicity

MTT assay is a widely used test for evaluating cytotoxicity of various molecules and drugs in cell cultures (Berridge, Herst and Tan 2005 : 127). The lipophilic positive charged 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide (MTT) dye added to the cell medium is taken up by endocytosis only in metabolically living cells. MTT assay is based on the capability of viable cells to reduce the MTT tetrazolium salt to produce insoluble purple-colored formazan that has to be dissolved subsequently in organic solvent for spectrophotometric dosage measurement (Mosmann et al. 1983 : 55). *In vitro* studies of HeLa cells showed that the *in vitro* cytotoxicity assay, approximately 50% of HeLa cells was killed after incubation with the agent (concentrations of incubation ~100 mg/L). In addition, conjugation of low molecular weight PEI (600 kDa and 12 kDa) with chitosan by using a coupling reagent. Though the actual cytotoxicity of the product is subjected to changes in the grafting ratio of PEI, based MTT assay *in vitro*, no significant decrease in cell viability was observed after incubation with the synthesized copolymer (up to 500 mg/L) in the presence of 10% FBS (Lai and Lin 2009 : 161). Huang et al. compared the cytotoxicity of the new polymers, low molecular weight PEI cross-

linked by (2-hydroxypropyl)-β cyclodextrin or (2-hydroxypropyl)-γ-cyclodextrin with PEI 25 kDa and PEI 600 kDa using a MTT assay in SKOV-3 cells. The new polymers showed significantly lower cytotoxicity than that of PEI 25 kDa (Huang et al. 2006 : 2383). Putnam et al. studied *in vitro* cytotoxicity of the polylysine-graft imidazole acetic acid polymers, measured as a function of polymer concentration using the MTT assay. Results from the CRL 1476 smooth muscle cell line shown that for both polylysine and PEI, cytotoxicity increases with increasing polymer concentration, with greater than half the cell population metabolically inactive at the highest polymer concentration (55 mg/mL). In contrast, cells incubated with polylysine conjugated with 4-imidazole acetic acid 73.5, 82.5 and 86.5 mole % retained greater than 80% of their metabolic activity. The results are consistent among all of the cell lines tested (smooth muscle cells (CRL 1476), macrophages (P388D1), and hepatoblastoma (HepG2) (Putnam et al. 2001 : 1203).

4.2.2 Transfection efficiency

Transfection is the expressing a foreign or modified gene in an organism. Thus, method of transfection for identifying a successful gene uptake event is necessary. Reporter genes have become an important method in studies of gene expression. They are widely used in biomedical and pharmaceutical research and also in molecular biology and biochemistry to study gene expression and other cellular events coupled to gene expression, such as receptor activity, intracellular signal transduction (Figure 7). Reporter gene consists of two functional parts : One is a DNA-sequence that gives the information about the protein that is produced (coding region). The other part is a specific DNA-sequence linked to the coding region; it regulates the transcription of the gene (promoter). The promoter is either activating or suppressing the expression of the gene. The purpose of the reporter gene assay is to measure the regulatory potential of an unknown DNA-sequence. This can be done by linking a promoter sequence to an easily detectable reporter gene such as that encoding for the firefly luciferase. Common reporter genes are β-galactosidase, β-glucuronidase and luciferase. Various detection methods (Table 4) are used to measure expressed reporter gene protein. These include luminescence, absorbance and fluorescence.

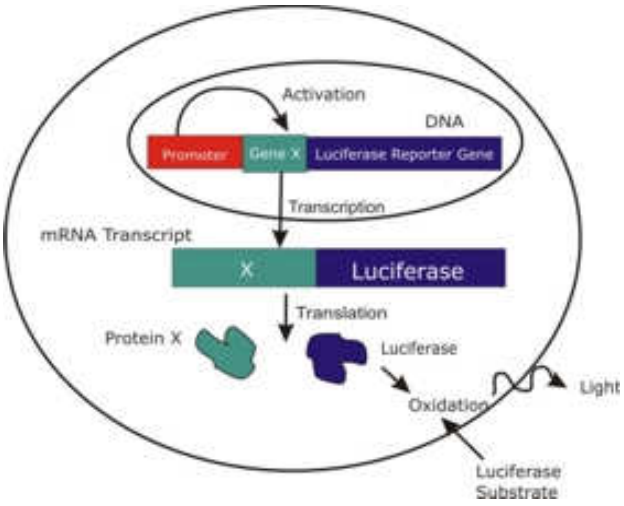

Figure 7 Composition of reporter gene.

Source : Reporter gene assay [Online], accessed 24 March 2009. Available from http://www.berthold.com/ww/en/pub/bioanalytik/applikation/reporter_gene.cfm

Table 4 Detection method for reporter gene.

Reporter	**Detection method**		
	Luminescence	Fluorescence	Absorbance
Luciferase	+	+	
β -Galactocidase (GUS)	+	+	+
β -Glucuronidase(b-Gal)	+	+	
Secreted placental alkaline phosphatase (SEAP)	+		+
Green Fluorescent Protein (GFP)		+	

Source : Reporter gene assay [Online], accessed 24 March 2009. Available from http://www.berthold.com/ww/en/pub/bioanalytik/applikation/reporter_gene.cfm

Thomas et al. studied the transfection efficiency by using assay for *β*-gal expression. They found that removal of the residual *N*-acyl moieties from commercial linear 25 kDa PEI enhanced its transfection efficiency 21 fold *in vitro*. The results suggested that the DNA (gWiz β-gal) trasfection efficiency of linear PEIs *in vitro* was affected by their extent of deacylation. In addition, commercial linear PEI 25 kDa had ability to deliver DNA to the mouse lung 10,000 fold, and the lung-versus-spleen specificity increased 1,500 fold (Thomas et al. 2005 : 5679). Ahn et al. studied gene transfection efficiency of DNA/PEI nano-particles in rat bone marrow stem cells

(rBMSCs). The formation of nanoparticles from DNA and PEI was performed by the addition of the PEI solution to the DNA solution. After transfection, enhanced green fluorescence protein (EGFP) was monitored by flow cytometry. These results demonstrated that naked DNA showed no EGFP expression, whereas delivery of DNA/PEI nanoparticles to rBMSCs showed EGFP expression and resulted in 2-10% transfection (Ahn et al. 2008 : 116). Kim et al. synthesized PEI with acid-labile linkages as a biodegradable gene carrier. PEI with acid-labile/pCMV-Luc complexes were transfected to 293T or A75R cells. The transfeciton efficiency was measured by luciferase assay. Transfection assay showed that, in 293T cells, the transfection efficiency increased with the N/P ratio. In A7R5 cells, transfection efficiency of acid-labile PEI was saturated at a 20:1 N/P ratio, suggesting that the transfection efficiency of acid-labile PEI may be dependent on cell lines. In addition, the transfection efficiency of the acid-labile PEIs was comparable to that of PEI 25 kDa and PEI 1.8 kDa. Acid labile PEI showed much higher transfection efficiency than PEI 1.8 kDa and slight lower transfection efficiency than PEI 25 kDa (Kim et al. 2005 : 215).

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Materials for purification of pDNA (Table 5)

Table 5 Composition of buffers for QIAGEN® Plasmid Midi Kits.

Buffer	Composition
Buffer P1 (resuspension buffer)	50 mM Tris. Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A
Buffer P2 (lysis buffer)	200 mM NaOH, 1% SDS (w/v)
Buffer P3 (neutralization buffer)	3.0 M potassium acetate, pH 5.5
Buffer QBT (equilibration buffer)	750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v); 0.15%Triton® X-100 (v/v)
Buffer QC (wash buffer)	1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)
Buffer QF (elution buffer)	1.25 M NaCl; 50 mM Tris. Cl, pH 8.5; 15% isopropanol (v/v)

1.2 Liposomes reagents

- 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, CHAPS (Sigma®, St Louis, MO, USA)
- Branched polyethylenimine, PEI (Sigma-Aldrich Chemie. GmbH, Germany)
- Cetylpyridinium chloride, CPC (Sigma®, St Louis, MO, USA)
- Chitosan lactate with molecular weight (MW) of 20, 45, 200 and 460 kDa
- Cholesterol, Chol (Carlo Erba)
- Dodecyl trimethylammoniumbromide, DTAB (Sigma®, St Louis, MO, USA)
- Egg yolk phosphatidyl choline, EPC (Wako Pure Chemical Osaka, Japan)
- Lipofectamine2000™ (Invitrogen, NY, USA)

- Methylated *N*-(4-pyridylmethyl) chitosan (TM_{69}-Py_{62} –CS)
- Sodium oleate, NaO (Sigma®, St Louis, MO, USA)
- Sodium taurocholate, NaT (Sigma®, St Louis, MO, USA)
- Stearylamine, SA (Sigma®, St Louis, MO, USA)

1.3 Tissue culture reagents

Tissue culture reagents (GIBCO™ Grand Island, NY, USA).

- RPMI Medium 1640
- Fetal bovine serum EU Approved origin (FBS)
- L-Glutamine
- MEM non-essential amino acids 100X w/o

1.4 All other chemicals

- 0.25% trypsin-EDTA (GIBCO™)
- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma® company, St. Louis, MO, USA)
- Blue/Orange 6x loading dye (Promega)
- Casein enzyme hydrolysate, type-1; Tryptone (HIMEDIA® Himedia Labortories Pvt. Ltd.)
- Chloroform (VWR International Ltd. England analytical reagent grade)
- Dimethyl sulfoxide (Fisher Scientific; analytical reagent grade)
- Ethanol absolute (Scharlau® ET0016 Scharlau® Chemie SPAIN analytical reagent grade)
- GenePure LE Agarose 500 g (ISC BioExpress® , USA)
- Lambda DNA/*Hin*d III Markers (Promega)
- Methanol (Labscan Asia Co., Ltd. Thailand analytical reagent grade)
- Sodium bicarbonate (Analar® bDH; VWR International Ltd.)
- Sodium chloride (UNIVAR® Ajax Finechem; analytical reagent grade)
- Sterile water for irrigation
- Tris(hydroxymethyl)aminomethane (Pacific Science, Thailand molecular biology grade)
- Trypan blue stain 0.4% (GIBCO™)
- Yeast extract powder (HIMEDIA® Himedia Laboratories Pvt. Ltd.)

2. Equipment

- 1.5 mL, 2 mL Eppendrof® tubes
- 15 mL, 50 mL centrifuge tubes-sterile (Biologic research company)
- 24-well tissue culture test plates (TPP®; Switzerland)
- 25 cm^2 and 75 cm^2 cell culture flask (Corning®; Corning Incorporated)
- 96-well cell culture cluster (Costar®; Corning Invorporated)
- Analytical balance (Satorius CP224S, Sartorius CP3202S; Scientific promotion Co., Ltd.)
- Automatic autoclave (Model : LS-2D; Scientific promotion CO., Ltd.)
- Bacterial incubator (Contherm; Lab Focus CO., Ltd)
- Cellulose acetate filter 0.2 μm (Sartorius AG. 37070 Goettingen, Germany)

- Centrifuge (Hermle Z300K; Labnet®; Lab Focus CO., Ltd.)
- CO_2 incubator (HERA Cell 240 Heraeus)
- DyNA Vap centrifuge evaporator (Labnet®; Lab Focus CO., Ltd.)
- Fluorescence microscope (Model : GFP-B, wavelengths : excitation filter 480/40 and emission filter 535/50)
- Fusion universal microplate analyzer (Model No : AOPUS01 and A153601 ; A Packard bioscience company)
- GeneRay UV-Photometer (Biometra® λ260/280 nm)
- Inverted microscope (Eclipse TE 2000-U; Model : T-DH Nikon® Japan)
- Laminar air flow (BIO-II-A)
- Magnetic stirrer and magnetic bar
- Measuring pipettes (1, 2, 5, 10 mL)
- Micropipette 0.1-2 μL, 2-20 μL, 10-100 μL, 20-100 μL, 100-1000 μL (Masterpette®; Bio-Active Co., Ltd.)
- Micropipette tip
- NIPRO Hypodermic needle 25G× 1″ (0.5×25 mm) thin wall
- pH meter (HORIBA compact pH meter B-212)
- Pipette aid (Powerpette Plus; Bio-Active Co., Ltd.)
- Protein and nucleic acid electrophoresis (MyRUN intelligent electrophoresis unit; Cosmobio CO., Ltd.)
- Sartorius® filter set (Sartorius BORO 3.3 Goettingen, Germany)
- Shaking incubator (GFL 3031)
- TERUMO® Syringe 50 mL
- Transmission electron microscope
- Water bath (Hetofrig CB60; Heto High Technology)
- Zetasizer Nano ZS (Malvern instruments Ltd., Malvern, UK)

3. Methods

3.1 Preparation of liposomes

3.1.1 Preparation of anionic liposomes coated by cationic polymer

EPC:NaO, EPC:CHAPS, or EPC:NaT (10:1, 10:1.5 or 10:2 molar ratio) anionic liposomes were prepared by sonication method. Briefly, EPC, CHAPS and NaT were separately dissolved in chloroform : methanol (2:1 v/v) mixture. NaO was dissolved in methanol. The materials were deposited in a test tube and the solvents were evaporated with nitrogen gas. The lipid film was placed in a desiccator connected to a vacuum pump at least 6 hours to remove remaining organic solvent. The dried lipid film was hydrated with Tris buffer (20 mM Tris and 150 mM NaCl, pH 7.1). Following hydration, the dispersion was sonicated in bath sonication for 10 minutes and then in probe sonicator for each of 30 minutes, 2 cycles. Titanium fragments and multilamellar aggregate were removed by centrifugation at 15,000 rpm for 10 minutes at 4 °C. The top phase was separated from the bottom phase. For cationic polymer-coated liposomes, the suspension of the top phase was pipetted and mixed with cationic polymer solution (1 mg/mL) at the ratios of anionic liposomes : cationic polymer = 1:1 (w/w) with a magnetic stirrer for 30 minutes.

3.1.2 Preparation of cationic liposomes

Lipid compositions of liposomes were EPC:Chol:SA, EPC:Chol:DTAB or EPC:Chol:CPC (10:2:1, 10:2:2, 10:2:3 and 10:2:4 molar ratio). Cationic liposomes were prepared by sonication method. Briefly, EPC, Chol, SA, DTAB and CPC were separately dissolved in chloroform : methanol (2:1 v/v). The materials were deposited in a test tube and the solvents were evaporated with nitrogen gas. The lipid film was placed in a desiccator connected to a vacuum pump at least 6 hours to remove remaining organic solvent. The dried lipid film was hydrated with Tris buffer (20 mM Tris and 150 mM NaCl, pH 7.1). Following hydration, the dispersion was sonicated in bath sonication for 10 minutes and then in probe sonicator for each of 30 minutes, 2 cycles. Titanium fragments and multilamellar aggregate were removed by centrifugation at 15,000 rpm for 10 minutes at 4°C.

3.2 Purification and quantification of pDNA

The pDNA encoding green fluorescence protein (pEGFP-C2) was used as gene in gene expression study. To amplify the pDNA, pDNA was transformed into *Escherichia coli* DH5-α (*E. Coli*). After amplification of the *E. Coli*, the plasmids were isolated by using QIAGEN® Plasmid Midi Kits.

3.2.1 Purification of pDNA

The pDNA were isolated by using QIAGEN® Plasmid Midi Kits according to the manufacturer's directions. The process of purification of pDNA is shown as follows :

1. Pick a single colony from a freshly streaked selective plate and inoculated a starter culture of 9 mL LB medium containing 10 mg/mL kanamycin 45 μL, Incubate for approximately 8 hours at 37°C with vigorous shaking (approximately 250 rpm).
2. Dilute the starter culture into selective LB medium. Pipette 3 mL of a starter culture in 100 mL of LB medium containing 10 mg/mL kanamycin 500 μL. Growth at 37°C for 12-16 hours with vigorous shaking (approximately 200 rpm).
3. Harvest the bacterial cells by centrifugation at 4000 rpm for 5 minutes at 4 °C.
4. Resuspend the bacterial pellet in 4 mL Buffer P1.
5. Add 4 mL Buffer P2, mix throughly by vigorously inverting the sealed tube 4-6 times, and incubate at room temperature (15-25 °C) for 5 minutes.
6. Add 4 mL of chilled Buffer P3, mix immediately and throughly by vigorously inverting 4-6 times, and incubate on ice for 15 minutes.
7. Centrifuge at 15,000 rpm for 40 minutes at 4°C. Remove supernatant containing plasmid DNA promptly.
8. Centrifuge the supernatant again at 15,000 rpm for 40 minutes at 4 °C. Remove supernatant containing plasmid DNA promptly.
9. Equilibrate a QIAGEN-tip 100 by applying 4 mL Buffer QBT, and allow the column to empty by gravity flow.

10. Apply the supernatant from step 8 by to the QIAGEN-tip and allow it to enter the resin by gravity flow.

11. Wash the QIAGEN-tip with 2 × 10 mL Buffer QC.

12. Elute DNA with 5 mL Buffer QF.

13. Precipitate DNA by adding 3.5 mL (0.7 volumes) room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at 20,000 rpm for 30 minutes at 4 °C. Carefully decant the supernatant.

14. Wash DNA pellet with 2 mL of room-temperature 70% ethanol, and centrifuge at 15,000 rpm for 10 minutes at 4 °C. Carefully decant the supernatant without disturbing the pellet.

15. Air-dry the pellet for 5-10 minutes, and redissolve the DNA in a suitable volume of TE buffer, pH 8.0.

3.2.2 Quantification of pDNA concentration

The concentration of pDNA was determined by GeneRay UV Photometer (Biometra® λ260/280 nm). The process of quantification of pDNA is shown as follows : 5 μL of the pure plasmid was diluted into 995 μL of distilled water and gently mixed. Then, the diluted plasmid was measured at λ_{260} nm and λ_{280} nm with GeneRay UV Photometer (Biometra® λ260/280 nm). The concentration of pDNA was calculated using equation :

$$\text{Plasmid concentration} = 50\ \mu g/mL \times OD_{260\ nm} \times DF$$

- A solution of 50 μg/mL of an average double-stranded DNA has an $OD_{260\ nm}$ of 1.
- $OD_{260\ nm}$ is the optical density from the absorbance reading.
- DF is the dilution factor (in the above it would be 200).

DNA maximally absorbs ultraviolet light at a wavelength of about 260 nm. It is the bases that are principally responsible for this absorption, while absorption at 280 nm indicates protein contamination. A ratio of 1.8 A_{260}/A_{280} is given as means of determining purify (Sullivan et al., quoted in Torchilin and Weissig 2003 : 299).

3.3 Preparation of sprayed dried chitosan lactate

Chitosan (CS) with MW of 20, 45, 200, and 460 kDa and 85% degree of deacetylation was purchased from Seafresh Chitosan (lab) Co., Ltd in Thailand. CSL was prepared as previously described (Nunthanid et al. 2004 : 15-26). CS of different MWs was dissolved in distilled water containing lactic acid in a 1:1.3 molar ratio. The solution was adjusted with distilled water to make a 1% w/w solution and stirred for 12 hours. This solution was spray-dried under the following conditions : the inlet temperature was maintained at 125 ± 2°C by using a spray dryer (Minispray Dryer, Büchi 190, Postfach, Switzerland). The obtained powder was collected and stored in a desiccator containing dry silica gel prior to use in each experiment.

3.4 Synthesis of Methylated *N*-(4-pyridylmethyl) chitosan (TM_{69}-Py_{62} –CS)

3.4.1 Synthesis of Py-CS

The synthesis protocol for *N*-pyridinylmethyl chitosans (Py-CS) is shown in Figure 8 (Opanasopit et al. 2008 : 128). In brief, chitosan was deacetylated to obtain 94% degree of deacetylation (DD; determined by ^{1}H-NMR). 1.00 g of chitosan was dissolved in 70 mL of 1% acetic acid solution. The solution was diluted with 70 mL ethanol, and then 0.58-1.16 mL of 4-pyridinecarboxaldehyde was added to the solution. The reaction mixture was stirred at room temperature for 24 hours. At this point the pH of the solution was adjusted to 5 by adding 15% NaOH. Then, 1.54 g of $NaCNBH_3$ was added, and the resulting solution was allowed to stir at room temperature for 24 hours, followed by adjusting the pH to 7 with 15% NaOH. The aqueous solution was dialyzed against de-ionized (DI) water using dialysis tubing with MW cut-off of 12,000-14,000 g/mole (Aldrich, Germany) for 3 days, followed by freeze drying.

3.4.2 TM_{69}-Py_{62} –CS

1.00 g of *N*-(4-pyridinylmethyl) chitosans (Py-CS) was dispersed in 50 mL of 1-methyl-2-pyrrolidone (NMP) at room temperature and the mixture was stirred for 12 hours. Then 8 mL of 15% aqueous NaOH was dropped slowly in the solution (For high degree of quaternization of CS, 20% aqueous NaOH was used instead of 15% aqueous NaOH). Sodium iodide (3.0 g) was added and the mixture was stirred at 60°C for 15 minutes. Subsequently, 3 mL, 3 mL and 2 mL of iodomethane was added three times, respectively every four hours and the mixture was stirred at 60°C for 12 hours. After methylation, methylated chitosan were precipitated in 300 mL of acetone. The precipitate was dissolved in 15% NaCl solution in order to replace the iodide counter-ion with a chloride counter-ion. The suspension was dialyzed with deionized water for 3 days to remove inorganic materials and then freeze-dried. The synthetic pathway of TM_{69}-Py_{62}–CS is shown in Figure 8 and the percent quaternization of TM_{69}-Py_{62} –CS used in this study is show in Table 6.

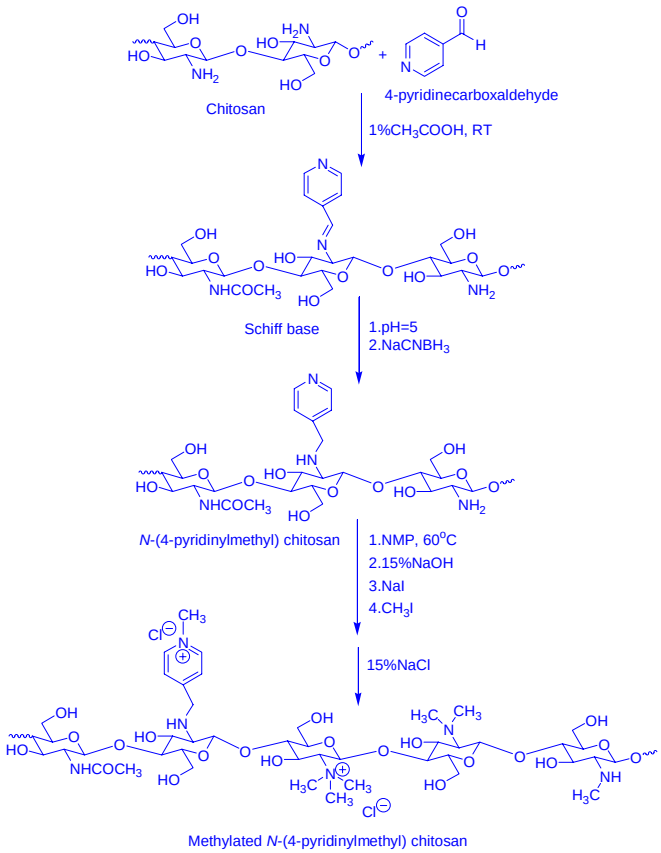

Figure 8 The synthesis of TM_{69}-Py_{62}–CS.

Table 6 The percent quaternization of TM_{69}-Py_{62}–CS used in this study.

Samples	ES(%)	DQ_{Py} (%)	DQ_{CS} (%)	DQ_{Total} (%)	$N(CH_3)_2$ (%)	$NHCH_3$ (%)	Recovery (%)
TM_{69}-Py_{62}–CS	62	62	7	69	10	13	70

ES is the extent of *N*-substitution; DQ_{Py} is degree of quaternization at N atom of pyridine ring; DQ_{CS} is degree of quaternization at GlcN of chitosan; $NHCH_3$ is *N*-methylation at GlcN of chitosan; Recovery (%) is dried weight of methylated product (g)/dired weight of chitosan (g) or dried weight of *N*-(4-pyridinylmethyl) chitosan (g) × 100.

3.5 Cell culture, routine maintenance

The human hepatoma cell lines (Huh7 cells) were chosen as model in this transfection efficiency and cytotoxicity study.

3.5.1 The cultivation of Huh 7 cells

Huh7 cells were maintained in complete growth medium (RPMI Medium 1640) in a humidified air atmosphere (5% CO_2, 95% RH, 37 $^{\circ}$C). Cultivated cells were visualized using an inverted microscope to detect cross-contamination or visible microbial contamination. The cells were passage every 3-5 days as spit ratio 1:5 to 1:10.

3.5.2 Preparation of media

Complete culture medium is prepared as follows; fetal bovine serum (FBS) was heat inactivated by incubating for 30 minutes in a 56 $^{\circ}$C water bath. RPMI Medium 1640 powder was dissolved in 5% less sterile water than desired total volume of medium with gentle stirring, and 2.0 g of $NaHCO_3$ per L of medium was added for adjust pH to 0.2-0.3 below desired final working pH (pH 7.4). The solution was sterilized immediately by membrane filtration. Then the solution was supplemented with : 1% v/v Non-essential Amino Acids, 10% v/v heat inactivated FBS.

3.5.3 Thawing frozen cell

The cells were thawed quickly in 37 $^{\circ}$C water bath and one vial of cells was diluted into 10 mL of complete growth medium, gently mixed. It was centrifuged at 750 rpm 5 minutes 25 $^{\circ}$C for removing DMSO containing cell culture freezing medium that can be toxic to cell. The medium above the pellet was removed and the cells in complete growth medium were resuspended. The cell suspension was diluted to appropriate concentration, and transferred into a tissue culture (TC) flask.

3.5.4 Subculture

Cultivated Huh7 cells were visualized using an inverted microscope to 70-80% confluency in the TC flasks. The media was aspirated and rinsed one time with PBS. 0.25% trypsin/EDTA solution was added, the cells were incubated at 37 $^{\circ}$C for approximately 1 minute or until the cells detached and floated. This can be confirmed by periodic visual inspection of the flasks or observing cells under an inverted microscope. Trypsin/EDTA was inactivated by adding excess serum-containing medium. Cell suspension was removed to a conical tube and cell was pelleted by centrifugation at 750 rpm for 5 minutes. Complete culture medium was added to detached cells. The cells were resuspended by using pipette up and down. The aliquots of the cell suspension were added to new TC flasks. Subcultivation ratio was 1:5. The complete culture medium was added for a total of 15 mL per 75 cm^2 TC flask. The cell culture was then placed in incubator.

3.5.5 Freezing cell

Cells to be frozen should be in late log phase growth. After the subculturing cell culture, pellet of cells was diluted in complete growth medium. 1 mL of the cell suspension transferred into cryoprotective tube and 50 μL DMSO was added for cryoprotectant. Cells were stored at -20 °C, -80 °C overnight, respectively. For long term storage, the cells were stored in liquid nitrogen.

3.6 Preparation of polymer-coated liposomes/DNA complexes

Complexes of cationic liposomes and polymer-coated liposomes/DNA were formulated at weight ratio of 0.05, 0.1, 0.5, 1, 5 and 10 by adding 1 μg of DNA solution to the polymer-coated liposomes solution and diluting with distilled water. The mixture was gently pipetted and vortexed for 3-5 seconds to initiate the complex formation and left at room temperature for 30 minutes to complete the process.

3.7 Characterization of polymer-coated liposomes/DNA complexes

3.7.1 Gel electrophoresis

Complex formation was confirmed by electrophoresis. Agarose gels were prepared with 0.8% agarose solution in Tris-borate-EDTA buffer with ethidium bromide (0.5μg/mL). The electrophoresis was carried out for 45 minutes at 100 V. The volume of the sample loaded in the well was 12 μL of cationic liposomes and polymer-coated liposomes/DNA complex containing 1 μg of DNA.

3.7.2 Particle size and zeta-potential of the polymer-coated liposomes/DNA complexes

The particle size and surface charge of polymer-coated liposomes/DNA complexes were measured by using the Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) at room temperature. The complexes were diluted with distilled water that was passed through 0.22 μm membrane filter prior to use. All samples were measured in triplicate.

3.7.3 Morphology

The morphology of polymer-coated liposomes/DNA complexes were observed using transmission electron microscope (TEM-JEOL-1230 80 KV). Polymer-coated liposomes/DNA complexes was diluted with Tris buffer pH 7.4 and was then sonicated with sonicator for 10 minutes. One drop of polymer-coated liposomes/DNA complexes was placed on a copper grid. The grid was allowed to dry for 15 minutes further and was then examined under the electron microscope.

3.8 *In vitro* transfection on Huh7 cells

Huh7 cells were seeded for 24 hours into 24-well plates at a density of 2×10^4 cells/cm^2 in 1 mL of growth medium (RPMI 1640 containing 10% FBS, supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, 1% nonessential amino acid solution). Prior to transfection, the medium was removed and the cells were rinsed with phosphate-buffered saline (PBS, pH 7.4), and then supplied with 500 μL of fresh culture medium without FBS. Five hundred microlitres of the polymer-coated liposomes or cationic polymer/DNA complexes containing 1 μg of pEGFP-C2 plasmid DNA were incubated with the cells for 24 hours at 37 °C under 5% CO_2 atmosphere. Non-treated cells and cells transfected with naked plasmid used as controls were incubated at the same time. After 24 hours, the transfection media were removed and the cells were further cultivated with 1 mL of culture medium for 3 days at 37 °C under 5% CO_2 atmosphere. All transfection experiments were performed in triplicate. For fluorescence assay of transfection, the cells were directly viewed under a fluorescence microscope (Model : GFP-B; wavelengths : excitation filter 480/40 and emission filter 535/50). The transfection efficiency was calculated by transfected-cells (green color) per area of a 24-well tissue culture plate (1.9 cm^2).

3.9 Evaluation of cytotoxicity of cationic polymer and polymer-coated liposomes/DNA complexes

Evaluation of polymer-coated liposomes or cationic liposomes/DNA complexes' cytotoxicity was performed by MTT assay (Smith et al. 1992 : 191-194). Huh7 cells were seeded at a cell density of 8,000 cells/well in 96-well plates with 100 μL of medium and incubated for 24 hours prior to experiment. Cationic polymer and polymer-coated liposomes were applied on to the cell in culture medium without serum at weight ranging from 0.001-1000 μg (100 μL/well) and cell viability was compared to cells treated with culture medium without serum only. In case of the cationic polymer or polymer-coated liposomes/DNA complexes, 100 μL of complex containing media at the same weight ratio as the transfection efficiency (equivalent to 0.2 μg of pEGFP-C2) was added and incubated with the cells for 24 hours at 37 °C under 5% CO_2 atmosphere. After 24 hours, 100 μL of culture with serum was added to each well. After 3 hours, 100 μL of MTT (5 mg/mL) was added to each well and the incubation was continued for 4 hours. Then, the medium was removed, and formazan crystals formed in the living cells were dissolved in 100 μL DMSO per well. Relative viability (%) was calculated based on absorbance at 550 nm using a microplate reader (A Packard BioScience Company). The percentage of cell viability compared to control cells containing cell culture medium without liposomes was calculated using following eqution :

$$\% \text{ cell viability} = \frac{[OD]_{test} - [OD]_{DMSO}}{[OD]_{control} - [OD]_{DMSO}} \times 100$$

3.10 Statistical analysis

All experiment measurments were performed in triplicate. The data were expressed as mean ± standard deviation (SD). Statistical significance of difference in transfection efficiency and cell viability was examined using one-way analysis of variance (ANOVA) followed by LSD post hoc test.

CHAPTER IV

RESULTS AND DISCUSSION

1. Results

1.1 Complex formation

In order to investigate the optimal conditions for the complex formation, it was necessary to evaluate the condensation capability of cationic polymer-coated anionic liposomes and cationic liposomes with DNA. The formation of complexes between polymer-coated anionic liposomes, cationic liposomes and pEGFP-C2 plasmid DNA was visualized by agarose gel electorphoresis.

1.1.1 Cationic polymer-coated anionic liposomes

Small unilamellar anionic liposomes were prepared using ultrasonic generator, and then coated with cationic polymer solution i.e. CSL with the MW of 20, 45, 200 and 460 kDa, TM_{69}-Py_{62}-CS and PEI. After the preparation, the liposomes formed complexes with plasmid DNA encoding green fluorescent protein (pEGFP-C2) at various weight ratios. The complexes were characterized by agarose gel electrophoresis. Figures 9-11 illustrate gel electrophoresis results of CSL coated anionic liposomes (EPC:NaO = 10:2)/DNA complexes (Figure 9), TM_{69}-Py_{62}-CS coated anionic liposomes (EPC:NaO = 10:2)/DNA complexes (Figure 10) and PEI coated anionic liposomes (EPC:NaO, EPC:CHAPS and EPC:NaT)/DNA complexes (Figure 11). The complete complexes were formed, evidenced by the retention of DNA within the wells. The weight ratio at which complete complexes were formed of each formulation is shown in Table 7. Figure 9 (A-D) shows that the naked DNA (Lane 1) and anionic liposomes (EPC:NaO=10:2) coated with CSL with molecular weight of 20, 45, 200 and 460 kDa (EPC:NaO = 10:2)/DNA complexes at weight ratios of 0.05, 0.1, 0.5, 1, 5 and 10 (Lane 2-7). Compared with the naked DNA, all formulations showed DNA-polymer complex since the migration of DNA was retarded. For CSL-coated liposomes/DNA complexes (all MW of CS), the migration of DNA was completely retarded when the weight ratio was above 0.05. For TM_{69}-Py_{62}-CS-coated anionic liposomes (EPC:NaO = 10:2), the migration of DNA was completely retarded when the weight ratio of TM_{69}-Py_{62}-CS coated liposomes (EPC:NaO = 10:2)/DNA was above 0.1 (Figure 10). In PEI-coated liposomes, the migration of DNA was completely retarded when the weight ratio of PEI-coated liposomes/DNA was above 0.1 (Figure 11).

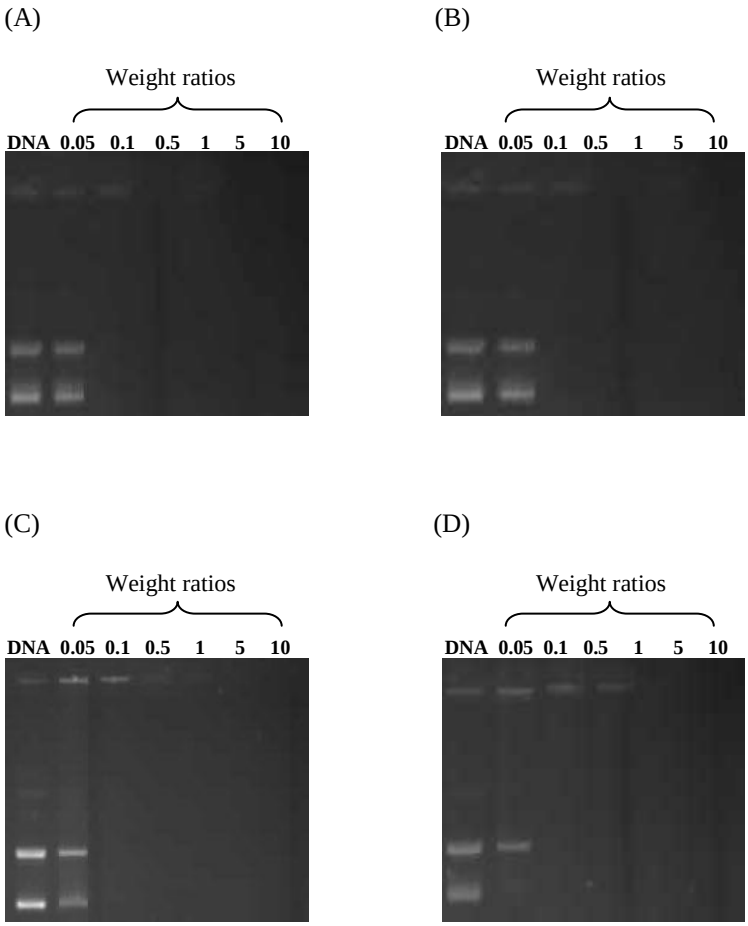

Figure 9 Gel retarding analysis of CSL-coated liposomes/DNA complexes on an agarose gel; (A) CSL20-coated liposomes (EPC:NaO = 10:2)/DNA complexes, (B) CSL45-coated liposomes (EPC:NaO = 10:2)/DNA complexes, (C) CSL200-coated liposomes (EPC:NaO = 10:2)/DNA complexes and (D) CSL460-coated liposomes (EPC:NaO = 10:2)/DNA complexes.

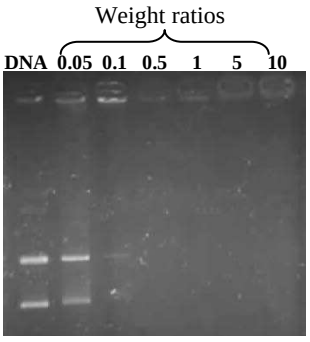

Figure 10 Gel retarding analysis of TM_{69}-Py_{62}-CS-coated liposomes (EPC:NaO = 10:2)/DNA complexes on an agarose gel.

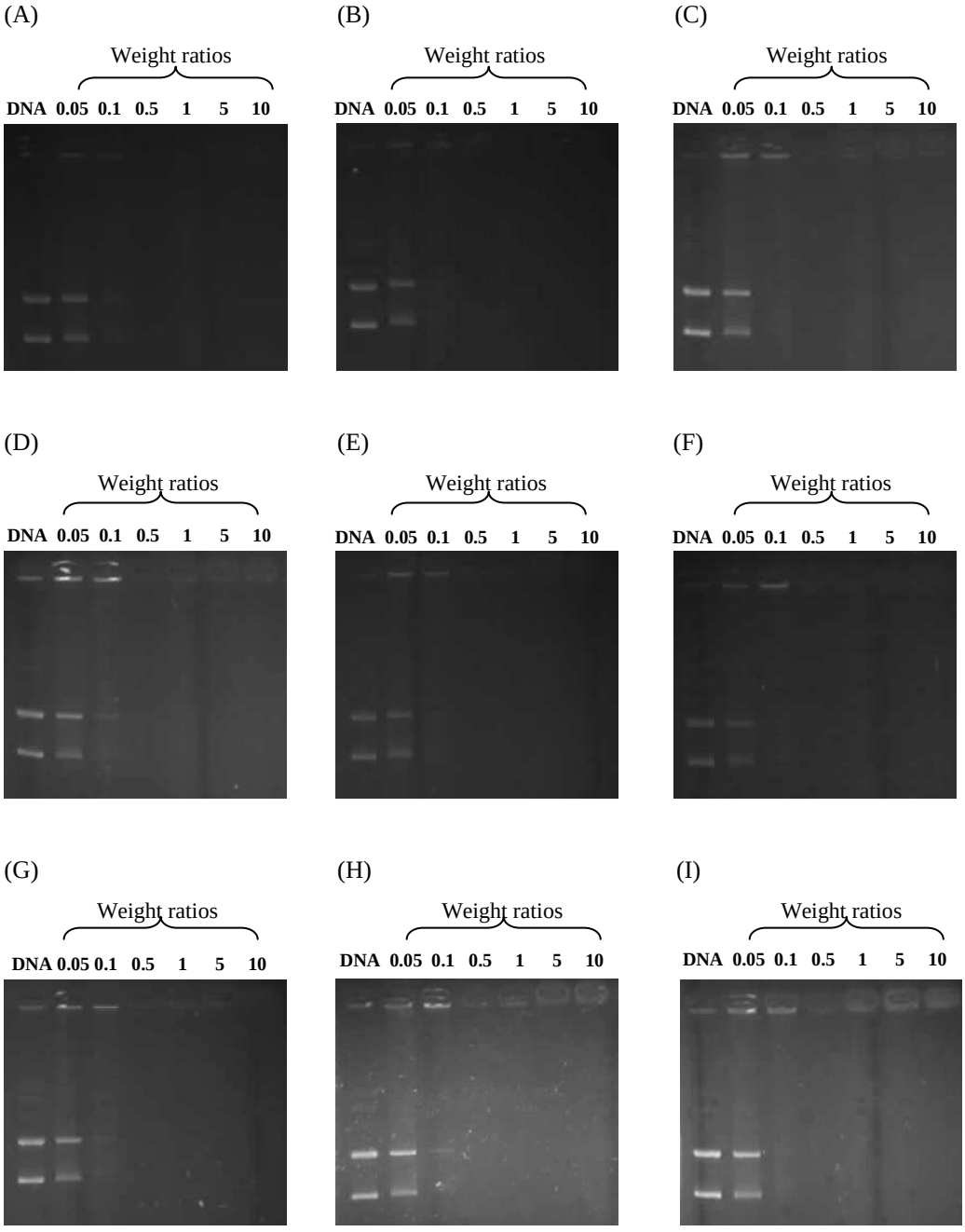

Figure 11 Gel retarding analysis of PEI-coated liposomes/DNA complexes on an agarose gel; (A) PEI-coated liposomes (EPC:NaO = 10:1), (B) PEI-coated liposomes (EPC:NaO = 10:1.5), (C) PEI-coated liposomes (EPC:NaO = 10:2), (D) PEI-coated liposomes (EPC:CHAPS = 10:1), (E) PEI-coated liposomes (EPC:CHAPS = 10:1.5), (F) PEI-coated liposomes (EPC:CHAPS = 10:2), (G) PEI-coated liposomes (EPC:NaT = 10:1), (H) PEI-coated liposomes (EPC:NaT = 10:1.5) and (I) PEI-coated liposomes (EPC:NaT = 10:2).

1.1.2 Cationic liposomes

The cationic liposomes used in this study were composed of EPC, cholesterol (Chol) and SA at the molar ratio of 10:2:1, 10:2:2, 10:2:3 and 10:2:4. The liposomes formed complexes with pEGFP-C2 at various weight ratios. The complexes were analyzed by agarose gel electrophoresis. Figures 12 illustrated gel electrophoresis of cationic liposomes (EPC:Chol: SA)/DNA complexes. The results showed that the retardation of migration of DNA on an agarose gel was higher when the amount of SA added to liposomes increased. The weight ratio at which complete complexes were formed of each formulation are shown in Table 7.

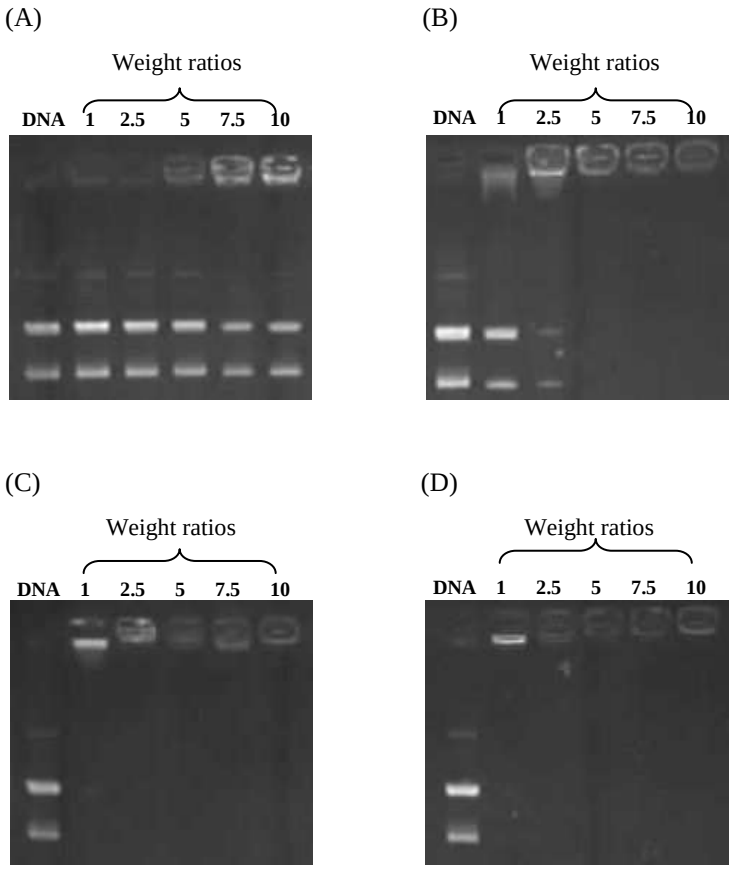

Figure 12 Gel retarding analysis of cationic liposomes/DNA complexes on an agarose gel; (A) EPC:Chol:SA = 10:2:1, (B) EPC:Chol:SA = 10:2:2, (C) EPC:Chol:SA = 10:2:3 and (D) EPC:Chol:SA = 10:2:4.

Table 7 Weight ratio of liposomes : DNA, at which the complete complexes were formed in various liposomes formulations.

Formulation	Weight ratio
CSL20-coated liposomes (EPC:NaO = 10:2)	0.1
CSL45-coated liposomes (EPC:NaO = 10:2)	0.1
CSL200-coated liposomes (EPC:NaO = 10:2)	0.1
CSL460-coated liposomes (EPC:NaO = 10:2)	0.1
TM_{69}-Py_{62}-CS-coated liposomes (EPC:NaO = 10:2)	0.5
PEI-coated liposomes (EPC:NaO = 10:1)	0.5
PEI-coated liposomes (EPC:NaO = 10:1.5)	0.5
PEI-coated liposomes (EPC:NaO = 10:2)	0.5
PEI-coated liposomes (EPC:CHAPS = 10:1)	0.5
PEI-coated liposomes (EPC:CHAPS = 10:1.5)	0.5
PEI-coated liposomes (EPC:CHAPS = 10:2)	0.5
PEI-coated liposomes (EPC:NaT = 10:1)	0.5
PEI-coated liposomes (EPC:NaT = 10:1.5)	0.5
PEI-coated liposomes (EPC:NaT = 10:2)	0.5
Cationic liposomes (EPC:Chol:SA=10:2:1)	> 10
Cationic liposomes (EPC:Chol:SA=10:2:2)	5
Cationic liposomes (EPC:Chol:SA=10:2:3)	2.5
Cationic liposomes (EPC:Chol:SA=10:2:4)	2.5

1.2 The particle size and zeta-potential of the polymer-coated anionic liposomes and cationic liposomes/DNA complexes

Particle size and surface charge of the complex, are necessary to assure its uptake by cells. Particularly, the particle size of a complex is an important factor that influences the access and passage of the complex through the targeting site. The results of particle size and zeta-potential of the polymer-coated anionic liposomes and cationic polymer/DNA complexes at various weight ratios are shown in Figure 13.

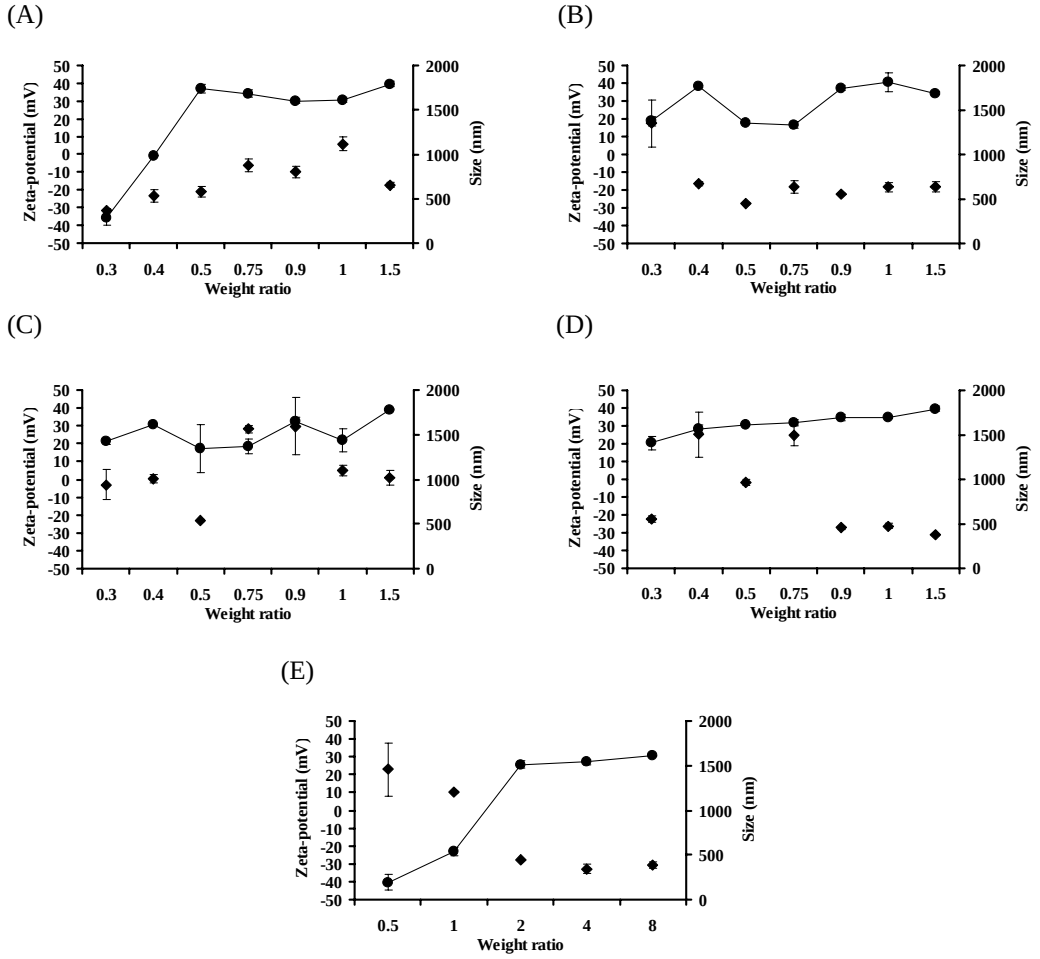

Figure 13 Zeta-potential (●) and particle size (◆) at various weight ratios of polymer-coated anionic liposomes/DNA complexes; (A) PEI-coated liposomes (EPC:NaO = 10:2)/DNA complexes, (B) PEI-coated liposomes (EPC:CHAPS = 10:2)/DNA complexes, (C) PEI-coated liposomes (EPC:NaT = 10:1.5)/DNA complexes, (D) PEI/DNA complexes and (E) TM_{69}-Py_{62}-CS-coated liposomes (EPC:NaO = 10:2)/DNA complexes.

The particle size of PEI-coated liposomes (EPC:NaO = 10:2)/DNA complexes increased with an increasing weight ratio from 0.3 to 1. Initially, negative values of zeta-potentials were observed at lower weight ratio (0.3 and 0.4). However, when the complete complexes were formed, the zeta-potential values became approximately zero. The zeta-potential of the complexes was found to be more positive with the higher weight ratios.

The particle size of PEI-coated liposomes (EPC:CHAPS = 10:2)/DNA complexes decreased with an increasing weight ratio from 0.3 to 0.5 and slightly increased to constant value of about 630 nm after a weight ratio of 0.5, (Figure 13 (B)). At the weight ratio around 0.3 to 0.5, the complete complexes were formed. The zeta-potential were positive and increased with an increasing in weight ratio from 0.75 to 1.

The particle size of PEI-coated liposomes (EPC:NaT = 10:1.5)/DNA complexes increased with an increasing in weight ratio from 0.75 to 0.9 and decreased to constant in the range of 1013 to 1100 after a weight ratio of 0.9 (Figure 13 (C)). At the weight ratio around 0.3 to 0.5, the complete complexes were formed, and the zeta-potential increased with an increasing in weight ratio from 0.5 to 0.9.

The particle size of PEI/DNA complexes increased with an increasing in weight ratio from 0.3 to 0.75 and decreased to constant in the range of 375 to 454 nm after a weight ratio of 0.75 (Figure 13 (D)). At the weight ratio around 0.3 to 0.5, the complete complexes were formed, and the zeta-potential increased with an increasing in weight ratio in the range of 20.3 to 39.7 mV.

The particle size of TM_{69}-Py_{62}-CS-coated liposomes (EPC:NaO = 10:2)/DNA complexes decreased with an increasing in weight ratio from 0.5 to 8 and decreased to constant in the range of 388 to 1458 nm (Figure 13 (E)). At the weight ratio lower than 2, the complex had a negative value of zeta-potential. However, at weight ratio higher than 2, where complete complexes were formed, the zeta-potential was positive and value was constant in the range of 25.5 to 30.4 mV.

1.3 Morphology

The formation of polymer-coated liposomes was also proven by the morphological examination of the polymer-coated liposomes/DNA complex under transmission electron microscope (TEM). The TEM images revealed a spherical in the structure of PEI-coated liposomes/DNA complexes (Figure 14).

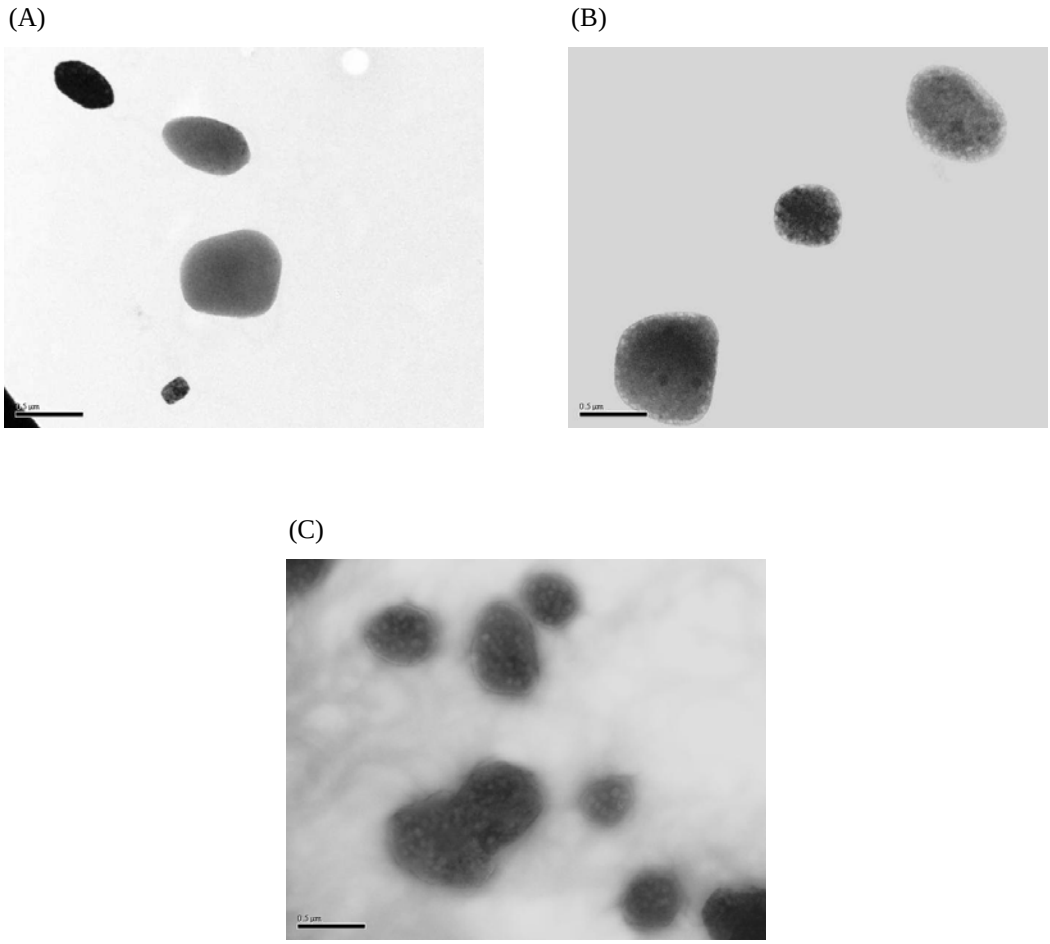

Figure 14 TEM image of polymer-coated liposomes/DNA complexes; (A) PEI-coated liposomes (EPC:CHAPS = 10:2)/DNA complexes (weight ratio of 0.75), (B) PEI-coated liposomes (EPC:NaT = 10:1.5)/DNA complexes (weight ratio of 0.75) and (C) PEI-coated liposomes (EPC:NaO = 10:2)/DNA complexes (weight ratio of 0.5).

1.4 *In vitro* transfection

To evaluate the polymer-coated anionic liposomes and cationic liposomes mediated gene transfection efficiencies, *in vitro* gene transfection assay was performed in Huh7 cells using pEGFP-C2. In this study, the factors affecting *in vitro* transfection efficiency i.e. type of cationic polymers, type of anionic surfactants and lipid surfactant ratio, weight ratio, pH and the presences of serum were investigated.

1.4.1 Cationic polymer coated anionic liposomes

At first, the optimum condition for gene transfection was investigated with different cationic polymers i.e. CSL with MW of 20, 45, 200 and 460 kDa, TM_{69}-Py_{62}-CS, PEI coated–anionic liposomes (EPC:NaO = 10:2) and cationic polymers. Cells transfected with naked DNA (1 μg DNA/well) were used as a negative control. Huh7 cells were seeded in 24-well plate and incubated for 24 hours, then, transfected with the complexes of cationic polymers-coated anionic liposomes or cationic

polymers for 24 hours. Transfection efficiency was evaluated by transfected-cell counting using images obtained by fluorescence microscope.

The fluorescence images are shown in Figure 15. In case of intact cells (Figure 15 (A)), and cells transfected with pDNA, no gene expression were observed. In contrast, gene expression was found in the transfection of PEI/DNA complexes (weight ratio of 0.75) (Figure 15 (C)), Lipofectamine 2000™/DNA complexes (weight ratio of 2) and PEI-coated liposomes (EPC:NaO = 10:2)/DNA complexes (weight ratio of 0.5) and PEI-coated liposomes (EPC:NaO = 10:2)/DNA complexes (weight ratio of 0.3). The PEI-coated liposomes (EPC:NaO = 10:2)/DNA complexes (weight ratio of 0.5) (Figure 15 (E)) gave higher gene expression than PEI-coated liposomes (EPC:NaO = 10:2)/DNA complexes (weight ratio of 0.3) (Figure 15 (F)).

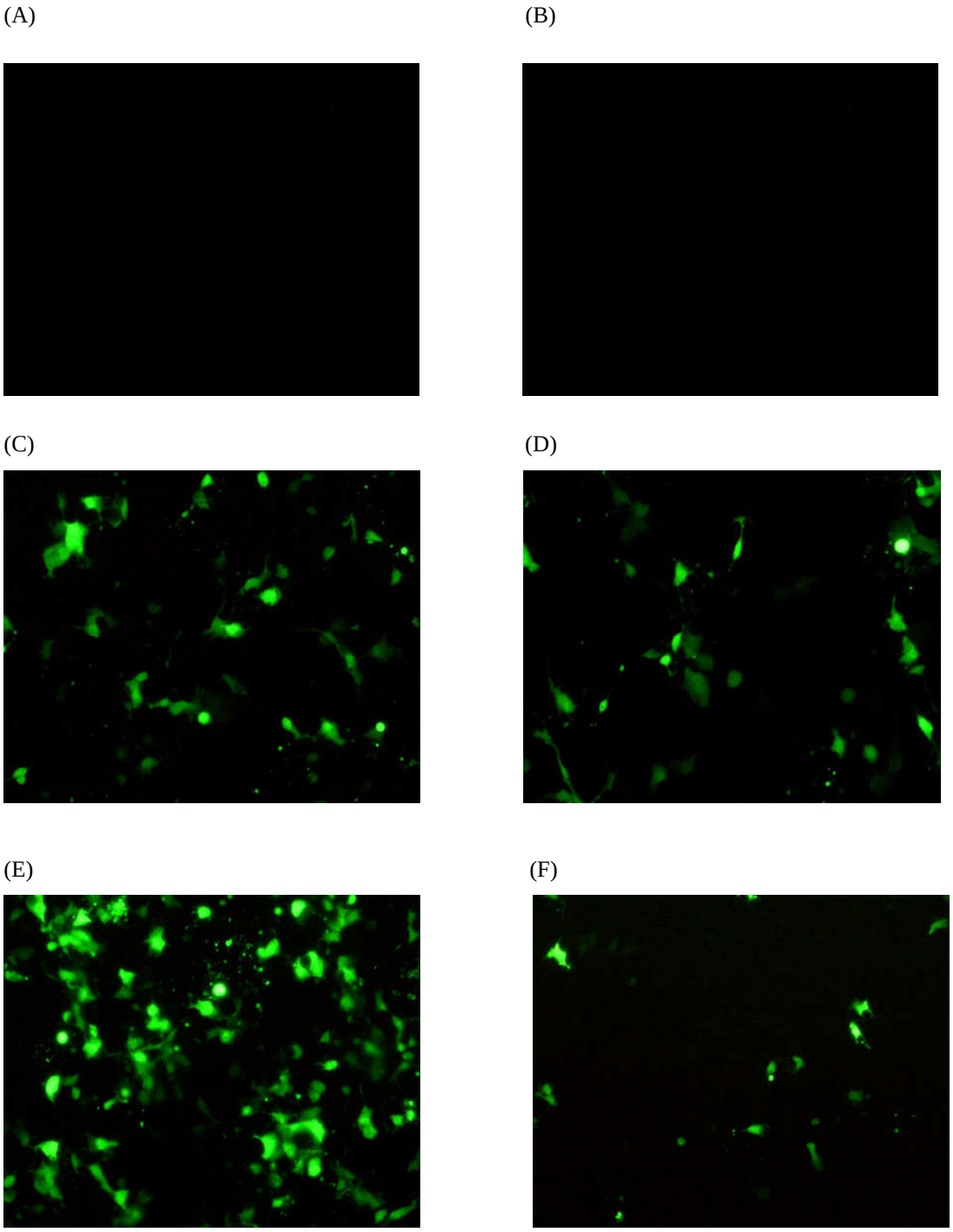

Figure 15 Fluorescence images observations for pEGFP-C2 expression in Huh7 cells culture with mediated by; (A) cell without complexes, (B) free DNA, (C) PEI/DNA complexes (weight ratio of 0.75), (D) Lipofectamine 2000™/DNA complexes (weight ratio of 2), (E) PEI-coated liposomes (EPC:NaO = 10:2)/DNA complexes (weight ratio of 0.5) and (F) PEI-coated liposomes (EPC:NaO = 10:2)/DNA complexes (weight ratio of 0.3).

1.4.1.1 Effect of polymers

The type of cationic polymers might affect the gene transfection efficiencies. Therefore, the gene transfection efficiency of each polymer-coated anioic liposomes (EPC:NaO = 10:2) were investigated.

In CSL-coated anionic liposomes, there was no tranfection in control (cell without complexes) and cells transfected with naked DNA. The transfection efficiency was studied at the transfection medium pH 6.2 because no transfection occured in medium pH 7.4. CSL/DNA complexes were formulated with chitosan of various MWs (20, 45, 200 and 460 kDa) in order to investigate the effect of MW on transfection efficiency. There was no significant difference in the tranfection efficiency between CSL and CSL-coated anionic liposomes in all MW of CSL. As shown in Figure 16, the transfection efficiencies of CSL/DNA complexes and CSL-coated anionic liposomes/DNA complexes increased as the weight ratio increased. The maximum transfection efficiency of all MW was found in the weight ratios of 1 and 2.

In TM_{69}-Py_{62}-CS-coated anionic liposomes (Figure 17), the maximum transfection efficiency was found at a weight ratio of 2 (260 ± 28 cell/cm^2). The transfection efficiency of TM_{69}-Py_{62}-CS-coated anionic liposomes was significantly lower than the TM_{69}-Py_{62}-CS /DNA complexes (744 ± 55 cell/cm^2).

In PEI-coated anionic liposomes (Figure 18), the maximum transfection efficiency was found at a weight ratio of 2 (4314 ± 336 cell/cm^2) and it was significantly higher than that of the PEI/DNA complexes (1278 ± 88 cell/cm^2).

It can be ranked the maximum transfection efficiency of different types of cationic polymer-coated anionic liposomes as PEI > TM_{69}-Py_{62}-CS > CSL.

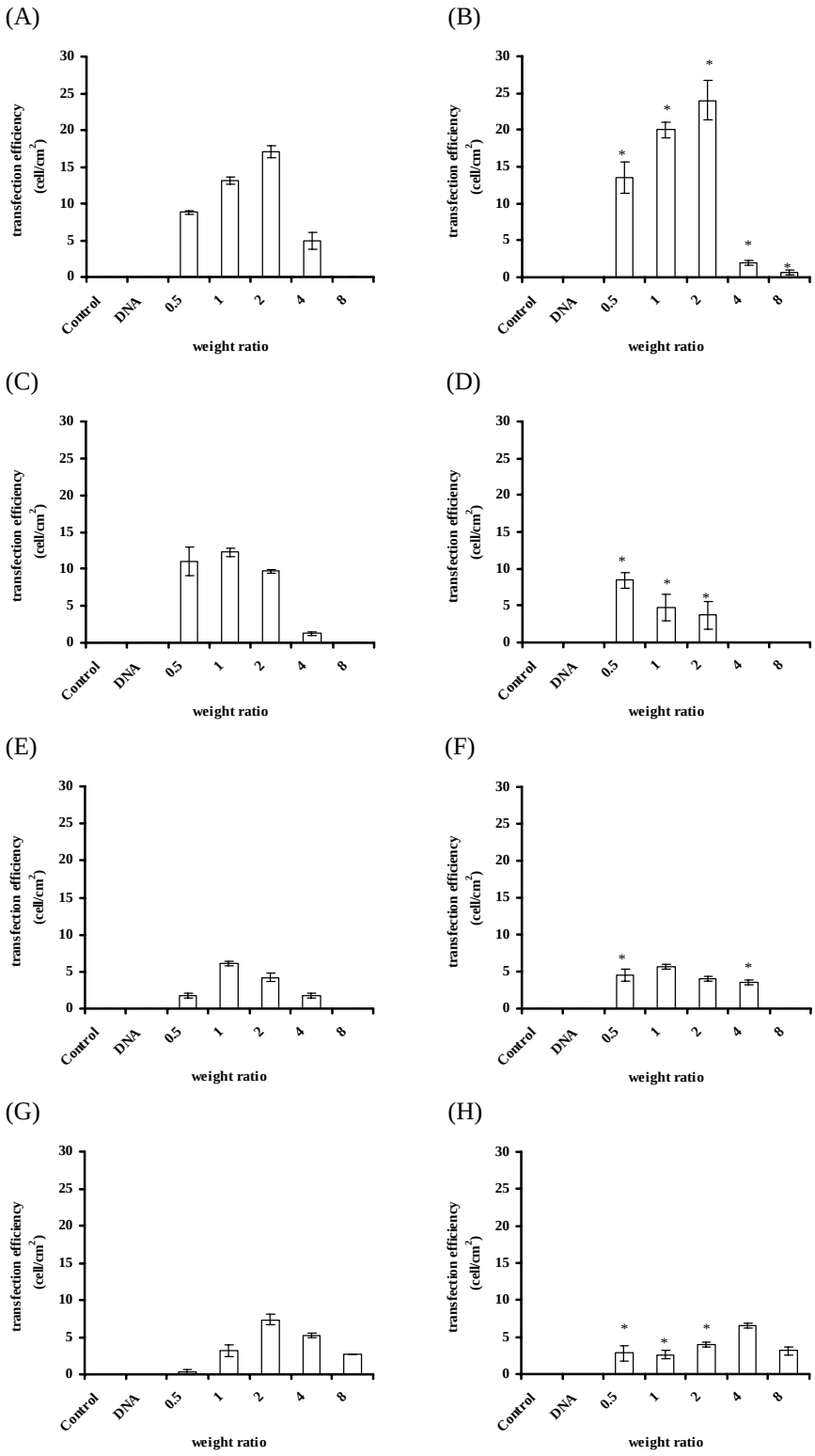

Figure 16 Effect of MW of CSL on transfection efficiency of CSL-coated anionic liposomes (EPC:NaO = 10:2) and CSL in transfection medium pH 6.2; (A) CSL20, (B) CSL20-coated anionic liposomes, (C) CSL45, (D) CSL45-coated anionic liposomes, (E) CSL200, (F) CSL200-coated anionic liposomes, (G) CSL460 and (H) CSL460-coated anionic liposomes. Values shown are the means $\pm$ SD of triplicated experiment (* indicate $p \leq 0.05$).

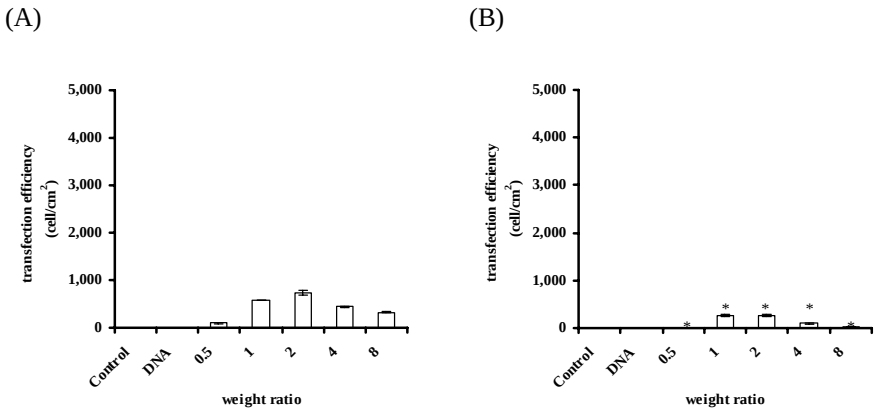

Figure 17 Effect of chitosan derivatives (TM_{69}-Py_{62}-CS) on transfection efficiency of TM_{69}-Py_{62}-CS-coated anionic liposomes (EPC:NaO = 10:2) in transfection medium pH 7.4; (A) TM_{69}-Py_{62}-CS and (B) TM_{69}-Py_{62}-CS-coated anionic liposomes. Values shown are the means $\pm$ SD of triplicated experiment (* indicate $p \leq 0.05$).

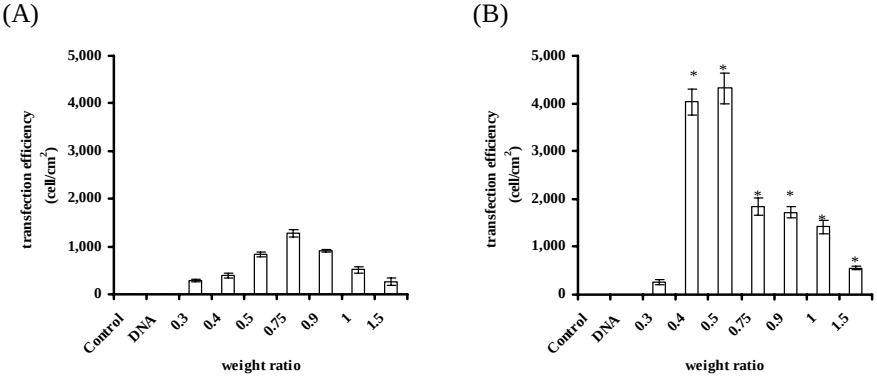

Figure 18 Effect of PEI on transfection efficiency of PEI-coated anionic liposomes (EPC:NaO = 10:2); (A) PEI and (B) PEI-coated anionic liposomes in transfection medium pH 7.4. Values shown are the means $\pm$ SD of triplicated experiment (* indicate $p \leq 0.05$).

1.4.1.2 Effect of anionic surfactants and ratio of lipid : surfactant

In this study, the optimal type of surfactants and lipid to surfactant ratio on the transfection efficiency were investigated. Anionic surfactants (NaO and NaT) and switterionic surfactants (CHAPS) were selected and lipid to surfactant ratios were varried (10:1, 10:1.5 and 10:2). PEI was used to coat on anionic liposomes. The PEI/DNA complexes (weight ratio of 0.75) and Lipofectamine 2000^{TM} (weight ratio of 2) were used as positive control.

Anionic liposomes were prepared by EPC:NaO, EPC:NaT and EPC:CHAPS at a molar ratio of 10:1, 10:1.5 and 10:2 and then coated with PEI. The results of transfection efficiency of EPC:NaO liposomes, EPC:NaT liposomes, and EPC:CHAPS liposomes are shown in Figure 19, 20 and 21, respectively. The gene transfection efficiencies (at pH 7.4) were significantly influenced by the weight ratios, the types of surfactants and lipid to surfactant ratios. The transfection efficiencies

increased to reach the maximum when the weight ratio increased, then decreased by further increment of the ratios. Among surfactants tested, NaO-liposomes showed the highest transfection efficiency (Figure 19). The highest transfection efficiency at weight ratio of 0.5, was 4314 ± 336 cell/cm^2, which was higher than that of the highest transfection efficiency of NaT-liposomes at weight ratio of 0.75 (1526 ± 157 cell/cm^2) and of CHAPS-liposomes at the weight ratio of 0.75 (1736 ± 107 cell/cm^2). When the EPC:surfactant ratio (10:1, 10:1.5 and 10:2) increased, the gene transfection efficiency increased. The highest transfection efficiency of NaO-liposomes and CHAPS-liposomes was obtained with the formulation with EPC:surfactant ratio of 10:2, whereas of NaT-liposomes was 10:1.5.

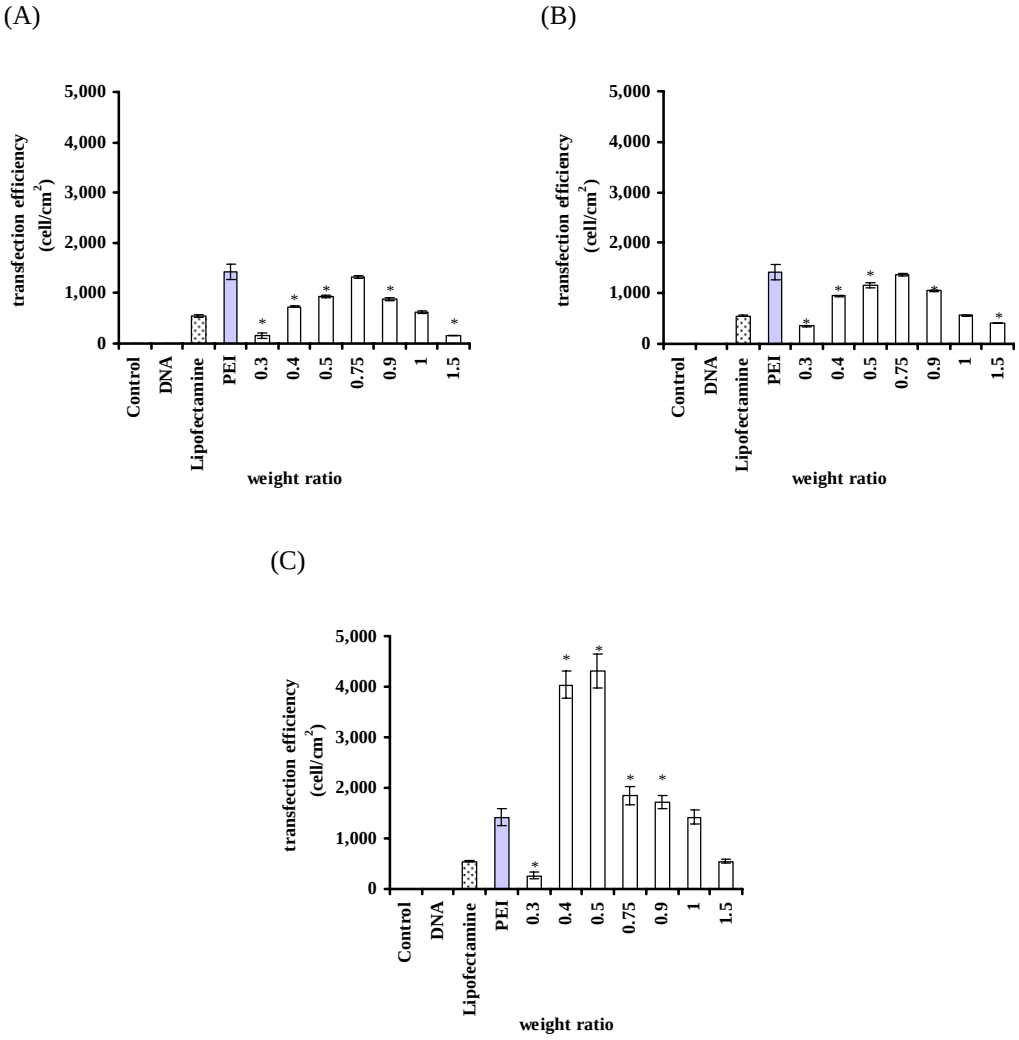

Figure 19 Effect of type of anionic surfactants and lipid surfactant ratio on transfection efficiency of cationic polymer-coated anionic liposomes (EPC:NaO) and; (A) EPC:NaO = 10:1, (B) EPC: NaO = 10:1.5 and (C) EPC:NaO = 10:2. Values shown are the means ± SD of triplicated experiment (* indicate $p \leq 0.05$).

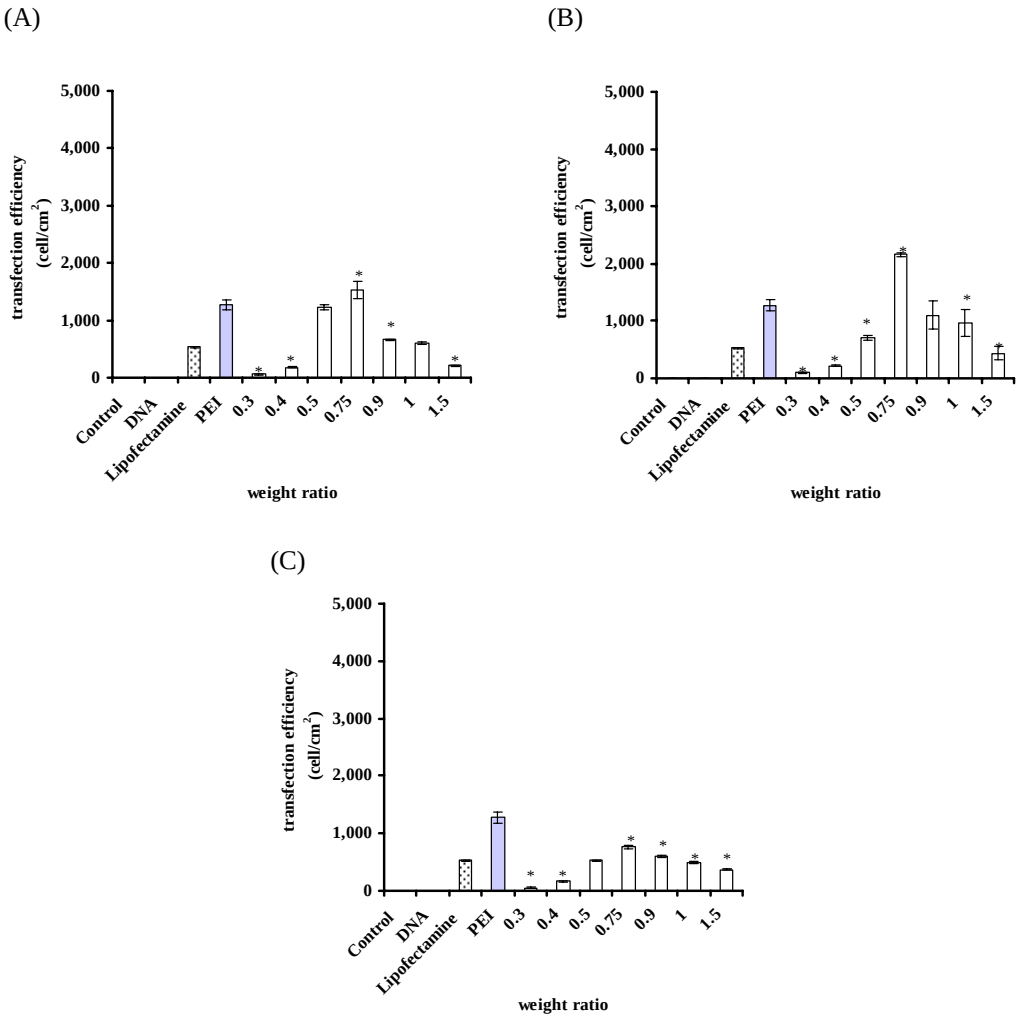

Figure 20 Effect of type of anionic surfactants and lipid surfactant ratio on transfection efficiency of cationic polymer-coated anionic liposomes (EPC:NaT) and; (A) EPC:NaT = 10:1, (B) EPC: NaT = 10:1.5 and (C) EPC:NaT = 10:2. Values shown are the means $\pm$ SD of triplicated experiment (* indicate $p \leq 0.05$).

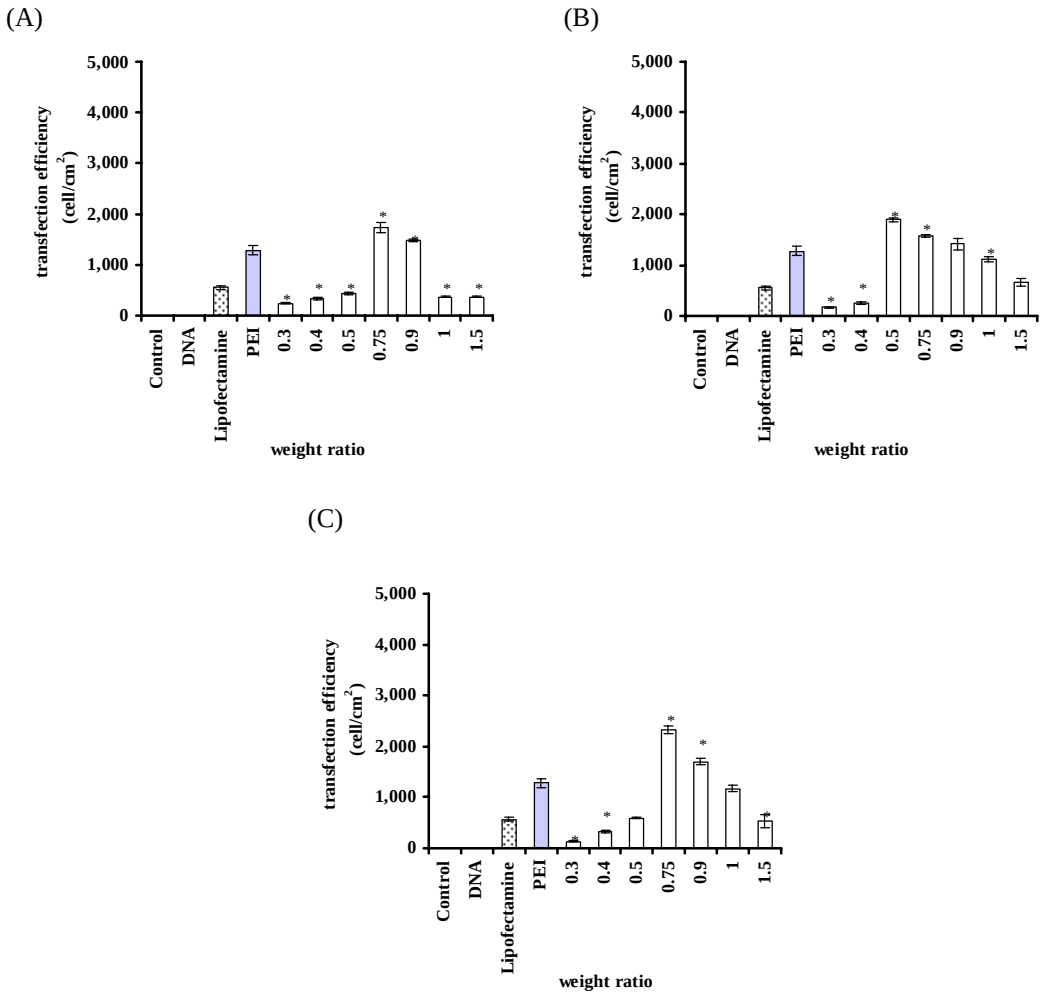

Figure 21 Effect of type of anionic surfactants and lipid surfactant ratio on transfection efficiency of cationic polymer-coated anionic liposomes (EPC:CHAPS) and; (A) EPC:CHAPS = 10:1, (B) EPC:CHAPS = 10:1.5 and (C) EPC:CHAPS = 10:2. Values shown are the means $\pm$ SD of triplicated experiment (* indicate $p \leq 0.05$).

1.4.1.3 Effect of weight ratio

The gene transfection efficiencies were significantly influenced by weight ratios. The pattern of gene transfection efficiency with weight ratio is bell-shape. The gene transfection efficiencies gradually increased as the weight ratio increased to a maximum value, and then decreased. However, the maximum transfection efficiency of each polymer-coated liposomes and cationic liposomes formulation were different at different weight ratios.

1.4.1.4 Effect of pH

PEI-coated liposomes were chosen for the study of the effect of culture medium pH because the gene expression was relatively high. In the study, PEI-coated liposomes prepared from EPC:NaO = 10:2, EPC:CHAPS = 10:2 and EPC:NaT = 10:1.5 complexed with DNA at weight ratio of 0.5, 0.75, 0.75,

respectively were compared. The transfection efficiency was investigated at pH 6.2 and 7.4, and the results are shown in Figure 22. The results showed that transfection efficiency of PEI, Lipofectamine, PEI-coated liposomes complexes (EPC:NaO = 10:2, EPC:CHAPS = 10:2 and EPC:NaT = 10:1.5) at pH 6.2 was significantly lower than that of pH 7.4.

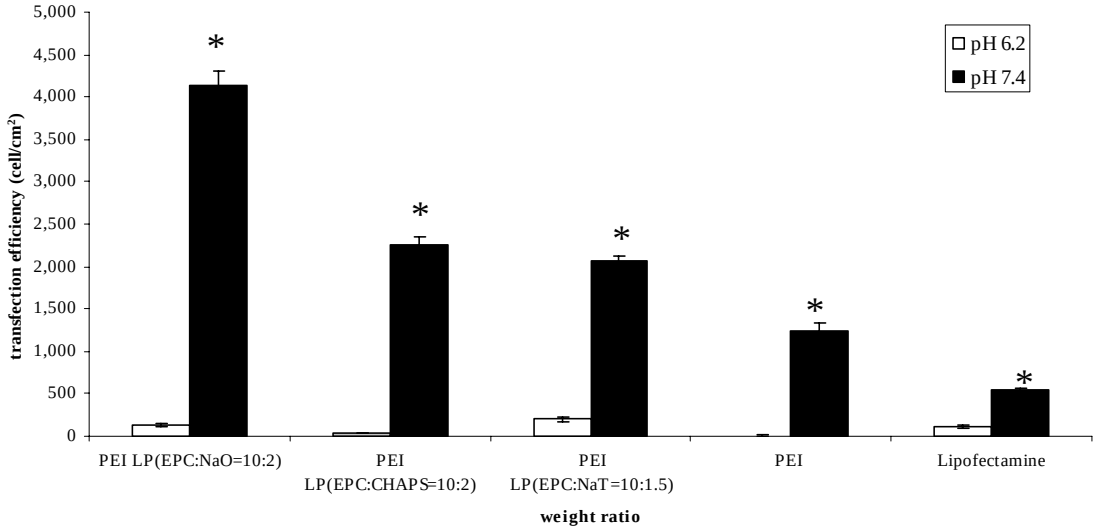

Figure 22 Effect of pH of culture medium on the transfection efficiency of PEI-coated liposomes/DNA complexes (EPC:NaO = 10:2 at weight ratio of 0.5, EPC:CHAPS = 10:2 at weight ratio of 0.75 and EPC:NaT = 10:1.5 at weight ratio of 0.75), PEI/DNA complexes at weight ratio of 0.75 and Lipofectamine/DNA complexes at weight ratio of 2.

1.4.1.5 Effect of serum

In order to evaluate the effect of serum to transfection, the transfection efficiency of PEI-coated liposomes in the absence or presence of serum was investigated. As shown in Figure 23, the transfection efficiency in cells transfected in serum containing media (10% FBS) was significantly lower than that in serum-free media. However, there was no significant difference in transfection efficiency in PEI-coated liposomes (EPC:CHAPS = 10:2) in the absence or presence of serum.

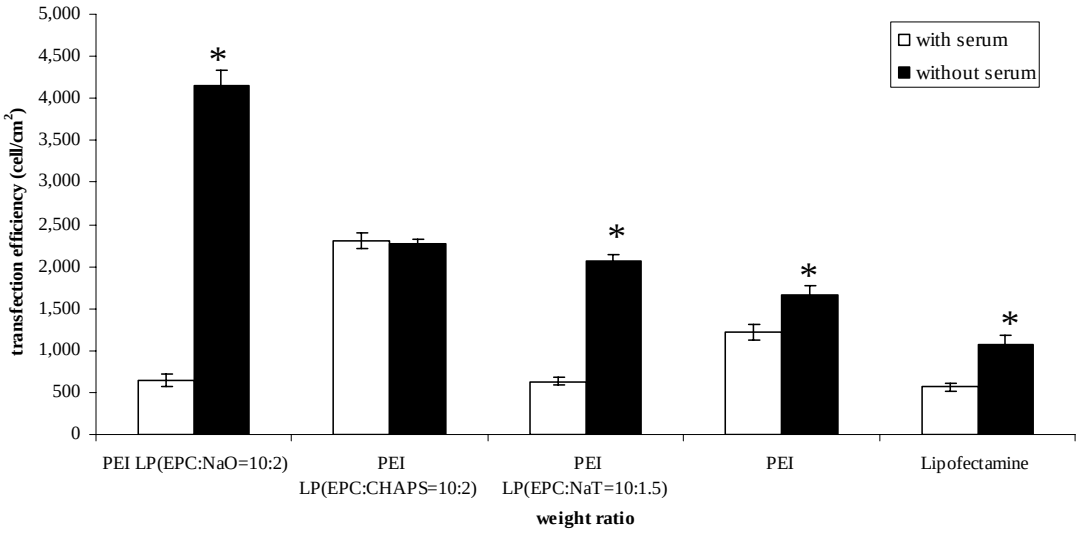

Figure 23 Effect of serum on gene transfection efficiency. Huh7 cells were incubated in the absence or presence of 10% serum with PEI-coated liposomes/DNA complexes.

1.4.2 Cationic liposomes

In this study, the optimal type of surfactants, lipid to surfactant ratio and cationic liposomes to DNA weight ratio were investigated. Cationic surfactants were stearylamine (SA), dodecyl trimethylammoniumbromide (DTAB) and cetylpyridinium chloride (CPC). The lipid surfactant molar ratios were varied as EPC:Chol:cationic surfactant = 10:2:1, 10:2:2, 10:2:3 and 10:2:4. The PEI/DNA complexes (weight ratio of 0.75) and Lipofectamine 2000^{TM} (weight ratio of 2) were used as positive control. The results showed that there were no tranfection at all molar ratios of EPC:Chol:DTAB and EPC:Chol:CPC liposomes/DNA complexes. Only the cells transfected with EPC:Chol:SA liposomes/DNA complexes showed gene expression. The results of transfection efficiency of EPC:Chol:SA liposomes/DNA complexes are shown in Figure 24.

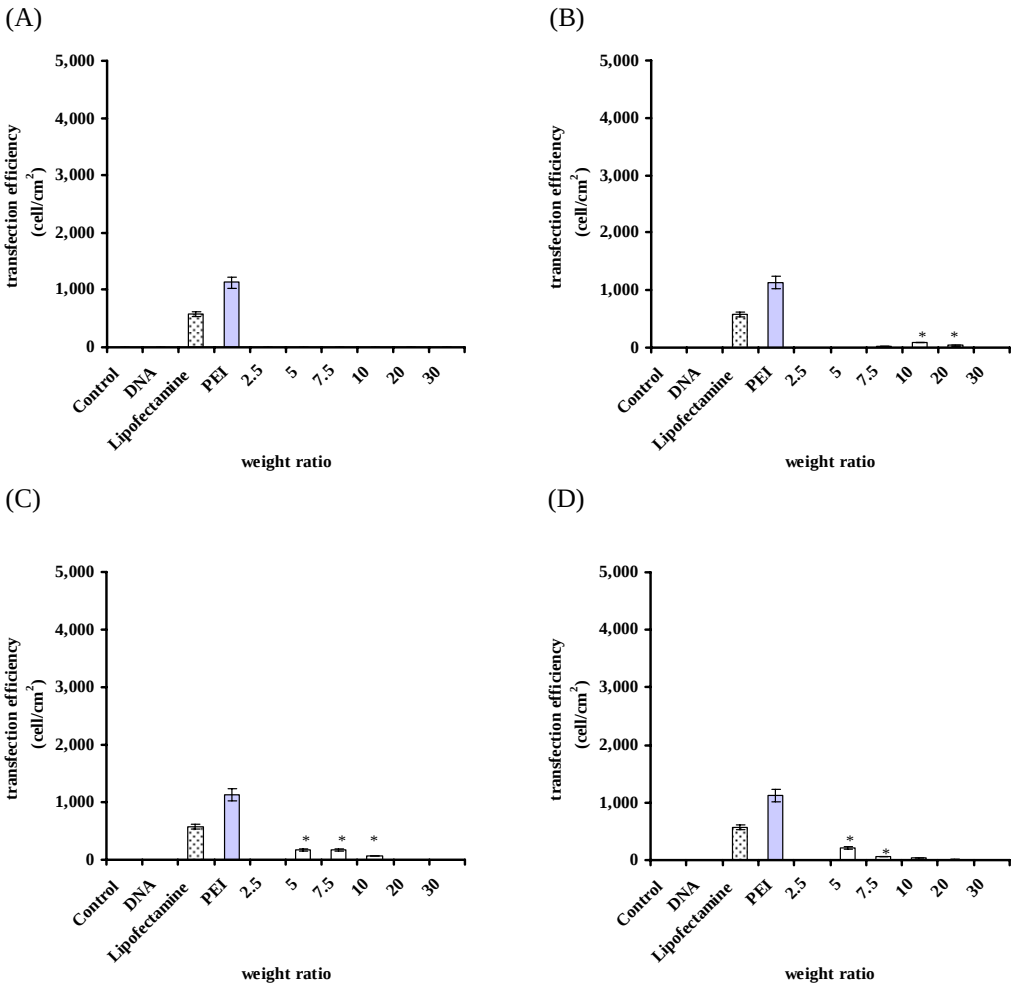

Figure 24 Effect of molar ratio of lipid:SA and liposomes/DNA weight ratio on transfection efficiency of cationic liposomes (EPC:Chol:SA); (A) molar ratio of 10:2:1, (B) molar ratio of 10:2:2, (C) molar ratio of 10:2:3 and (D) molar ratio of 10:2:4. Values shown are the means $\pm$ SD of triplicated experiment (* indicate $p \leq 0.05$).

The gene transfection efficiencies were significantly influenced by molar ratio of lipid:SA and liposomes/DNA weight ratio. EPC:Chol:SA at the molar ratio of 10:2:4 showed the highest transfection efficiency. As the EPC:Chol:SA molar ratios were increased, the gene transfection efficiency increased. The highest value of transfection efficiency of EPC:Chol:SA liposomes (10:2:4) at weight ratio of 5 was 210 $\pm$ 26 cell/cm^2. The transfection efficiency can be ranked as follows: EPC:Chol:SA liposomes (10:2:4) at weight ratio of 5 (210 $\pm$ 26 cell/cm^2), EPC:Chol:SA liposomes (10:2:3) at weight raio of 7.5 (173 $\pm$ 28 cell/cm^2), EPC:Chol:SA liposomes (10:2:2) at weight ratio of 10 (84.04 $\pm$ 0.8 cell/cm^2). There were no transfection of EPC:Chol:SA (10:2:1) liposomes/DNA complexes.

1.5 Cell viability

The major requirement for the development of gene carriers is low cytotoxicity. In this study the cytotoxicity of carriers and carrier/DNA complexes on cell viability was investigated by MTT assay.

1.5.1 Cytotoxicity of carriers

Gene carriers (cationic polymer coated-anionic liposomes, cationic liposomes and PEI) were applied to the cells at concentration ranging from 0.001 to 1000 μg/mL for 24 hours and the effect of amount of gene carriers on cell viability was measured. The results of cell viability (IC_{50}) of all gene carriers tested are shown in Table 8. The results showed that the cytotoxicities of cationic polymer coated-anionic liposomes and cationic liposomes were concentration-dependent. The cationic polymer-coated anionic liposomes and cationic liposomes were less toxic than PEI. The highest toxicity was observed in PEI. This results revealed that the formulation as liposomes reduced cytotoxicity of PEI. TM_{69}-Py_{62}-CS-coated liposomes (EPC:NaO = 10:2) showed less cytotoxicity than PEI-coated liposomes (EPC:NaO = 10:2). The effect of anionic surfactants revealed that EPC:NaO liposomes showed the higher cytotoxicity than EPC:CHAPS liposomes and EPC:NaT liposomes. The cytotoxicity of each formulation can be ranked as EPC:NaO liposomes > EPC:CHAPS liposomes ~ EPC:NaT liposomes ~ EPC:Chol:SA liposomes. The cytotoxicity had a tendency to increase as the molar ratio of surfactants increased.

Table 8 Cytoxicity (IC_{50}) of cationic polymer, cationic polymer coated anionic liposomes and cationic liposomes on Huh7 cells.

Samples	IC_{50} (μg/mL)
Cationic polymer	
PEI	136
Cationic polymer coated anionic liposomes	
PEI-coated liposomes (EPC:NaO = 10:1)	925
PEI-coated liposomes (EPC:NaO = 10:1.5)	342
PEI-coated liposomes (EPC:NaO = 10:2)	417
TM_{69}-Py_{62}-CS-coated liposomes (EPC:NaO = 10:2)	1515
PEI-coated liposomes (EPC:CHAPS = 10:1)	3296
PEI-coated liposomes (EPC:CHAPS = 10:1.5)	2869
PEI-coated liposomes (EPC:CHAPS = 10:2)	1135
PEI-coated liposomes (EPC:NaT = 10:1)	5180
PEI-coated liposomes (EPC:NaT = 10:1.5)	1158
PEI-coated liposomes (EPC:NaT = 10:2)	1036
Cationic liposomes	
EPC:Chol:SA=10:2:1	1862
EPC:Chol:SA=10:2:2	1402
EPC:Chol:SA=10:2:3	1525
EPC:Chol:SA=10:2:4	3713

1.5.2 Effect of carriers/DNA complexes on cell viability

The naked DNA did not show any cytotoxicity effect on the cells, and the cell viability was maintained around 100%. However, approximately 80% of cells were viable after the incubation with PEI/DNA complexes and Lipofectamine2000™/DNA complexes (Figure 25). The effect of cationic polymer-coated liposomes and cationic liposomes/DNA complexes on cell viability are shown in Figure 26. The results showed that cationic polymer-coated liposomes, cationic liposomes and PEI showed a significant decrease in cell viability with increasing in weight ratio of carriers/DNA complexes.

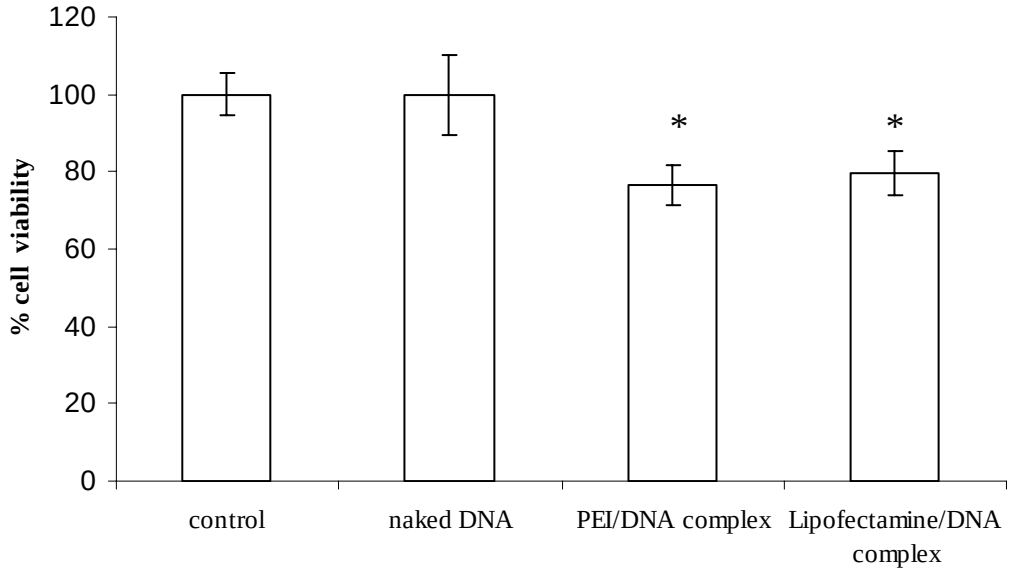

Figure 25 Effect of naked DNA, PEI/DNA complexes (weight ratio of 0.75) and Lipofectamine2000^{TM}/ DNA complexes (weight ratio of 2) on cell viability (* indicated $p \leq 0.05$).

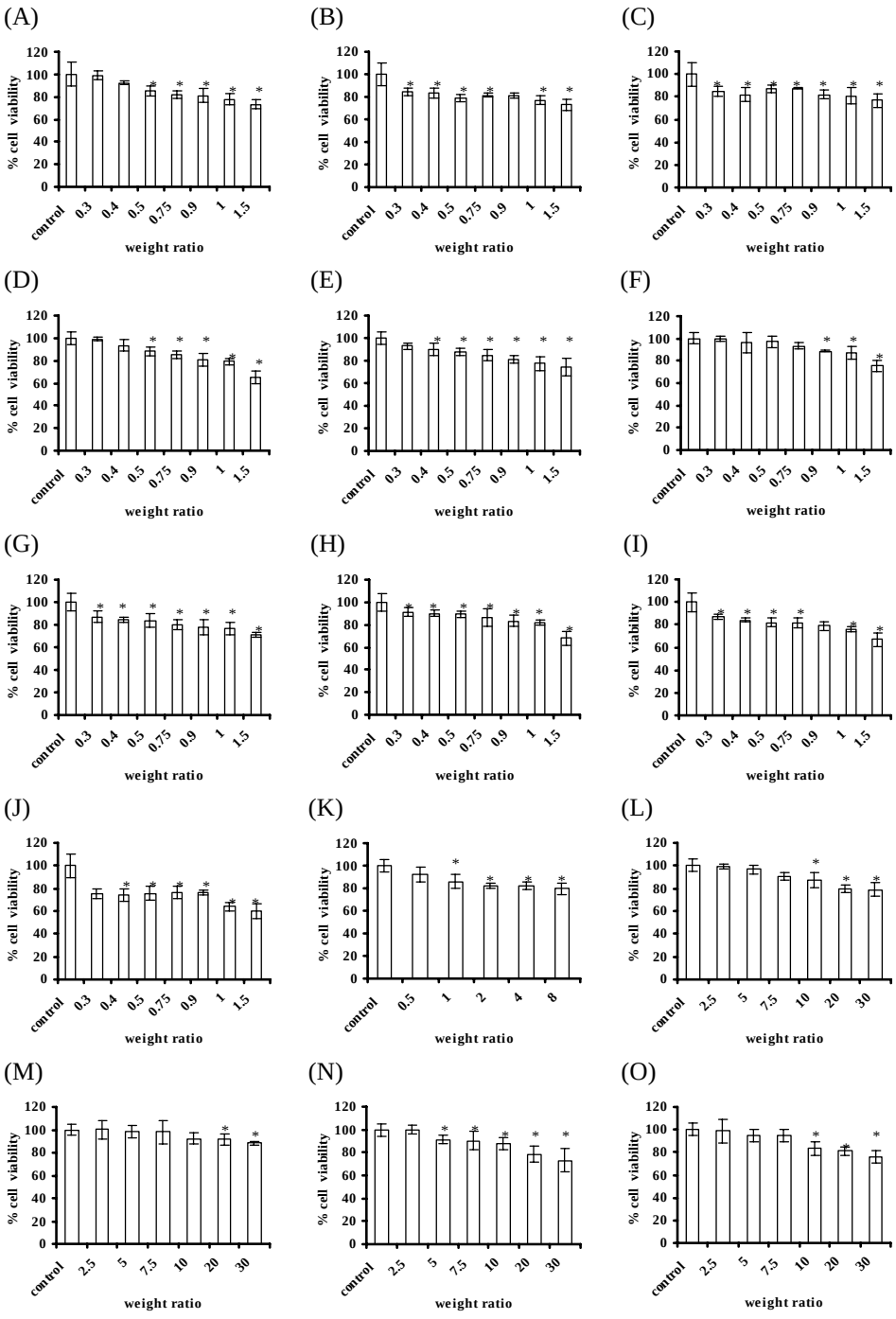

Figure 26 Effect of carriers/DNA complexes on cell viability; (A) PEI-coated liposomes (EPC:NaO = 10:1), (B) PEI-coated liposomes (EPC:NaO=10:1.5), (C) PEI-coated liposomes (EPC:NaO = 10:2), (D) PEI-coated liposomes (EPC:CHAPS = 10:1), (E) PEI-coated liposomes (EPC: CHAPS = 10:1.5), (F) PEI-coated liposomes (EPC:CHAPS = 10:2), (G) PEI-coated liposomes (EPC:NaT = 10:1), (H) PEI-coated liposomes (EPC:NaT = 10:1.5), (I) PEI-coated liposomes (EPC:NaT = 10:2), (J) PEI/DNA complexes, (K) TM_{69}-Py_{62}-CS-coated liposomes (EPC:NaO = 10:2), (L) EPC:Chol:SA = 10:2:1, (M) EPC:Chol:SA = 10:2:2, (N) EPC:Chol:SA = 10:2:3, and (O) EPC:Chol:SA = 10:2:4.

2. Discussion

Cationic polymers have been used as tools for gene carriers. The main reason for the using cationic polymers is that cationic polymers enable compaction of DNA (Dunlap et al. 1997 : 3095). However, sometimes the high toxicity of cationic polymer such as PEI limited application in gene therapy. However, in these cationic polymers, cationic liposomes have the advantages in terms of safety, simplicity of preparation and high gene encapsulation capability. However, they have some disadvantages such as low efficiency of transfection (El-Aneed 2004 : 1). Therefore, the combination of liposomes and cationic polymer might be a promising approach for enhancing transfection efficiency while decreasing the cytotoxicity. In the present study, liposomes used for gene carriers can be characterized in two types. The first type is cationic polymer-coated anionic liposomes. Polymer coating is an alternative method to modify the surface of liposomes. It is a characteristic of the polymer coating method that the manner of coating is very simple. Liposome suspension and polymer solution are mixing together, without chemically linking between the polymers and the lipid molecules. The second type is cationic liposomes prepared with EPC, cholesterol and cationic surfactants to modify surface of liposomes to achieve positively charge. The modified colloidal carriers with surfactants are useful pharmaceutical techniques to improve gene delivery (Huang et al. 2006 : 158). However, a few studies focusing on the effect of surfactants on the non-viral gene delivery system were available.

The ability of cationic liposomes to condense DNA was studied by gel retardation assay. All liposomal formulations were able to condense DNA at different weight ratios. CSL (MW 20, 45, 200 and 460 kDa)-coated liposomes were completely retarded when the weight ratio was about 0.1. PEI-coated liposomes (EPC:NaO, EPC:CHAPS, EPC:NaT) and TM_{69}-Py_{62}-CS-coated liposomes (EPC:NaO = 10:2) were very effective in condensing DNA, leading to a complete condensation of DNA at weight ratios about 0.5. While, SA liposomes with lipid:Chol:SA molar ratio of 10:2:2,10:2:3 and 10:2:4 condensed DNA at weight ratio 5, 2.5, and 2.5, respectively.

The size of condensed DNA is a with critical factor for *in vivo* delivery, because the particle size influences not only the biodistribution but also the efficiency of cellular uptake through endocytosis (Sun, Zhang and Zhang 2004 : 533). Therefore, particle size determination is a useful tool for characterizing colloidal drug delivery systems. In this study, the particles size of carriers/DNA complexes determined by light-scattering measurements, were in the rage of 300 to 1500 nm. Initially, negative values of zeta-potentials were observed at low weight ratio in liposomal formulations of PEI-coated liposomes (EPC:NaO = 10:2) and TM_{69}-Py_{62}-CS-coated liposomes (EPC:NaO = 10:2). However, when the complete complexes were formed, the zeta-potential values became approximately zero. The zeta- potential of the complexes was found to be more positive when the weight ratio of polymer-coated liposomes was increased. This might be due to the higher density of cationic polymer. The positive surface charge of lipoplexes is necessary for binding to anionic cell surfaces, which consequently facilitates uptake by the cell (Jiang et al. 2006 : 277).

To investigate the liposomes mediated gene transfection efficiencies, *in vitro* gene transfection assay was performed with Huh 7 cells using pEGFP-C2 plasmid encoding green fluorescent protein (GFP). PEI and Lipofectamine 2000^{TM} complexed with DNA were used as a positive control. In all studies, there were no transfection in

control (cells without complexes) and naked DNA. The gene transfection efficiencies of cationic polymer-coated anionic liposomes were influenced by cationic polymers, anionic surfactants, lipid surfactant ratio, carriers/DNA weight ratio, pH of transfection medium and serum. PEI showed higher transfection efficiencies than TM_{69}-Py_{62}-CS and CSL (MW 20, 45, 200 and 450 kDa), respectively. However, there were no significant difference in the transfection efficiency between CSL and CSL-coated anionic liposomes in all MW of CSL. Whereas, the transfection efficiency of TM_{69}-Py_{62}-CS-coated anionic liposomes was significantly lower than TM_{69}-Py_{62}-CS/DNA complexes. Only the transfection efficiency of PEI-coated anionic liposomes was significantly higher than the PEI/DNA complexes. The transfection efficiency of CSL coated liposomes/DNA complexes was dependent on pH of transfection medium. The transfection efficiency of CSL at the transfection medium pH 6.2 was higher than medium pH 7.4. This results was consistent with the previous studies reported that the transfection efficiency of CS was dependent on pH. Chitosan-mediated high gene transfection was observed at the medium pH value below 6.5 (Weecharangsan et al. 2008 : 161). These results might be due to the protonation of amines in CS. The pK_a of primary amines of CS is about 6.3-6.4. Therefore, CS/DNA complexes are positively charge, and can bind with negatively charge of cells through electrostatic interaction at below pH 7. In case of TM_{69}-Py_{62}-CS, independence on pH of transfection medium might be due to increment of solubility of the quaternized CS derivatives as well as permanent positive charge on CS backbone, making the quaternized CS derivatives be soluble over a wider pH range. In case of PEI, the weight ratio between polymer and DNA, and zeta-potential, dramatically influenced the efficacy of the gene delivery system. It has been estimated that every fifth or sixth amino nitrogen of PEI is protonated at physiological pH (Suh, Paik and Hwang 1994: 318) and only these positively charged amino groups ionically interact with the negatively charged DNA. The pK_a value of the individual nitrogen atoms within the PEI molecules can not be determined and the absolute amount of positively charged amino groups is not known. At high PEI/DNA weight ratios, the positive net charge of the corresponding complexes increased, improving cell interaction and enhancing the cellular and nuclear uptake and retention. The branched form of PEI contains 1°, 2°, and 3° amines, and they have a potential to be protonated, making PEI to be an effective buffer through a wide pH range. With nitrogens appearing as one out of every three atoms in the PEI backbone, any benefits of branching and protonability quickly accumulate in relation to the overall polymer size. Our results showed that transfection efficiency of PEI, Lipofectamine, PEI-coated liposomes complexes (EPC:NaO = 10:2, EPC:CHAPS = 10:2 and EPC:NaT = 10:1.5) at pH 6.2 was significant lower than that of pH 7.4 (Figure 22). This low transfection efficiency might be due to the cytotoxicity of these polymers at pH 6.2. The different in pHs of PEI solutions do not make any significant difference in transfection efficiency (Boussif et al. 1995 : 7299). This might be that PEI nitrogen having its own local environment, which influenced its protonability.

NaO-liposomes showed higher transfection efficiency than NaT-liposomes and CHAPS-liposomes, respectively. Increasing the EPC:surfactant ratio (10:1, 10:1.5 and 10:2) increased the gene transfection efficiency. The highest transfection efficiency of NaO-liposomes and CHAPS-liposomes was EPC:surfactant ratio of 10:2, whereas the highest transfection efficiency of NaT-liposomes was 10:1.5. In comparison, the transfection efficiency of PEI coated-liposomes (EPC:NaO = 10:2) at the weight ratio

of 0.5, (EPC:CHAPS = 10: 1.5) at the weight ratio of 0.75 and (EPC:NaT = 10:1.5) at the weight ratio of 0.75 were 4314, 2320 and 2158 cell/cm^2, respectively. Their transfection efficiency was higher than PEI/DNA complexes and Lipofectamine 2000^{TM}/DNA complexes. PEI-coated liposomes have been previously referred as promising transfection agents. Liposomes modified with PEI also showed remarkable transfection efficiency such as Cetylated PEI600/DOPE (Yamazaki et al. 2000 : 1148), PEI800-Chol/soybean phospholipid liposomes (Chen et al. 2007 : 256) and PEI25/Dosper lipopolyplexes (Hanzlikova et al. 2009 : 16).

To evaluate the effect of serum, the transfection efficiency were carried out in the presence of 10% serum. The poor transfection efficiency of cationic liposomes in the presence of serum is due to the non-specific interactions of the cationic particles with serum proteins that mask the cationic charges at the surface the liposomes (Nicolazzi et al. 2003 : 126). In case of PEI-coated liposomes, the results showed that all PEI coated liposomes decreased transfection efficiency in the presence of serum. However, PEI-coated liposomes (EPC:CHAPS = 10:2) in the absence of serum showed similar transfection efficiency to the presence of serum. The result indicated that PEI-coated liposomes (EPC:CHAPS = 10:2) has potential to apply for *ex vivo* gene transfer and intra-tissue administration.

In a case of cationic liposomes, there were no transfection when all molar ratios of EPC:Chol:DTAB and EPC:Chol:CPC liposomes/DNA complexes were used. Only EPC:Chol:SA liposomes/DNA complexes showed gene expression. This might be due to the cytotoxicity of CPC and DTAB (Scholer et al. 2001 : 62-63; Vlachy et al. 2009 : 279). EPC:Chol:SA at the molar ratio of 10:2:4 showed the highest transfection efficiency among other molar ratio. Increasing the EPC:Chol:SA molar ratios (10:2:1, 10:2:2, 10:2:3 and 10:2:4) increased the gene transfection efficiency. At high SA composition, the positive net charge of the corresponding liposome complexes increased, cell interaction improved and the transfection efficiency enhanced.

The cytotoxicity of cationic polymer-coated anionic liposomes and cationic liposomes were determined by MTT assay. All liposomal formulations had lower cytotoxicity than PEI. Although the exact mechanism still remains unclear, it is considered that the interaction of liposomes with PEI might increase the safety of PEI. This result was similar to Chen et. al. that PEI linked with cholesterol and developed liposomes modified with PEI decreased the cytotoxicity (Chen et al. 2007 : 255). SA liposomes showed low cytoxicity. Audera et al. also revealed that SA liposomes prepared from dipalmitoylphosphatidylcholine-Chol-SA gave low toxicity (Audera et al. 1998 : 139).

CHAPTER V
CONCLUSIONS

In this study, the cationic polymer coated anionic liposomes and cationic liposomes were assessed for their transfection efficiency and cytotoxicity on Huh7 cells. The factors affecting the transfection efficiency such as particle size, surface charge, type of anionic surfactants, lipid/surfactant ratio, carriers/DNA weight ratio, pH of culture medium and serum were investigated. The results of this study could be concluded as follow:

1. Physicochemical properties of cationic polymer-coated anionic liposomes and cationic liposomes /DNA complexes

1.1 Complex formation of polymer-coated liposomes and cationic liposomes with pDNA

Gel electrophoresis illustrated that cationic polymer-coated anionic liposomes and cationic liposomes were able to form complex with DNA. The weight ratio at which complete complexes were formed as rank of CSL-coated anionic liposomes < TM_{69}-Py_{62}-CS-coated anionic liposomes ~ PEI-coated liposomes < cationic liposomes (EPC:Chol:SA).

1.2 Particle size and surface charge of the polymer-coated liposomes and cationic liposomes with pDNA

The particle size of the liposomes/DNA complexes were in the range of 300 to 1500 nm depending on the weight ratio. The zeta-potential of these complexes were positive charge after the complete complexes were formed.

1.3 Morphology by TEM

The shape of cationic polymer-coated anionic liposomes/DNA complexes were spherical.

2. *In vitro* transfection efficiency

2.1 Cationic polymer coated anionic liposomes

2.1.1 Effect of polymers

The transfection efficiencies were influenced by the type of cationic polymers coating on anionic liposomes (EPC:NaO = 10:2). It can be ranked the maximum transfection efficiencies of cationic polymers as PEI > TM_{69}-Py_{62}-CS > CSL.

2.1.2 Effect of anionic surfactants and ratio of lipid:surfactant

NaO-liposomes showed a higher transfection efficiency than NaT-liposomes and CHAPS-liposomes. The highest transfection efficiency of NaO-liposomes and CHAPS-liposomes was obtained at the EPC:surfactant molar ratio of 10:2, whereas that of NaT-liposomes was 10:1.5.

2.1.3 Effect of weight ratio

The gene transfection efficiencies were significantly influenced by weight ratios. By increasing weight ratios, transfection efficiencies showed a graph like bell shaped pattern.

2.1.4 Effect of pH

The transfection efficiency of PEI, Lipofectamine 2000^{TM}, PEI-coated liposomes (EPC:NaO = 10:2, EPC:CHAPS = 10:2 and EPC:NaT = 10:1.5) at pH 6.2 was significantly lower than that pH 7.4. This result might be due to the cytotoxicity at this low pH.

2.1.5 Effect of serum

The transfection efficiency of PEI-coated liposomes (EPC:CHAPS = 10:2) in the media which containing serum was not significantly different from absence of serum. While, the transfection efficiency of others PEI-coated liposomes decreased in the presence of serum.

2.2 Cationic liposomes

There were no transfection when used all molar ratios of EPC:Chol:DTAB and EPC:Chol:CPC liposomes/DNA complexes. While, EPC:Chol:SA liposomes/DNA complexes at the molar ratio of 10:2:4 showed the highest gene expression than others molar ratio. Increasing the EPC:Chol:SA molar ratios increased the transfection efficiency.

3. Cell viability

3.1 Cytotoxicity of carriers

The cationic polymer-coated anionic liposomes and cationic liposomes were less toxic than PEI. The highest toxicity was observed in PEI. In addition, TM_{69}-Py_{62}-CS-coated liposomes (EPC:NaO = 10:2) showed less cytotoxicity than PEI-coated liposomes (EPC:NaO = 10:2). The effect of anionic surfactants revealed that NaO liposomes showed a higher cytotoxicity than CHAPS liposomes and NaT liposomes. The cytotoxicity of liposomal formulations can be ranked as EPC:NaO liposomes > EPC:CHAPS liposomes ~ EPC:NaT liposomes ~ EPC:Chol:SA liposomes.

3.2 Effect of carriers/ DNA complexes on cell viability

The cytotoxicity of polymer-coated anionic liposomes and cationic liposomes/DNA complexes were determined by MTT assay. The results showed that all cationic polymer coated anionic liposomes/DNA complexes and cationic liposomes/DNA complexes had lower cytotoxicity than PEI/DNA complexes. By increasing weight ratios, the cell viability decreased.

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APPENDIX

APPENDIX I

Calculation of liposomes/pDNA ratio (weight ratio)

Calculation of anionic liposomes (EPC:NaO = 10:1, molar ratio) : 1mg/mL PEI = 1:1 weight ratio

Calculation of 10 mM EPC

pipette 200 mM EPC 300 μL = 200 mmole/1000 mL × 0.3 mL = 0.06 mmole = 60 μmole (Add 6 mL of Tris buffer) ∴ concentration of EPC in Tris buffer = 60 μmole/6 mL = 10 mM

Calculation of 1 mM NaO

pipette 20 mM of NaO 300 μL = 20 mmole/1000 mL × 0.3 mL = 0.006 mmole = 6 μmole (Add 6 mL of Tris buffer) ∴ concentration of NaO in Tris buffer = 6 μmole/6 mL = 1 mM

Calculation of concentration of liposomes

pipette 200 mM EPC 300 μL; 200 mmole/1000 mL × 0.3 mL = 0.06 mmole = 0.06×10^{-3} mole × 773 g/mole = 46.38 mg
pipette 20 mM NaO 300 μL; 20 mmole/1000 mL × 0.3 mL = 0.006 mmole = 0.006×10^{-3} mole × 304.4 g/mole = 1.8264 mg

EPC + NaO (total lipid) = 48.2064 mg/6 mL = 8.0344 mg/mL (approx. 8 mg/mL)
Because the ratio of $\frac{4mg/0.5mL(Lipid)}{4mg/4mL(PEI)}$ = 1:1 (w/w) or 0.5:4 (v/v)
∵ Totol volume of lipid + PEI = 4.5 mL (but, total volume of formulation is 5 mL)
∴ Add 0.5 mL of Tris buffer pH 7.1
Concentration of PEI (cationic polymer) = 4mg/5mL = 0.8 mg/mL (or 0.8 μg/μL)
Pipette suspension of polymer-coated liposomes 0.375, 0.5, 0.625, 0.938, 1.125, 1.25 and 1.875 μL, which has the content of polymer = 0.3, 0.4, 0.5, 0.75, 0.9, 1 and 1.5 μg, respectively and form complexes with DNA 1 μg
∴ PEI/DNA ratio (weight ratio) = 0.3, 0.4, 0.5, 0.75, 0.9, 1 and 1.5 respectively

APPENDIX II

The particle size and zeta-potential of the PEI-coated anionic liposomes/DNA complexes

Table 9 The particle size and zeta-potential of the PEI-coated liposomes (EPC:NaO = 10:2)/DNA complexes.

weight ratio	size (nm)	AVG	SD	PDI	AVG	SD	Zeta-potential (mV)	AVG	SD
0.3	345	363	24.476	0.266	0.292	0.024	-39.8	-35.7	4.378
	354			0.313			-36.3		
	391			0.298			-31.1		
0.4	607	532	68.609	0.720	0.472	0.216	-1.0	-0.9	0.175
	472			0.320			-1.0		
	516			0.377			-0.7		
0.5	631	578	57.240	0.434	0.449	0.050	37.9	37.1	2.498
	517			0.408			34.3		
	585			0.505			39.1		
0.75	802	878	70.803	0.514	0.539	0.095	35.1	34.3	1.929
	892			0.460			35.7		
	941			0.644			32.1		
0.9	734	803	65.824	0.529	0.505	0.025	29.7	30.0	1.222
	865			0.508			31.3		
	811			0.479			28.9		
1	1103	1116	77.777	0.559	0.610	0.064	28.9	30.7	1.607
	1199			0.681			31.4		
	1045			0.589			31.9		
1.5	667	653	28.534	0.515	0.488	0.030	41.3	39.6	1.480
	672			0.494			38.9		
	620			0.456			38.6		

Table 10 The particle size and zeta-potential of the PEI-coated liposomes (EPC:CHAPS = 10:2)/DNA complexes.

weight ratio	size (nm)	AVG	SD	PDI	AVG	SD	Zeta-potential (mV)	AVG	SD
0.3	1127	1348.667	264.186	0.573	0.699	0.178	20.8	18.6	2.706
	1278			0.621			15.6		
	1641			0.903			19.5		
0.4	702	674.033	24.492	0.391	0.374	0.027	38.0	38.4	0.635
	656			0.389			38.0		
	665			0.343			39.1		
0.5	430	443.967	12.566	0.362	0.356	0.015	17.1	17.9	0.907
	455			0.367			17.8		
	447			0.339			18.9		
0.75	555	637.533	73.711	0.368	0.394	0.057	17.9	16.5	1.582
	662			0.355			14.8		
	696			0.459			16.9		
0.9	551	556.033	7.071	0.725	0.648	0.108	36.1	37.2	1.474
	554			0.695			36.7		
	564			0.525			38.9		
1	572	631.000	53.362	0.560	0.555	0.048	36.5	40.5	5.125
	646			0.601			46.3		
	675			0.505			38.8		
1.5	696	632.433	57.097	0.513	0.543	0.046	34.0	34.4	0.907
	616			0.520			33.7		
	586			0.596			35.4		

Table 11 The particle size and zeta-potential of the PEI-coated liposomes (EPC:NaT = 10:1.5)/DNA complexes.

weight ratio	size (nm)	AVG	SD	PDI	AVG	SD	Zeta-potential (mV)	AVG	SD
0.3	1087	940.433	168.257	0.739	0.583	0.146	22.4	21.1	1.464
	757			0.451			19.5		
	978			0.558			21.3		
0.4	1048	1006.533	49.591	0.723	0.719	0.003	30.7	30.9	0.569
	952			0.718			31.5		
	1020			0.717			30.4		
0.5	556	553.333	10.617	0.451	0.431	0.026	32.0	17.1	13.503
	542			0.440			13.7		
	563			0.401			5.7		
0.75	1592	1566.667	40.464	0.530	0.667	0.127	13.6	18.3	4.230
	1588			0.690			19.5		
	1520			0.780			21.8		
0.9	1966	1595.333	323.132	0.942	0.765	0.164	29.8	32.5	2.524
	1373			0.736			32.9		
	1447			0.618			34.8		
1	1134	1100.667	55.157	0.805	0.799	0.050	28.9	22.1	6.516
	1037			0.747			21.6		
	1131			0.846			15.9		
1.5	1104	1013.367	80.217	0.795	0.711	0.075	39.6	39.1	0.503
	985			0.686			39.2		
	952			0.652			38.6		

Table 12 The particle size and zeta-potential of the PEI/DNA complexes.

weight ratio	size (nm)	AVG	SD	PDI	AVG	SD	Zeta-potential (mV)	AVG	SD
0.3	519	555	31.904	0.382	0.416	0.043	23.7	20.3	3.808
	567			0.401			16.2		
	580			0.465			21.1		
0.4	1591	1504	252.417	0.574	0.646	0.189	26.2	28.4	1.997
	1702			0.860			30.1		
	1220			0.503			28.9		
0.5	927	965	35.059	0.609	0.690	0.071	30.6	30.5	0.404
	971			0.718			30.1		
	996			0.742			30.9		
0.75	1440	1496	119.643	0.847	0.659	0.169	33.2	31.9	1.916
	1414			0.613			32.8		
	1633			0.518			29.7		
0.9	442	454	21.835	0.377	0.387	0.013	35.4	34.7	1.629
	441			0.401			32.8		
	479			0.382			35.8		
1	476	475	25.964	0.397	0.393	0.029	34.1	34.6	0.436
	501			0.419			34.8		
	449			0.362			34.9		
1.5	376	375	4.969	0.361	0.362	0.030	40.3	39.7	1.308
	380			0.392			40.6		
	370			0.333			38.2		

Table 13 The particle size and zeta-potential of the TM_{69}-Py_{62}-CS-coated liposomes (EPC:NaO = 10:2)/DNA complexes.

weight ratio	size (nm)	AVG	SD	PDI	AVG	SD	Zeta-potential (mV)	AVG	SD
0.5	1435	1458	296.689	0.931	0.977	0.040	-45.1	-40.4	4.143
	1174			1.000			-37.4		
	1766			1.000			-38.6		
1	1196	1204	9.074	0.670	0.646	0.023	-20.7	-23.0	2.470
	1214			0.624			-22.6		
	1203			0.645			-25.6		
2	449	445	4.844	0.489	0.428	0.054	23.3	25.5	2.107
	440			0.386			25.7		
	447			0.409			27.5		
4	402	344	50.218	0.444	0.480	0.032	28.6	27.0	1.600
	319			0.502			27.0		
	312			0.495			25.4		
8	401	388	33.812	0.893	0.641	0.226	30.0	30.4	0.404
	413			0.575			30.8		
	349			0.455			30.5		

APPENDIX III

In vitro transfection efficiency

1. *In vitro* transfection study

1.1 Cationic polymer-coated anionic liposomes

1.1.1 Effect of polymers

1.1.1.1 CSL

Table 14 Effect of polymers on transfection efficiency of CSL20 in medium without serum, pH 6.2.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	16	8.421	17	8.947	17	8.947	8.772	0.304
1	24	12.632	26	13.684	25	13.158	13.158	0.526
2	31	16.316	34	17.895	32	16.842	17.018	0.804
4	7	3.684	11	5.789	10	5.263	4.912	1.096
8	0	0.000	0	0.000	0	0.000	0.000	0.000

Table 15 Effect of polymers on transfection efficiency of CSL20-coated anionic liposomes (EPC:NaO = 10:2) in medium without serum, pH 6.2.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	30	15.789	25	13.158	22	11.579	13.509	2.127
1	36	18.947	40	21.053	38	20.000	20.000	1.053
2	51	26.842	41	21.579	45	23.684	24.035	2.649
4	4	2.105	4	2.105	3	1.579	1.930	0.304
8	1	0.526	2	1.053	1	0.526	0.702	0.304

Table 16 Effect of polymers on transfection efficiency of CSL45 in medium without serum, pH 6.2.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	17	8.947	24	12.632	22	11.579	11.053	1.898
1	24	12.632	24	12.632	22	11.579	12.281	0.608
2	19	10.000	18	9.474	18	9.474	9.649	0.304
4	3	1.579	2	1.053	2	1.053	1.228	0.304
8	0	0.000	0	0.000	0	0.000	0.000	0.000

Table 17 Effect of polymers on transfection efficiency of CSL45-coated anionic liposomes (EPC:NaO = 10:2) in medium without serum, pH 6.2.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	14	7.368	18	9.474	16	8.421	8.421	1.053
1	11	5.789	5	2.632	11	5.789	4.737	1.823
2	9	4.737	3	1.579	9	4.737	3.684	1.823
4	0	0.000	0	0.000	0	0.000	0.000	0.000
8	0	0.000	0	0.000	0	0.000	0.000	0.000

Table 18 Effect of polymers on transfection efficiency of CSL200 in medium without serum, pH 6.2.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	3	1.579	3	1.579	4	2.105	1.754	0.304
1	12	6.316	11	5.789	12	6.316	6.140	0.304
2	7	3.684	9	4.737	8	4.211	4.211	0.526
4	3	1.579	4	2.105	3	1.579	1.754	0.304
8	0	0.000	0	0.000	0	0.000	0.000	0.000

Table 19 Effect of polymers on transfection efficiency of CSL200-coated anionic liposomes (EPC:NaO = 10:2) in medium without serum, pH 6.2.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	7	3.684	10	5.263	9	4.737	4.561	0.804
1	11	5.789	10	5.263	11	5.789	5.614	0.304
2	8	4.211	7	3.684	8	4.211	4.035	0.304
4	7	3.684	7	3.684	6	3.158	3.509	0.304
8	0	0.000	0	0.000	0	0.000	0.000	0.000

Table 20 Effect of polymers on transfection efficiency of CSL460 in medium without serum, pH 6.2.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	1	0.526	0	0.000	1	0.526	0.263	0.372
1	5	2.632	7	3.684	6	3.158	3.158	0.744
2	13	6.842	15	7.895	14	7.368	7.368	0.744
4	10	5.263	10	5.263	9	5.263	5.263	0.304
8	5	2.632	5	2.632	5	2.632	2.632	0.000

Table 21 Effect of polymers on transfection efficiency of CSL460-coated anionic liposomes (EPC:NaO = 10:2) in medium without serum, pH 6.2.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	3	1.579	7	12.632	6	3.158	2.807	1.096
1	4	2.105	6	12.632	5	2.632	2.632	0.526
2	8	4.211	7	9.474	8	4.211	4.035	0.304
4	13	6.842	12	1.053	12	6.316	6.491	0.304
8	6	3.158	7	3.684	5	2.632	3.158	0.526

1.1.1.2. TM_{69}-Py_{62}-CS

Table 22 Effect of polymers on transfection efficiency of TM_{69}-Py_{62}-CS in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	206	108.421	172	90.526	189	99.474	99.474	8.947
1	1104	581.053	1124	591.579	1102	584.211	584.211	6.403
2	1573	796.316	1304	686.316	1422	743.684	743.684	55.153
4	837	440.526	845	444.737	798	435.088	435.088	13.235
8	624	328.421	617	324.737	602	323.333	323.333	5.916

Table 23 Effect of polymers on transfection efficiency of TM_{69}-Py_{62}-CS-coated anionic liposomes (EPC:NaO = 10:2) in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	11	5.789	16	8.421	20	10.526	8.246	2.373
1	547	287.895	431	226.842	488	256.842	257.193	30.528
2	555	292.105	454	238.947	476	250.526	260.526	27.954
4	211	111.053	158	83.158	189	99.474	97.895	14.014
8	45	23.684	64	33.684	54	28.421	28.596	5.002

1.1.1.3 PEI

Table 24 Effect of polymers on transfection efficiency of PEI in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.3	528	277.895	496	261.053	601	316.316	285.088	28.325
0.4	702	369.474	699	367.895	856	450.526	395.965	47.258
0.5	1502	790.526	1662	874.737	1604	844.211	836.491	42.633
0.75	2384	1254.737	2287	1203.684	2614	1375.789	1278.070	88.393
0.9	1768	930.526	1687	887.895	1690	889.474	902.632	24.170
1	926	487.368	887	466.842	1104	581.053	511.754	60.885
1.5	460	242.105	398	209.474	657	345.789	265.789	71.177

Table 25 Effect of polymers on transfection efficiency of PEI-coated anionic liposomes (EPC:NaO = 10:2) in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	Cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.3	454	238.947	630	331.579	450	236.842	269.123	54.099
0.4	8042	4232.632	7898	4156.842	7067	3719.474	4036.316	276.998
0.5	8684	4570.526	8432	4437.895	7475	3934.211	4314.211	335.705
0.75	3638	1914.737	3757	1977.368	3117	1640.526	1844.211	179.154
0.9	3420	1800.000	3378	1777.895	2997	1577.368	1718.411	122.654
1	2898	1525.263	2785	1465.789	2389	1257.368	1416.140	140.679
1.5	1016	534.737	1126	592.632	991	521.579	549.649	37.801

1.1.2 Effect of anionic surfactants and ratio of lipid:surfactant

1.1.2.1 EPC:NaO at the molar ratio of 10:1, 10:1.5 and 10:2

Table 26 Effect of type of anionic surfactants and lipid surfactant ratio on transfection efficiency of PEI-coated liposomes (EPC:NaO = 10:1) in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
Lipofectamine	1004	528.421	1056	555.789	1077	566.842	530.531	19.779
PEI	2614	1375.789	3044	1602.105	2452	1290.526	1422.807	161.023
0.3	254	133.684	231	121.579	429	160.351	160.351	56.994
0.4	1352	711.579	1401	737.368	1422	732.456	732.456	18.906
0.5	1742	916.842	1806	950.526	1752	929.825	929.825	18.120
0.75	2504	1317.895	2459	1294.211	2554	1318.772	1318.772	25.012
0.9	1674	881.053	1698	893.684	1599	872.105	872.105	27.181
1	1125	592.105	1195	628.947	1204	618.246	618.246	22.762
1.5	278	146.316	285	150.000	298	151.053	151.053	5.342

Table 27 Effect of type of anionic surfactants and lipid surfactant ratio on transfection efficiency of PEI-coated liposomes (EPC:NaO = 10:1.5) in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
Lipofectamine	1004	528.421	1056	555.789	1077	566.842	530.531	19.779
PEI	2614	1375.789	3044	1602.105	2452	1290.526	1422.807	161.023
0.3	644	338.947	664	349.474	679	357.368	348.596	9.242
0.4	1834	965.263	1769	931.053	1798	946.316	947.544	17.138
0.5	2254	1186.316	2112	1111.579	2306	1213.684	1170.526	52.852
0.75	2654	1396.842	2568	1351.579	2602	1369.474	1372.632	22.796
0.9	2004	1054.737	2019	1062.632	1978	1041.053	1052.807	10.918
1	1046	550.526	1065	560.526	1100	578.947	563.333	14.417
1.5	788	414.737	807	422.105	788	414.737	417.193	4.254

Table 28 Effect of type of anionic surfactants and lipid surfactant ratio on transfection efficiency of PEI-coated liposomes (EPC:NaO = 10:2) in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
Lipofectamine	1004	528.421	1056	555.789	1077	566.842	530.531	19.779
PEI	2614	1375.789	3004	1602.105	2452	1290.526	1422.807	161.023
0.3	454	238.947	630	331.579	450	236.842	269.123	54.099
0.4	8042	4232.632	7898	4156.842	7067	3719.474	4036.316	276.998
0.5	8684	4570.526	8432	4437.895	7475	3934.211	4314.211	335.705
0.75	3638	1914.737	3757	1977.368	3117	1640.526	1844.211	179.154
0.9	3420	1800.000	3378	1777.895	2997	1577.368	1718.411	122.654
1	2898	1525.263	2785	1465.789	2389	1257.368	1416.140	140.679
1.5	1016	534.737	1126	592.632	991	521.579	549.649	37.801

1.1.2.2 EPC:NaT at the molar ratio of 10:1, 10:1.5 and 10:2

Table 29 Effect of type of anionic surfactants and lipid surfactant ratio on transfection efficiency of PEI-coated liposomes (EPC:NaT = 10:1) in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
Lipofectamine	1024	538.947	1012	532.632	989	520.526	530.702	9.361
PEI	2436	1282.105	2594	1365.263	2245	1181.579	1276.316	91.979
0.3	138	72.632	81	42.632	112	58.947	58.070	15.019
0.4	368	193.684	327	172.105	331	174.211	180.000	11.898
0.5	2344	1233.684	2390	1257.895	2224	1170.526	1220.702	45.108
0.75	2676	1408.421	3238	1704.211	2784	1465.263	1525.965	156.960
0.9	1246	655.789	1286	676.842	1224	644.211	658.947	16.543
1	1156	608.421	1198	630.526	1120	589.474	609.474	20.547
1.5	424	223.158	392	206.316	376	197.895	209.123	12.863

Table 30 Effect of type of anionic surfactants and lipid surfactant ratio on transfection efficiency of PEI-coated liposomes (EPC:NaT = 10:1.5) in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
Lipofectamine	1024	538.947	1012	532.632	989	520.526	530.702	9.361
PEI	2436	1282.105	2594	1365.263	2245	1181.579	1276.316	91.979
0.3	207	108.947	182	95.789	196	103.158	102.632	6.595
0.4	398	209.474	444	233.684	412	216.842	220.000	12.410
0.5	1368	720.000	1404	738.947	1256	661.053	706.667	40.623
0.75	4168	2193.684	4022	2116.842	4108	2162.105	2157.544	38.624
0.9	1868	983.158	1782	937.895	1659	1382.500	1101.184	244.676
1	1602	843.158	1558	820.000	1498	1248.333	970.497	240.892
1.5	720	378.947	690	363.158	688	573.333	438.480	117.053

Table 31 Effect of type of anionic surfactants and lipid surfactant ratio on transfection efficiency of PEI-coated liposomes (EPC:NaT = 10:2) in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
Lipofectamine	1024	538.947	1012	532.632	989	520.526	530.702	9.361
PEI	2436	1282.105	2594	1365.263	2245	1181.579	1276.316	91.979
0.3	105	55.263	69	36.316	112	58.947	50.175	12.143
0.4	331	174.211	328	172.632	298	156.842	167.895	9.604
0.5	1006	529.474	998	525.263	982	516.842	523.860	6.432
0.75	1506	792.632	1398	735.789	1446	761.053	763.158	28.479
0.9	1196	629.474	1122	590.526	1088	572.632	597.544	29.064
1	968	509.474	870	457.895	952	501.053	489.474	27.670
1.5	716	376.842	688	362.105	688	362.105	367.018	8.508

1.1.2.3 EPC:CHAPS at the molar ratio of 10:1, 10:1.5 and 10:2

Table 32 Effect of type of anionic surfactants and lipid surfactant ratio on transfection efficiency of PEI-coated liposomes (EPC:CHAPS = 10:1) in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
Lipofectamine	1024	538.947	1012	532.632	1138	598.947	556.842	36.601
PEI	2384	1254.737	2287	1203.684	2614	1375.789	1278.070	88.393
0.3	478	251.579	454	238.947	421	221.579	237.368	15.062
0.4	671	353.158	645	339.474	597	314.211	335.614	19.758
0.5	808	425.263	884	465.263	788	414.737	435.088	26.657
0.75	3100	1631.579	3506	1845.263	3290	1731.579	1736.140	106.915
0.9	2856	1503.158	2758	1451.579	2768	1456.842	1470.526	28.382
1	710	373.684	687	361.579	698	367.368	367.544	6.055
1.5	704	370.526	654	344.211	688	362.105	358.947	13.439

Table 33 Effect of type of anionic surfactants and lipid surfactant ratio on transfection efficiency of PEI-coated liposomes (EPC:CHAPS = 10:1.5) in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	Cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
Lipofectamine	1024	538.947	1012	532.632	1138	598.947	556.842	36.601
PEI	2384	1254.737	2287	1203.684	2614	1375.789	1278.070	88.393
0.3	284	149.474	354	186.316	312	164.211	166.667	18.543
0.4	426	224.211	508	267.368	498	262.105	251.228	23.545
0.5	3682	1937.895	3587	1887.895	3522	1853.684	1893.158	42.351
0.75	3048	1604.211	2938	1546.316	3001	1579.474	1576.667	29.049
0.9	2812	1480.000	2789	1467.895	2448	1288.421	1412.105	107.285
1	2228	1172.632	2119	1115.263	2012	1058.947	1115.614	56.843
1.5	1142	601.053	1388	730.526	1222	643.158	658.246	66.042

Table 34 Effect of type of anionic surfactants and lipid surfactant ratio on transfection efficiency of PEI-coated liposomes (EPC:CHAPS = 10:2) in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	Cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
Lipofectamine	1024	538.947	1012	532.632	1138	598.947	556.842	36.601
PEI	2384	1254.737	2287	1203.684	2614	1375.789	1278.070	88.393
0.3	218	114.737	291	153.158	265	139.474	135.789	19.474
0.4	628	330.526	657	345.789	578	304.211	326.842	21.033
0.5	1162	611.579	1121	590.000	1098	577.895	593.158	17.063
0.75	4514	2375.789	4478	2356.842	4234	2228.421	2320.351	80.175
0.9	3362	1769.474	3245	1707.895	3094	1628.421	1701.930	70.715
1	2306	1213.684	2276	1197.895	2087	1098.421	1170.000	62.490
1.5	768	404.211	1268	667.368	978	514.737	528.772	132.139

1.1.3 Effect of weight ratio

Table 35 Effect of weight ratio on transfection efficiency of PEI-coated liposomes (EPC:NaO = 10:2) in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
Lipofectamine	1004	528.421	1056	555.789	1077	566.842	530.531	19.779
PEI	2614	1375.789	3004	1602.105	2452	1290.526	1422.807	161.023
0.3	454	238.947	630	331.579	450	236.842	269.123	54.099
0.4	8042	4232.632	7898	4156.842	7067	3719.474	4036.316	276.998
0.5	8684	4570.526	8432	4437.895	7475	3934.211	4314.211	335.705
0.75	3638	1914.737	3757	1977.368	3117	1640.526	1844.211	179.154
0.9	3420	1800.000	3378	1777.895	2997	1577.368	1718.411	122.654
1	2898	1525.263	2785	1465.789	2389	1257.368	1416.140	140.679
1.5	1016	534.737	1126	592.632	991	521.579	549.649	37.801

Table 36 Effect of weight ratio on transfection efficiency of PEI-coated liposomes (EPC:CHAPS = 10:2) in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
Lipofectamine	1024	538.947	1012	532.632	1138	598.947	556.842	36.601
PEI	2384	1254.737	2287	1203.684	2614	1375.789	1278.070	88.393
0.3	218	114.737	291	153.158	265	139.474	135.789	19.474
0.4	628	330.526	657	345.789	578	304.211	326.842	21.033
0.5	1162	611.579	1121	590.000	1098	577.895	593.158	17.063
0.75	4514	2375.789	4478	2356.842	4234	2228.421	2320.351	80.175
0.9	3362	1769.474	3245	1707.895	3094	1628.421	1701.930	70.715
1	2306	1213.684	2276	1197.895	2087	1098.421	1170.000	62.490
1.5	768	404.211	1268	667.368	978	514.737	528.772	132.139

Table 37 Effect of weight ratio on transfection efficiency of PEI-coated liposomes (EPC:NaT = 10:1.5) in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
Lipofectamine	1024	538.947	1012	532.632	989	520.526	530.702	9.361
PEI	2436	1282.105	2594	1365.263	2245	1181.579	1276.316	91.979
0.3	207	108.947	182	95.789	196	103.158	102.632	6.595
0.4	398	209.474	444	233.684	412	216.842	220.000	12.410
0.5	1368	720.000	1404	738.947	1256	661.053	706.667	40.623
0.75	4168	2193.684	4022	2116.842	4108	2162.105	2157.544	38.624
0.9	1868	983.158	1782	937.895	1659	1382.500	1101.184	244.676
1	1602	843.158	1558	820.000	1498	1248.333	970.497	240.892
1.5	720	378.947	690	363.158	688	573.333	438.480	117.053

1.1.4 Effect of pH

Table 38 Effect of pH on transfection efficiency of PEI-coated liposomes (EPC:NaO = 10:2) in medium without serum, pH 6.2.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	204	107.368	283	148.947	245	128.947	128.421	20.794

Table 39 Effect of pH on transfection efficiency of PEI-coated liposomes (EPC:CHAPS = 10:2) in medium without serum, pH 6.2.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	56	29.474	62	32.632	58	30.526	30.877	1.608

Table 40 Effect of pH on transfection efficiency of PEI-coated liposomes (EPC:NaT = 10:1.5) in medium without serum, pH 6.2.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	418	220.000	330	173.684	378	198.947	197.544	23.190

Table 41 Effect of pH on transfection efficiency of PEI in medium without serum, pH 6.2.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	Cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0.000	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0.000	0.000	0.000	0.000
0.5	15	7.895	18	9.474	16	8.421	8.596	0.804

Table 42 Effect of pH on transfection efficiency of Lipofectamine 2000™ in medium without serum, pH 6.2.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0.000	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0.000	0.000	0.000	0.000
Lipofectamine	195	102.632	235	123.684	204	107.368	111.228	11.044

1.1.5 Effect of serum

Table 43 Effect of serum on transfection efficiency of PEI-coated liposomes (EPC:NaO = 10:2) in medium with serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	Cell/well	cell/cm^2	cell/well	cell/cm^2	Cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	1250	657.895	1365	718.421	1098	577.895	651.404	70.488

Table 44 Effect of serum on transfection efficiency of PEI-coated liposomes (EPC:CHAPS = 10:2) in medium with serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	Cell/well	cell/cm^2	cell/well	cell/cm^2	Cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	4374	2302.105	4570	2405.263	4222	2222.105	2309.825	91.823

Table 45 Effect of serum on transfection efficiency of PEI-coated liposomes (EPC:NaT = 10:1.5) in medium with serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	Cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	1292	680.000	1212	637.895	1121	590.000	635.965	45.031

Table 46 Effect of serum on transfection efficiency of PEI in medium with serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	2532	1332.632	2235	1176.316	2208	1162.105	1223.684	94.618

Table 47 Effect of serum on transfection efficiency of Lipofectamine2000™ in medium with serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	1004	528.421	1056	555.789	1178	620.000	568.070	47.008

1.2 Cationic liposomes

Table 48 Transfection efficiency of cationic liposomes (EPC:Chol:SA = 10:2:1) in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
Lipofectamine	1177	619.474	1021	537.368	1088	572.632	576.491	41.188
PEI	2143	1127.895	1949	1025.789	2342	1232.632	1128.772	103.424
2.5	0	0.000	0	0.000	0	0.000	0.000	0.000
5	0	0.000	0	0.000	0	0.000	0.000	0.000
7.5	0	0.000	0	0.000	0	0.000	0.000	0.000
10	0	0.000	0	0.000	0	0.000	0.000	0.000
20	0	0.000	0	0.000	0	0.000	0.000	0.000
30	0	0.000	0	0.000	0	0.000	0.000	0.000

Table 49 Transfection efficiency of cationic liposomes (EPC:Chol:SA = 10:2:2) in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
Lipofectamine	1177	619.474	1021	537.368	1088	572.632	576.491	41.188
PEI	2143	1127.895	1949	1025.789	2342	1232.632	1128.772	103.424
2.5	0	0.000	0	0.000	0	0.000	0.000	0.000
5	11	5.789	11	5.789	12	6.316	5.965	0.304
7.5	52	27.368	52	27.368	54	28.421	27.719	0.608
10	158	83.158	161	84.737	160	84.211	84.035	0.804
20	58	30.526	96	50.526	88	46.316	42.456	10.544
30	6	3.158	3	1.579	5	2.632	2.456	0.804

Table 50 Transfection efficiency of cationic liposomes (EPC:Chol:SA = 10:2:3) in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
Lipofectamine	1177	619.474	1021	537.368	1088	572.632	576.491	41.188
PEI	2143	1127.895	1949	1025.789	2342	1232.632	1128.772	103.424
2.5	0	0.000	3	1.579	3	1.579	1.053	0.912
5	261	137.368	347	182.632	326	171.579	163.860	23.598
7.5	274	144.211	382	201.053	328	172.632	172.632	28.421
10	117	61.579	116	61.053	110	57.895	60.175	1.993
20	15	7.895	19	10.000	18	9.474	9.123	1.096
30	0	0.000	1	0.526	1	0.526	0.351	0.304

Table 51 Transfection efficiency of cationic liposomes (EPC:Chol:SA = 10:2:4) in medium without serum, pH 7.4.

Samples	Transfection efficiency (cell/cm^2)						AVG (cell/cm^2)	SD
	1		2		3			
	cell/well	cell/cm^2	cell/well	cell/cm^2	cell/well	cell/cm^2		
Cell	0	0.000	0	0.000	0	0.000	0.000	0.000
DNA	0	0.000	0	0.000	0	0.000	0.000	0.000
Lipofectamine	1177	619.474	1021	537.368	1088	572.632	576.491	41.188
PEI	2143	1127.895	1949	1025.789	2342	1232.632	1128.772	103.424
2.5	2	1.053	1	0.526	2	1.053	0.877	0.304
5	449	236.316	350	184.211	398	209.474	210.000	26.057
7.5	129	67.895	123	67.737	132	69.474	67.368	2.412
10	87	45.789	37	19.474	68	35.789	33.684	13.284
20	21	11.053	14	7.368	18	9.474	9.298	1.848
30	2	1.053	0	0.000	1	0.526	0.526	0.526

APPENDIX IV

Cytotoxicity

1. Cytotoxicity of carriers

Table 52 Cytotoxicity of PEI-coated liposomes (EPC:NaO = 10:1) on Huh7 cells.

n	Weight of PEI-coated liposomes (EPC:NaO=10:1) (μg)					
	0.001	0.01	0.1	1	10	100
1	51.890	42.899	58.569	60.110	52.404	47.266
2	66.532	71.413	72.440	70.899	41.872	46.239
3	72.183	71.156	80.661	65.761	51.119	45.211
4	74.239	74.495	75.780	62.679	49.064	48.037
5	73.468	72.697	63.193	72.183	54.202	48.294
6	80.661	82.972	84.514	85.284	59.853	47.266
7	81.431	83.229	92.734	76.550	55.743	152.844
8	88.624	85.798	91.706	112.257	57.028	386.862
AVG	78.434	77.394	72.526	70.495	52.661	47.052
SD	6.310	6.337	10.024	8.658	5.535	1.154

Table 53 Cytotoxicity of PEI-coated liposomes (EPC:NaO = 10:1.5) on Huh7 cells.

n	Weight of PEI-coated liposomes (EPC:NaO=10:1.5) (μg)					
	0.001	0.01	0.1	1	10	100
1	64.734	77.321	75.780	66.275	50.349	47.523
2	71.670	79.633	84.257	80.147	41.872	47.780
3	72.440	79.633	66.532	72.954	45.468	42.642
4	81.174	78.862	72.697	59.596	44.440	41.615
5	85.798	81.174	64.734	77.835	48.294	42.385
6	91.193	78.862	82.716	69.615	54.459	46.495
7	75.780	86.569	88.110	88.367	47.009	35.450
8	85.798	85.541	87.339	99.924	49.578	23.633
AVG	82.031	79.248	74.453	71.070	47.683	44.740
SD	6.987	1.266	8.080	7.595	3.903	2.821

Table 54 Cytotoxicity of PEI-coated liposomes (EPC:NaO = 10:2) on Huh7 cells.

n	Weight of PEI-coated liposomes (EPC:NaO=10:2) (μg)					
	0.001	0.01	0.1	1	10	100
1	98.822	97.609	81.561	60.676	53.421	40.890
2	100.997	73.647	87.497	68.590	49.904	38.692
3	98.580	96.950	87.561	74.086	46.167	48.585
4	101.238	107.502	92.993	70.569	44.847	42.649
5	97.856	107.722	86.617	89.035	49.684	45.507
6	99.547	100.687	98.928	97.389	52.542	48.585
7	101.722	151.690	156.307	119.813	51.443	49.244
8	118.152	172.355	160.264	124.869	54.520	58.038
AVG	99.698	97.353	88.193	76.724	50.316	44.151
SD	8.308	12.521	6.771	13.763	3.407	4.095

Table 55 Cytotoxicity of PEI-coated liposomes (EPC:CHAPS = 10:1) on Huh7 cells.

n	Weight of PEI-coated liposomes (EPC:CHAPS=10:1) (μg)					
	0.001	0.01	0.1	1	10	100
1	92.298	86.983	103.654	70.553	60.646	57.022
2	101.238	100.272	107.037	89.882	55.572	57.022
3	101.480	97.856	98.097	88.674	61.613	53.639
4	103.171	104.621	78.043	70.069	59.921	52.190
5	105.346	113.319	86.258	95.681	64.029	56.780
6	108.487	108.245	98.580	108.970	65.237	58.713
7	129.266	107.520	95.439	100.272	65.479	58.472
8	108.245	108.970	90.124	111.870	69.103	61.371
AVG	100.707	97.433	97.433	79.795	60.356	55.331
SD	4.979	7.508	7.508	10.964	3.091	2.267

Table 56 Cytotoxicity of PEI-coated liposomes (EPC:CHAPS = 10:1.5) on Huh7 cells.

n	Weight of PEI-coated liposomes (EPC:CHAPS=10:1.5) (μg)					
	0.001	0.01	0.1	1	10	100
1	101.238	99.305	81.184	79.976	62.096	55.089
2	101.963	93.990	88.916	86.741	53.398	56.297
3	99.064	96.648	90.607	86.500	59.438	51.223
4	99.064	94.956	93.265	70.069	55.089	49.532
5	105.829	100.272	95.439	86.500	62.096	56.297
6	108.487	109.937	94.231	105.346	62.338	56.780
7	109.453	117.668	100.997	100.030	66.928	57.989
8	105.104	101.963	101.238	104.138	67.895	58.230
AVG	100.332	97.034	90.607	81.957	59.076	53.035
SD	1.494	2.710	1.090	7.235	3.928	3.184

Table 57 Cytotoxicity of PEI-coated liposomes (EPC:CHAPS = 10:2) on Huh7 cells.

n	Weight of PEI-coated liposomes (EPC:CHAPS=10:2) (μg)					
	0.001	0.01	0.1	1	10	100
1	98.822	115.735	106.554	73.694	69.828	46.874
2	100.997	108.245	98.339	80.942	59.921	47.599
3	98.580	93.506	107.037	74.660	56.056	51.706
4	101.238	106.796	78.768	79.493	62.096	51.465
5	97.856	85.050	85.533	76.835	66.445	46.874
6	99.547	85.775	84.083	86.500	56.780	40.109
7	101.722	82.875	79.009	76.110	52.915	47.841
8	118.152	86.500	78.768	82.150	59.680	45.183
AVG	99.823	92.678	81.232	77.698	60.465	47.206
SD	1.499	10.665	3.306	3.217	5.568	3.657

Table 58 Cytotoxicity of PEI-coated liposomes (EPC:NaT = 10:1) on Huh7 cells.

n	Weight of PEI-coated liposomes (EPC:NaT = 10:1) (μg)					
	0.001	0.01	0.1	1	10	100
1	98.580	78.768	66.204	70.069	57.264	52.190
2	93.023	91.547	72.486	85.050	49.290	51.465
3	83.117	97.372	75.143	88.191	56.780	48.565
4	89.882	94.231	79.976	87.708	58.718	46.874
5	93.990	88.191	78.285	80.942	61.613	53.639
6	108.245	111.628	97.614	87.708	65.962	54.123
7	102.446	109.212	85.533	101.238	58.955	55.814
8	73.935	106.796	87.224	114.769	60.616	59.680
AVG	91.719	90.027	85.726	83.278	61.718	50.547
SD	5.729	7.144	7.617	7.021	2.931	2.762

Table 59 Cytotoxicity of PEI-coated liposomes (EPC:NaT = 10:1.5) on Huh7 cells.

n	Weight of PEI-coated liposomes (EPC:NaT = 10:1.5) (μg)					
	0.001	0.01	0.1	1	10	100
1	102.688	92.540	97.856	80.217	56.539	46.391
2	101.480	94.473	92.298	79.009	56.539	52.915
3	95.439	94.956	88.432	80.701	51.465	51.948
4	98.097	106.312	88.674	75.143	50.740	46.632
5	103.896	97.614	89.641	79.251	55.089	44.699
6	100.272	99.789	97.614	81.909	58.713	45.183
7	99.064	94.956	97.782	94.956	55.814	50.740
8	100.755	95.198	86.016	91.090	59.921	52.673
AVG	100.211	96.980	91.664	79.372	55.603	48.898
SD	2.677	4.355	4.322	2.321	3.188	3.504

Table 60 Cytotoxicity of PEI-coated liposomes (EPC:NaT = 10:2) on Huh7 cells.

n	Weight of PEI-coated liposomes (EPC:NaT = 10:2) (μg)					
	0.001	0.01	0.1	1	10	100
1	95.439	90.365	85.775	79.009	56.539	48.324
2	91.574	94.473	86.016	82.634	50.498	48.807
3	91.090	87.708	84.567	83.600	52.673	44.216
4	90.365	88.816	82.392	70.794	51.465	43.975
5	97.614	97.131	96.164	83.600	55.089	48.324
6	95.681	91.815	94.231	84.083	53.156	49.532
7	99.547	96.164	81.184	95.923	55.089	51.465
8	102.205	95.439	85.291	101.480	58.472	52.673
AVG	94.473	92.751	86.953	80.620	54.123	46.729
SD	3.529	3.540	5.380	5.157	2.669	2.414

Table 61 Cytotoxicity of PEI on Huh7 cells.

n	Weight of PEI (μg)					
	0.001	0.01	0.1	1	10	100
1	72.954	65.761	65.761	66.018	47.780	43.413
2	70.899	66.275	73.468	64.220	44.183	45.468
3	70.899	67.560	72.183	62.679	41.101	45.211
4	66.532	68.587	72.183	66.532	43.413	40.073
5	69.872	69.872	73.725	64.477	42.642	36.220
6	72.679	67.046	59.083	67.560	44.697	46.495
7	66.275	73.211	78.862	61.394	39.560	49.064
8	69.358	71.927	86.055	57.798	38.532	34.936
AVG	70.642	69.700	69.401	65.248	43.969	42.813
SD	2.332	2.457	5.838	1.780	2.254	3.942

Table 62 Cytotoxicity of TM_{69}-Py_{62}-CS coated liposomes (EPC:NaO = 10:2) on Huh7 cells.

n	Weight of TM_{69}-Py_{62}-CS -coated liposomes (EPC:NaO = 10:2) (μg)					
	0.001	0.01	0.1	1	10	100
1	100.295	91.178	75.306	74.293	70.916	49.304
2	100.633	73.618	88.476	71.929	60.110	49.641
3	100.295	73.955	80.371	84.424	67.201	46.940
4	100.971	76.319	69.228	83.073	66.188	45.927
5	100.971	77.670	74.968	72.942	67.201	52.005
6	99.958	79.358	67.201	75.306	62.474	54.369
7	101.646	85.775	87.801	81.385	52.005	52.005
8	98.269	83.073	73.955	71.591	49.304	43.900
AVG	100.380	80.118	77.163	76.868	60.640	49.261
SD	1.001	6.144	7.858	5.247	7.344	3.516

Table 63 Cytotoxicity of cationic liposomes (EPC:Chol:SA = 10:2:1) on Huh7 cells.

n	Weight of cationic liposomes (EPC:Chol:SA = 10:2:1) (μg)					
	0.01	0.1	1	10	100	1000
1	76.822	72.281	72.281	62.111	51.578	41.771
2	79.183	83.905	83.360	68.468	51.033	40.136
3	79.364	82.633	64.109	68.286	50.670	39.773
4	80.999	80.454	78.456	65.562	49.035	38.502
5	90.806	91.169	81.544	75.551	51.033	38.320
6	80.636	83.905	88.263	62.656	52.667	42.134
7	94.620	80.454	64.472	62.293	70.647	43.587
8	98.797	72.100	66.652	80.272	74.642	42.134
AVG	89.171	80.863	74.892	66.418	51.003	40.795
SD	8.134	6.309	9.312	4.857	1.190	1.897

Table 64 Cytotoxicity of cationic liposomes (EPC:Chol:SA = 10:2:2) on Huh7 cells.

n	Weight of cationic liposomes (EPC:Chol:SA = 10:2:2) (μg)					
	0.01	0.1	1	10	100	1000
1	66.470	76.822	82.452	63.564	42.679	31.237
2	78.638	83.360	86.810	63.746	45.766	41.044
3	77.730	76.640	78.638	77.548	42.316	35.596
4	80.454	92.804	80.999	68.831	38.138	39.047
5	86.266	82.452	78.275	75.551	49.943	39.773
6	87.719	93.167	95.346	84.268	48.672	34.143
7	91.532	83.541	100.613	98.615	20.341	20.522
8	100.431	84.449	99.160	98.615	21.975	36.504
AVG	89.280	84.154	83.753	72.251	44.586	36.807
SD	7.397	6.214	6.468	8.281	4.408	3.778

Table 65 Cytotoxicity of cationic liposomes (EPC:Chol:SA = 10:2:3) on Huh7 cells.

n	Weight of cationic liposomes (EPC:Chol:SA = 10:2:3) (μg)					
	0.01	0.1	1	10	100	1000
1	73.916	80.817	76.459	70.829	36.686	77.367
2	88.263	80.999	89.353	75.369	41.407	37.230
3	82.089	92.985	74.824	72.100	51.941	36.867
4	104.790	87.174	79.364	91.169	46.493	34.688
5	98.615	98.797	96.617	84.268	41.952	36.141
6	104.790	102.792	97.344	92.804	41.407	33.780
7	111.146	111.146	111.146	96.799	72.463	61.930
8	109.330	109.149	109.149	94.983	66.288	76.640
AVG	95.709	88.154	85.660	78.747	43.314	35.741
SD	10.178	7.788	10.119	8.705	5.246	1.466

Table 66 Cytotoxicity of cationic liposomes (EPC:Chol:SA = 10:2:4) on Huh7 cells.

n	Weight of cationic liposomes (EPC:Chol:SA = 10:2:4) (μg)					
	0.01	0.1	1	10	100	1000
1	94.620	86.084	80.091	77.003	48.490	41.771
2	87.900	96.981	77.548	83.541	53.757	46.493
3	86.266	94.257	89.353	80.454	47.037	36.686
4	98.070	87.719	90.624	80.636	47.219	41.589
5	100.795	89.353	79.364	84.449	46.856	44.313
6	106.606	92.985	93.893	81.362	54.665	43.768
7	106.606	90.443	80.091	76.822	40.499	40.136
8	104.972	86.447	81.725	81.725	42.497	40.499
AVG	93.530	90.533	84.086	80.749	47.628	41.907
SD	6.306	3.914	6.201	2.735	4.871	2.995

Calculation of toxicity value of polymer-coated liposomes and cationic liposomes on Huh 7 cells.

The toxicity values were expressed as IC_{50} (50% Inhibitory Concentration) The IC_{50} were calculated by using the following example : For Figure 27, the following calculations were performed

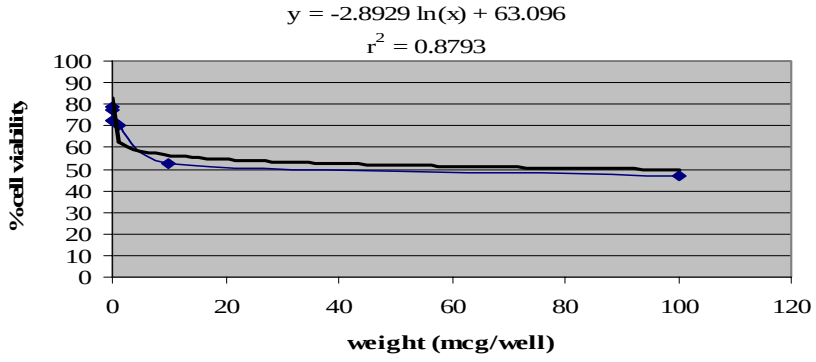
$$y = -2.8929\ln(x) + 63.096 \qquad (y = 50)$$

$$\ln(x) = (50-63.096)/-2.8929$$

$$x = e^{(50-63.096)/-2.8929}$$

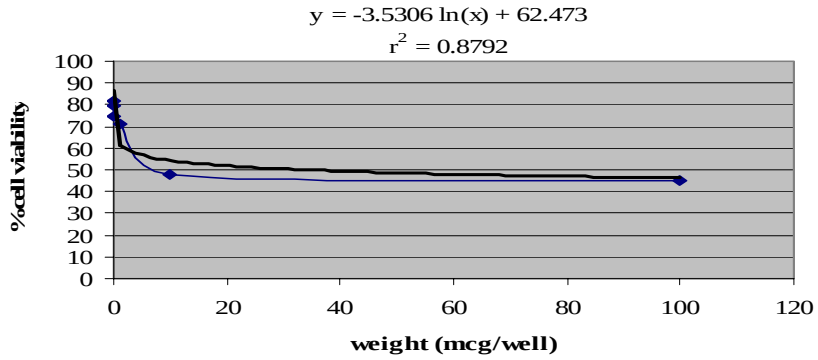
$$x = 92.4756\ \mu g/well = 92.4756\ \mu g/100\ \mu L$$

$\therefore IC_{50}$ of PEI-coated liposomes (EPC:NaO = 10:1) = 924.756 μg/mL



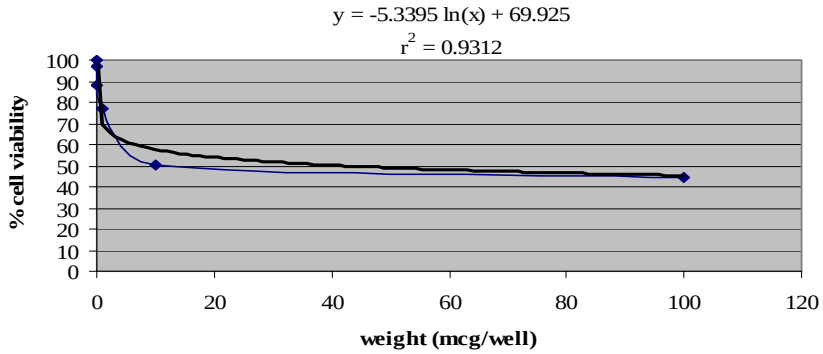
IC_{50} = 924.756 μg/mL

Figure 27 IC_{50} data of PEI-coated liposomes (EPC:NaO = 10:1) on Huh7 cells.



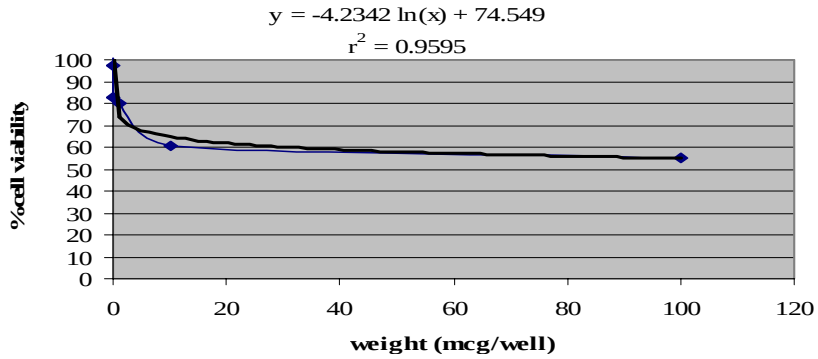
IC_{50} = 342.206 μg/mL

Figure 28 IC_{50} data of PEI-coated liposomes (EPC:NaO = 10:1.5) on Huh7 cells.



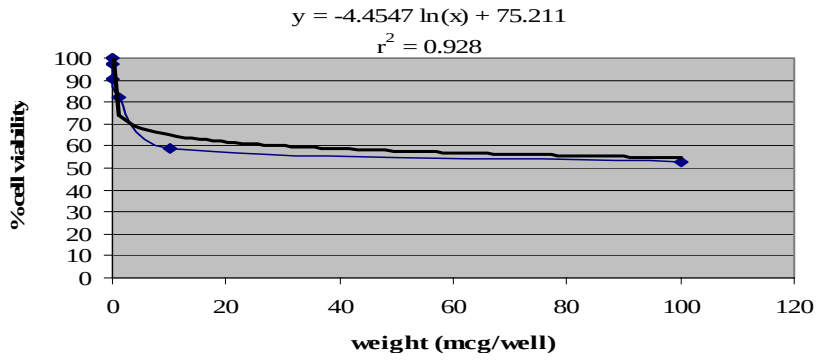
IC_{50} = 417.680 μg/mL

Figure 29 IC_{50} data of PEI-coated liposomes (EPC:NaO = 10:2) on Huh7 cells.



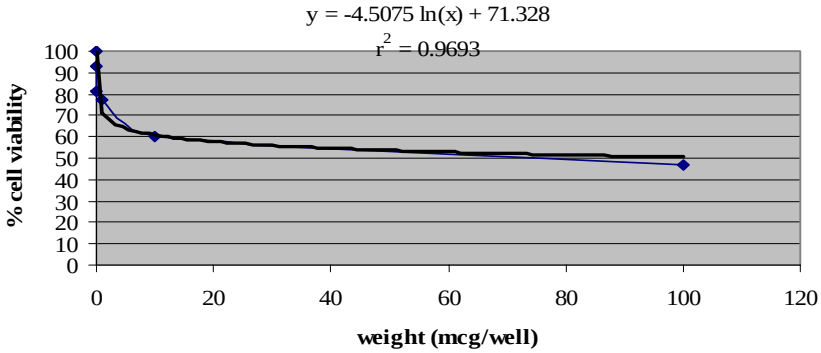
IC_{50} = 3295.702 μg/mL

Figure 30 IC_{50} data of PEI-coated liposomes (EPC:CHAPS = 10:1) on Huh7 cells.



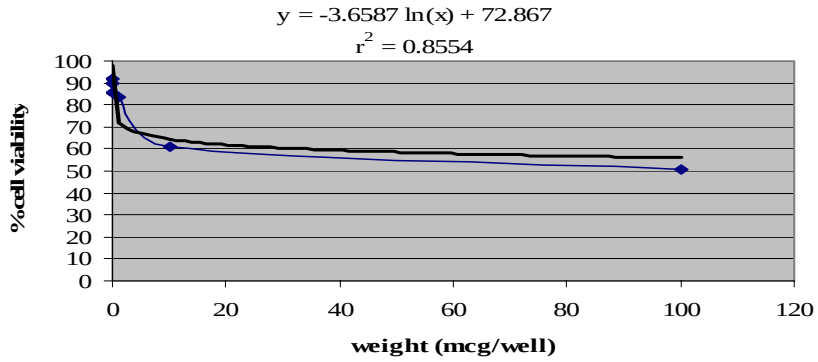
IC_{50} = 2869.810 μg/mL

Figure 31 IC_{50} data of PEI-coated liposomes (EPC:CHAPS = 10:1.5) on Huh7 cells.



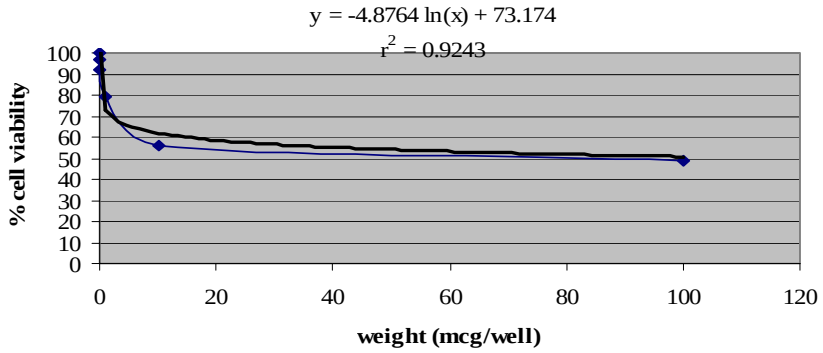
IC_{50} = 1134.849 μg/mL

Figure 32 IC_{50} data of PEI-coated liposomes (EPC:CHAPS = 10:2) on Huh7 cells.



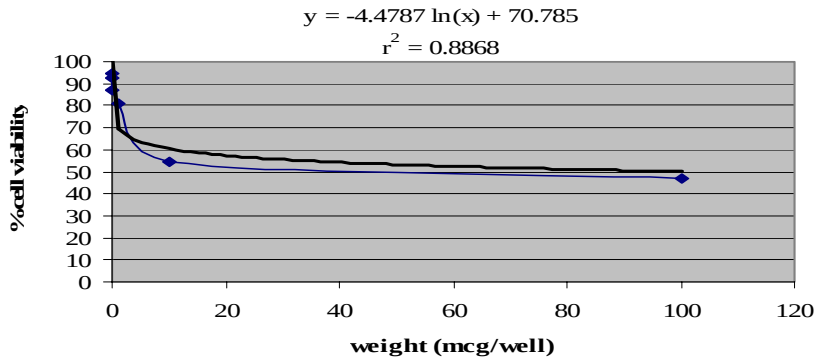
IC_{50} = 5180.305 μg/mL

Figure 33 IC_{50} data of PEI-coated liposomes (EPC:NaT = 10:1) on Huh7 cells.



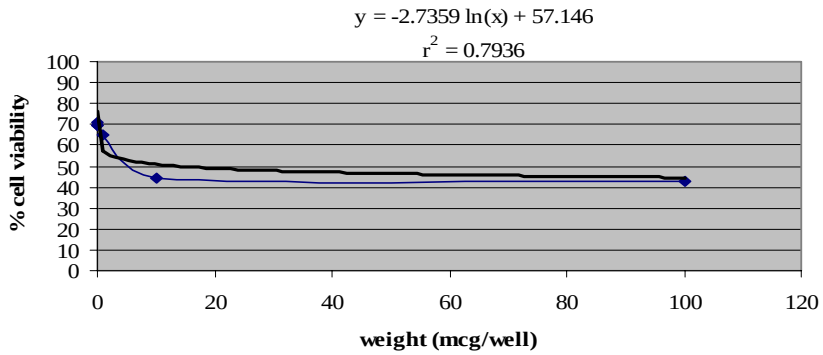
IC_{50} = 1158.477 μg/mL

Figure 34 IC_{50} data of PEI-coated liposomes (EPC:NaT = 10:1.5) on Huh7 cells.



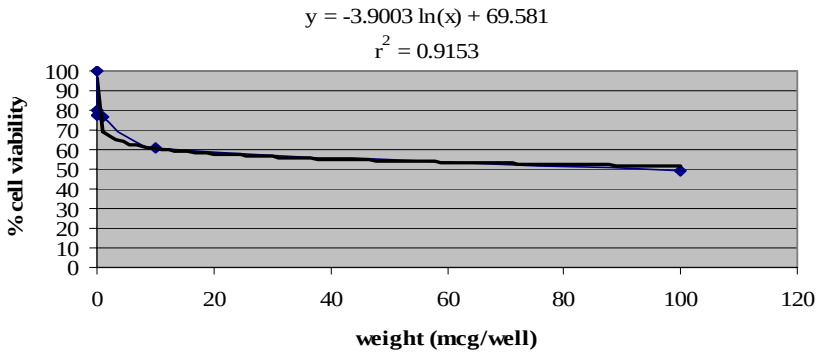
IC_{50} = 1036.330 μg/mL

Figure 35 IC_{50} data of PEI-coated liposomes (EPC:NaT = 10:2) on Huh7 cells.



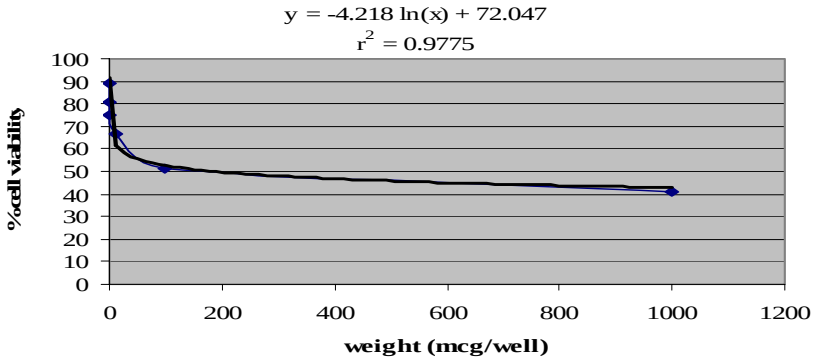
IC_{50} = 136.254 μg/mL

Figure 36 IC_{50} data of PEI on Huh7 cells.



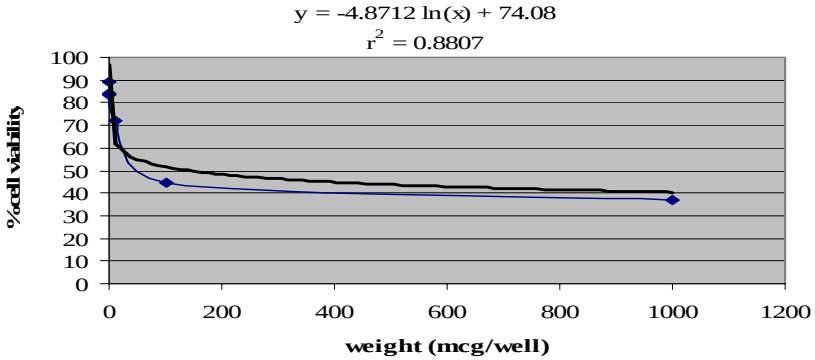
IC_{50} = 1514.693 μg/mL

Figure 37 IC_{50} data of TM_{69}-Py_{62}-CS-coated liposomes (EPC:NaO = 10:2) on Huh7 cells.



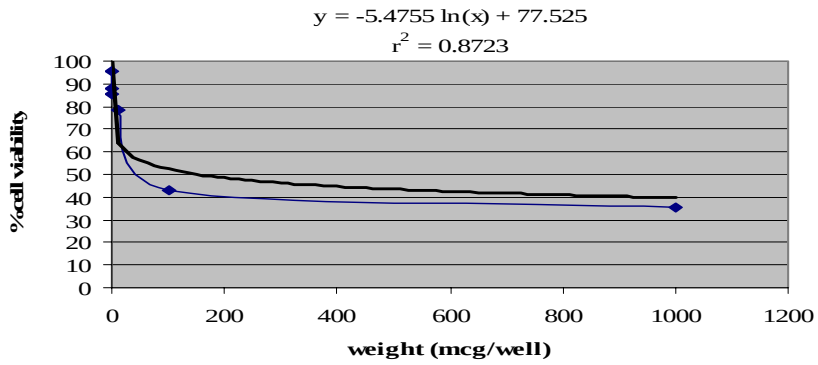
IC_{50} = 1862.141 μg/mL

Figure 38 IC_{50} data of cationic liposomes (EPC:Chol:SA = 10:2:1) on Huh7 cells.



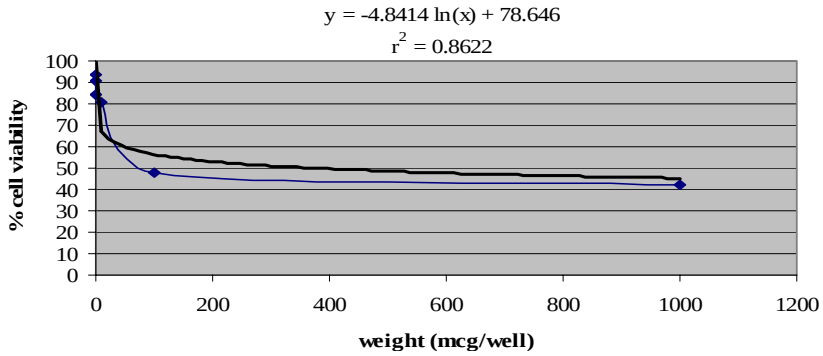
IC_{50} = 1402.379 μg/mL

Figure 39 IC_{50} data of cationic liposomes (EPC:Chol:SA = 10:2:2) on Huh7 cells.



IC_{50} = 1524.655 μg/mL

Figure 40 IC_{50} data of cationic liposomes (EPC:Chol:SA = 10:2:3) on Huh7 cells.



IC_{50} = 3712.529 μg/mL

Figure 41 IC_{50} data of cationic liposomes (EPC:Chol:SA = 10:2:4) on Huh7 cells.

2. Effect of carriers/DNA complexes on cell viability

Table 67 Cytotoxicity of PEI-coated liposomes (EPC:NaO = 10:1)/DNA complexes on Huh7 cells.

n	Weight ratio							
	control	0.3	0.4	0.5	0.75	0.9	1	1.5
1	117.497	78.424	73.279	71.054	91.078	112.630	110.684	74.392
2	94.693	79.954	85.794	84.681	92.885	104.983	82.735	70.081
3	97.335	83.847	74.114	86.767	95.110	96.362	79.954	65.771
4	87.323	86.906	82.317	76.755	91.773	101.506	85.933	75.365
5	106.512	90.243	73.696	80.510	91.217	94.276	86.072	78.980
6	96.640	86.072	76.338	85.655	91.773	104.009	82.317	72.862
7	165.052	89.409	72.167	88.992	99.838	96.501	77.451	72.723
8	333.581	90.521	72.723	104.009	100.255	101.784	77.868	73.557
AVG	100.000	85.672	77.590	80.904	92.306	99.073	81.761	72.966
SD	10.543	4.608	5.235	6.092	1.514	3.863	3.518	3.863

Table 68 Cytotoxicity of PEI-coated liposomes (EPC:NaO = 10:1.5)/DNA complexes on Huh7 cells.

n	Weight ratio							
	control	0.3	0.4	0.5	0.75	0.9	1	1.5
1	117.497	77.590	82.178	77.590	79.119	79.119	86.767	65.492
2	94.693	84.820	83.152	81.483	81.900	76.616	89.270	73.001
3	97.335	80.927	76.477	80.927	83.847	81.622	75.365	76.338
4	87.323	82.178	79.119	73.279	80.927	82.596	72.306	79.537
5	106.512	88.992	88.297	80.649	81.622	79.119	74.253	76.756
6	96.640	83.152	81.622	80.510	81.483	83.569	82.457	69.108
7	165.052	81.344	87.184	92.746	87.045	82.596	77.034	68.969
8	333.581	88.158	88.575	99.838	89.548	83.291	79.676	74.809
AVG	100.000	84.224	83.326	79.073	81.483	81.066	76.848	73.001
SD	10.543	0.023	4.408	3.148	1.528	2.495	3.716	4.766

Table 69 Cytotoxicity of PEI-coated liposomes (EPC:NaO = 10:2)/DNA complexes on Huh7 cells.

n	Weight ratio							
	control	0.3	0.4	0.5	0.75	0.9	1	1.5
1	117.497	85.516	87.462	86.906	90.382	82.596	103.453	81.622
2	94.693	86.628	81.344	88.436	87.879	75.782	95.110	65.214
3	97.335	79.397	69.108	87.462	85.794	87.323	82.596	74.809
4	87.323	77.868	77.034	81.483	87.184	82.735	90.660	79.815
5	106.512	84.125	84.820	90.660	87.462	78.980	79.954	79.676
6	96.640	85.794	81.205	85.794	88.436	83.152	84.125	78.841
7	165.052	90.382	87.323	91.912	87.462	78.841	69.942	61.212
8	333.581	88.436	86.072	103.175	88.158	84.403	76.338	61.322
AVG	100.000	84.768	81.796	86.790	87.482	81.727	80.603	76.663
SD	10.543	4.258	6.260	3.076	0.863	3.655	7.068	6.047

Table 70 Cytotoxicity of PEI-coated liposomes (EPC:CHAPS = 10:1)/DNA complexes on Huh7 cells.

n	Weight ratio							
	control	0.3	0.4	0.5	0.75	0.9	1	1.5
1	96.700	82.155	105.859	99.125	92.660	108.822	90.774	88.620
2	98.586	108.283	96.970	94.276	90.774	96.162	86.734	80.808
3	103.165	102.357	94.815	96.431	96.970	94.815	83.771	74.882
4	95.354	100.741	97.778	86.734	89.428	78.384	75.152	60.606
5	96.431	96.970	99.663	89.158	85.657	89.158	81.616	70.034
6	111.785	99.933	89.158	92.660	84.040	85.118	77.306	71.380
7	97.239	97.239	89.158	89.428	81.077	75.690	80.269	62.222
8	100.741	100.471	87.542	82.963	86.734	78.114	81.077	60.067
AVG	100.000	99.071	93.583	88.189	85.387	81.293	79.084	64.862
SD	5.402	1.821	4.885	3.602	3.106	5.622	2.757	5.415

Table 71 Cytotoxicity of PEI-coated liposomes (EPC:CHAPS = 10:1.5)/DNA complexes on Huh7 cells.

n	Weight ratio							
	control	0.3	0.4	0.5	0.75	0.9	1	1.5
1	96.700	83.822	84.039	83.822	80.782	76.221	74.267	68.838
2	98.586	98.806	87.731	116.178	82.953	83.388	76.221	75.136
3	103.165	95.331	89.034	82.736	92.942	75.136	79.696	74.050
4	95.354	93.160	100.326	90.771	88.382	83.605	68.404	84.908
5	96.431	92.942	88.599	90.771	83.822	80.565	80.999	71.010
6	111.785	90.337	92.291	90.337	91.205	82.302	84.908	80.565
7	97.239	91.422	91.640	83.388	78.610	82.085	74.701	69.707
8	100.741	96.417	86.645	84.691	89.868	79.696	77.524	67.535
AVG	100.000	92.856	90.038	87.991	84.777	80.968	77.307	74.745
SD	5.402	2.301	5.237	3.641	5.043	2.933	6.275	7.558

Table 72 Cytotoxicity of PEI-coated liposomes (EPC:CHAPS = 10:2)/DNA complexes on Huh7 cells.

n	Weight ratio							
	control	0.3	0.4	0.5	0.75	0.9	1	1.5
1	96.700	97.778	77.845	92.660	107.744	88.620	83.502	72.189
2	98.586	98.855	100.202	102.357	103.704	88.350	86.195	77.037
3	103.165	98.316	99.933	101.549	103.704	89.158	83.771	83.502
4	95.354	102.626	97.508	95.354	94.276	88.350	94.815	68.418
5	96.431	103.165	100.741	92.121	96.162	87.811	84.040	78.923
6	111.785	99.663	100.471	108.283	89.966	90.505	98.316	71.919
7	97.239	114.209	101.818	110.438	94.276	93.737	83.771	90.236
8	100.741	110.976	102.357	107.744	89.966	99.125	86.465	92.121
AVG	100.000	100.067	96.117	96.808	92.929	88.799	87.609	75.331
SD	5.402	2.284	9.026	4.862	2.812	0.943	5.719	5.511

Table 73 Cytotoxicity of PEI-coated liposomes (EPC:NaT = 10:1)/DNA complexes on Huh7 cells.

n	Weight ratio							
	control	0.3	0.4	0.5	0.75	0.9	1	1.5
1	88.810	87.383	85.421	80.963	94.160	76.505	81.676	84.173
2	87.740	92.020	83.638	82.211	90.415	79.893	72.225	79.180
3	103.611	90.593	85.778	75.078	93.981	82.746	83.460	77.040
4	97.013	80.428	80.250	89.523	79.715	64.021	71.333	74.721
5	101.115	84.530	85.065	90.058	76.861	75.078	74.008	69.371
6	107.713	69.550	88.988	67.945	82.033	85.956	90.058	71.690
7	106.286	65.626	91.663	66.875	86.670	80.785	91.306	71.155
8	107.713	71.511	86.491	66.875	75.078	78.645	88.810	68.836
AVG	100.000	86.991	84.030	83.567	80.071	77.954	76.540	71.155
SD	8.082	4.678	2.264	6.291	4.548	6.587	5.622	2.322

Table 74 Cytotoxicity of PEI-coated liposomes (EPC:NaT = 10:1.5)/DNA complexes on Huh7 cells.

n	Weight ratio							
	control	0.3	0.4	0.5	0.75	0.9	1	1.5
1	88.810	89.345	91.306	93.981	94.160	88.631	79.893	62.238
2	87.740	89.880	85.778	86.135	76.861	82.033	82.925	58.315
3	103.611	92.198	85.778	89.701	80.606	78.645	82.390	64.021
4	97.013	87.205	91.306	88.275	85.956	78.823	78.823	68.658
5	101.115	87.205	89.345	88.810	78.288	81.855	78.823	75.791
6	107.713	98.975	91.485	90.593	95.230	88.096	82.390	70.798
7	106.286	92.733	93.090	85.421	84.530	90.415	81.498	72.225
8	107.713	104.503	92.555	92.020	94.338	79.180	84.886	72.046
AVG	100.000	91.077	90.080	89.367	86.246	83.460	81.453	68.012
SD	8.082	4.099	2.871	2.865	7.512	4.844	2.137	5.928

Table 75 Cytotoxicity of PEI-coated liposomes (EPC:NaT = 10:2)/DNA complexes on Huh7 cells.

n	Weight ratio							
	control	0.3	0.4	0.5	0.75	0.9	1	1.5
1	88.810	88.453	90.415	87.561	89.523	83.638	79.001	62.238
2	87.740	90.771	90.236	86.670	82.033	76.683	76.505	58.315
3	103.611	86.135	86.135	81.676	77.396	75.256	76.078	64.021
4	97.013	83.281	84.708	80.071	78.110	74.721	74.721	68.658
5	101.115	84.530	84.173	81.855	80.428	78.828	72.760	76.861
6	107.713	85.956	81.676	79.536	93.090	80.963	92.733	72.581
7	106.286	85.956	84.351	76.326	92.555	85.956	83.638	66.161
8	107.713	87.561	85.065	81.676	90.415	75.435	81.855	66.340
AVG	100.000	86.580	83.995	81.922	81.498	78.868	75.613	66.897
SD	8.082	2.333	1.340	3.682	4.851	4.218	2.318	5.845

Table 76 Cytotoxicity of PEI/DNA complexes on Huh7 cells.

n	Weight ratio							
	control	0.3	0.4	0.5	0.75	0.9	1	1.5
1	117.497	71.611	80.232	72.028	90.939	78.007	81.761	54.786
2	94.693	71.194	69.247	83.013	88.297	77.868	72.584	62.294
3	97.335	79.954	66.605	76.756	72.862	79.397	59.791	59.096
4	87.323	71.750	73.140	81.205	79.537	76.060	67.578	47.694
5	106.512	79.258	77.451	68.969	73.279	75.643	66.744	69.664
6	96.640	77.451	79.119	72.445	71.750	77.868	68.273	67.022
7	165.052	86.906	83.430	101.645	75.782	71.611	62.572	62.155
8	333.581	86.628	81.761	112.213	86.767	77.034	60.209	57.706
AVG	100.000	75.203	74.299	75.736	76.663	76.686	64.195	60.052
SD	10.543	4.122	5.558	5.555	5.677	2.368	3.808	6.942

Table 77 Cytotoxicity of TM_{69}-Py_{62}-CS-coated liposomes (EPC:NaO = 10:2)/DNA complexes on Huh7 cells.

n	Weight ratio					
	control	0.5	1	2	4	8
1	97.932	88.138	90.165	82.735	78.008	76.657
2	96.581	102.322	88.476	80.371	83.411	78.345
3	97.932	91.178	74.293	80.371	85.775	74.293
4	94.555	85.437	81.385	81.385	82.060	74.631
5	95.568	89.489	81.385	86.112	78.345	78.008
6	108.062	94.555	89.489	86.450	83.411	89.489
7	102.659	100.633	92.866	80.034	87.463	82.735
8	106.712	87.125	86.788	80.709	83.073	83.411
AVG	100.000	92.360	85.606	82.271	82.693	79.696
SD	5.164	6.271	6.116	2.615	3.266	5.178

Table 78 Cytotoxicity of cationic liposomes (EPC:Chol:SA = 10:2:1)/DNA complexes on Huh7 cells.

n	Weight ratio						
	control	2.5	5	7.5	10	20	30
1	96.700	106.128	106.128	106.128	76.229	111.246	65.993
2	98.586	103.165	118.519	92.391	88.350	82.155	83.771
3	103.165	103.165	110.707	89.697	86.465	74.343	79.192
4	95.354	97.778	113.939	91.044	81.347	77.037	83.502
5	96.431	100.471	94.276	82.963	86.734	80.808	78.923
6	111.785	102.896	102.626	95.892	94.007	80.000	82.155
7	97.239	98.586	94.276	91.044	94.007	82.694	78.653
8	100.741	97.778	95.892	89.158	91.852	80.808	79.192
AVG	100.00	99.502	96.768	90.313	87.374	79.692	78.923
SD	5.402	2.193	3.979	3.921	6.219	2.981	5.635

Table 79 Cytotoxicity of cationic liposomes (EPC:Chol:SA = 10:2:2)/DNA complexes on Huh7 cells.

n	Weight ratio						
	control	2.5	5	7.5	10	20	30
1	96.700	113.939	92.660	114.209	86.465	82.694	87.273
2	98.586	107.744	97.508	93.737	92.929	90.774	91.582
3	103.165	107.205	97.508	100.741	95.623	91.313	87.273
4	95.354	98.586	95.354	83.232	99.125	95.623	86.465
5	96.431	86.734	98.586	102.357	86.465	89.966	88.350
6	111.785	99.933	109.091	93.737	92.660	98.047	87.811
7	97.239	86.734	113.670	126.869	102.896	89.428	105.859
8	100.741	97.778	100.471	120.135	102.357	89.697	104.781
AVG	100.00	100.040	98.451	98.002	92.211	91.403	88.126
SD	5.402	8.513	5.624	10.427	5.024	5.297	1.806

Table 80 Cytotoxicity of cationic liposomes (EPC:Chol:SA = 10:2:3)/DNA complexes on Huh7 cells.

n	Weight ratio						
	control	2.5	5	7.5	10	20	30
1	96.700	107.744	108.283	107.744	78.384	77.037	74.882
2	98.586	119.865	91.582	97.508	93.737	82.424	81.077
3	103.165	105.859	94.815	94.276	85.657	64.646	74.074
4	95.354	96.431	92.391	86.734	91.313	83.771	55.488
5	96.431	103.704	92.660	99.663	92.391	80.539	68.148
6	111.785	96.970	83.771	76.229	91.852	84.040	84.040
7	97.239	98.855	92.121	87.003	84.310	104.781	89.158
8	100.741	98.316	92.660	89.428	83.771	104.512	89.697
AVG	100.00	100.022	91.429	90.120	87.677	78.743	72.952
SD	5.402	3.850	3.525	7.933	5.433	7.372	10.218

Table 81 Cytotoxicity of cationic liposomes (EPC:Chol:SA = 10:2:4)/DNA complexes on Huh7 cells.

n	Weight ratio						
	control	2.5	5	7.5	10	20	30
1	96.700	85.387	85.502	89.428	77.576	81.616	71.380
2	98.586	103.434	95.084	99.933	84.848	79.192	73.535
3	103.165	85.657	93.199	90.236	84.579	76.768	80.269
4	95.354	93.199	97.778	92.391	80.269	88.620	75.421
5	96.431	94.276	95.623	101.010	76.498	83.771	74.074
6	111.785	112.323	102.088	102.088	94.545	79.192	87.273
7	97.239	106.936	95.354	110.438	84.040	78.114	72.727
8	100.741	107.744	96.970	110.707	104.242	105.589	71.380
AVG	100.00	98.620	94.949	94.599	83.194	81.039	75.758
SD	5.402	10.384	5.316	5.482	6.044	4.069	5.456

APPENDIX V

LIST OF ABBREVIATIONS

$^{\circ}C$	degree celsius
μg	microgram
μL	microlitre
μm	micrometre
approx.	approximately
AVG	average
cell/cm^2	cell per centimetre square
DNA	deoxyribonucleic acid
ed.	edition
e.g.	example given, for example
et al.	and others
etc.	for example, such as
g	gram
IC_{50}	Inhibition Concentration Fifty
i.e.	in other words
kbp	kilo base pair
kDa	kilo-Daltons
L	litre
mcg	microgram
mg/mL	milligram per millilitre
mL	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
mV	millivolt
MW	molecular weight
nm	nanometre
nmol	nanomole
N/P ratio	number of polymer nitrogens per DNA phosphate
p.	page
PBS	phosphate-buffered saline

LIST OF ABBREVIATIONS

pDNA	plasmid of deoxyribonucleic acid
PI	polydisperse index
pK_a	coefficient for the acidity substance
QC	quality control
RNA	ribonucleic acid
rpm	round per minute
SD	standard deviation
siRNA	small interfering ribonucleic acid
V	volt
v/v	volume by volume
w/w	weight by weight

BIOGRAPHY

Name	Kingkan Subsantisuk, Miss
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Institution Attended	
2001 - 2006	Silpakorn University. Bachelor of Pharmacy
2007 - 2009	Silpakorn University. Master of Pharmacy (Pharmaceutical Technology)
Presentation	
	Kingkan Subsantisuk, Praneet Opanasopit, Tanasait Ngawhirunpat, Theerasak Rojanarata and Uracha Ruktanonchai "A novel gene carrier using anionic liposomes coated by chitosan lactate." The 2nd Thailand Nanotechnology Conference 2008, Phuket Graceland Resort & Spa Hotel. Patong, Phuket, Thailand, August 13 – 15, 2008.
	Kingkan Subsantisuk, Praneet Opanasopit, Tanasait Ngawhirunpat, Theerasak Rojanarata "A novel gene carrier using anionic liposomes coated by PEI and water soluble chitosan derivatives." The CGI Award for Young Scientists 2008, November 21, 2008, Chulaphorn Research Institute Convention Center, Bangkok, Thailand.
Working Experience	
2006 - 2007	Eye Ear Nose Throat Hospital Pharmacist (Full-time)