ENHANCEMENT OF GENE DELIVERY MEDIATED BY CATIONIC POLYPEPTIDES

By

Jintana Tragulpakseerojn

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

Program of Pharmaceutical Sciences

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การเพิ่มการนำส่งยีนโดยอาศัยพอลิเพปไทด์ประจุบวก

โดย

นางสาวจินตนา ตระกูลปักษีโรจน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร
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The Graduate School, Silpakorn University has approved and accredited the thesis title of “Enhancement of Gene Delivery Mediated by Cationic Polypeptides” submitted by MissJintana Tragulpakseerode as a partial fulfillment of the requirements for the degree of Master of Science in Pharmaceutical Sciences.

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The aim of this research was to investigate the effect of cationic polypeptides mixed with cationic polymers (chitosan hydrochloride, CSHCl) or anionic liposomes on *in vitro* transfection efficiency and cytotoxicity in human cervical carcinoma cells (HeLa cells) and human hepatoma cells (Huh7 cells). The effect of the type and molecular weight of polypeptides, the weight ratio of carrier: DNA, the pH of transfection medium, order of mixing and the serum on transfection efficiency and cytotoxicity were evaluated. The results showed that three types of cationic polypeptides including poly-L-lysine (PLL), poly-L-arginine (PLA) and poly-L-ornithine (PLO) were able to form complete complex with DNA at weight ratio 0.5. The PLA showed a higher transfection efficiency than PLL and PLO. Hela cells showed higher transfection efficiency than that of Huh7 cells. The transfection efficiency of PLA depended on the MW. PLA with high MW (> 70 kDa) showed the highest transfection efficiency. Therefore, the PLA MW > 70 kDa was used to mixed with CSHCl or coated anionic liposome. The order of mixing between CSHCl, PLA and DNA affected the transfection efficiency. The transfection efficiency on HeLa cells at pH 6.4 was ranked as: PLA/DNA/CSHCl > PLA/CSHCl/DNA > CSHCl/DNA/PLA > PLA/DNA. The highest transfection efficiency of PLA/DNA/CSHCl complexes was equal to PEI/DNA complex and higher than PLA/DNA complex about 2.39 times. In PLA-coated liposomes, the transfection efficiency on HeLa cells was about 1.83 and 1.49 times higher than the PLA/DNA complexes at pH 7.4 and 6.4, respectively. In conclusion, PLA/DNA/CSHCl showed elevated potential as gene carrier by efficiently condensing the DNA, mediating the highest level of gene transfection and lowering the cytotoxicity, compared with PEI, in HeLa cells.

Program of Pharmaceutical Sciences Graduate School, Silpakorn University Academic year 2009
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การวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของการผสมพอลิเพปไทด์ประจุบวก กับพอลิเมอร์ประจุบวก (ไคโตซานไฮโดรคลอไรด์, CSHCl) หรือลิโพโซมประจุลบต่อประสิทธิภาพในการถ่ายโอนยีนและความเป็นพิษในเซลล์เพาะเลี้ยง human cervical carcinoma cells (HeLa cells) และ human hepaatoma cells (Huh7 cells) โดยศึกษาปัจจัยของชนิดและน้ำหนักโมเลกุลของพอลิเพปไทด์ประจุบวก, อัตราส่วนโดยน้ำหนักของตัวพาต่อดีเอ็นเอ, พีเอชของตัวกลางในการนำส่งยีน, ลำดับการผสม และผลของซีรัม จากการศึกษาพบว่าพอลิเพปไทด์ประจุบวกทั้งสามชนิดประกอบด้วยพอลิแอลไ ลซีน (PLL), พอลิแอลอาร์จินีน (PLA) และพอลิแอลออร์นิทีน (PLO) สามารถเกิดสารประกอบเชิงซ้อนกับดีเอ็นเอได้ที่อัตราส่วนโดยน้ำหนัก 0.5 PLA มีประสิทธิภาพในการถ่ายโอนยีนสูงกว่า PLL และ PLO ตามลำดับ ในเซลล์ HeLa แสดงประสิทธิภาพในการถ่ายโอนยีนที่สูงกว่าใน Huh7 ประสิทธิ- ภาพในการถ่ายโอนยีนของ PLA ขึ้นอยู่กับน้ำหนักโมเลกุล โดยจะพบว่าที่น้ำหนักโมเลกุลสูง (> 70 kDa) จะให้ประสิทธิภาพในการถ่ายโอนยีนสูงที่สุด ดังนั้นจึงเลือกใช้ PLA น้ำหนักโมเลกุล > 70 kDa ในการผสมกับไคโตซานไฮโดรคลอไรด์ หรือนำมาเคลือบกับลิโพโซม ลำดับการผสมระหว่างไคโตซานไฮโดรคลอไรด์, PLA และดีเอ็นเอส่งผลต่อประสิทธิภาพในการถ่ายโอนยีน เมื่อเปรียบ เทียบประสิทธิภาพในการถ่ายโอนยีนเข้าสู่เซลล์ HeLa พบว่า PLA/DNA/CSHCl > PLA/CSHCl /DNA > CSHCl/DNA/PLA > PLA/DNA ที่ pH 6.4 สารประกอบเชิงซ้อน PLA/DNA/CSHCl มีประสิทธิภาพในการถ่ายโอนยีนสูงที่สุดซึ่งไม่แตกต่างจาก PEI/DNA และมีประสิทธิภาพสูงกว่า PLA/DNA ประมาณ 2.39 เท่า ในกรณีของ PLA ที่เคลือบบนลิโพโซม พบว่าประสิทธิภาพในการถ่ายโอนยีนเข้าสู่เซลล์ HeLa สูงกว่าสารประกอบเชิงซ้อน PLA/DNA ประมาณ 1.83 และ 1.49 เท่า ที่ pH 7.4 และ 6.4 ตามลำดับ จากผลการศึกษาทั้งหมดสรุปได้ว่า PLA/DNA/CSHCl เป็นตัวพาที่มีประสิทธิภาพในการถ่ายโอนยีนสูงที่สุดโดยสามารถเกิดสาร ประกอบเชิงซ้อนกับดีเอ็นเอได้ และมีความเป็นพิษต่อเซลล์ HeLa ค่อนข้างต่ำเมื่อเทียบกับ PEI

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ลายมือชื่อนักศึกษา .....

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

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CHAPTER 1
INTRODUCTION

1. Statement and significance of the research problem

Gene therapy holds enormous promise for correcting genetic defects and treating countless diseases (Sato et al. 2001: 2075). However, perhaps the single biggest challenge that many scientists face today in gene therapy is delivery. Two general approaches have been used for gene delivery. The first is viral vectors, which are highly effective. In this type of approach the viruses can act so as to sneak foreign genes into cells. Unfortunately, viruses, even disabled ones, can cause serious side effects (Byrnes et al. 1995 : 1015). The second approach involves using a nonviral vector such as a polymer (Wong, Pelet and Putnam 2007 : 799-803), lipid, or liposome (Patil, Rhodes and Burgess 2004 : 1). While potentially safer, these synthetic systems are not as effective as viral vectors. Thus, it is critically important that efforts toward creating synthetic gene therapy vectors such as polymers be developed.

The use of natural polymers and oligomers derived from chitosan to complex and condense plasmids was first described by Mumper et al. (1995). Chitosan, a (1→4) 2-amino-2-deoxy-β-D-glucan, is a linear cationic polysaccharide derived by partial alkaline deacetylation of chitin, a polymer abundant in nature. Chitin is mostly obtained from exoskeletons of crustacean; main sources are shell wastes of shrimp and crab. Chitosan is commercially available in a wide variety of molecular weights having a degree of deacetylation usually from 70-90%. The backbone of the copolymer consists of two subunits, D-glucosamine and *N*-acetyl-D-glucosamine, which are linked by 1→4 glycosidic bonds (Torchilin 2001 : 137-138). Chitosans, except for high molecular weight chitosans of specific salt forms, are generally shown to be nontoxic, partially due to their biodegradability. This polymer is a weak base with a pK_a value of the D-glucosamine residue of about 6.2-7.0 and is therefore insoluble at neutral or basic pH (Mansouri et al. 2004 : 3). To increase charge density of chitosan at physiological pH, and to overcome solubility problems,

chitosan derivative chitosan sulfate, N-methylene phosphonic chitosan, alkylate chitosan, N-carbosymethy chitosan (Rinaudo 2006 : 619), chitosan salts (Weecharangsan et al. 2008 : 161) etc., are introduced.

Polyethylenimine (PEI) is well known in the chemical industry as a polymer with high positive charge density. PEI exists in two principal forms, branched and linear. After internalization, gene transfer complexes are mostly found in intracellular compartments, which can be potentially acidified, such as endosomes or lysosomes, and their subsequent release into the cytoplasm represents a major bottleneck for gene delivery (Zabner et al. 1995 : 18997). The protonation profile of PEI, bearing an intrinsic buffer capacity, and its high transfection capacity led to development of the “proton sponge” hypothesis: the buffering of the endosome by PEI causes proton accumulation and subsequent influx of chloride into the vesicle. Osmotic swelling by influx of water can thereafter lead to disruption of the vesicle and release of the polyplex into the cytoplasm. PEI has different molecular weights associated with different transfection efficiencies. Only the PEIs larger than 10 kDa have been used successfully in transfection studies (Zhang et al. 2004 : 174). However, toxicity, which is another important factor for the *in vivo* gene delivery, increases sharply as the molecular weight increases. Therefore, there have been many efforts to combine the merits of these two types of PEI-high transfection efficiency of high molecular weight PEI and low toxicity of low molecular weight PEI for an ideal gene delivery vector. For example, derivatives of low molecular weight PEI were modified primary amines into guanidines for enhancing transfection efficiency by maintain its low cytotoxicity (Lee et al. 2008 : 666).

In this study, the gene carriers using cationic polypeptides with chitosan hydrochloride (CSHCl) or anionic liposome are selected to investigate. *In vitro* transfection efficiency and cytotoxicity were also evaluated. A number of variables that influenced transfection efficiency such as type of cationic polypeptides (poly-L-arginine, poly-L-lysine and poly-L-ornithine), carrier/DNA weight ratio, particle size, surface charge and pH of culture medium were determined.

2. Objective of this research

2.1 To study cationic polypeptides complexing with CS or liposome as non-viral gene carrier.

2.2 To study the physicochemical properties such as cationic polypeptides/CS or liposome/DNA weight ratio, order of mixing, cationic polypeptides and DNA, particle size, surface charge, and pH of culture medium affecting the transfection efficiency to cell culture model.

2.3 To study the cytotoxicity of cationic polypeptides and cationic polypeptides with CS or liposome and carriers/DNA complexes.

3. The research hypothesis

3.1 Cationic polypeptides with CS or liposome can be used as gene carrier.

3.2 Formulation factors such as type of cationic polypeptides with CS or liposome and carrier/DNA weight ratio, order of mixing, cationic polypeptide and DNA, and pH of culture medium significantly influence physicochemical properties (particle size, surface charge, and DNA condensation), transfection efficiency and cytotoxicity.

CHAPTER 2

LITERATURE REVIEWS

1. Introduction to Gene delivery

The basic concept underlying gene therapy is that human disease may be treated by the transfer of genetic material into specific cells of a patient in order to enhance gene expression or to inhibit the production of a target protein (Latchman 2001 : 1; Wunderbaldinger, Jr and Weissleder 2000 : 156). For a genetic disorder in general, a rational strategy for gene-based therapy requires identification of the defective gene, elucidation of the functional properties of the protein product, and understanding of the mechanism by which the mutation alters the phenotype (Mochizuki, Yasuda and Mouradian 2008 : 260).

Human genetic disease is the result of mutation or deletion of genes that impair normal metabolic pathways, ligand/receptor function, regulation of cell cycle, or structure and function of cytoskeletal or extracellular proteins. Diseases that are suitable for treatment by gene therapy can be divided into genetic and acquired diseases. Genetic diseases are typically caused by a single gene mutation or deletion. The acquired diseases are those for which no single gene has been identified as the only cause of the disease state. However, expression of a single gene delivered by a gene delivery system to the correct cell type(s) can potentially lead to the elimination of the diseases state. Some examples for each type of disease are listed in Table 1 and 2 (Sullivan 2003 : 1).

A gene can be transferred to the cells in the body by two general strategies, *ex vivo* and *in vivo*. In *ex vivo* gene transfer, the cells are removed from the patient. These cells are cultured in the laboratory and then used as the recipient for a desired gene. The cells that have the desired gene are then cultured and returned to the patient and continue to divide and develop into a number of different cells in the body (Evans et al. 2006: 243-258; Bouragaize, Jewell and Buiser 2000 : 224-230). For *in vivo* gene delivery, genes are administered directly to the body. By this way, genes

can be encountered with biological barriers from the point of injection to the surface of the cellular target such as degradation in the blood circulation, scavenging by circulating or resident macrophage, extravasation from vascular later, permeation across endothelial barrier and distribution within tissue (Pouton and Seymour 2001 : 187-203). For each strategy of gene transfer, efficient delivery of gene to the cells can be aided by the application of vectors.

Table 1 Candidate genetic diseases for gene therapy

Disease	Defect	Incidence	Target
Severe combined immunodeficiency	Adenosine deaminase	Rare	Bone marrow or T cells
Hemophilia	Clotting Factors VIII and IX	1/10,000	Liver and muscle
Familial hypercholester-olemia	Low-density lipoprotein receptor	1/1,000,000	Liver
Cystic fibrosis	Loss of cystic fibrosis transmembrane conductance regulator	1/3,000	Lung
Hemoglobinopathies	α and β globin	1/600	Red blood cell precursors
Gaucher's disease	Glucocerebrosidase	1/450	Liver
Inherited emphysema	α1 Antitrypsin	1/3,500	Lung, liver
Muscular dystrophy	Dystrophin	1/3,500	Muscle

Soruce : Sean M. Sullivan, Pharmaceutical gene delivery systems, Volume 131. (USA : Marcel Dekker, 2003), 2.

Table 2 Candidate acquired diseases for gene therapy

Disease	Defect	Incidence	Target
Cancer	Defects in tumor suppressors and /or presence of oncogenic factors	1 million/yr	Liver, lung, brain, pancreas, breast, prostate, kidney
Neurological diseases (Parkinson's and Alzheimer's)	Neurotransmitter release; structural defect of β-amyloid protein	1 million Parkinson's 4 million Alzheimer's*	Brain, neurons, glial cells, Schwann cells
Cardovascular diseases	Defect in blood vescles	13 million Alzheimer's*	Arteries, vascular endothelial cells
Infectious diseases	Suppressed immune system (HIV), liver destruction (HBV)	Increasing numbers	Liver, T cells, antigen-presenting cells, macrophages,

Remark : * = in United states

Soruce : Sean M. Sullivan, Pharmaceutical gene delivery systems, Volume 131. (USA : Marcel Dekker, 2003), 2.

2. Biological Barriers

2.1 The extracellular barriers (Philipp and Wagner 2009 : 350-352)

Upon in vivo application polyplexes face both intracellular and extracellular barriers. A schematic diagram of the potential intracellular and extracellular fate of a polyplex is shown in Figure 1. Strategies have to be developed in designing polyplexes that are able to survive blood or other biological fluids, and to escape extracellular physical barriers in order to reach the target cells. The specific strategies must consider the physicochemical properties of polyplexes e.g. size, shape/flexibility, overall charge, charge density and nonelectrostatic interactions at the surface. Besides considering these physical properties, one could imagine to use active endogenous cellular transport mechanisms such as transcytosis.

In systemic administration, i.e., intravenously application of the vector, the circulatory pathway/environment and various nontarget cells and organs, encountered by the polyplex, are major challenges. Local administration methods such as direct

injection of the vectors into the target region or topical administration are not confronted with the circulation problems, but, nevertheless, still confronted with barriers such as extracellular matrix or inflammatory and immune responses.

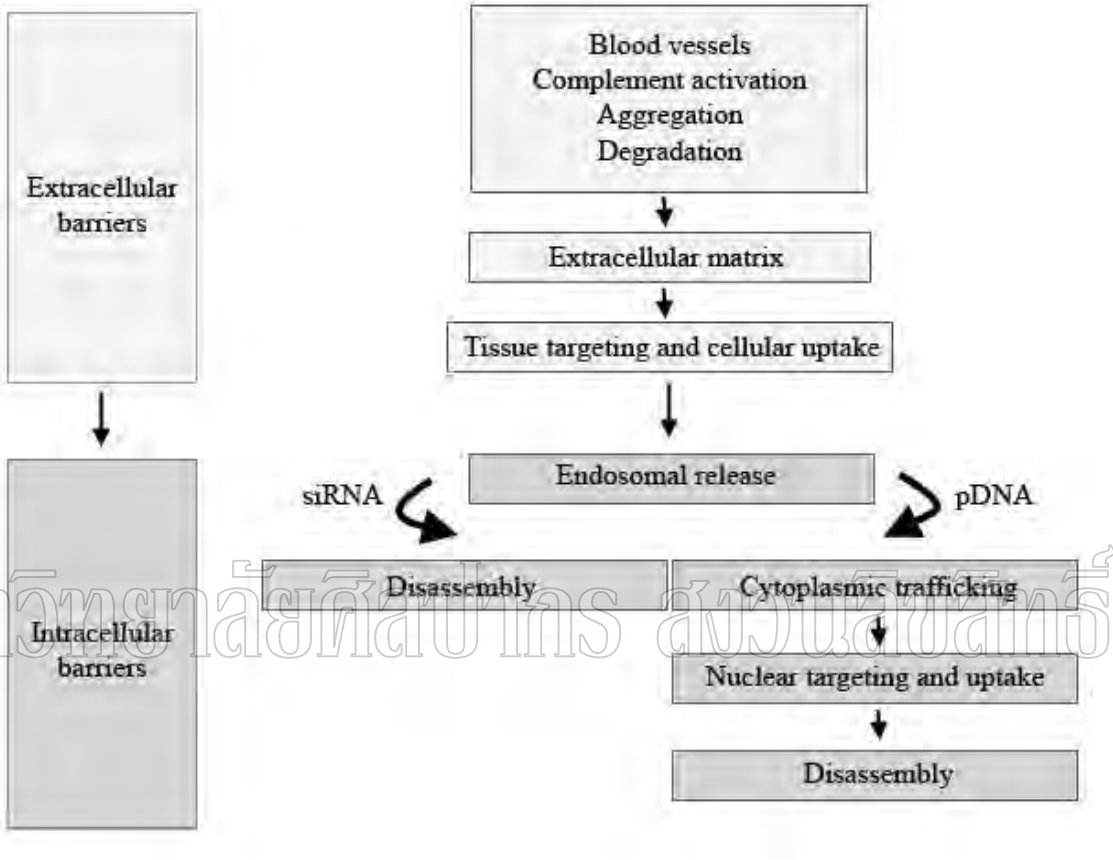


Figure 1 Extracellular and intracellular barriers to gene delivery

2.1.1 Physical restrictions of transfection particles

Size seems to be a general critical factor for drug targeting. Because of size restriction, several hundred nanometer large polyplexes are not able to penetrate endothelial and epithelial barriers, or extravasate from the vascular to the interstitial space. Particle size is also an important factor when considering organ clearance and intraorgan distribution. For example, particles that are too large to pass through the vascular endothelium to the liver parenchyma are engulfed and degraded by liver Kupffer cells, i.e., phagocytic cells located next to the vascular epithelial cells.

The structure of condensed nucleic acid polyplexes has been analyzed in several reports. Polyplexes have been characterized by electron microscopy and atomic force microscopy (shape, size), laser light scattering (size), their electrophoretic mobility (reflects charge and size of complexes), zeta potential measurements (charge), circular dichroism (conformation of pDNA), or centrifugation techniques (molecular weight and condensation). The results give some insight on generating small polyplexes. In addition, the compact polyplexes are more stable against enzymatic or mechanical degradation, which takes place during nucleic acid transport to the target cells/tissue.

However, preparation of effective polyplexes for *in vivo* delivery of nucleic acids remains a major hurdle. The extent of nucleic acid condensation depends on a number of variables including the ratio of positively charged nucleic acid-binding element (cationic carrier) to negatively charged nucleic acid. Also the size and modification of the nucleic acid-binding element, size and sequence of the nucleic acid, and even the procedure of complex formation are strongly influencing the *in vitro* and *in vivo* gene transfer efficiency. The net charge of the nucleic acid/cationic carrier complex affects its solubility. Polyplexes with either an excess of nucleic acid or positively charged carrier are stabilized in solution by the negative or positive charges. At molar charge ratios (positive charges of carrier nitrogens to negative charges of nucleic acid phosphates) close to electroneutrality, often aggregation of polyplexes is observed unless the carrier contains hydrophilic domains such as PEG for stabilization. The procedure of complex formation still has to be improved in generating homogenous and stable polyplexes capable of overcoming the physical barriers.

For example, Wagner et al. described one approach for polyplex formation. Flash mixing of dilute compounds in physiological phosphate-free buffer results in formation of kinetically controlled polyplexes. At ratios of electroneutrality or higher, donut- and rod-like particles of 80-120 nm in diameter are formed for PLL polyplexes. Polyplexes containing Tf-conjugated PLL have increased solubility compared to unmodified PLL. For this type of polyplex formation a strong influence of parameters such as pDNA concentration or charge ratio and ionic strength of solution was observed. Mixing pDNA/PEI polyplexes at N/P (PEI nitrogen: DNA

phosphate) molar ratios below 6/1 in 150 mM saline results in rapid aggregation, which can be avoided by complex formation at low ionic strength (e.g. glucose balanced buffer) generating particles with an average diameter of hydrophilic PEG residues into polyplexes stabilize and prevent their aggregation.

2.1.2 Undesired interactions with plasmid, degradative enzyme, matrix and nontarget tissue

Polyplexes, when administered in vivo, are surrounded by a variety of compounds present in blood plasma, Salts, lipids, carbohydrates, proteins, or enzymes contribute to changes in the physicochemical properties of the polyplex. Some of these factors (opsonins) may coat the polyplex, causing aggregation, dissociation, or degradation of the polyplex. This may influence the composition of the polyplex as well as the bioavailability. Thus, the polyplexes, even when reaching the target cells/tissue, may no longer exhibit the physical properties necessary for efficient gene transfer into cells.

Previous studies have demonstrated the inactivation of pDNA/PLL polyplexes y blood components, whereby one of the factors was identified as the complement system. Upon incubation of polyplexes with human plasma, specific proteins (IgM, fibrinogen, fibronectin and complement C3) bind to the complexes. By coating pDNA/PEI polyplexes with PEG through covalent coupling to PEI, interaction with blood components was found to be strongly reduced.

Once the polyplex has traveled across the vascular barrier into the interstitial space, it has to avoid interactions with the extracellular matrix in order to reach the target cells/tissue. The extracellular matrix comprises different combinations of collagens, proteoglycans, hyaluronic acid, fibronectin and other glycoproteins. These components could act as barriers by binding the polyplexes e.g. hyaluronic acid binds cations very effectively. This implies that polyplexes resulting in a net positive charge could also interact with the extracellular matrix like binding, dissociation or aggregation. Thus, optimizing the polyplexes bearing an overall low charge ratio close to neutrality is a possibility to avoid such interactions.

2.1.3 Inflammatory and immunological responses

As a result of introducing foreign molecules into the body, individual immune cells are stimulated to produce antibodies (humoral immunity). In addition

to this humoral response, specific T cells may also be activated (cellular immunity). These two processes are the specific immune response. There is, however, also the nonspecific immune response, including phagocytosis, inflammation and other nonspecific host resistance mechanisms such as the complement system. These nonspecific mechanisms are developed immediately against any foreign molecule, even though the host has never encountered. Thus, the nonspecific immune response is a major extracellular barrier for the polyplex.

Inflammatory response is a major problem for any gene delivery system. During an inflammatory response, neutrophil granulocytes and to a lesser extent macrophages migrate out of the capillaries into the surrounding tissue. At the site of inflammation the phagocytes recognize the foreign molecules via receptors on their surface leading to subsequent phagocytosis. Attachment is greatly enhanced and specified upon opsonization of foreign molecules, e.g. by the C3b component of complement. Both neutrophil granulocytes and macrophages have receptors which specifically bind to C3b allowing them to recognize their target.

Positively charged polyplexes have the ability to activate the complement system. The positive charges are responsive to the complement protein C3b. Opsonization of such particles by C3b leads to the initiation of a cascade of events presumably resulting in the clearance of polyplexes by the reticuloendothelial system. Coating the positive charges of the polyplexes with other macromolecules may inhibit interactions with components of the complement system, hence decrease complement activation and clearance of the complexes from the blood circulation.

2.2 Intracellular barriers to gene delivery

The cellular association of naked DNA molecules is very poor, since negative charges on both the cell surface and the surface and the DNA molecules interrupt contact with each other via electrostatic interactions. Thus, in order to enhance cellular association, DNA was condensed with cationic polymers and cationic liposomes that neutralize the effect of the negative charge. Cationic polymer and lipid-mediated transfection is a relatively inefficient process, which is initiated by binging of the plasmid expression vector to the cell. While a significant portion of the cell-associated DNA is internalized, only a small percentage of DNA is released from endosomes. Therefore, only a small amount of DNA will be available as free DNA in

the cytoplasm and this DNA will still be susceptible to degradation. This free DNA must still find its way to the nucleus. An unlikely alternative involves the complex itself entering the nucleus and subsequently dissociating in the nucleus in order for the DNA to be available for transcription. Figure 2 shown targeted cell penetration and trafficking properties of the viral cycle mimicked by an artificial virus. The coating material condenses tightly while binds reversibly the target nucleic acid, usually in vitro (A). The final nanocomplex recognizes specific receptors at the cell membrane and is internalized via endocytic pathways (B). Once located within early endosomes (C), the artificial virus escapes from the endosomal route (D) and thus avoids lysosomal targeting (E). Finally, the nanocomplex enters into the cell nucleus (F) and permits transgene expression (G). For the delivery of siRNA, nuclear localization signals are absent and the nucleic acid is released in the cytoplasm (H). However, others have suggested that cationic polymer and lipid dissociation occurs during transport into the nucleus (Bally et al. 1999 : 310).

A cationic vector enhances cell-surface binding through interactions with the negative constituents of the cell surface (e.g. heparin sulfate proteoglycans) or through selective binding to specific receptors, resulting in a strong transgene expression. This method of condensation also enables targeting of the cells by modulating different ligands to the surfaces of the gene vectors (Table 3).

Currently, several ligands are used for recognition by their specific receptors and uptake via cellular receptor-mediated endocytosis. In this pathway, however, lysosomal degradation presents a second barrier for gene vectors. Before the endosomal membrane fuses with lysosome, the gene vectors must be released into the cytosol. The importance of endosomal escape is clearly evidenced by the drastically enhanced transfection efficiency in the presence of lysosomotropic reagents, such as chloroquine (Farhood, Serbina and Huang 1995 : 293; Pouton et al. 1998 : 289), which accumulate in the acidic lysosome and destabilize the membrane by causing it to swell. Various devices, such as pH-sensitive fusiogenic lipids (Hui et al. 1996 : 590), polycations that have proton sponge ability (Kircheis, Wightman and Wagner 2001 : 343) and pH-sensitive membrane lytic peptides (Wagner 1998 : 155-158) can also aid in endosomal escape.

The final barrier to the intracellular trafficking of DNA is the nuclear membrane. Since the size threshold for freely passing through the nuclear pore complex is 50 kDa, commonly used plasmid DNA (pDNA) is too large to pass, unassisted, through the nuclear-pore complexes. Therefore, plasmid DNA is thought to enter into the nucleus when the nuclear membrane structure disappears during the M-phase of mitosis. In fact, the percent of plasmid DNA, reaching the nucleus has been reported to be less than 1% of the total cytoplasmically microinjected DNA. Therefore, in order to improve entry into the nucleus, nuclear localization signal peptides (NLS) are attached either to the plasmid DNA itself or to counter-polycations. Various reports on the use of NLS peptides as polycations have recently been published. Although these approaches succeed in enhancing transgene expression, the technology for delivering plasmid DNA to the nucleus in non-dividing cells needs further development (Akita et al. 2005 : 136).

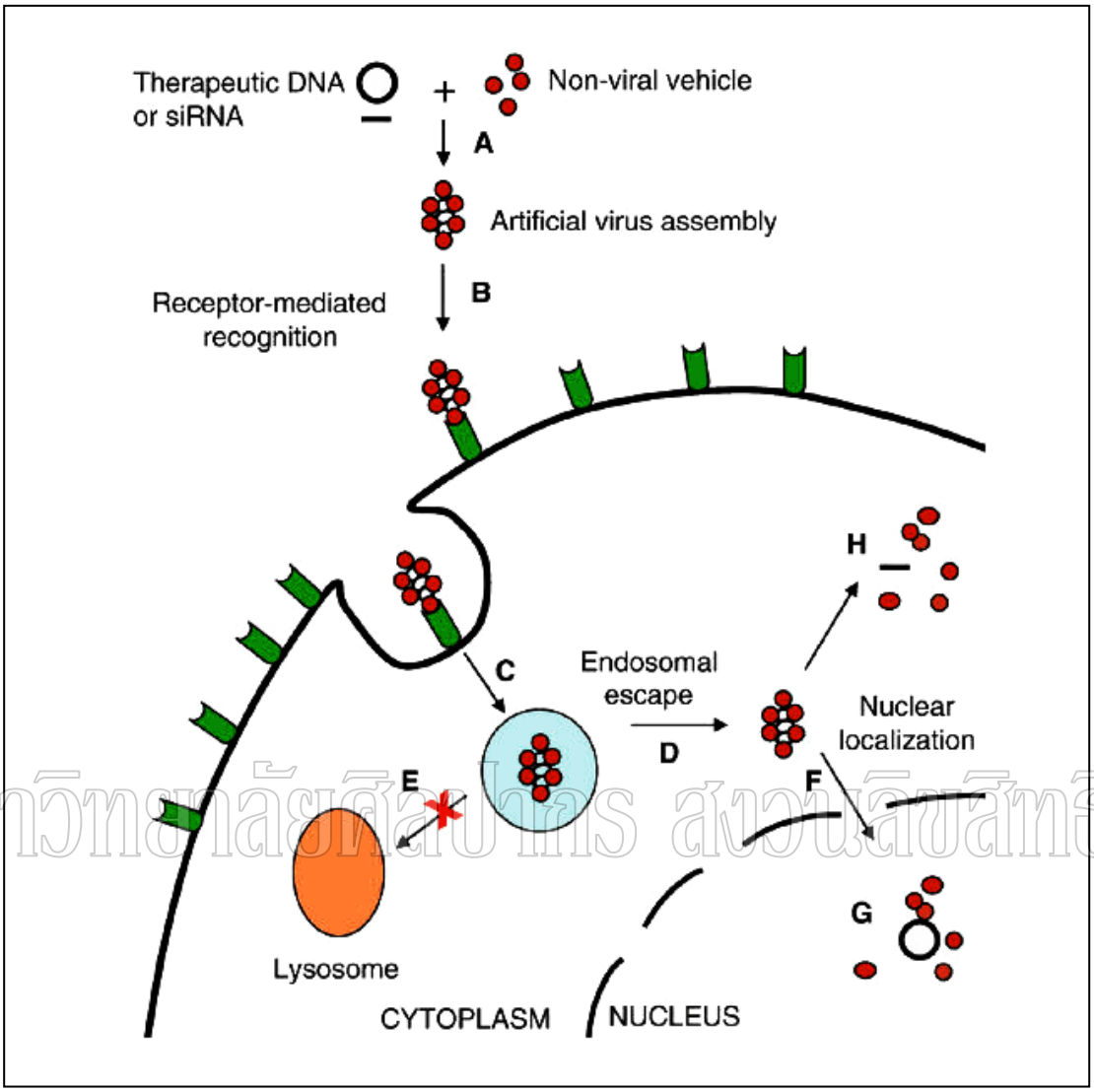


Figure 2 Targeted cell penetration and trafficking properties of the viral cycle mimicked by an artificial virus

Source : Paolo Saccardo, Antonio Villaverde and Nuria Gonzalex-Montalban, “Peptide-mediated DNA condensation for non-viral gene therapy,” Biotechnology Advances 27 (2009) : 433.

Table 3 Target receptors/molecules employed for cells-specific gene delivery using a ligand-polymer-plasmid DNA complex

Target receptor/molecule	Ligand-polymer	Target cell
Asialoglycoprotein receptor	Asialoorosomucoid-PLL	Hepatocytes
	Lactosylated PLL	Hepatoma cells
	Galactosylated PLL	Hepatocytes
	Galactosylated poly (L-ornithine)	Hepatocytes
	Galactosylated PEI	Hepatocytes
Mannose receptor	Mannosylated PLL	Macrophages
	Mannosylated PEI	Dendritic cells
Transferrin receptor	Transferrin-PLL	Various cells
Polymeric immunoglobulin	Antibody-PLL	Respiratory epithelial cells
Platelet endothelial cell adhesion molecule	Antibody-PEI	Pulmonary endothelial cells
Surfactant protein A receeptor	Surfactant protein A-PLL	Airway cells

Source : Makiya Nishikawa, Yoshinobu Takakura and Mitsuru Hashida, "Pharmacokinetics of polymer-plasmid DNA complex," In Polymeric gene delivery : principles and applications (Florida : CRC Press LLC, 2005), 71.

3. Methods of DNA delivery

3.1 Physical methods

3.1.1Cell membrane-parmeabilizing gene delivery techniques

3.1.1.1 Electroporation

The hydrophobic pores size range of 1.10 nm makes the explanation of plasmid uptake difficult, because the plasmid size is much larger than the size of the average pore. The intracellular entry of many macromolecules typically impeded by the cell membrane has been made possible by electroporation. Electroporation or electropermeabilization involves the use of an electric field to open up pores in the cell. During the time that the pores are open, DNA can enter the cell

directly into the cytoplasm and ultimately into the nucleus. This process is a physical process, not dependent upon special characteristics of the cell, and therefore can be used with virtually any cell type. The optimal amplitude and length of pulse will vary for each cell type, so this procedure should be optimized for each cell type. Increasing the voltage would increase the amount of DNA per cell due to the induction of a larger pore size; however, the toxicity would also be higher. This method has been used to successfully transfer genes *in vivo* (Lin et al. 2002 : 1-4; Tjelle et al 2006 : 4667-4670) and *in vitro* (Espinos et al. 2001 : 341-349).

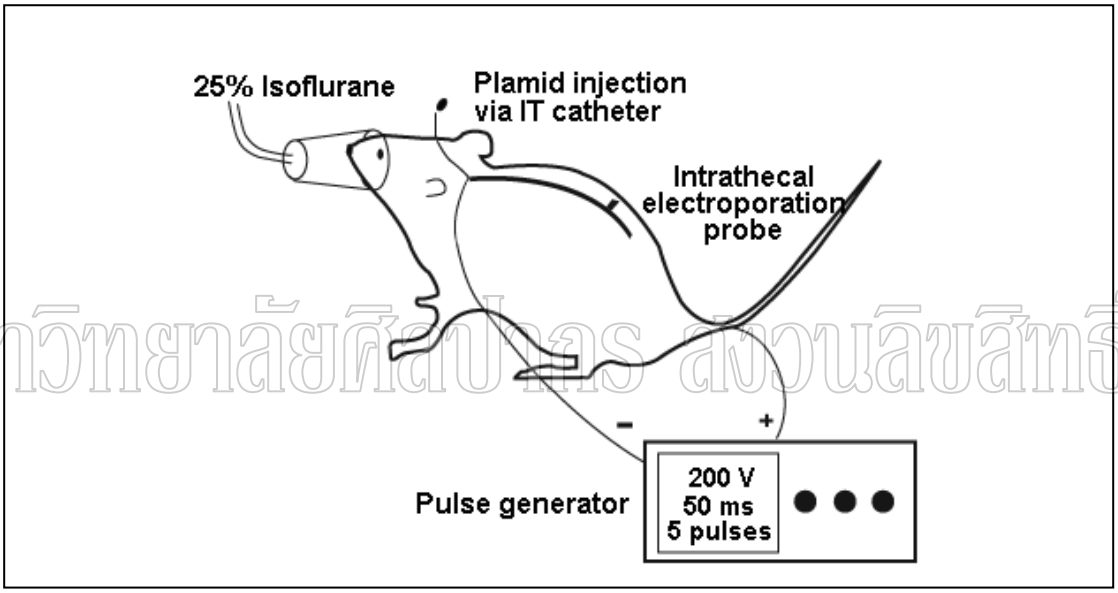


Figure 3 The simplified diagram of the experimental set up for direct gene transfer by intrathecal electroporation. The electric pulses of 200 V, 50 ms were applied by electroporator

3.1.1.2 Sonoporation

Sonoporation or ultrasound utilizes ultrasonic energy to permeabilize cell membranes enabling the uptake of plasmid. Cavitation resulting from the ultrasonic waves increases the permeability of the cells, allowing for more efficient gene delivery. Ultrasonic energy essentially is delivered as a sound wave. Like any wave, transmission through a heterogeneous medium results in the loss of intensity due to absorption and deflection or refraction within the medium or tissue. Absorption of the wave itself varies with the protein, fat and water content and the

acoustic impedance of the tissue. Proteins absorb ultrasound well but fat and skin do not. The interfaces formed with other media such as air or blood reflect the change of refraction of the wave. Provided the influences of each tissue upon a wave can be established, ultrasound can be focused on a target organ or region.

3.1.1.3 Laser irradiation

Laser irradiation requires a laser source (e.g. neodymium-ytrium-aluminium garnet, argon ion, holmium-YAG, titanium sapphire), the power of which controlled by a pulse generator. The laser beam is commonly focused onto the target cell via a lens. Predictably, the permeability of the cell membrane is modified at the site of the beam impact (probably) by a local thermal effect. This perturbation is sufficient to allow a gene present in the surrounding medium to be transferred into the cell; the efficiency depends to some extent upon the difference in osmotic pressure between the cytoplasm and the medium. At present, gene delivery via laser irradiation is not widely used and remains a relatively novel approach. The high cost and the physical size of the laser sources required, as well as the need of appropriate "know-how", are contributory, limiting factors.

3.2 Mechanical methods

3.2.1 Direct DNA injection and microinjection

The most direct method to introduce DNA into cells is microinjection, either into the cytoplasm or into the nucleus. This is a microsurgical procedure that is conducted on a single cell, using a glass needle, a precision positioning device to control the movement of the micropipette and a microinjector. The advantages of this procedure are as follows (Dokka and Rojanasakul 2005 : 54):

It is independent of the length and sequence of the DNA

DNA is applied directly to the site of action

Culture environment can be controlled

The amount of DNA reaching the target site can be controlled

It is highly efficient

The disadvantages of this procedure are as follows:

It requires specialized equipment

It is technically demanding and hence not reproducible all the time

It is a monocellular technique (it can be performed only in a single cell)

It has limited applications *in vivo*

3.2.2 Particle bombardment (gene gun)

The idea behind gene-mediated particle bombardment is to move naked DNA plasmid into target cells on an accelerated particle carrier. This is a method of introducing DNA using metal particles as a carrier. It has been shown that particle bombardment is an effective means of gene transfer both *in vitro* and *in vivo*. In this method, pDNA is first coated onto the surface of gold particles and then propelled using a gene gun to accelerate the DNA-coated particles into target cells. Gene transfer mediated by particle bombardment has an advantage in that it can be applied to various tissue cells and cancer cells *in vivo* with relatively high efficiency. Disadvantages of particle-bombardment-mediated gene transfer are that it requires specific equipment and that only the surface portion of organs can be transfected.

4. Vector for gene delivery

4.1 Viral vector

These are by far the most efficient vectors and hence 70% of clinical trials utilize these vectors. Currently, the viral vectors in clinical trials include retroviruses, adenovirus, pox virus, adeno-associated virus and herpes simplex virus. These vectors have been used for a wide range of indications from cancer to infectious diseases. Unfortunately, these vectors have a number of disadvantages, including limited DNA carrying capacity, lack of specificity, production and packaging problems, replication and recombination potential and high cost. For these reasons, nonviral systems, especially synthetic DNA delivery systems, have become increasingly desirable Table 4 lists the advantages and disadvantages of current viral vectors.

Table 4 Viral gene delivery systems

Vector	Advantages	Disadvantages
Retrovirus	Integration into host genome	Low transduction efficiency
	1-30% transduction efficiency	Packaging cell line required
		No targeting
		Replication competence
		Insert size 9-12 kb
		Infects only dividing cells
		Low titer and unstable in blood
		Random DNA insertion
Adenovirus	High transduction efficiency	No integration/temporary effect
	Infection cell types of man	Packaging cell line required
	Infection does not require cell division	Safety/toxicity/immunogenicity
		Replication competence
		No targeting
		Insert size 4-5 kb
Adeno-associated virus	Integration into host genome	No targeting
	Infection does not require cell division	Packaging cell line required
		Safety
		Insert size 5 kb
		Produced in low titers
		Good for small scale only
Herpes simplex virus	Infects wide range of cell types	No targeting
	Large insert size, 40-50 kb	Packaging cell line required
	Relatively prolonged espression	Toxicity
	Very high titers	Difficult to develop due to complexity
Vaccinia virus	Large insert size, 25 kb	Immunogenicity/toxicity/safety
		No targeting
Avipox virus	Infection does not require cell division	Immunogenicity/toxicity/safety
	Large insert size, >4 kb	No targeting

Source : Sujatha Dokka and Yon Rojanasakul “Cellular uptake and trafficking,” In Polymeric gene delivery (New York : CRC Press, 2005), 52.

4.1.1 Retroviral vectors

Retroviruses have long been recognized as a powerful means of delivering foreign genes to target cells since they are relatively simple to construct as a vector-delivery system. Retroviruses are enveloped, single-stranded RNA viruses that enter target cells by a receptor-mediated endocytotic process. Once inside a host cell, the viral RNA is converted into DNA and is integrated into the genetic material of that cell. Since the retroviral DNA is incorporated into the genome of the host cell, the gene(s) encoded by this DNA will be maintained for the life of the host cell and upon division of the host cells, retroviral-encoded DNA will be inherited by the resulting daughter cells. Thus, the integration of the retroviral- encoded DNA into the host genome has the potential to permit the enduring expression of a therapeutic gene in a targeted cell.

Retroviral vectors used in gene therapy are replication deficient, such that they are unable to replication in the host cell and can infect only one cell. This characteristic, although essential for the safety of viral vectors in gene therapy, imposes restrictions on the amounts of virus that can safely be administered.

4.1.2 Adenoviral vectors

Adenoviruses are non-enveloped, double-stranded DNA viruses that normally elicit mild respiratory tract infections in humans upon infection. During adenovirus infection, adenovirus particles enter host cells by receptor-mediated endocytosis and once inside the cytoplasm of the cell, the adenoviral DNA enters the host nucleus where viral reproduction occurs.

One of the main concerns in using adenoviral vectors for the delivery of therapeutic genes in the clinic is the potential for patients to develop an immune response to the adenoviral vector, viral proteins or infected cells. A pre-existing immune response to adenoviral infection could block adenoviral-driven gene therapy treatment completely or might result in an adverse reaction to the treatment, thereby threatening the life of the patient.

4.1.3 Adeno-associated viral vectors

The use of adeno-associated viral (AAV) vectors provides an alternative to adenoviral vector for gene therapy and means for long-term gene expression with a reduced risk of adverse reactions upon administration of vector.

AAV viruses are linear, single-stranded DNA parvoviruses that are not associated with any disease in humans. Unlike the adenovirus DNA, the AAV genome is able to insert itself into the genetic material of the infected cell at a specific location.

Retroviral- and adenoviral-based vectors provide well-characterized and highly efficient methods for DNA delivery into cells for gene therapy purposes. Both methods for gene delivery have advantages and disadvantages that affect their applications in gene therapy approaches.

4.1.4 Other viral

In view of their potential limitations, research into alternative viral vectors continues. Herpes simplex virus (HSV) vectors provide an alternative to previously described vectors. HSV is a large and relatively complexes enveloped, double-stranded DNA virus that has the capacity to encode large therapeutic genes. Like AAV, it can remain latent in infected cells providing the potential for long-term expression of the therapeutic gene. Although able to infect many cell types, HSV vectors are currently limited in their use by vector toxicity (Bicknell and Brooks, in Brooks 2002 : 23-35).

4.2 Non-viral vector

Despite the ability to efficiently transfect cells, viral methods for human gene therapy have several limitations: (1) viral particles are often unstable and can have low titers; (2) viruses can be immunogenic; (3) a recombinant replication-competent virus can be generated; and (4) oncogene activation and cancer development may occur (Kean, Roth and Thanou 2005 : 643-644; Srinivasan and Burgess 2009 : 62). To circumvent these potential problems, transfection methods using non-viral artificial vectors carrying plasmid DNA have been developed. Plasmid DNA vectors have the following major structural elements: (1) a human gene under the control of an appropriate promoter; (2) a polyadenylation site; (3) an origin of replication necessary for plasmid amplification after transfection (usually not in mammalian cells except EBNA1 and SV40 ori in COS-1); and (4) a selective marker to confer drug resistance so that transfected cells can be selected in appropriate medium (Wunderbaldinger et al. 2000 : 158).

4.2.1 Polylysine

The lysine of PLL has ε-amine groups with positive charges that contribute to the condensation of the negative charged phosphate groups of DNA by charge interaction. PLL condensed DNA into small complexes with donuts or rods structures. Complex formation is dependent on the molecular weight of PLL. PLL residues greater than 20 will bind DNA in physiological saline. However, a smaller chain of PLL will not bind DNA in physiological saline. When the PLL-DNA charge ratio is below 0.5, there is no consistent size of complex. At more than 0.5 charge ratio, DNA condenses into a more compact form. In addition, the size of the PLL-DNA complex gradually increases in a time-dependent manner at a charge ratio of 1.0. However, above or below 1.0 charge ratio, the complex size is stable (Kwoh et al. 1999 : 171-190). PLL is synthesized by polymerization of the *N*-carboxy-anhydride of lysine (Figure 5). They are linear polypeptides with the amino acid lysine as the repeat unit; thus, they possess a biodegradable nature.

Poly (L-lysine) (PLL) has been popularly used as a gene delivery vehicle due to its excellent ability to condense DNA. PLL also provides DNA with effective protection from extracellular enzymatic attacks by forming polyelectrolyte complexes (Jeong, Kim and Park 2007 : 1244). PLL shows the highest transfection efficiency at a 2:1 weight ratio of PLL-DNA. However, the transfection efficiency of PLL is lower than that of PEI. The lower transfection efficiency of PLL compared to PEI may be due to a difference of intracellular trafficking. First, the efficiency of the endosomal escape of the PLL-DNA complex is lower than PEI. PEI has many protonation sites, which contribute to the escape from the endosomal compartment (Lee and Kim 2005 : 81). Since the entire primary ε-amine groups of lysine in PLL are protonated at physiological pH, PLL does not have any intrinsic proton-buffering effect at endosomal pH, which makes it difficult to achieve maximum transfection efficiency, presumably due to inefficient endosomal escape of the PLL/DNA complexes. The average endosomal pH of DNA delivered by the PLL complexes was reported as 4.5, suggesting that most of delivered DNA was localized in the lysosomal compartment. In contrast, the endosomal pH values of the DNA delivered by cationic lipid and PEI were 7.1 and 5.9, respectively (Jeong, Kim and Park 2007 : 1244). The plasmid delivered by PLL is broken down in the

lysosomes, which is one of the reasons why PLL has a lower transfection efficiency compared to PEI. In another hypothesis, the PEI-DNA complex escapes from late endosomes, which are closer to the nucleus than early endosomes. However, the PLL-DNA complexes were localized in early endosomes, which were far from the nucleus (Lee and Kim 2005 : 81).

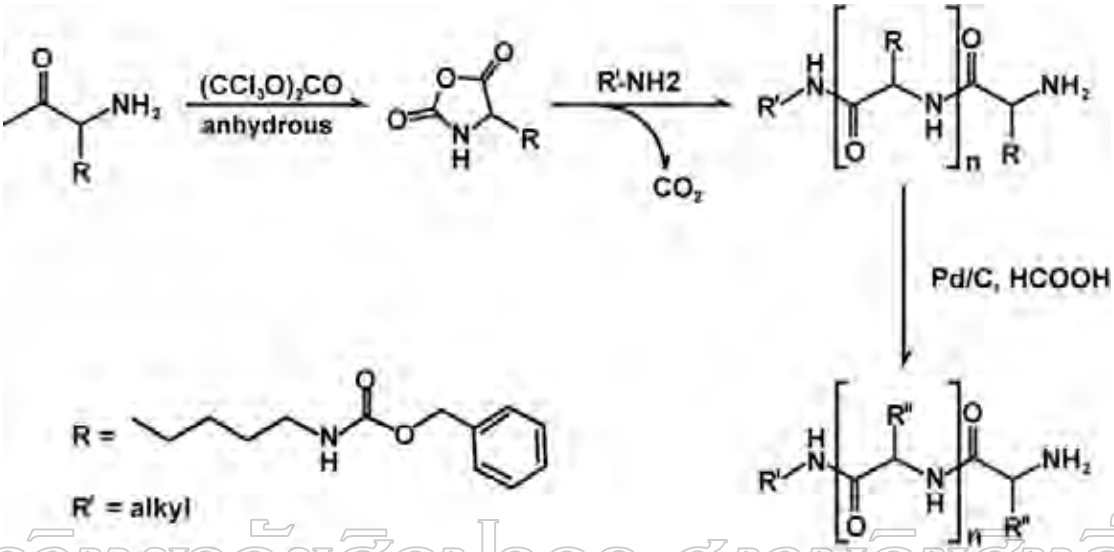


Figure 4 Synthesis of poly (L-lysine) based on *N*-carboxy anhydride chemistry.
Source: Ji Hoon Jeong, Sung Wan Kim and Tae Gwan Park, "Molecular design of functional polymers for gene therapy," Prog. Polym. Sci. 32 (2007) : 1245.

The cytotoxicity of PLL is relatively high. The cell viabilities decreased to 40-60% after the addition of the PLL-DNA complexes. The cytotoxicity of the cationic particles is derived from the charge density and the shape of the particles. The PLL-DNA complex has been formulated to have a positive charge, which shows the highest transfection efficiency to various types of cells. Although the positively charged complex interacts easily with the cellular membrane, it is the positive charge that induces the cytotoxicity. It is generally accepted that the cytotoxicity of the cationic polymers is related to the transfection efficiency. In addition, the low degradable rate of PLL suggests that PLL will be excreted from the body at a slow rate (Lee and Kim 2005 : 81). For low cytotoxicity, two main approaches have been employed. The first is to conjugate polyethylene glycol (PEG)

moiety to PLL, and the second is to introduce degradable bonds or moieties to PLL. PEG conjugation reduced the cytotoxicity of PLL and also increased the solubility of the PLL-DNA complex, which is favorable for *in vivo* gene delivery.

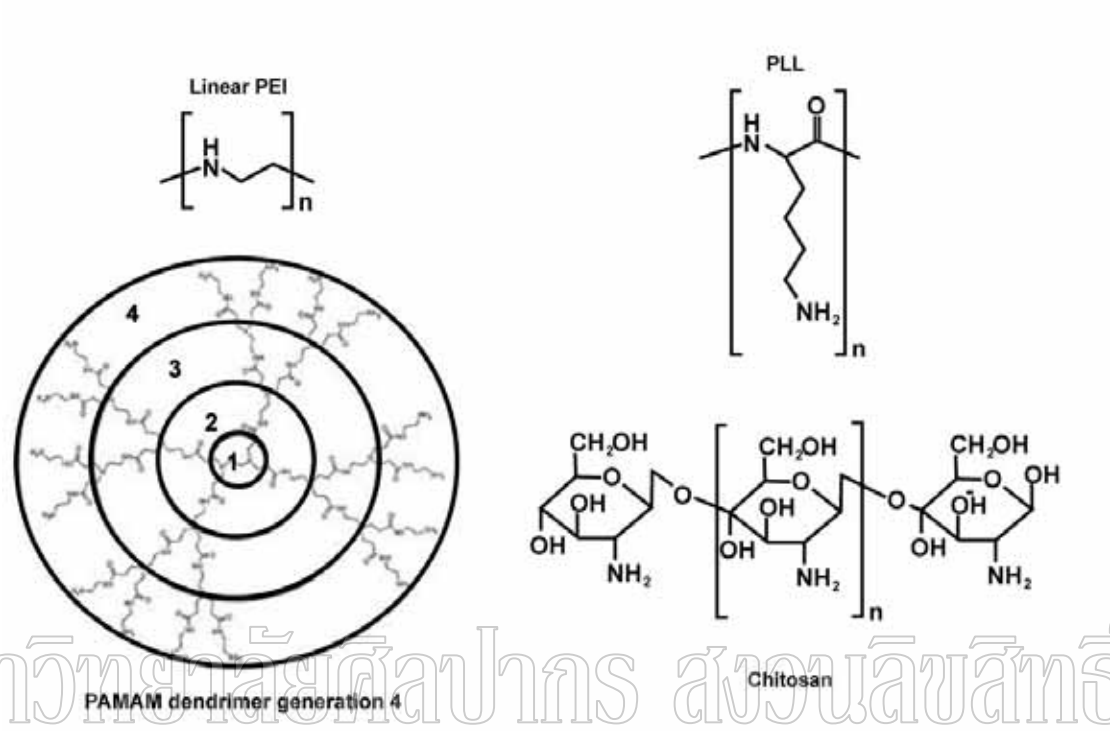


Figure 5 Structures of cationic polymers used in gene therapy
Source : Marie Morille et al., "Progress in Developing Cationic Vectors for Non-Viral Systemic Gene Therapy Against Cancer," Biomaterials 29 (2008) : 3478.

4.2.2 Chitosan and chitosan derivative

Chitosan is a carbohydrate polymer, derived from chitin; the latter a by-product of the shell fish industry and was first reported as a gene delivry agent in the mid-1990s. Chitosan is composed of N-acetyl-D-glucosamine and D-glucosamine monomers linked by β(1,4) glycosidic bonds. The presence of amino groups in chitosan gives chitosan the ability to condense DNA. Chitosan is attractive as a gene delivery tool because of its good biocompatibility profile when compared to polyamine dendrimers. Chitosan is not as amine dense as PEI or poly (L-lysine) (Figure 5) and this property could be responsible for its good biocompatibility and also possibly for its relatively poor gene transfer ability. Chotosan's favorable

biocompatibility has prompted researcher to find ways to optimize gene transfer with this agent. Controlling molecular weight appears to be the key and an optimum gene transfer activity lies between a degree of polymerization of 7 and 635. Additionally, increasing the charge density by incorporating a permanent positive charge in the molecule in the form of trimethyl quaternary ammonium group appears to offer some marginal benefit and the incorporation of targeting groups such as galactose improves targeting to hepatocytes. Urocanic acid groups have aided gene transfer, the latter possibly by aiding endosomal escape. However chitosan, while being able to achieve gene transfer to some extent, appears to lack the required level of efficiency that would be needed to allow it to be developed for the clinical delivery of genes (Uchegbu et al. 2009 : 331).

4.2.2.1 Deoxycholic acid-modified CS

Deoxycholic acid was conjugated to CS in methanol and water media using EDC as a coupling agent. The degree of substitution (DS) was determined to be 5.1 (5.1 deoxycholic acid groups substituted per 100 anhydrousglucose units). Hydrophobically modified CS provides colloidally stable self-aggregates in aqueous media having mean diameter of ~160 nm with a unimodal size distribution. Self-aggregates-DNA complexes were formed in aqueous media and found useful in transfecting in mammalian cells *in vitro.* The transfection efficiency of this system was relatively higher in comparison to naked DNA but significantly lower than the Lipofectamine®-DNA formulation.

4.2.2.2 Quaternized CS

Despite advantageous properties, CS is insoluble at physiological pH values. Free amine groups of CS can form ammonium salts with inorganic and organic acids. Reaction of CS with excess of methyl iodide in alkaline conditions gives *N*-trimethyl CS derivative. Such quaternary CS derivatives are useful for applications using their electronic properties, because the derivatives can keep their cationic character independent of the external conditions, including pH of medium. Trimethyl CS oligomers (TMO) of 40% (TMO-40) and 50% (TMO-50) degrees of quaternization were synthesized and examined for their transfection efficiencies in two cell lines: COS-1 and Caco-2. CS raises the transfection efficiency 2-4 times compared to the control value (i.e. naked DNA). TMO-50 markedly

increases the transfection efficiencies from 5-fold (for complexes with a DNA: oligomer ratio of 1: 6) to 52-fold (for a ratio of 1: 14). TMO-40 displays even higher transfection efficiencies, ranging from 26-fold (for a ratio of 1: 6) to 131-fold (for a ratio of 1: 14). However, none of the TMO-based vectors was able to increase the transfection efficiency in differentiated cells likes Caco-2. CS and TMO oligomers were found to exhibit significantly lower cytotoxicity than DOTAP, a well-known cationic lipid formulation commonly used in gene transfection.

4.2.2.3 CS modified with hydrophilic polymers

Modification of chitin and CS with a hydrophilic polymer such as polyethylene glycol (PEG) would be expected to result in hydrophilic chitin or CS while keeping the fundamental skeleton intact. Multiple methods have developed for the grafting of hydrophilic polymers onto chitin or CS to improve affinity to water or organic solvents. PEG-CS derivatives with various molecular weights (MW = 550, 2000, 5000) of PEG and DSs were synthesized, and the water solubility of these derivatives was evaluated at pH values of 4, 7.2, and 10. Almost all PEG-CS derivatives were soluble in acidic buffer (pH 4). Furthermore, some derivatives dissolved in neutral (pH 7.2) and alkaline buffers (pH 10). The weight ratio of PEG in the derivatives seems to dominate its water solubility. Higher molecular weight PEG was found to enhance water solubility of CS with a lower molecular weight PEG. PEG modification was found to minimize aggregation and prolong the transfection potency for at least 1 month in storage. Intravenous injection of CS-DNA nanoparticles and PEGylated-CS-DNA nanoparticles resulted in majority of nanoparticles to localize in kidney and liver within the first 15 min. The clearance of the PEGylated nanoparticles was slightly slower than that of non PEGylated nanoparticles.

4.2.2.4 Galactosylated CS

Although, in many cases, uptake of CS-DNA nano-particles appears to occur in the absence of ligand-receptor interaction. Park et al. prepared and examined galactosylated CS-graft-dextran-DNA complexes. Galactose groups were chemically bound to CS for liver-targeted delivery and dextran was grafted for enhancing the complex stability in aqueous media. This system was found to efficiently transfect Chang liver cells expressing asialoglycoprotein receptor

(ASGPR), which specifically recognize the galactose ligands on modified CS. In parallel work, galactosylated CS-graft-PEG (CGP) was developed for the same purpose. CGP-DNA complexes were found to be stable due to hydrophilic PEG shielding and increased the protection against DNase. Also, CGP-DNA complexes were found to enhance transfection in HepG2 cells having ASGP-R, indicating that galactosylated CS will be an effective hepatocyte-targeted gene carrier. A galactosylated CS-graft-poly(vinyl pyrolidone) (CGPVP) was also synthesized and showed improved physicochemical properties over the unmodified CS (Park et al. 2000 : 97). In some research have been synthesized lactosylated-modified CS derivatives (having various DSs) and tested their transfection efficiencies in many cell lines. However, the *in vitro* transfection was found to be cell-type dependent. HeLa cells were efficiently transfected by this modified carrier even in the presence of 10% serum, but neither CS nor lactosylated CS have been able to transfect HepG2 and BNL CL2 cells (Azzem and Domb 2005 : 283).

4.2.2.5 CS conjugated with transferrin, KNOB, and endosomolytic proteins

The transferrin receptor responsible for iron import to cells is found in many mammalian cells. As a ligand, transferrin could efficiently transfer low-molecular-weight drugs, macromolecules, and liposomes, through a receptor-mediated endocytosis mechanism. Transferrin has been applied to deliver plasmid DNA (pDNA) and oligonucleotide (Azzem and Domb 2005 : 284). Mao et al. explored two strategies to bind transferrin onto the surface of CS-DNA complex. In the first strategy, aldehyde groups were induced in transferrin (glycoprotein) after oxidation with periodate, and thereafter allowed to react with CS amine groups via the formation of Schiff-base linkages. The transfection efficiencies of transferrin-modified CS carrier (at varying degrees of modification) were examined in the HEK293 cell line and found to produce a two-fold transgene expression in comparison to unmodified CS carrier. In the second strategy, transferrin was introduced to the nanoparticle surface through a disulfide bond. The transferrin-conjugated carrier only resulted in a maximum of four-fold increase in transfection efficiency in HEK293 cells and only 50% increase in HeLa cells. The negligible increase in the transgene efficiencies as a result of ligand-modification (e.g. galactose

and transferrin) led the investigators to speculate that CS nanospheres may enter the cell via a unique endocytic pathway. To further enhance the transfection efficiency, KNOB (C-terminal globular domain of the adenoviral fiber protein) was conjugated to the CS by the disulfide linkages as well. Conjugation of KNOB to the CS-DNA nanoparticles was found to improve gene expression level in HeLa cells approximately 130-fold (Mao et al. 2001 : 404). MacLaughlin et al. found that the inclusion of pH-sensitive endosomolytic peptide GM227.3 in the formulation enhanced the level of expression in COS-1 cells. *In vivo* expression in rabbits involved anesthetizing the animal, opening region of the intestinal tract for direct administration into the intestinal lumen. Expression of a plasmid-CS-GMM225.1 formulation in rabbits after administration in the upper small intestine and colon was observed, in contrast with naked plasmid, which gave no expression. The lipidic formulation DOTMA-DOPE was used as control and was not expressed to as high as extent as the CS-lytic peptide formulation (MacLaughlin et al. 1998 : 269). Currently there are a number of methods for transferring therapeutic DNA into cells and tissues based on both viral and non-viral systems. No one system is ideal and all confer both advantages and disadvantages that might limit their clinical use.

4.2.3 PEI

Since its first use as a gene carrier in 1995, PEI has been considered as one of the most effective polymer-based gene carriers and used as a carrier for plasmid DNAs, oligonucleotides, and small interfereing RNAs (siRNA). PEI exists in either a branched or linear morphological structure. Branched PEI has a high density of amine groups, two thirds of which remain unprotonated in physiological environment. The unprotonated amines can absorb protons as the pH decreases. This unique property confers an extraordinary buffering capacity over a wide range of pH, which gives PEI-carrying nucleic acid drugs an opportunity to escape from the acidic endo-lysomal compartment via a hypothetical ‘‘proton sponge’’ effect (Kircheis, Wightman and Wagner 2001 : 343). Since the proton sponge effect plays an important role in the efficiency of PEI-based gene delivery systems, the further alteration in the proton-buffering capacity significantly enhances the overall transfection efficiency. Reduction of the number of protonable amines (thereby reducing the proton-buffering capacity of PEI) by the acetylation of primary

and secondary amines to secondary and tertiary amides, respectively, dramatically increases the transfection efficiency of PEI. PEI exists in a number of molecular weight formats (0.42-800 kDa) and transfection efficiency is highest at a molecular weight of between 12 to 70 kDa and the most commonly used PEI molecular weight is 22-25 kDa (Uchegbu et al. 2009 : 330).

4.2.4 Dendrimer

Dendrimers (from the Greek “dendron”: tree, and “meros”: part) are highly ordered, branched monodisperse macromolecules. Such dendritic structures first emerged in a new class of polymers named “cascade molecules,” initially reported by Vogtle and his group at the end of the 1970s. Further development by Tomalia’s group, and Newkome’s group gave rise to larger dendritic structures. These hyper-branched molecules were called “dendrimers” or “arborols” (from the Latin “arbor” for tree). For a historical view see review. Their unique molecular architecture means that dendrimers have a number of distinctive properties which differentiate them from other polymers; specifically they are not the result of statistical polymerization events but are built up in a stepwise fashion from a core group or by the addition of dendrimer arms to a core group (a divergent method of synthesis). The controlled synthetic approach means that dendrimers tend to be monodisperse with a well-defined size and structure. Dendrimers used in gene delivery are given in Figure 5. Dendrimers used for gene delivery contain amine functional groups and are usually protonated at physiological pH (cationic); such amine groups, which when protonated may be electrostatically bound to DNA phosphate groups to give dendriplexes. This electrostatic interaction protects DNA from degradation (Uchegbu et al. 2009 : 331).

Poly(amidoamine) (PAMAM) dendrimersare nanoscale structures with architecture characterized by dendritic branching and radial symmetry. PAMAM dendrimers range in size from 1.0-13.0 nm. Amine-terminateed PAMAM dendrimers develop high positive charge densities at their surfaces when at physiological pHs or when they are dissolved in water. Cationic dendrimers have been shown to complex with pDNA, RNA, single-strand oligonucleotides, and various sizes of double-stranded DNA. The PAMAM dendrimers were capable of transfecting many different cell types, including Jurkat and primary human

fibroblasts, which are typically difficult to transfect, with no specific generation optimal for every type (Table 5).

Table 5 Cells transfected with PAMAM dendrimers

V79	Chinese hamster lung fibroblast	Jurkat	Human acute T-cell leukemia
CHO	Chinest hamster ovary	CV-1	Monkey fibroblast
NHBE	Human brochial epithelium	COS-1	Monkey kidney fibroblast
HCS-2/8	Human Chondrosarcoma-Derived	COS-7	Monkey kidney fibroblast
SW480	Human colon adenocarcinoma	L-M(TK-)	Mouse connective tissue
HeLa	Human epithelial carcinoma	NIH3T3	Mouse embryonal
293T	Human epithelial kidney	EL4	Mouse lymphoma
K-562	Human erythroleukemia	RAW264.7	Mouse macrophage-like
HF1	Human fibroblast	D5	Mouse melanoma
U937	Human histiocytic lymphoma	YPE	Porcine Vascular endothelial
HepG2	Human hepatoma	Rat2	Rat embryonal fibroblast
A549	Human lung carcinoma		Rat hepatocytes
CCD-37LU	Human lung fibroblast	Clone 9	Rat liver epithelium
SW837	Human rectum adenocarcinoma	RMI	Rat mesothelial
SAEC	Human small airway epithelium	YB2/10	Rat myeloma
QS	Human synoviocyte		

Source : Lori A. Kubasiak and Donald A. Tomalia, “Cationic dendrimers as gene transfection vectors : Dendri-poly(amidoamines) and Dendri-poly(propylenimines),” In Polymeric gene delivery (New York : CRC Press, 2005), 144.

4.2.5 Cationic lipid

Most cationic lipids used for gene transfer have three parts: a hydrophobic lipid anchor group; a linker group, such as an ester, amide or carbamate; and a positively charged head-group, which interacts with DNA, leading to its condensation (Figure 6). Hydrophobic group lipid anchors can be either a cholesterol group or fatty acid chains. The linker group is a important component that determines the chemical stability and biodegradability of the lipid. The linker groups should be biodegradable yet strong enough to survive in a biological environment. Ester linkage between hydrophobic lipid anchors and cationic head-groups is likely to provide

biodegradability to cationic lipids. 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.

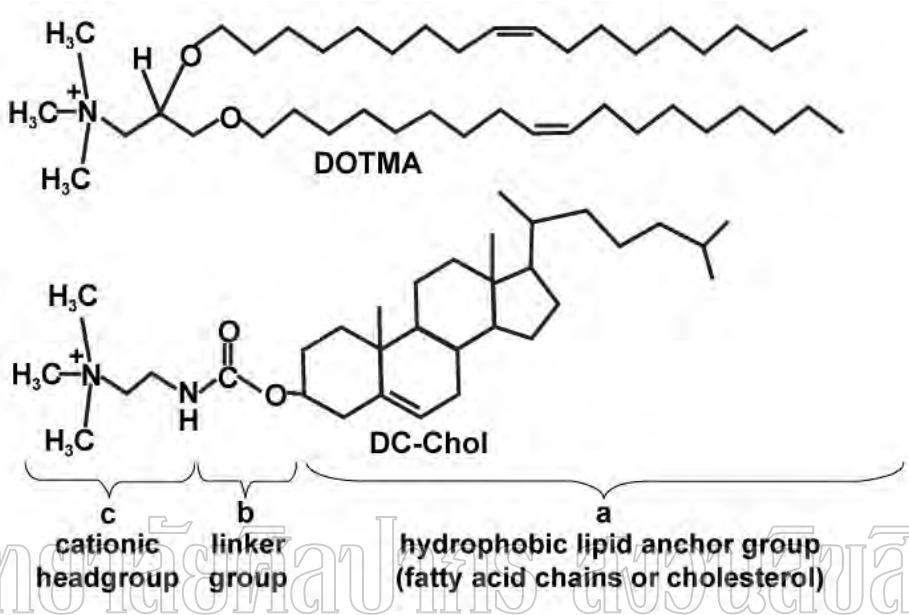


Figure 6 Basic components of cationic lipids (a) Hydrophobic lipid group; (b) linker group; (c) cationic head group.

Among all the basic components of the cationic lipid, the type of head-group has been shown to have a dominant role in transfection efficiency and cytotoxicity. Cholesterol is a naturally occurring lipid and is metabolized in the body. The early success of 3-(N [N′, N′-dimethylaminoethane]-carbamoyl cholesterol (DC-Chol) lipid-based gene delivery system spurred interest in the development of novel cholesterol-based cationic lipids. Cationic lipids in T-shape head-groups tend to be more effective than linear counterparts. The levels of gene expression obtained with spermine cholesteryl carbamate and spermidine cholesteryl carbamate in T-shape orientation was 50- to 100-fold higher both *in vitro* and *in vivo* than that observed with DC-Chol, which has only a single protonatable amine (Mahato and Kim in Amiji 2005 : 175-176). 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 the advantage of lipids and polycations, Zhou and Huang synthesized lipopolysine by mixing poly (L-lysine) of 3300 Da with two molar equivalents of N-hydroxysuccinimide ester of dipalmitoylsuccinylglycerol in dimethyl sulfoxide (Zhou and Huang 1994 : 195). Similarly, Choi et al. synthesized 3-(L-lysineamide carbamoyl) cholesterol (K-Chol) and 3-(L-ornithinamide carbamoyl) cholesterol (O-Chol) by the solid phase synthesis method. However, liposome preparation with colipid dioleoyl phosphatidylethanolamine (DOPE) was essential for enhanced gene transfer by these cationic amphiphiles (Choi et al. 2001 : 108-113). Yamazaki et al. grafted cetyl groups as hydrophobic lipid anchors onto PEI of 1,800 and 25,000 Da, and prepared polycation liposomes for gene transfer (Yamazaki et al. 2000 : 1148-1155).

4.2.6 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. Table 6 summarizes the various advantages and disadvantages of collagen as a biomaterial for delivery.

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).

Table 6 Summary of the advantages and disadvantages of collagen

advantages	**Disadvantages**
Available in abundance and can be purified from living organisms	Expensive nature of pure Type-I collagen
Nonantigenic	Variability of isolated collagen (cross-linking density, fiber size, impurities)
Bioreabsorbable and biodegradable	Increased hydrophillicity that leads to swelling and a more rapid release
Nontoxic and biocompatible	Variability in the enzymatic degradation rate as compared to hydrolytic degradation
High tensile strength, synergic with bioactive components	Complex handing properties
Hemostatic	Side effects, e.g., bovine spongioform encephalopathy (BSE) and mineralization
Biodegradability may be regulated by crosslinking	
Easily modified/derivatized using functional moieties	
Capable of conjugation with synthetic polymers	

Source : Goldie Kaul and Mansoor Amiji, “Protein Nanoparticles for gene delivery,” In Polymeric gene delivery (New York : CRC Press, 2005), 433.

CHAPTER 3
MATERIALS AND METHODS

1. Materials

1.1 Materials for purification of DNA (Table 7)

Table 7 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

Egg yolk phosphatidyl choline, EPC (Wako Pure Chemical Osaka, Japan)

Sodium oleate, NaO (Sigma®, St Louis, MO, USA)

Poly-L-arginine MW > 70,000 Da, PLA (S.M. Chemical Supplies Co., Ltd., Thailand)

Lipofectamine2000™ (Invitrogen, NY, USA)

1.3 Tissue culture reagents

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

RPMI Medium 1640 GIBCO™

MEM Medium GIBCO™

Foetal bovine serum EU Approved origin GIBCO™

L-Glutamine GIBCO™

MEM non-essential amino acids 100x w/o

1.4 All other chemicals

GenePure LE Agarose 500g (ISC BioExpress®, USA)

Lambda DNA / *Hin*d III Markers (Promega)

Blue/Orange 6x loading dye (Promega)

Sodium chloride (UNIVAR® Ajax Finechem; analytical reagent grade)

Casein enzyme hydrolysate, type-1; Tryptone (HIMEDIA® Himedia Laboratories Pvt. Ltd.)

Yeast extract powder (HIMEDIA® Himedia Laboratories Pvt. Ltd.)

Sterile water for irrigation (General Hospital Products Public Co., Ltd.)

Sodium bicarbonate (Analar® BDH ;VWR International Ltd.)

Dimethyl sulphoxide (Fisher Scientific; analytical reagent grade)

0.25% Trypsin-EDTA (GIBCO ™)

Trypan blue stain 0.4% (GIBCO™)

Chitosan (CS) MW 45 kDa with 85% degree of deacetylation (Seafresh company, Thailand)

Poly-L-arginine hydrochloride MW 5000-15000, 15000-70000, >70000 Da (S.M. Chemical Supplies Co., Ltd., Thailand)

Poly-L-ornithine hydrobromide MW 30000-70000 Da (S.M. Chemical Supplies Co., Ltd., Thailand)

Poly-L-lysine hydrobromide MW 30000-70000 Da (S.M. Chemical Supplies Co., Ltd., Thailand)

Branched polyethylenimine, PEI (Sigma-Aldrich Chemie. GmbH, Germany)

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Sigma-chemical company (St. Louis, MO, USA)

Ethanol absolute (Scharlau® ET0016 Scharlau® Chemie SPAIN analytical reagent grade)

Tris(hydroxymethyl)aminomethane (Pacific Science, Thailand molecular biology grade)

Chloroform (VWR International Ltd. England analytical reagent grade)

2. Equipments

1. Spray dryer (Minispray Dryer, Büchi 190, Postfach, Switzerland)
2. 1.5 mL, 2 mL Eppendrof® tubes
3. 15 mL, 50 mL centrifuge tubes-sterile (Biologic research company)
4. 24-well tissue culture test plates (TPP®; Switzerland)
5. 25 cm^2 and 75 cm^2 cell culture flask (Corning®; Corning Incorporated)
6. 96-well cell culture cluster (Costar®; Corning Invorporated)
7. Analytical balance (Satorius CP224S, Sartorius CP3202S; Scientific promotion Co., Ltd.)
8. Automatic autoclave (Model : LS-2D; Scientific promotion CO., Ltd.)
9. Bacterial incubator (Contherm; Lab Focus CO., Ltd)
10. Cellulose acetate filter 0.2 μm (Sartorius AG. 37070 Goettingen, Germany)
11. Centrifuge (Hermle Z300K; Labnet®; Lab Focus CO., Ltd.)
12. CO_2 incubator (HERA Cell 240 Heraeus)
13. DyNA Vap centrifuge evaporator (Labnet®; Lab Focus CO., Ltd.)
14. Fluorescence microscope (Model : GFP-B, wavelengths : excitation filter 480/40 and emission filter 535/50)
15. Fusion universal microplate analyzer (Model No : AOPUS01 and A153601 ; A Packard bioscience company)
16. GeneRay UV-Photometer (Biometra® λ260/280 nm)
17. Inverted microscope (Eclipse TE 2000-U; Model : T-DH Nikon® Japan)
18. Laminar air flow (BIO-II-A)
19. Magnetic stirrer and magnetic bar
20. Measuring pipettes (1, 2, 5, 10 mL)

21. Micropipette 0.1-2 μL, 2-20 μL, 10-100 μL, 20-100 μL, 100-1000 μL (Masterpette®; Bio-Active Co., Ltd.)

22. Micropipette tip

23. NIPRO Hypodermic needle 25G× 1″ (0.5×25 mm) thin wall

24. pH meter (HORIBA compact pH meter B-212)

25. Pipette aid (Powerpette Plus; Bio-Active Co., Ltd.)

26. Protein and nucleic acid electrophoresis (MyRUN intelligent electrophoresis unit; Cosmobio CO., Ltd.)

27. Sartorius® filter set (Sartorius BORO 3.3 Goettingen, Germany)

28. Shaking incubator (GFL 3031)

29. TERUMO® Syringe 50 mL

30. Water bath (Hetofrig CB60; Heto High Technology)

31. Zetasizer Nano ZS (Malvern instruments Ltd., Malvern, UK)

32. Spectrofluorometer (RF-1501, Shimadzu, Tokyo, Japan)

33. Heater and Magnetic stirrer (Heidolph®, Germany)

3. Methods

1. Preparation of anionic liposomes coated by cationic polypeptide

EPC: NaO (10: 2 molar ratio) anionic liposomes were prepared by sonication method. Briefly, EPC was 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 polypeptide-coated liposomes, the suspension of the top phase was pipetted and mixed with cationic polypeptide solution (1 mg/mL) at the ratios of anionic liposomes: cationic polypeptide = 1:1 (w/w) with a magnetic stirrer for 30 minutes. In the second step, the aqueous phase contained hydrophilic

surfactant and dispersed polymer. The primary emulsion was slowly added while the system was stirred at 1,000 rpm for 5 minutes.

2. Purification and quantification of DNA

The DNA encoding green fluorescence protein (pEGFP-C2) was used as observed gene expression. The plasmid DNA was purchased from Invitrogen. To amplify the pDNA, it 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.

2.1 Purification of DNA

The DNA was 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 in 100 ml of LB medium. Growth at 37°C for 12-16 hr with vigorous shaking (approx. 200 rpm).

2. Harvest the bacterial cells by centrifugation at 6000 *x g* for 15 min at 4°C.

3. Resuspend the bacterial pellet in 4 ml Buffer P1.

4. Add 4 ml Buffer P2, mix thoroughly by vigorously inverting the sealed tube 4-6 times, and incubate at room temperature (15 – 25 °C) for 5 min.

5. Add 4 ml of chilled Buffer P3, mix immediately and thoroughly by vigorously inverting 4-6 times, and incubate on ice for 15 min.

6. Centrifuge at ≥ 20,000 *x g* for 30 min at 4 °C. Remove supernatant containing plasmid DNA promptly.

7. Centrifuge the supernatant again at ≥ 20,000 *x g* for 15 min at 4 °C. Remove supernatant containing plasmid DNA promptly.

8. Equilibrate a QIAGEN-tip 100 by applying 5 ml Buffer QBT, and allow the column to empty by gravity flow.

9. Apply the supernatant from step 7 to the QIAGEN-tip and allow it to enter the resin by gravity flow.

10. Wash the QIAGEN-tip with 2 x 10 ml Buffer QC.

11. Elute DNA with 5 ml Buffer QF.

12. Precipitate DNA by adding 3.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at ≥ 15,000 *x g* for 30 min at 4 °C. Carefully decant the supernatant.

13. Wash DNA pellet with 2 ml of room-temperature 70% ethanol, and centrifuge at ≥ 15,000 *x g* for 10 min at 4 °C. Carefully decant the supernatant without disturbing the pellet.

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

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: A 35 μl of the pure plasmid was diluted into 665 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 follow equation:

$$\text{Plasmid concentration} = 50\ \mu g/ml \times OD_{260nm} \times DF \quad (1)$$

- A solution of 50 μg/ml of an average double-stranded DNA has an OD_{260nm} of 1.
- OD_{260nm} is the optical density from the absorbance reading.
- DF is the dilution factor (in the above in would be 20).

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. Often a ratio of 1.8 A_{260}/A_{280} is given as means of determining purity (Sullivan et al., in Torchilin and Weissig 2003 : 299).

3. Preparation of sprayed dried chitosan hydrochloride

Chitosan (CS) with MW of 45 kDa and 85% degree of deacetylation was purchased from Seafresh Chitosan (lab) Co., Ltd in Thailand. CSHCl was prepared as previously described (Nunthanid et al. 2004 : 15-26). CS was dissolved

in distilled water containing hydrochloric acid in a 1:0.800 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.

4. Cell culture, routine maintenance

The human cervical carcinoma cell lines (HeLa) and human hepatoma cell lines (Huh7 cells) were chosen as model in this transfection efficiency and cytotoxicity study. The cell lines observed under an inverted microscope in shown in Figure 7.

4.1 The cultivation of HeLa and Huh7 cells

HeLa and Huh7 cells were maintained in complete growth medium (MEM and RPMI Medium 1640) in a humidified air atmosphere (5% CO_2, 95% RH, 37 °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.

4.2 Preparation of media

The process of preparation of 1X complete culture medium is shown as follows: foetal bovine serum (FBS) was heat inactivated by incubating for 30 min in a 56 °C water bath. MEM or RPMI Medium 1640 powder was dissolved in 5% less sterile water than desired total volume of medium with gentle stirring and 2.2 or 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 Glutamax-I or L-Glutamine, 1% v/v Non-essential Amino Acids, 10% v/v heat inactivated FBS.

4.3 Thawing frozen cell

The cells were thawed quickly in a 37 °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 min 25 °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.

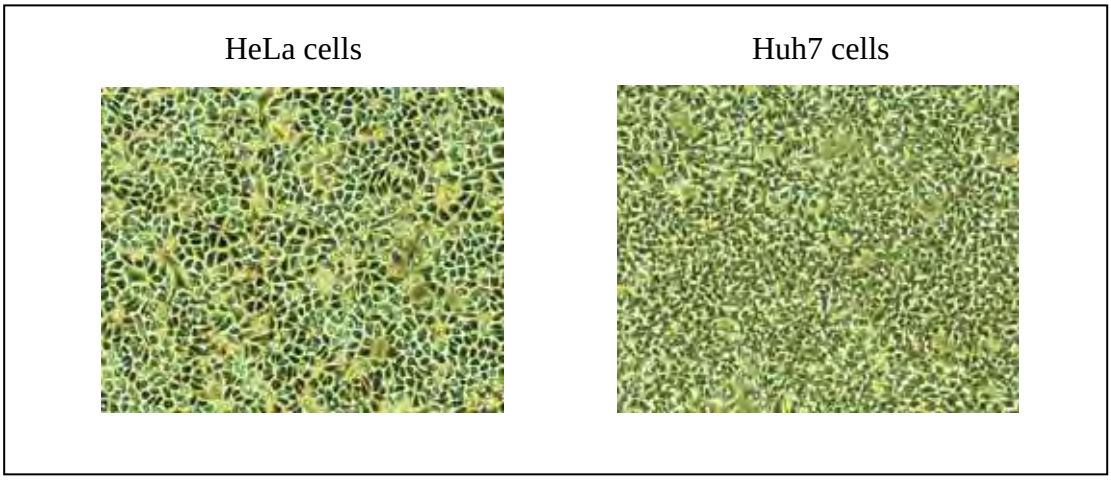


Figure 7 Cultivated HeLa and Huh7 on 75 cm^2 TC-flask, image 10× objective using an inverted microscope

4.4 Subculturing

Cultivated HeLa and 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 °C for approximately 1 min 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 min. 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 flasks. The cell culture was then placed in incubator.

4.5 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. For one 75 cm^2 cell culture flask, resuspended in 5 ml of complete culture medium. The

cell suspension tube and 5 labeled cryotubes were pre-cooled on ice for 5 minutes before adding 250 µl of DMSO into the suspension cell and mixing gently. 1ml of cell suspension was transferred into cryoprotective tubes. The cryostocks were stored at -20°C, -80°C overnight, respectively. For long term storage, the cells were stored in liquid nitrogen.

5. Preparation of the DNA-cationic polypeptide-CSHCl complexes

The DNA-cationic polypeptide-CSHCl complexes were prepared by charge interaction induced self-assembly at various weight ratios ranging from 0 to 50 and CSHCl 4 µg as described above. The complexes were prepared by adding CSHCl solution, polypeptides and DNA follow the order of mixing;

Polypeptides ⟶ DNA ⟶ CSHCl
CSHCl ⟶ DNA ⟶ polypeptides
Polypeptides ⟶ CSHCl ⟶ DNA

Then pipetting up and down and tapping the tubes gently. They were then incubated for 30 min at room temperature to ensure complex formation.

5.1 Gel electrophoresis

Complexes formation was confirmed by electrophoresis on a 1.0 % agarose gel with tris-acetate-EDTA (TAE) running buffer at 100 V for 45 min. UV transillumination of the gel was employed with ethidium bromide (0.5 µg/ml) to visualize DNA (Lee et al. 2005 : 8108).

5.2 Binding affinity

Ethidium bromide (EtBr) displacement assay was performed in order to study the ability of CS and polypeptide to bind the DNA. EtBr (0.1 mg/ml) was dissolved in 0.01 M phosphate buffered saline (PBS, pH 7.4). 20 µl of EtBr solution was added to a 20 µl of 500 µg/ml DNA solution. The steady state fluorescent measurement was performed on a spectrofluorometer (RF-1501, Shimadzu, Tokyo, Japan) at an excitation wavelength of 560 and an emission wavelength of 605 nm. An aliquot CS solution (1 mg/ml) or polypeptide (1 mg/ml) was then titrated into the DNA/EtBr solution to the varied weight ratios of DNA

/CS/polypeptide complexes (1:4:0 to 50) with vary order of mixing. The fluorescent intensity calculated based on the fluorescent intensity of the DNA/EtBr solution is shown in Equation 2. The recorded fluorescent intensity (FI) was expressed relative to the fluorescent intensity of the DNA/EtBr solution in the absence of CS or polypeptide (FI_0), after substracting the fluorescence of EtBr in the absence of DNA under the same buffer conditions (FI_{buff}). Data are presented as mean±SD. The assay was performed in triplicate.

$$FI(\%) = [(FI-FI_{buff})/(FI_0-FI_{buff})] \times 100 \quad (2)$$

5.3 Particle size and zeta-potential

The mean particle size of the DNA-CSHCl-polypeptide complexes, vary order of mixing, was determined by photon correlation spectroscopy (PCS) using the Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) at room temperature. According to obtain the optimum scattering intensity, the DNA/CS/polypeptide complexes were diluted with distilled water which had been passed through 0.2-μm membrane filter before used. All samples were measured in triplicate using the refractive index and viscosity of pure water in calculations. The zeta-potential (surface charge) was measured on the same instrument, and with the viscosity and dielectric constant of pure water for calculations (Lavertu et al. 2006 : 2817).

6. Preparation of the DNA/cationic polypeptide-coated liposome complexes

Complexes of cationic polypeptides and cationic polypeptide-coated liposomes/DNA were formulated at weight ratio of 1, 2, 2.5, 3 and 4 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.

6.1 Gel electrophoresis

Complexes formation was confirmed by electrophoresis on a 1.0 % agarose gel with tris-acetate-EDTA (TAE) running buffer at 100 V for 45 min. UV transillumination of the gel was employed with ethidium bromide (0.5 μg/ml) to visualize DNA (Lee et al. 2005 : 8108).

6.2 Particle size and zeta-potential

The mean particle size of the DNA/Cationic polypeptide-coated liposome complexes was determined by photon correlation spectroscopy (PCS) using the Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) at room temperature. According to obtain the optimum scattering intensity, the pDNA/CS complexes were diluted with distilled water which had been passed through 0.2-μm membrane filter before used. All samples were measured in triplicate using the refractive index and viscosity of pure water in calculations. The zeta-potential (surface charge) was measured on the same instrument, and with the viscosity and dielectric constant of pure water for calculations (Lavertu et al. 2006 : 2817).

7. *In vitro* transfection on HeLa and Huh7 cells

The day before transfection, HeLa or Huh7 Cells were seeded into a 24-well plates at a density of 2 x 10^4 cells/well in 1 ml of complete medium. The cell number seeded should produce 40–80% confluence on the day of transfection. The cells were incubated overnight in an incubator at 37°C and 5% CO_2. On the day of transfection experiments, the media in each well were removed and the cells were washed once with 1 ml of PBS. The DNA/cationic polypeptide-coated liposome or DNA/CS or DNA/cationic peptides or DNA/cationic peptide/CSHCl (vary order of mixing) complexes at various weight ratios (equivalent to 1 μg of pEGFP-C2) were added in 1 ml of serum free media and the cells were incubated with the complexes for 24 h at 37°C and 5% CO_2. The medium containing the remaining complexes was removed from the cells by gentle aspiration, and the cells were washed once with 1 ml of PBS. 1 ml of fresh complete medium was then added, and the incubation was continued for further 24-48 h. In case of the DNA/PEI complexes, the complexes were incubated with cells for 4 h, and further incubated with 1 ml of fresh complete medium for 20-48 h. All transfection experiments were performed in triplicate.

For the fluorescence assay of transfection, the cells were also 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-cell per area of a 24-well tissue culture plate (1.9 cm^2).

8. Evaluation of cytotoxicity

Evaluation of cytotoxicity was performed by MTT assay. The cells were seeded in a 96-well plate at a density of 8 x 10^3 cells/well in 100 µl of growth medium and grown overnight, prior to incubation with cationic polypeptides, cationic polypeptide-coated liposomes and cationic polypeptide-CSHCl growth medium was removed. Polypeptides, polypeptide-coated liposomes, polypeptide-CS and PEI were applied onto the cell in culture medium without serum at concentrations ranging from 0.001-1 mg/ml (100 µl/well) and cell viability was compared to cells treated with culture medium without serum only. In case of the DNA/polypeptides, DNA/polypeptide-coated liposomes and DNA/polypeptide/CS complexes, 100 µl of complex containing media at the same weight ratio as the transfection efficiency (equivalent to 0.2 µg of pEGFP-2) was added to each well and incubated for 24 h. After 24 h, 100 µl of MTT (1 mg/ml in culture medium without serum) was added to each well and incubated for 4 h under normal growing conditions. At this point, all media was removed and 100 µl DMSO was added. The formazen crystals were dissolved and then the absorbance was measured at 550 nm using a plate reader (A Packard BioScience Company). Viability of non-treated control cells was arbitrarily defined as 100%. The percentage of cell viability compared to control cells containing cell culture medium without polymer was calculated using follow equation:

$$\% \text{ cell viability} = \frac{[OD]_{test} - [OD]_{DMSO}}{[OD]_{control} - [OD]_{DMSO}} \times 100 \qquad (3)$$

9. Statistical analysis

All experimental measurements were collected in triplicate. Result values were expressed as mean value ± standard deviation (SD). Statistical significance of differences in transfection efficiency and cell viability were examined using one-way analysis of variance (ANOVA) followed by LSD post hoc test.

CHAPTER 4
RESULTS AND DISCUSSION

Results

1. Characterization of cationic polypeptide/DNA complexes

1.1 Physicochemical properties

Physicochemical properties of polypeptide/DNA complexes including complex formation, particle size and zeta potential were characterized because these properties might affect the transfection efficiency.

1.1.1 Effect of polypeptides type

Type of polypeptides is a formulation parameter affecting the physicochemical properties of polypeptide/DNA complexes. The interaction with DNA to each type of polypeptides was investigated by agarose gel electrophoresis technique. To vary the weight ratio of the each complex, the polypeptide concentration was changed and the DNA concentration was kept constant. The complexes formation between three type of cationic polypeptide and DNA was dependent on the weight ratio (Figure 8). When the concentration of polypeptide gradually increased, the DNA was gradually retained within the gel loading well. Migration of DNA on the agarose gel was retarded resulting from the charge neutralization of the complexes. The complete complexes were formed as the DNA was totally retained within the well. Migration of DNA on the agarose gel was retarded resulting from the charge neutralization of the complexes. The complete complexes were formed as the DNA was totally retained within the well (Figure 8: lane 8; the summary was showed in Table 8). The type of cationic polypeptides (PLL, PLA and PLO) did not affected. The complete complexes were formed at weight ratio above 0.1.

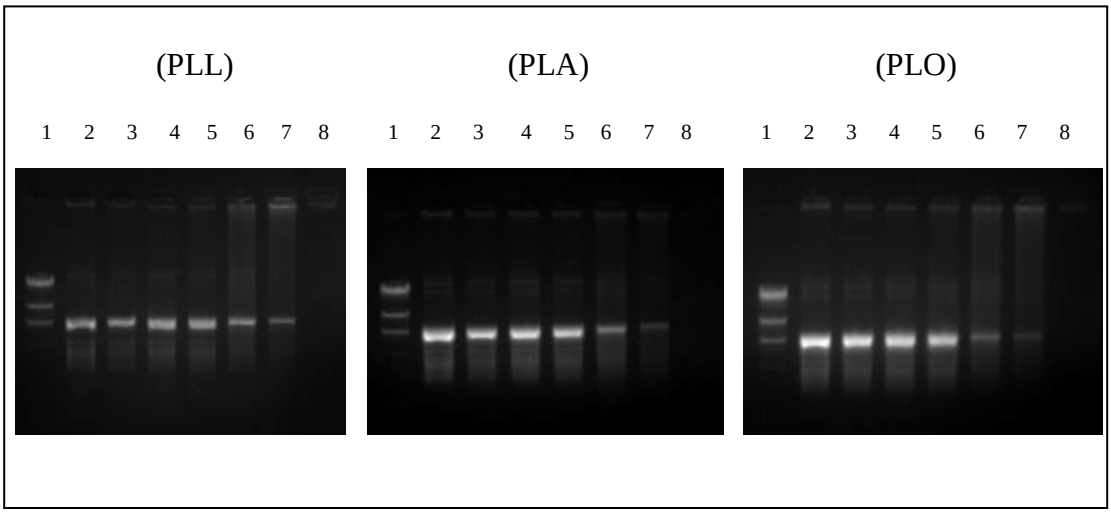


Figure 8 Agarose gel electrophoresis of polypeptides/DNA complexes with different type of peptides and weight ratio. Lane 1, λ*Hin*d III DNA marker, lanes 2-8, weight ratio of 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, respectively

Table 8 The weight ratio of three polypeptides/DNA complexes formed at pH 7.4

Samples	Wight ratio of complete complexes formed
PLL MW 30-70 kDa	0.5
PLA MW 15-70 kDa	0.5
PLO MW 30-70 kDa	0.5

Accordingly, the complete complexes were able to form at the weight ratio above 0.1, regardless to the type of polypeptide. To deliver DNA into cells, the particle size and zeta potential of a complex are the important factors that influence the access and passage of the complex to the targeting site. The particle size and zeta potential of complexes were investigated and it was found that their particle size and zeta potential were dependent on the weight ratio (Figure 9).

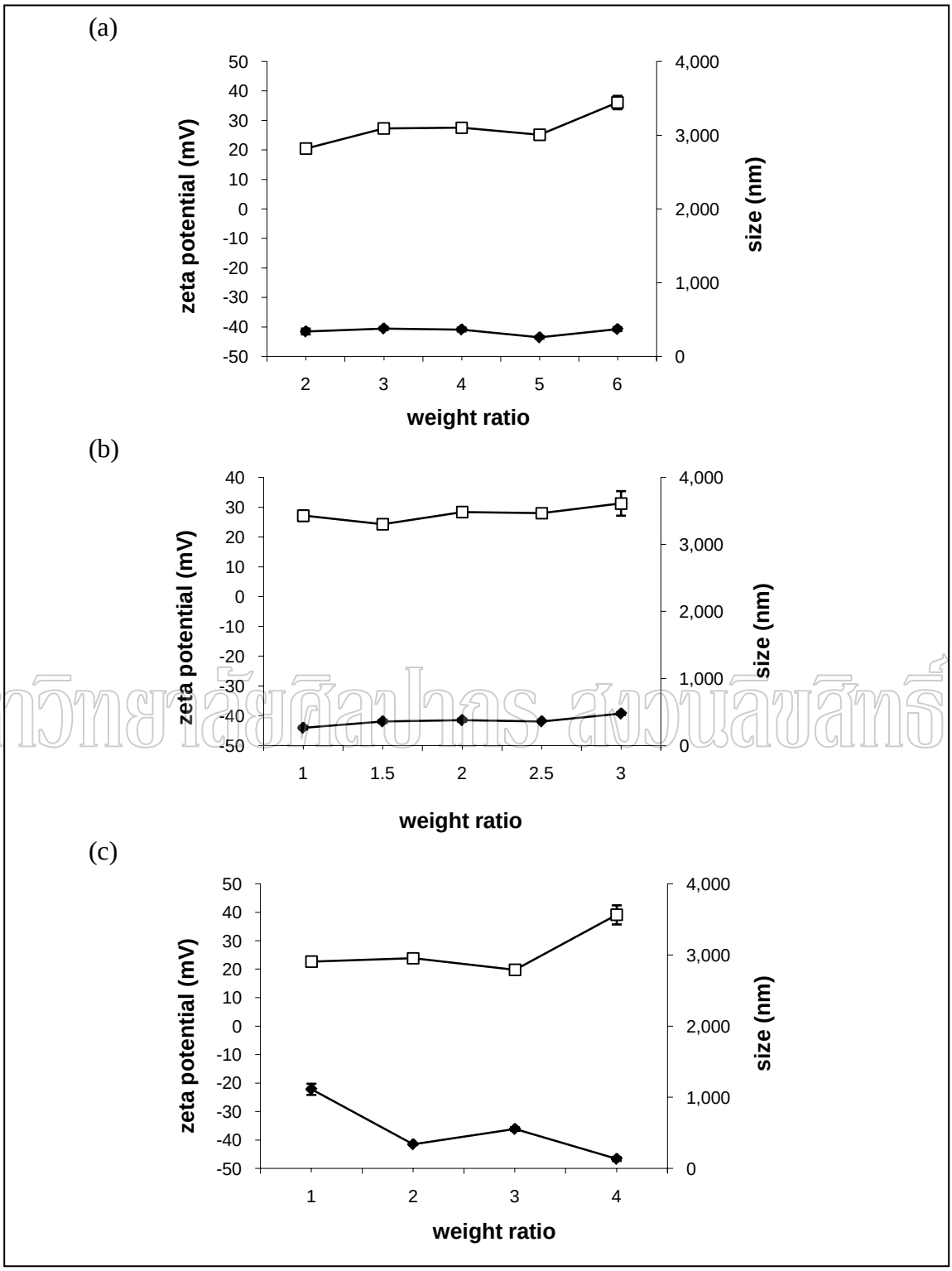


Figure 9 Zeta-potential (□) and particle size (♦) at various weight ratios of polypeptides/DNA complexes; (a) PLL MW 30-70 kDa/DNA complexes, (b) PLA MW 15-70 kDa/DNA complexes, (c) PLO MW 30-70 kDa/DNA complexes at pH 7.4

The particle size of PLL/DNA complexes slightly decreased with an increasing weight ratio from 1.0 to 2.5, as shown in Figure 9 (a). On the other hand, the zeta potential was positive and slightly increased with an increasing weight ratio from 1.0 to 2.5.

The particle size and zeta potential of PLA/DNA complexes remained constant at about 400 nm and +28 mV with weight ratio from 1.0-5.0, as shown in Figure 9 (b).

The particle size of PLO/DNA complexes decreased with an increasing weight ratio from 1 to 2. Then particle size slightly increased about 500 nm at weight ratio of 3 and decreased to constant at 134 nm after a weight ratio of 3 (Figure 9 (c). At weight ratio lower than 3, the complex had a positive value of zeta-potential and about value at 25 mV. The zeta-potential suddenly increased with weight ratio higher than 3.

1.1.2 Effect of pH

The effect of pH on the formation of polypeptide/DNA complexes was carried out in the complex solution at pH 6.4 by agarose gel electrophoresis. The results were shown in Figure 13. The complete complex were formed at weight ratio of 0.5 (Figure 10: lane 8; the summary was showed in Table 9). It indicated that the DNA binding was independent from the pH of complex solution.

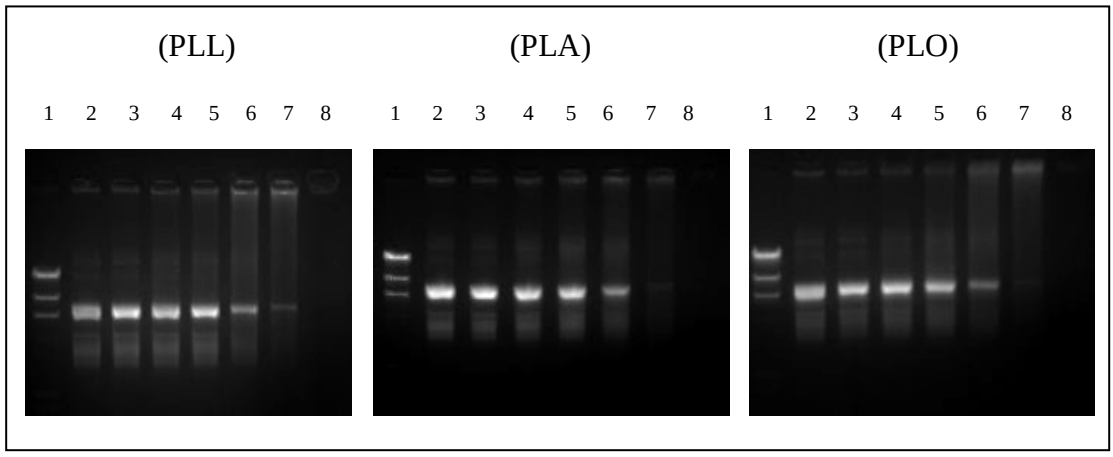


Figure 10 Agarose gel electrophoresis of polypeptides/DNA complexes at pH 6.4 with different type of peptides and weight ratio. Lane 1, λ*Hin*d III DNA marker, lanes 2-8, weight ratio of 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, respectively

Table 9 The weight ratio of three polypeptides/DNA complexes formed at pH 6.4

Samples	Wight ratio of complete complexes formed
PLL MW 30-70 kDa	0.5
PLA MW 15-70 kDa	0.5
PLO MW 30-70 kDa	0.5

Moreover, the effect of pH on the particle size and zeta potential of polypeptide/DNA complexes were investigated in the complex solution pH of 6.4. The particle size of PLL/DNA and PLA/DNA complexes at pH 6.4 was a little larger than those at pH 7.4 (Figure 11 (a) and (b)). However at pH 6.4, the all complexes were nanosize (200-800 nm). The particle sizes of PLO/DNA complexes were larger than PLL/DNA and PLA/DNA complexes.

The charge densities of polypeptides increased when the pH of the polypeptide solution decreased from high to lower pH. Due to the lower charge density of polypeptide at high pH, a weaker interaction between the DNA and polypeptide is expected.

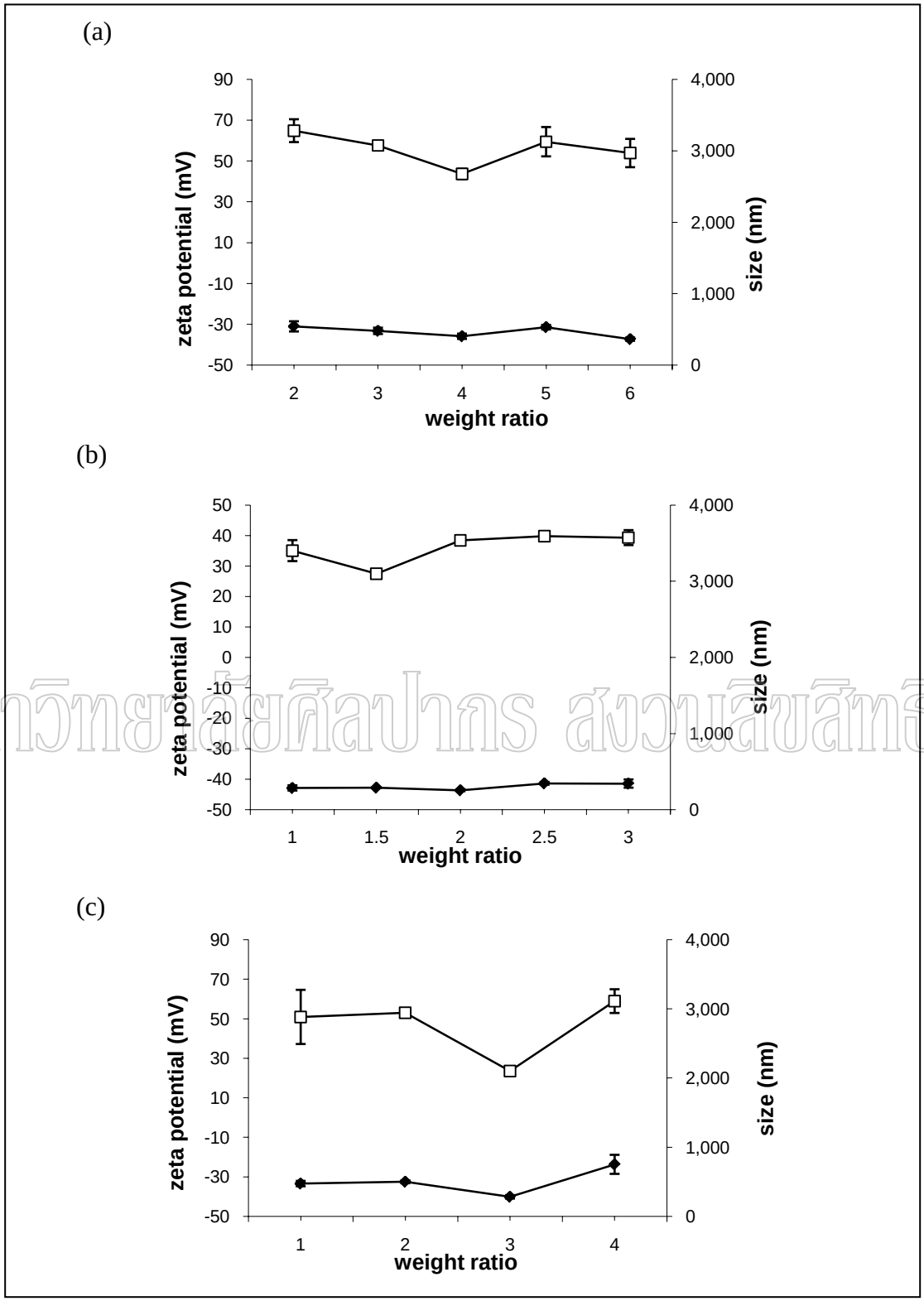


Figure 11 Zeta-potential (□) and particle size (♦) at various weight ratios of polypeptides/DNA complexes; (a) PLL MW 30-70 kDa/DNA complexes, (b) PLA MW 15-70 kDa/DNA complexes, (c) PLO MW 30-70 kDa/DNA complexes at pH 6.4

Effect of MW of PLA

The transfection efficiency of cationic polypeptides depended on the types with PLA showed the highest transfection efficiency and it is one of cationic polypeptide having been used for gene delivery with chitosan (Gao et al. 2008 : 241-246). Therefore, the PLA was used to study effect of MW. In order to investigate the effect of MW on the complex formation, PLA/DNA complexes with different MW (5-15, 15-70 and >70 kDa) were formulated. Figure 12 shows that the complexes were completely formed at weight ratios above 0.1 for all MW. The summary was also showed in Table 10.

Figure 12 Agarose gel electrophoresis of polypeptides/DNA complexes with different MW of PLA and weight ratio. Lane 1,8 and 16, λ*Hin*d III DNA marker; lanes 2-7, weight ratio of 0, 0.01, 0.05,0.1, 0.5, 1; lanes 9-15, weight ratio of 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5; lanes 17-21, weight ratio of 0, 0.01, 0.05,0.1, 0.5 at pH 7.4, respectively

Table 10 Weight ratio, which the complete complexes were formed of polypeptides at pH 7.4

Samples	Weight ratio of complete complexes formed
PLA MW 5-15 kDa	0.5
PLA MW 15-70 kDa	0.5
PLA MW >70 kDa	0.5

Additionally, the particle size and zeta potential of PLA/DNA complexes were determined. The particle size of the complexes had a trend showing a slight increase in the particle size as the MW of PLA was increased (Figure 13). This result might be expected that the high MW PLA could interact with DNA and thus condense the DNA more efficiently than the low MW PLA. The particle size of the complex with higher MW should be smaller as the weight ratio was increased.

The zeta potential of PLA/DNA complexes was contingent on the MW of PLA. The zeta potential of PLA/DNA complexes formulated with high MW PLA was fairly higher than those formulated with low MW PLA (Figure 13).

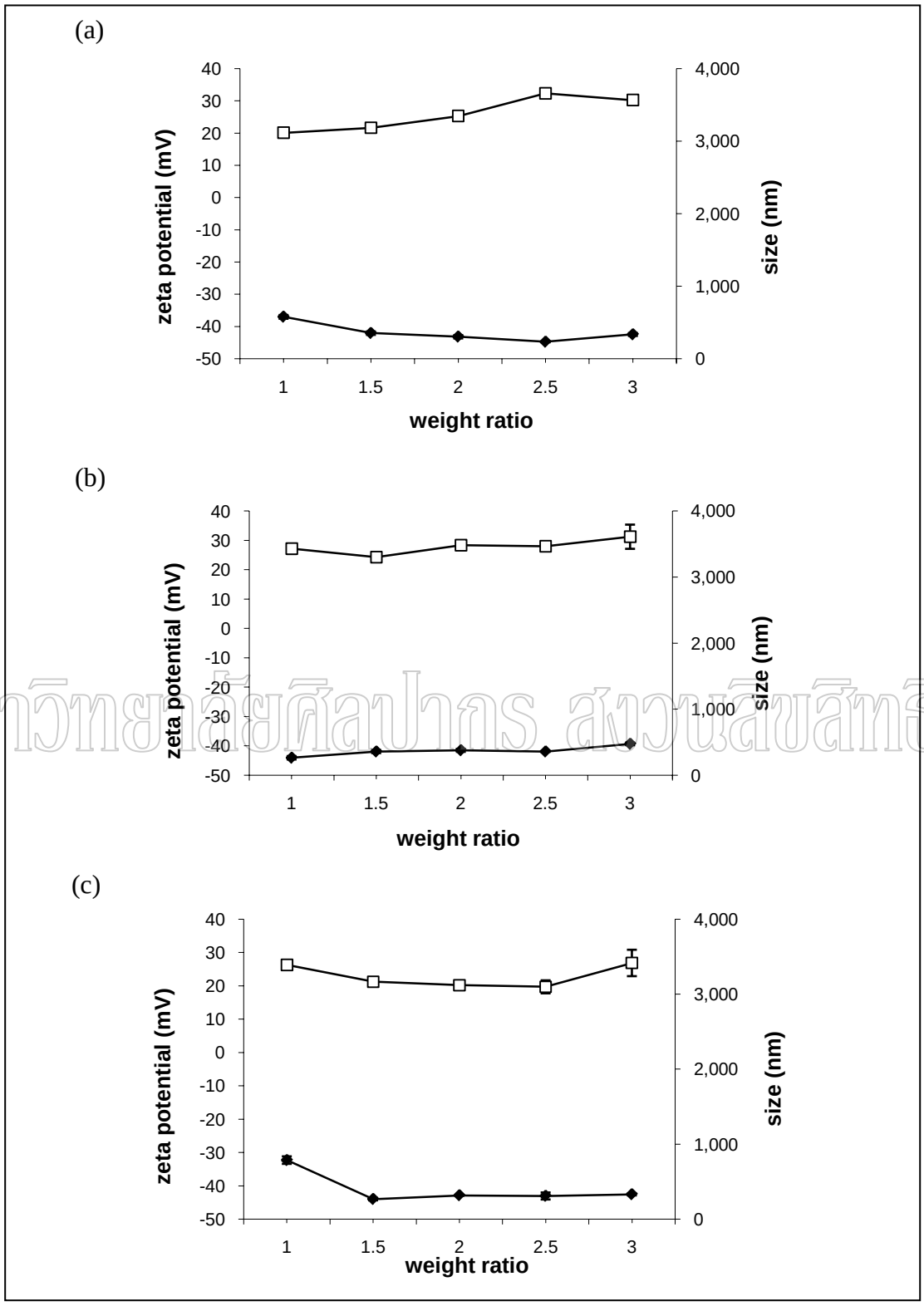


Figure 13 Zeta-potential (□) and particle size (♦) at various weight ratios of PLA/DNA complexes; (a) MW 5-15 kDa, (b) MW 15-70 kDa, (c) MW >70 kDa at pH 7.4

1.2 Transfection efficiency of polypeptide/DNA complexes

Because the physicochemical properties of the complexes directly connect with their bioactivities, investigating the transfection in different conditions in order to obtain the best transfection efficiency is necessary. Previous studies demonstrated that the gene delivery potential of polymer in mammalian cells depends on several factors such as type of polymer, polymer MW, weight ratio, pH of transfection medium/complex solution and cell type (Sato, Ishii and Okahata 2001 : 2075-2080; Haung et al. 2005 : 391-406; Zhao et al. 2006 : 223-228). Therefore, following studies including pH of transfection medium, cell type, type of polypeptides and polypeptide MW, transfection efficiency in these different conditions were investigated to find an optimal transfection condition and gene expression level. The gene transfection was evaluated by transfected-cell counting using images obtained by fluorescence microscope and the transfected-cell was calculated.

1.2.1 Effect of polypeptides type

To study the different type of polypeptide on transfection efficiency, HeLa and Huh7 cells were transfected with PLL/DNA, PLA/DNA and PLO/DNA complexes at pH 7.4. These complexes were used as a model of cationic polypeptide/DNA complexes. Firstly, the transfection was carried out in MEM medium for HeLa cells. PLL/DNA complexes were formulated at weight ratios of 0.1, 0.5, 1, 2, 4 and 6, PLA/DNA complexes were formulated at weight ratios of 1.5, 2, 2.5, 3 and 4, PLO/DNA complexes were formulated at weight ratios of 0.1, 0.5, 1, 2, 4 and 6. In HeLa cells, the maximum transfection efficiency achieved at the weight ratio of 4, 2.5 and 1 for PLL/DNA, PLA/DNA and PLO/DNA complexes, respectively (Figure 14). It was able to rank the maximum transfection efficiencies of polypeptides in HeLa cells as PLA > PLL > PLO. The reduced transfection efficiency when weight ratio increasing was also demonstrated by Pouton et al. (1998 : 295). Their study of transfection was done by delivering PLL/DNA complexes into B16 murine melanoma cells in the presence of 100 μM chloroquine.

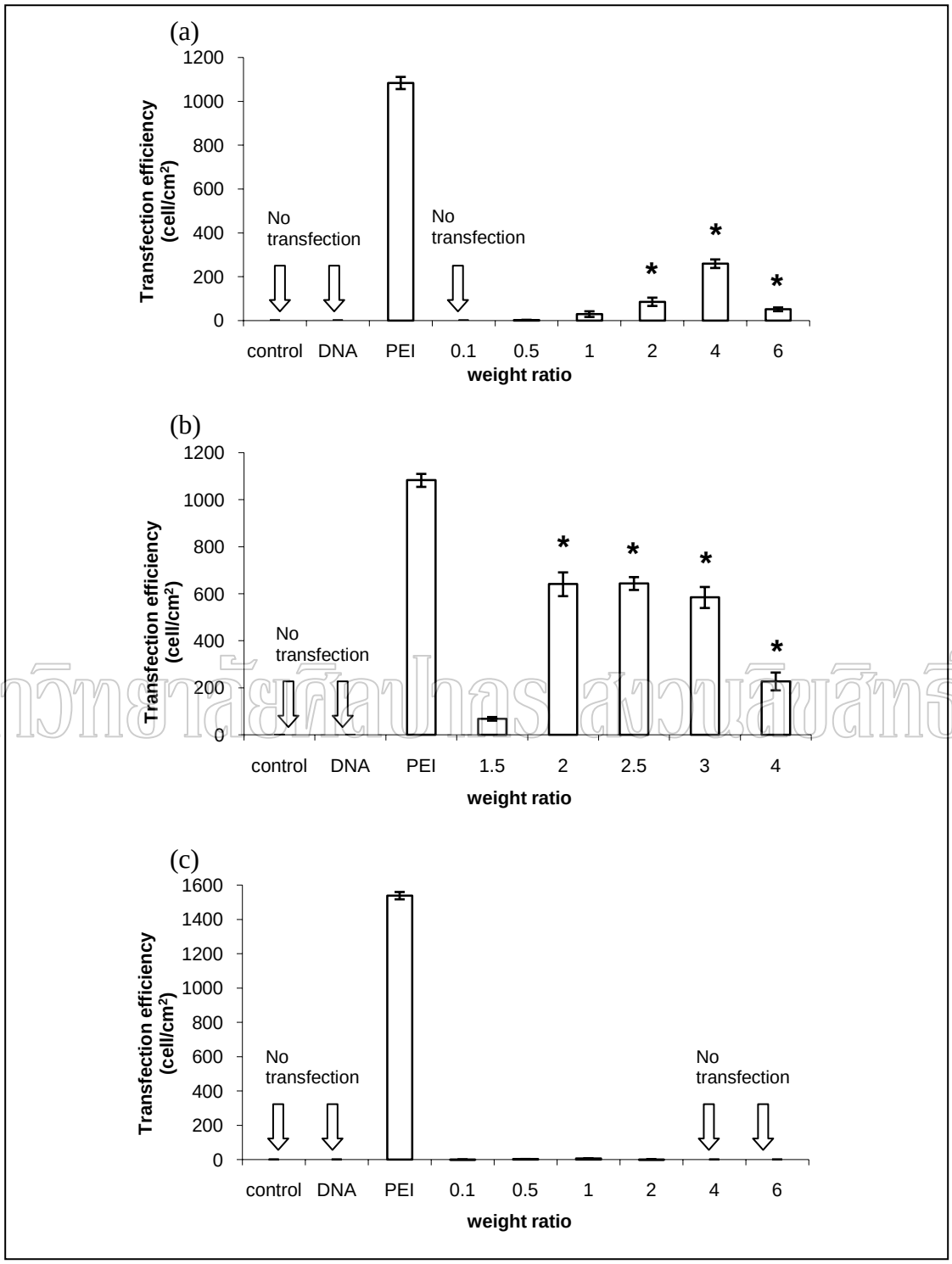


Figure 14 Effect of weight ratios on transfection efficiency of polypeptides with various weight ratios at pH 7.4 in HeLa cells; (a) poly-L-lysine MW 30-70 kDa, (b) poly-L-arginine MW 15-30 kDa (c) poly-L-ornithine MW 30-70 kDa. Values shown are the means ± SD of triplicate experiment (* indicate $p \leq 0.05$)

To further investigate, Huh7 cells were transfected with PLL/DNA complexes and PLA/DNA complexes. PLL/DNA complexes were formulated at weight ratios of 0.1, 0.5, 1, 2, 4 and 6. PLA/DNA complexes were formulated at weight ratios of 1.5, 2, 2.5, 3 and 4, and carried out the transfection in RPMI medium for Huh7 cells. The maximum transfection efficiency achieved at the weight ratio of 2 and 1.5 for PLL/DNA and PLA/DNA complexes, respectively (Figure 15).

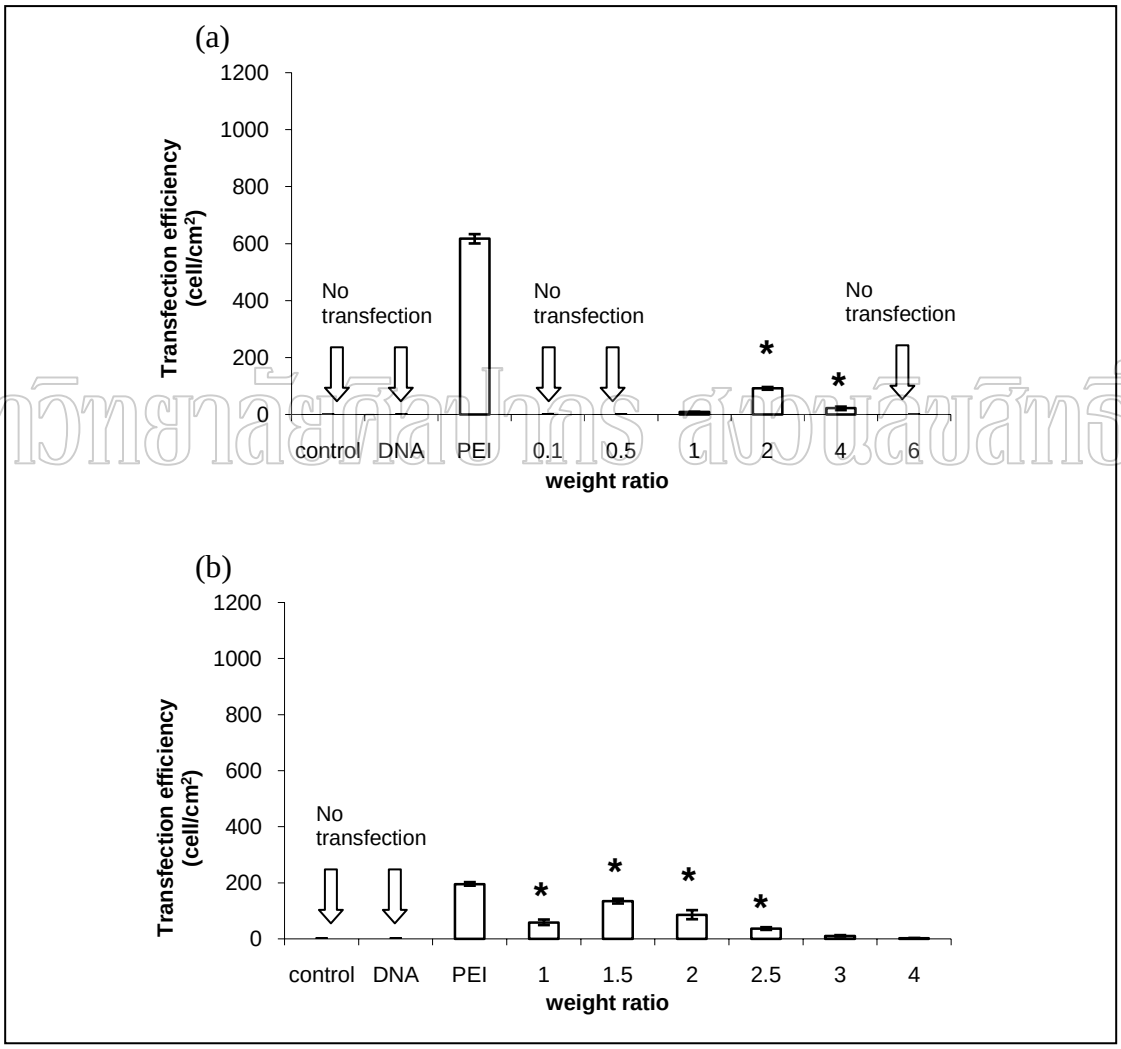


Figure 15 Effect of weight ratios on transfection efficiency of polypeptides with various weight ratios at pH 7.4 in Huh7 cells; (a) poly-L-lysine MW 30-70 kDa, (b) poly-L-arginine MW 15-30 kDa. Values shown are the means ± SD of triplicate experiment (* indicate $p \leq 0.05$)

1.2.2 Effect of pH

To test whether pH had an effect on transfection, both Hela and Huh 7 cells were transfected with PLL/DNA, PLA/DNA and PLO/DNA complexes at pH 6.4 using the various weight ratios as indicated in 1.2.1.

The results revealed that transfection efficiency of cationic polypeptides at pH 6.4 was not significant different to that of pH 7.4. The maximum transfection efficiency achieved at the weight ratio of 4, 2 and 2 for PLL/DNA, PLA/DNA and PLO/DNA complexes in HeLa, respectively (Figure 16) and 2 for PLL/DNA complexes in Huh7 cells (Figure 17).

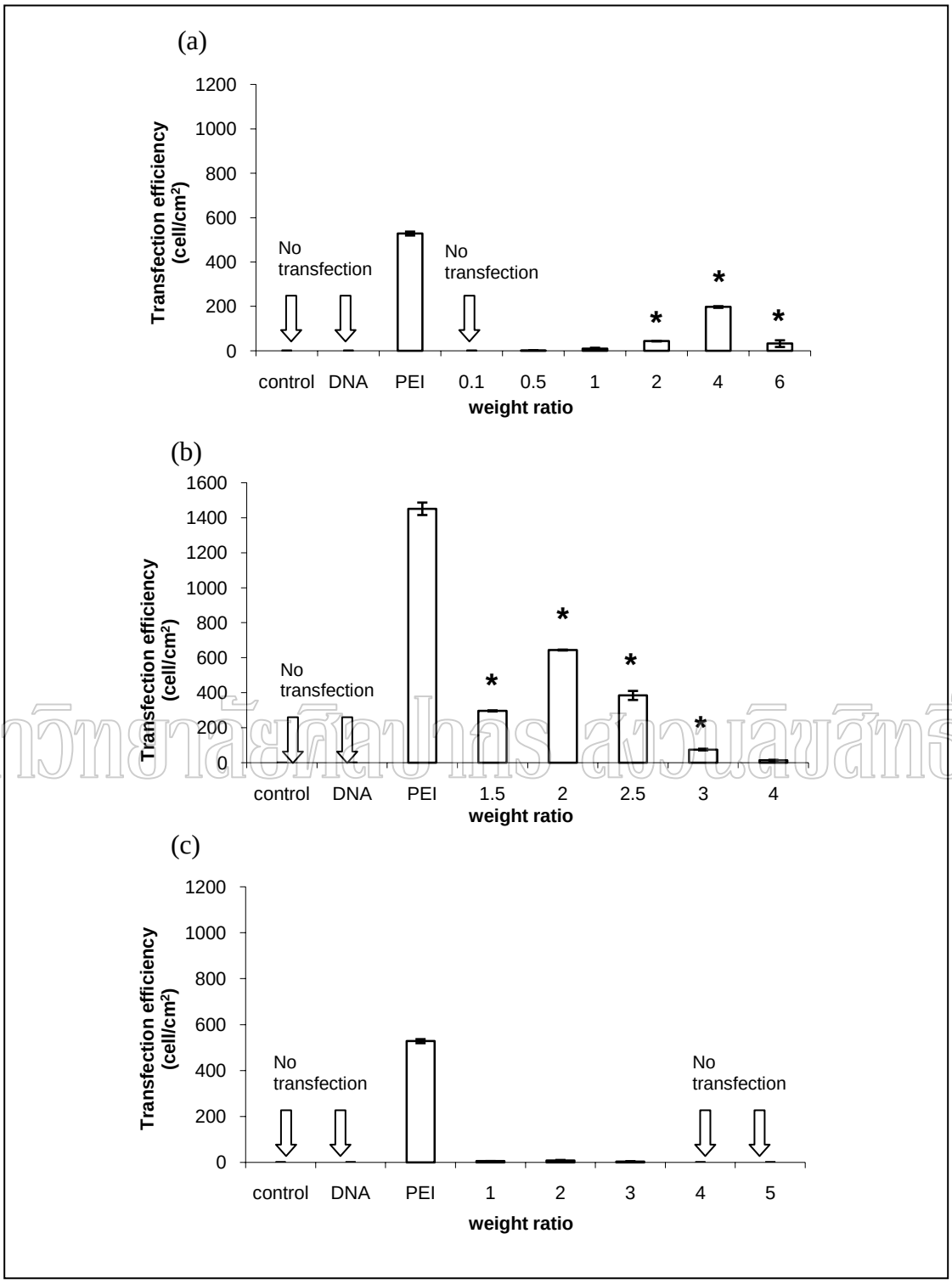


Figure 16 Effect of pH on transfection efficiency of polypeptides with various weight ratios at pH 6.4 in HeLa cells; (a) poly-L-lysine MW 30-70 kDa, (b) poly-L-arginine MW 15-70 kDa, (c) poly-L-ornithine MW 30-70 kDa. Values shown are the means ± SD of triplicate experiment (* indicate $p \leq 0.05$)

The transfection efficiency decreased when using Huh7 cells. The highest transfection efficiency was obtained with PLA/DNA complexes in HeLa cells at pH 7.4. It was able to rank the maximum transfection efficiency of cationic polypeptides as PLA>PLL>PLO. These results illustrated that there is the extreme importance of cell type and polypeptide type to gene transfer mediated, which is a unique property of this vector. Therefore, the PLA/DNA complexes, HeLa cells and pH 7.4 were the condition chosen for further exploring experiments.

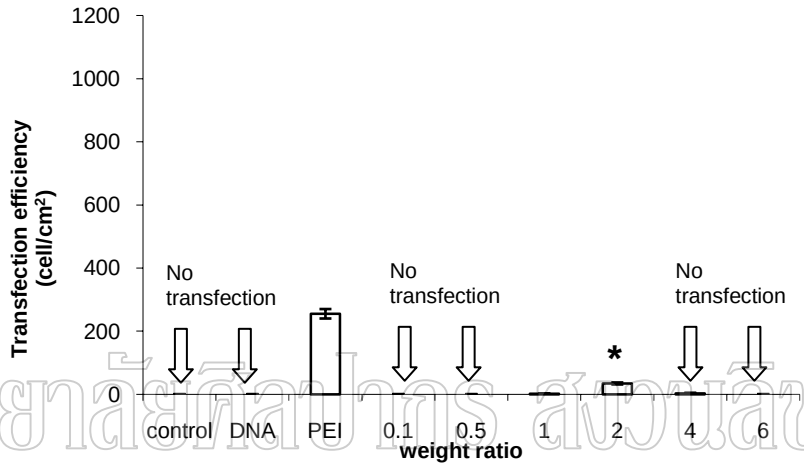


Figure 17 Effect of pH on transfection efficiency of poly-L-lysine MW 30-70 kDa with various weight ratios at pH 6.4 in Huh7 cells. Values shown are the means ± SD of triplicate experiment (* indicate $p \leq 0.05$)

1.2.3 Effect of MW of PLA

In order to investigate the effect of MW on the transfection efficiency, PLA with different MW (5-15, 15-70 and >70 kDa) were used. The transfection efficiencies of PLA/DNA complexes are shown in Figure 21. The transfection efficiency had a tendency to increase as the weight ratio increased. The minimum gene expressing efficiency was found in weight ratios 1, 6 and 8 in all MW. At the weight ratio higher than 2, the transfection efficiency of MW 15-70 and >70 kDa of PLA/DNA complexes were higher than for naked DNA. However, at different MW of PLA, the maximum transfection efficiency was found at different weight ratio. PLA/DNA complexes of MW 5-15, 15-70 and >70 kDa showed maximum transfection efficiency at the weight ratios of 3, 2 and 2.5, respectively.

Thus, the MW of PLA in the range studied affected the transfection efficiency. PLA MW of 15-70 and of >70 kDa had higher transfection efficiencies ($p \leq 0.05$) than PLA MW of 5-15 kDa. This might be due to the high transfection efficiency of high MW cationic polymer was attributed to the highly-positive charge complexes that be able to be uptake by the cells (Haung et al. 2005 : 391-406; Zhao et al. 2006 : 223-228). On the other hand, high transfection efficiency of cationic polymer/DNA formulated with low MW cationic polymer was observed by Mann et al. (2008 : 154). Most cationic peptides used for DNA condensation are low MW, usually containing a minimum of eight consecutive peptide residues, which are less toxic than their high MW counterparts. The main drawback of these low MW peptide-DNA formulations (formed through electrostatic interaction between peptide and DNA) is their instability during in vitro and in vivo gene delivery because of the poor peptide-DNA binding leads to easy DNA release.

The MW of PLA and weight ratio of PLA/DNA complexes is one of the important formulation parameters that affect transfection efficiency. In case of PLA/DNA complexes, the highest transfection efficiency observed in this study was PLA MW of > 70 kDa at the weight ratio of 2.5.

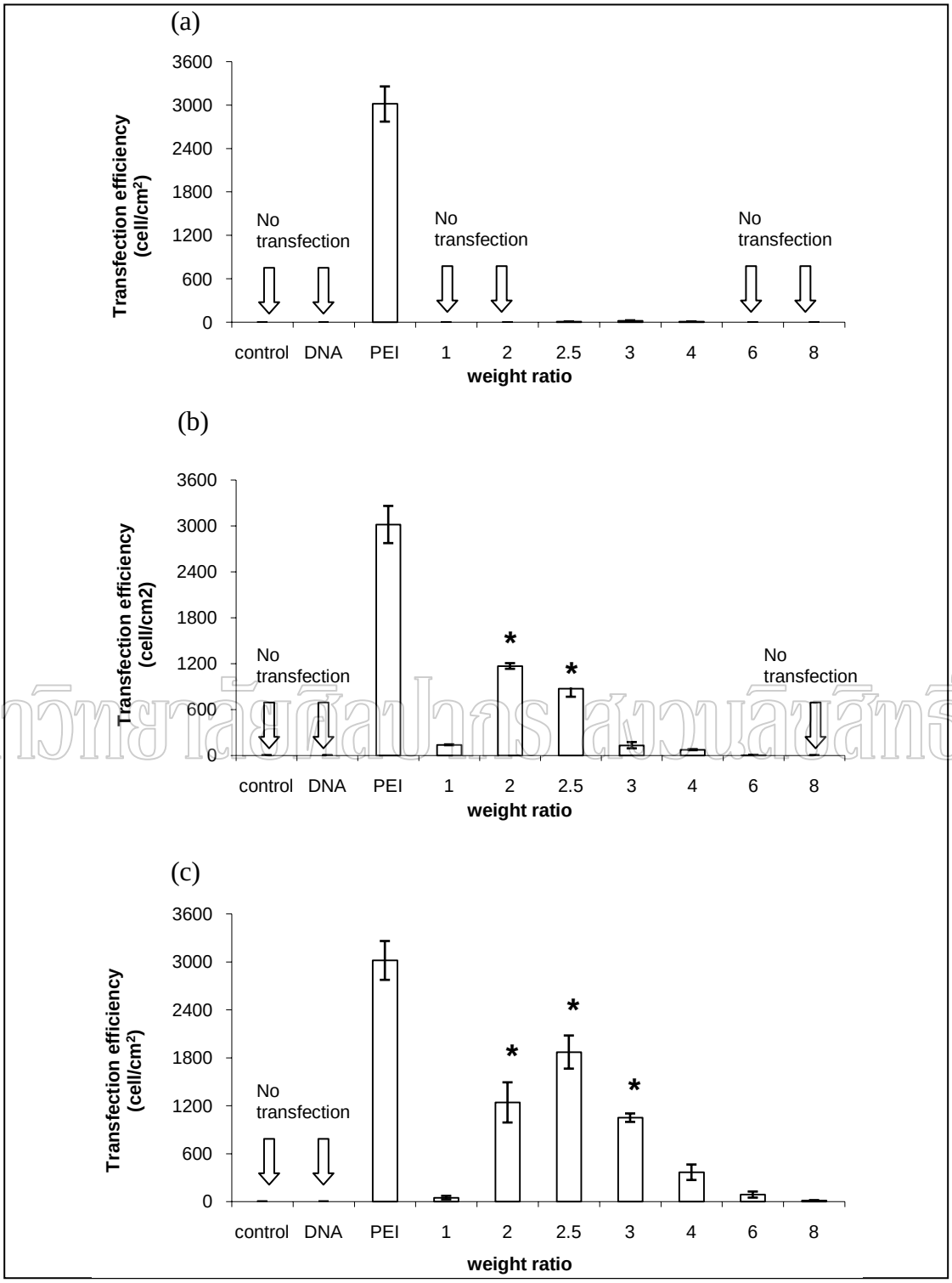


Figure 18 Effect of MW on transfection efficiency of poly-L-arginine with various weight ratios at pH 7.4 in HeLa cells; (a) MW 5-15 kDa, (b) MW 15-70 kDa, (c) MW > 70 kDa. Values shown are the means ± SD of triplicate experiment (* indicate $p \leq 0.05$)

1.3 Cytotoxicity of polypeptide and polypeptide/DNA complexes

1.3.1 Cytotoxicity of polypeptides

The effect on cell viability can be considered as one of the most important factors for the development of new gene carriers. In this study, MTT based cell proliferation assay was performed to evaluate the cytotoxic effect of the gene carriers. The viability of HeLa and Huh7 cells were tested in the presence of polypeptides at various weight ratios as studied in the transfection experiment. Cells without treatment of the polypeptide were considered as a positive control with a cell viability of 100%. Polypeptides were applied to the cells at concentration ranging from 0.001-1000 µg/ml for 24 h and then the effect on cell viability was measured.

The results showed that the cytotoxicity of PLL, PLA and PLO were concentration dependent. The cytotoxicity increased with increasing weight ratio of polypeptides. The cytotoxicity of the compounds could be ranked as PLA > PLL > PLO on HeLa cells (Table 11) and PLO > PLL > PLA on Huh7 cells (Table 12).

Table 11 Toxicity of polypeptides in HeLa cells at pH 7.4

Samples	IC_{50} (approximately) (µg/ml)
PLL	16.759
PLA	12.480
PLO	58.118
PEI	1.709

Table 12 Toxicity of polypeptides in Huh7 cells at pH 7.4

Samples	IC_{50} (approximately) (µg/ml)
PLL	57.002
PLA	117.811
PLO	29.351

1.3.2 Cytotoxicity of polypeptides/DNA complexes

The viability of HeLa and Huh7 cells were also tested in the presence of polypeptides/DNA complexes at various weight ratios as studied in the transfection experiment. The results are shown in Figure 19 to 21. 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 PLL/DNA complexes and PLA/DNA complexes at weight ratio less than 4 on HeLa cells (Figure 19 (a) and (b)). PLO/DNA complexes had high cytotoxicity level (Figure 19 (c)). The average cell viability was less than 60%. The results showed that cationic polypeptides showed a significant decrease in cell viability with increasing in weight ratio of polypeptides/DNA complexes. PEI was used as a control because it is a cationic polymer commercially available. The average cell viability of PEI/DNA complexes was about 50% (Figure 20).

The cytotoxicity of cationic polymer was probably caused by polymer aggregation on cell surfaces impairing the important membrane function (Jiang et al. 2007 : 278). Polypetides/DNA complexes had lower cytotoxicity than PEI 25 kDa. This might be a reason that polypeptides (PLL, PLA and PLO) are biodegradable polymer, it may degrade in cells and results in lower toxicity.

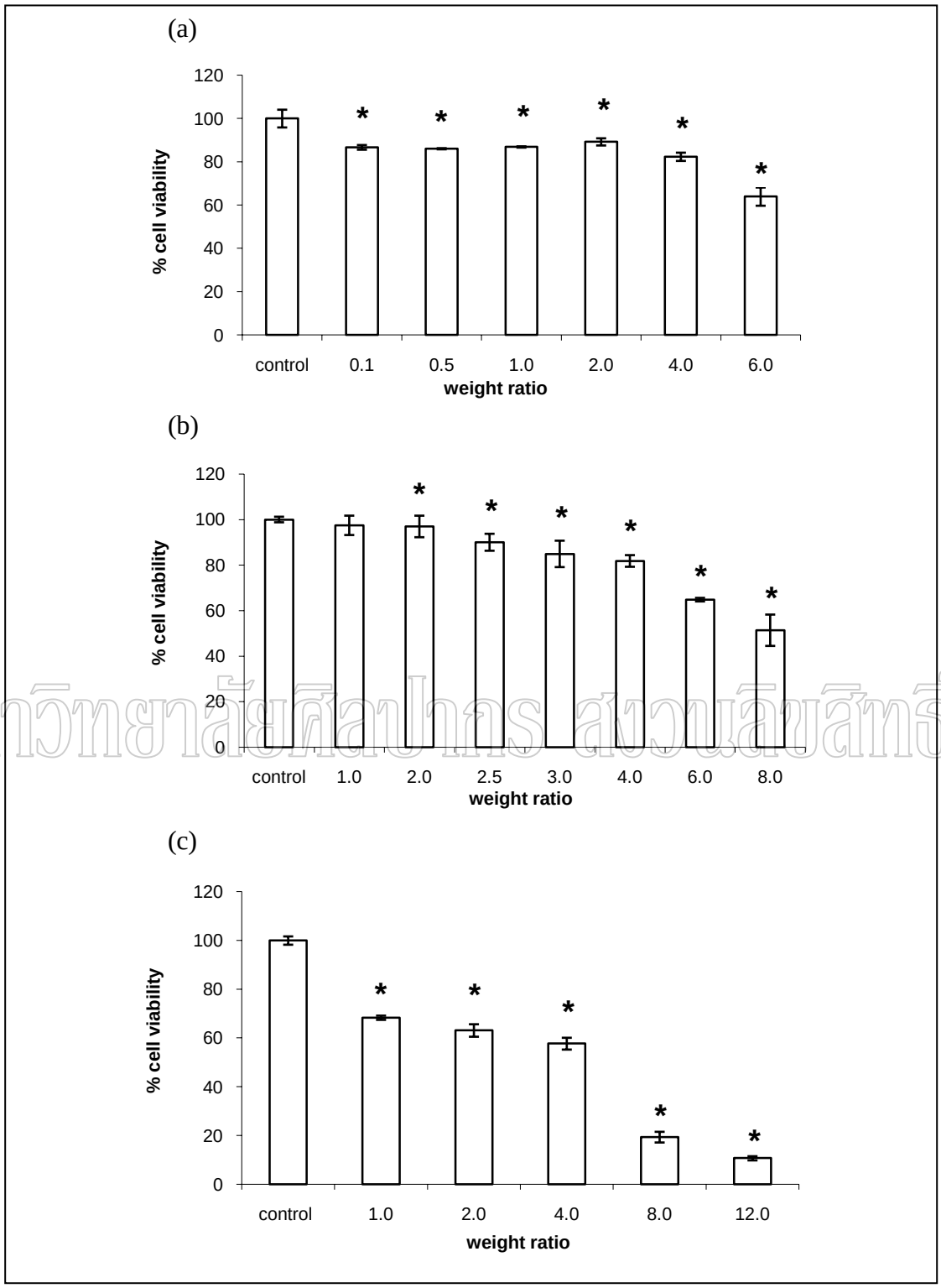


Figure 19 Effect of polypeptides/DNA complexes on cell viability at pH 7.4 in HeLa cells; (a) PLL MW 30-70 kDa, (b) PLA MW 15-70 kDa and (c) PLO MW 30-70 kDa

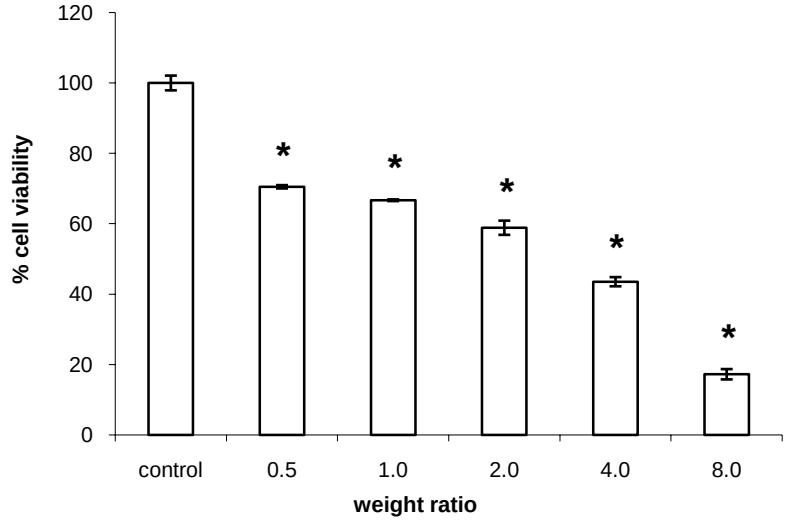


Figure 20 Effect of PEI25 kDa /DNA complexes on cell viability at pH 7.4 in HeLa cells

The cytotoxicity of Polypeptide/DNA complexes at pH 7.4 in Huh7 cells showed in Figure 21. The average cell viability of PLL/DNA and PLA/DNA complexes was over 60%. The average cell viability of PLO/DNA complexes was over 40%. The result suggested that that the cytotoxic effect of polypeptide/DNA complexes in Huh7 cells was higher than that in HeLa cells.

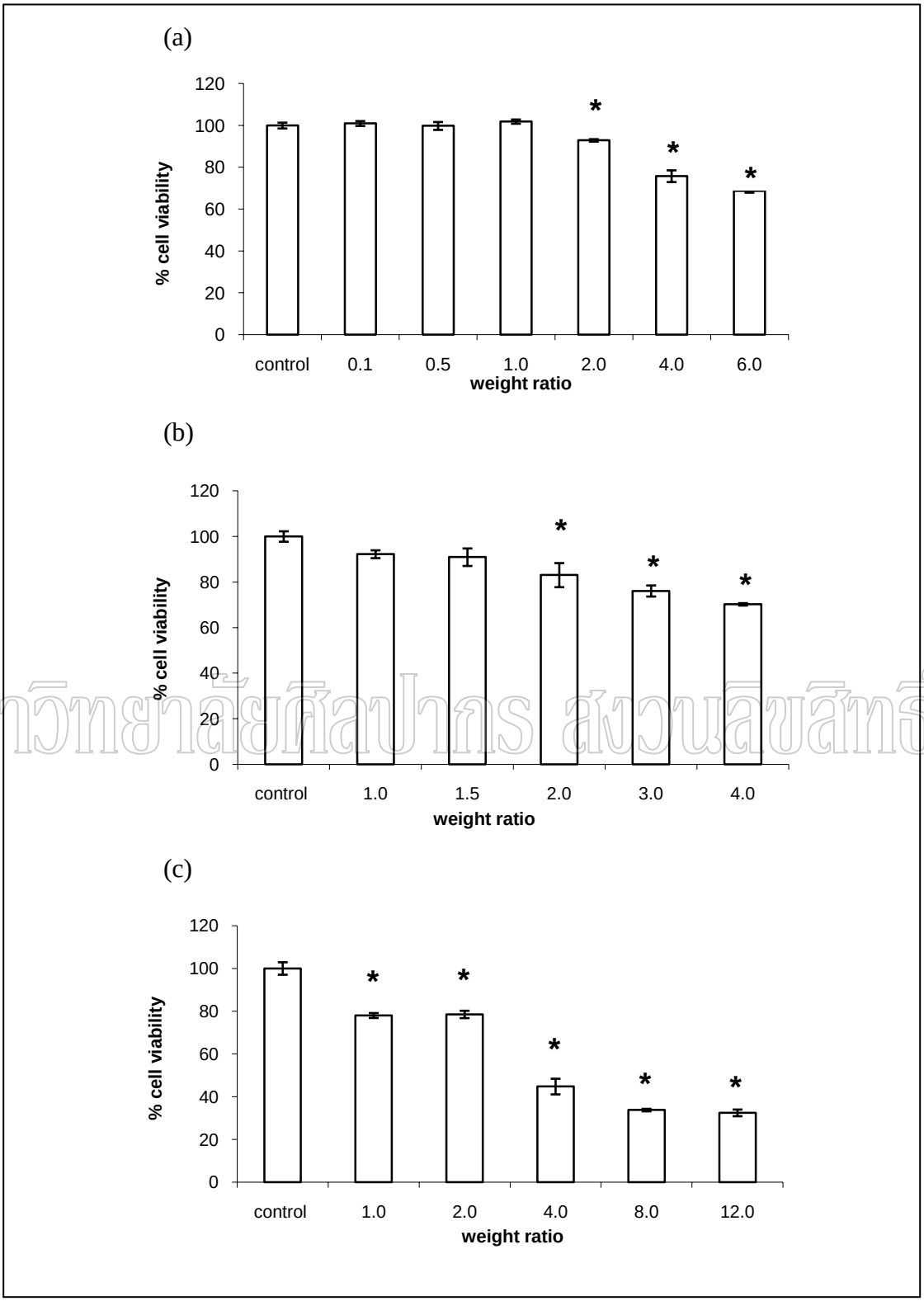


Figure 21 Effect of polypeptides/DNA complexes on cell viability at pH 7.4 in Huh7 cells; (a) PLL MW 30-70 kDa, (b) PLA MW 15-70 kDa and (c) PLO MW 30-70 kDa

1.3.2 Effect of pH

The cytotoxicity of polypeptides and polypeptide/DNA complexes in both cells were also done at pH 6.4, to evaluate the effect of pH. The results showed that the cytotoxicity of polypeptides was pH dependent. The cytotoxicities of all polypeptides at pH 6.4 were extremely higher toxicity than at pH 7.4 (Table 13 and 14).

Table 13 Toxicity of polypeptides in HeLa cells at pH 6.4

Samples	IC_{50} (approximately) (µg/ml)
PLL	30.872
PLA	1.535
PLO	1.358
PEI	0.927

Table 14 Toxicity of polypeptides in Huh7 cells at pH 6.4

Samples	IC_{50} (approximately) (µg/ml)
PLL	33.288
PLA	< 0.01
PLO	< 0.01

The viability of HeLa cells was tested in the presence of polypeptide/DNA complexes at various weight ratios as studied in the transfection experiment. The results are shown in Figure 22 to 24. All polypeptide/DNA complexes cytotoxicity at pH 6.4 was almost similar to that at pH 7.4.

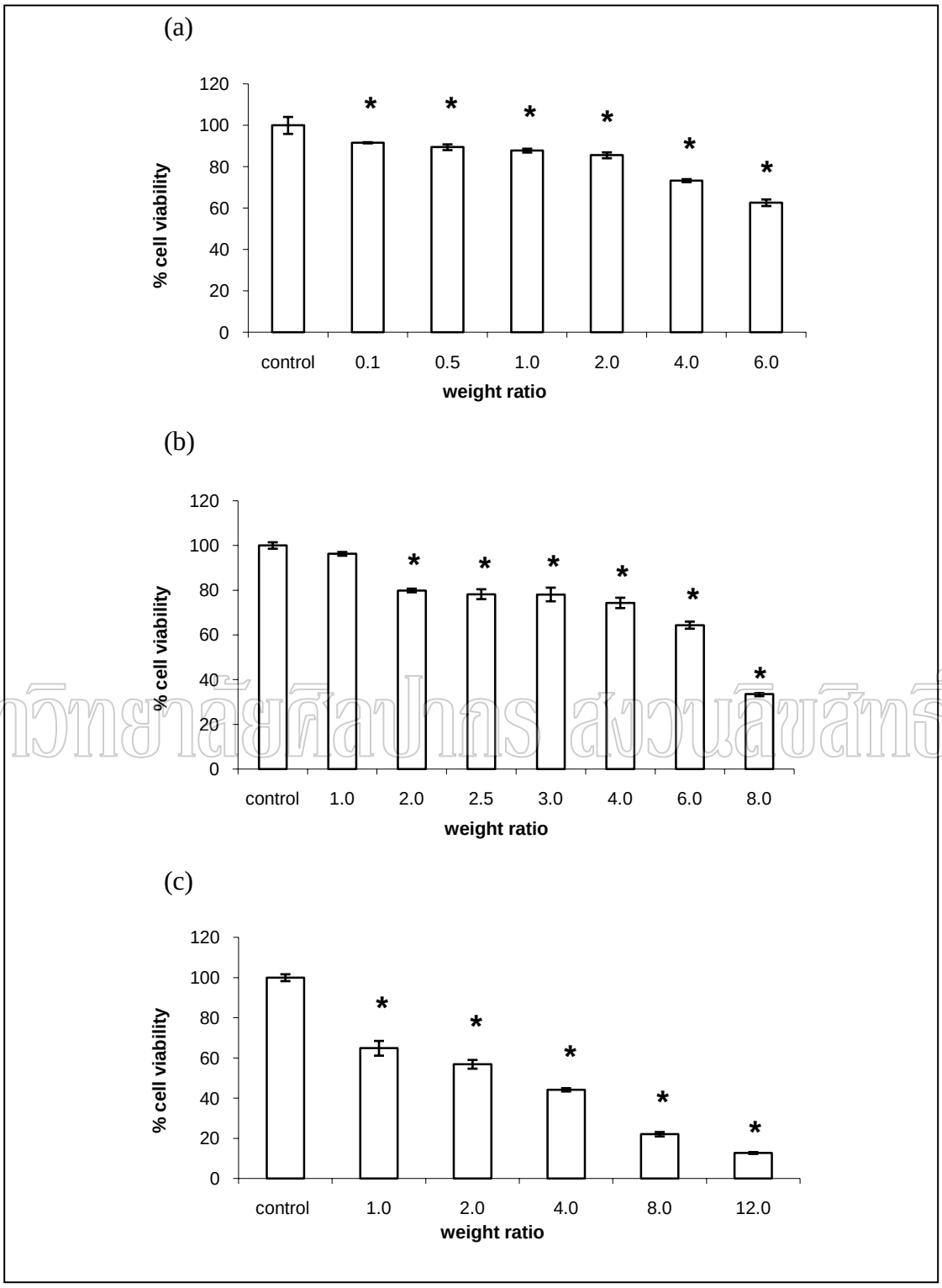


Figure 22 Effect of polypeptides/DNA complexes on cell viability at pH 6.4 in HeLa cells; (a) PLL MW 30-70 kDa, (b) PLA MW 15-70 kDa and (c) PLO MW 30-70 kDa

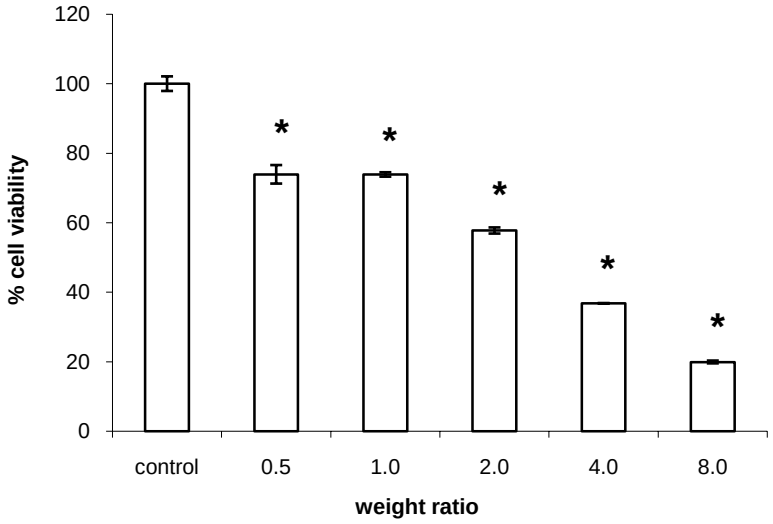


Figure 23 Effect of PEI25 kDa /DNA complexes on cell viability at pH 6.4 in HeLa cells

The cytotoxicity of polypeptide/DNA complexes at pH 6.4 in Huh7 cells showed in Figure 24. The average cell viability of PLL/DNA and PLO/DNA complexes were over 50%. The average cell viability of PLA/DNA complexes was over 70%. The result also suggested that the cytotoxic effect of polypeptide/DNA complexes in Huh7 cells was higher than that in HeLa cells.

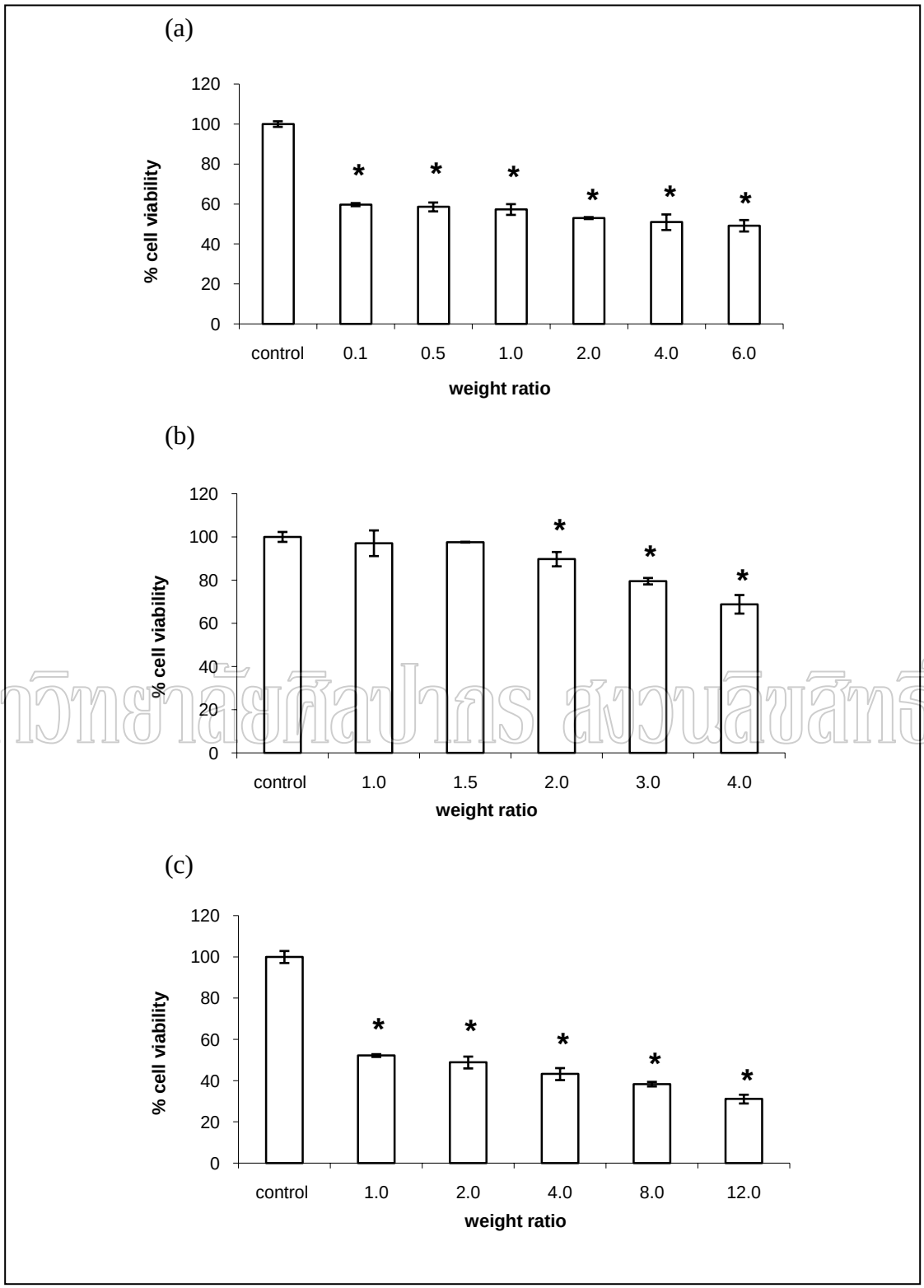


Figure 24 Effect of polypeptides/DNA complexes on cell viability at pH 6.4 in Huh7 cells; (a) PLL MW 30-70 kDa, (b) PLA MW 15-70 kDa and (c) PLO MW 30-70 kDa

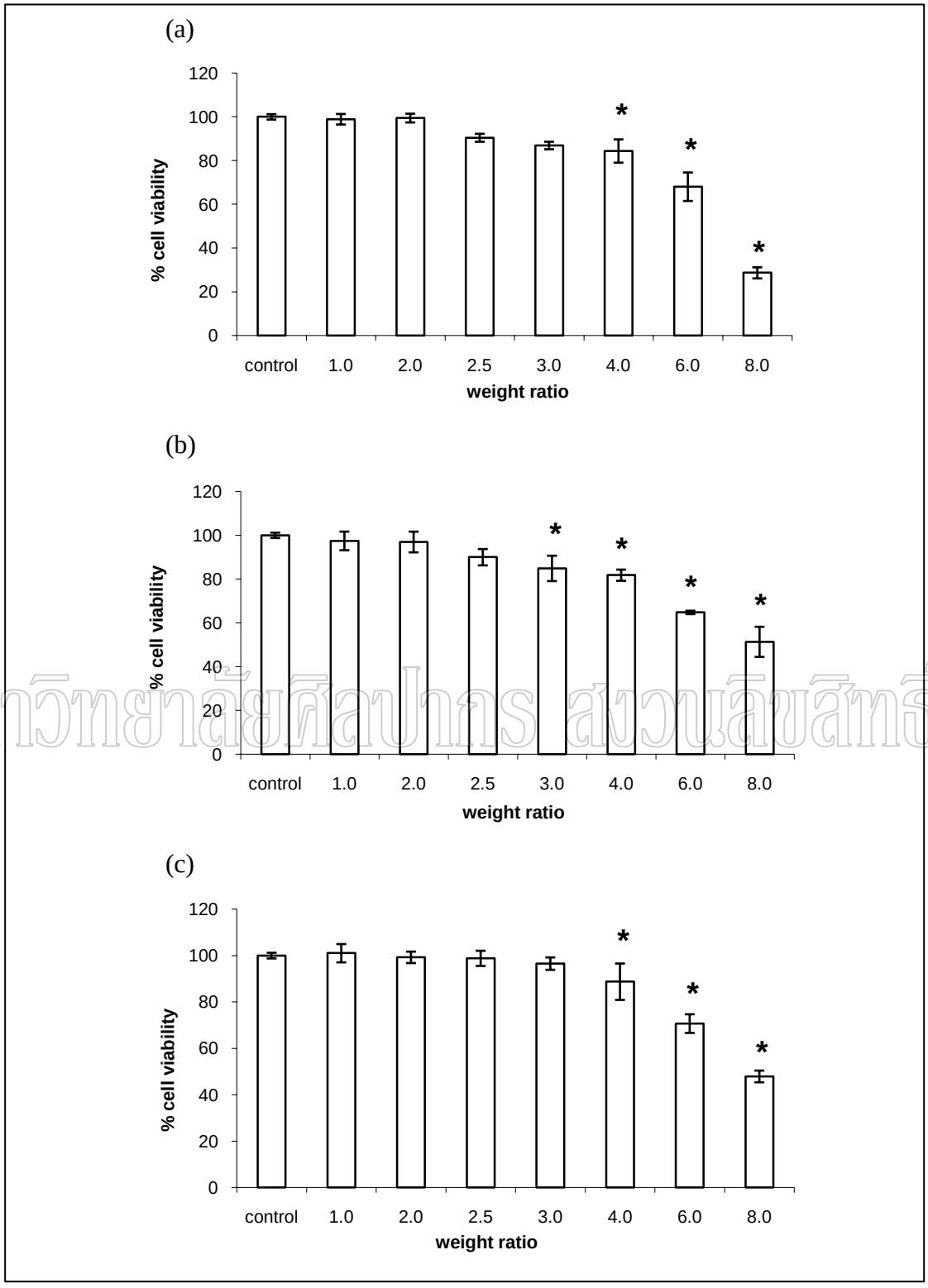


Figure 25 Effect of MW PLA on cell viability at pH 7.4 in HeLa cells; (a) MW 5-15 kDa, (b) MW 15-70 kDa and (c) >70 kDa

1.3.3 Effect of MW of PLA

The effect of MW on the cell viability is shown in Figure 25. The results showed that the PLA/DNA complexes significantly decreased in cell viability with increasing weight ratio.

2. Characterization of CSHCl on PLA/DNA complexes

2.1 Physicochemical properties

Physicochemical properties of CSHCl-PLA-DNA complexes including complex formation, binding affinity, particle size and zeta potential were characterized because these properties might affect the transfection efficiency.

2.1.1 Effect of mixing order

Regarding to the best efficiency transfected by PLA with MW of >70 KDa, this polypeptide was selected to form complexes with CSHCl and DNA. The CSHCl-PLA-DNA complexes were prepared by charge interaction induced self-assembly at various weight of PLA MW>70 kDa ranging from 0 to 50 whereas, DNA and CSHCl were fixed at 1 µg and 0.01 µg, respectively. The complexes of CSHCl, PLA and pDNA were prepared in the order of mixing as follows;

- 1) PLA ⟶ 2) DNA ⟶ 3) CSHCl, written as PLA/DNA/CSHCl
- 1) CSHCl ⟶ 2) DNA ⟶ 3) PLA, written as CSHCl/DNA/PLA
- 1) PLA ⟶ 2) CSHCl ⟶ 3) DNA, written as PLA/CSHCl /DNA

The weight ratio is one of formulation parameters that affect the physicochemical properties of CSHCl-PLA-DNA complexes. By using an agarose gel electrophoresis technique, the ability of chitosan and PLA to form complexes with DNA was investigated. Figure 26 is illustrated gel electrophoresis of CSHCl/PLA/DNA complexes with various order of mixing. For CSHCl/DNA complexes, the migration of DNA was completely retarded when the weight ratio was above 0.1. The migration of CSHCl/DNA/PLA, PLA/DNA/CSHCl and CSHCl/PLA/DNA complex formation was completely retarded when the weight ratio

was above 0.5, 0.5 and 0.1, respectively. Table 15 showed the weight ratio, which the complexes was formed at pH 6.4.

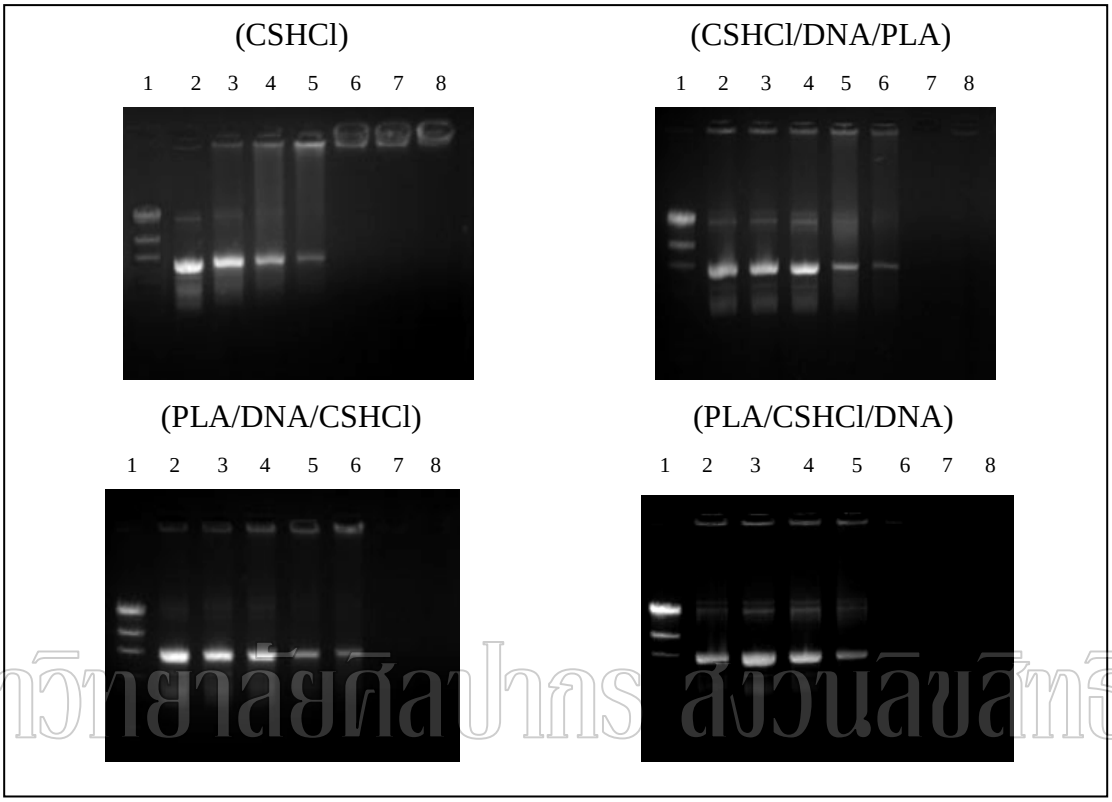


Figure 26 Agarose gel electrophoresis of CSHCl-PLA-DNA complexes with different weight of PLA. Lane 1, λ*Hin*d III DNA marker; lanes 2-8 of CSHCl, weight ratio of 0, 0.01, 0.05, 0.1, 0.5, 1, 4; lanes 2-8 of other complexes, weight ratio of 0, 0.01, 0.05, 0.1, 0.5, 1, 2, respectively

Table 15 The weight ratio of complete CSHCl-PLA-DNA complexes formed at pH 6.4

Samples	weight ratio of complete complexes formed
CSHCl/DNA	0.5
CSHCl/DNA/PLA	1.0
PLA/DNA/CSHCl	1.0
PLA/CSHCl/DNA	0.5

The binding affinity of CSHCl or PLA/DNA complexes was studied by ethidium bromide (EtBr) displacement assay. EtBr intercalates between

the base pairs of the DNA double helix, yielding a highly fluorescent DNA/EtBr complex, resulting in an increase in fluorescence. The degree of displacement of EtBr by CSHCl of PLA illustrates the binding affinity, indicating the relative strength of the interaction between CSHCl or PLA and DNA. Figure 27 shows relative fluorescent intensity of CSHCl/DNA complexes with various weight ratios of chitosan, PLA/DNA complexes with various weight ratios of PLA and CSHCl/DNA/PLA or PLA/DNA/ CSHCl complexes with various weight ratios of PLA and fixing weight of CSHCl at 4 µg. The DNA/EtBr complex without titration of CSHCl or PLA was considered as a control with a relative fluorescent intensity of 100%. With the increase in the amount of CSHCl or PLA, the intensity of fluorescence showed a decreasing trend, indicating EtBr was replaced by the added CSHCl or PLA, that is, CSHCl or PLA bound selectively to DNA. This could be the increase in the interaction of CSHCl or PLA and DNA when the amount of CSHCl or PLA increased. The result agreed with that of Douglas, Piccirillo and Tabrizian (2006: 354-361). They found that the binding affinity of chitosan to DNA increased with increasing the N/P ratio. In addition, the binding affinity reached a plateau at high weight ratio. The ranking order was order of mixing of PLA/DNA/CS ≈ CS/DNA/PLA > CS/DNA ≈ PLA/DNA.

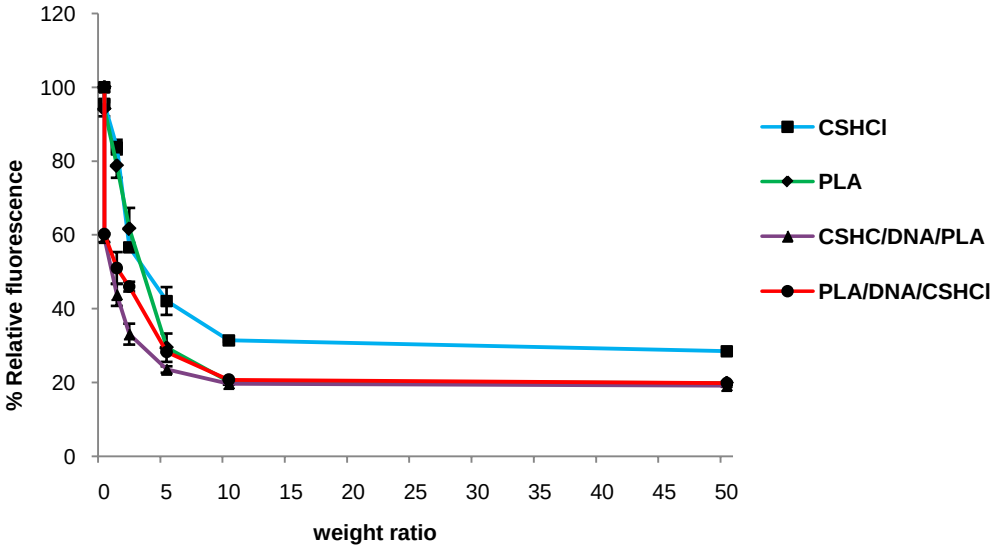


Figure 27 Relative fluorescent intensity of DNA/EtBr complex at varying weight ratio of PLA MW > 70 kDa and CSHCl, for CS-PLA fixing weight ratio of CS and varying weight ratio of PLA. Points represent mean ± S.D. (n = 3)

The CSHCl/PLA/DNA complexes were prepared by charge interaction induced self-assembly at various weight of PLA MW>70 kDa ranging from 0 to 4 whereas, DNA and CSHCl were fixed at 1 µg and 4 µg, respectively. Next, the particle size and zeta potential of those complexes were elucidated. As shown in Figure 28, the particle size was dependent on both weight ratio and order of mixing between CSHCl, PLA and DNA. The particle size of the all complexes was nanosized (230 to 400 nm), showing that complete complexes were formed. The zeta-potential was positive and about constant value in the range of 23 to 40 mV.

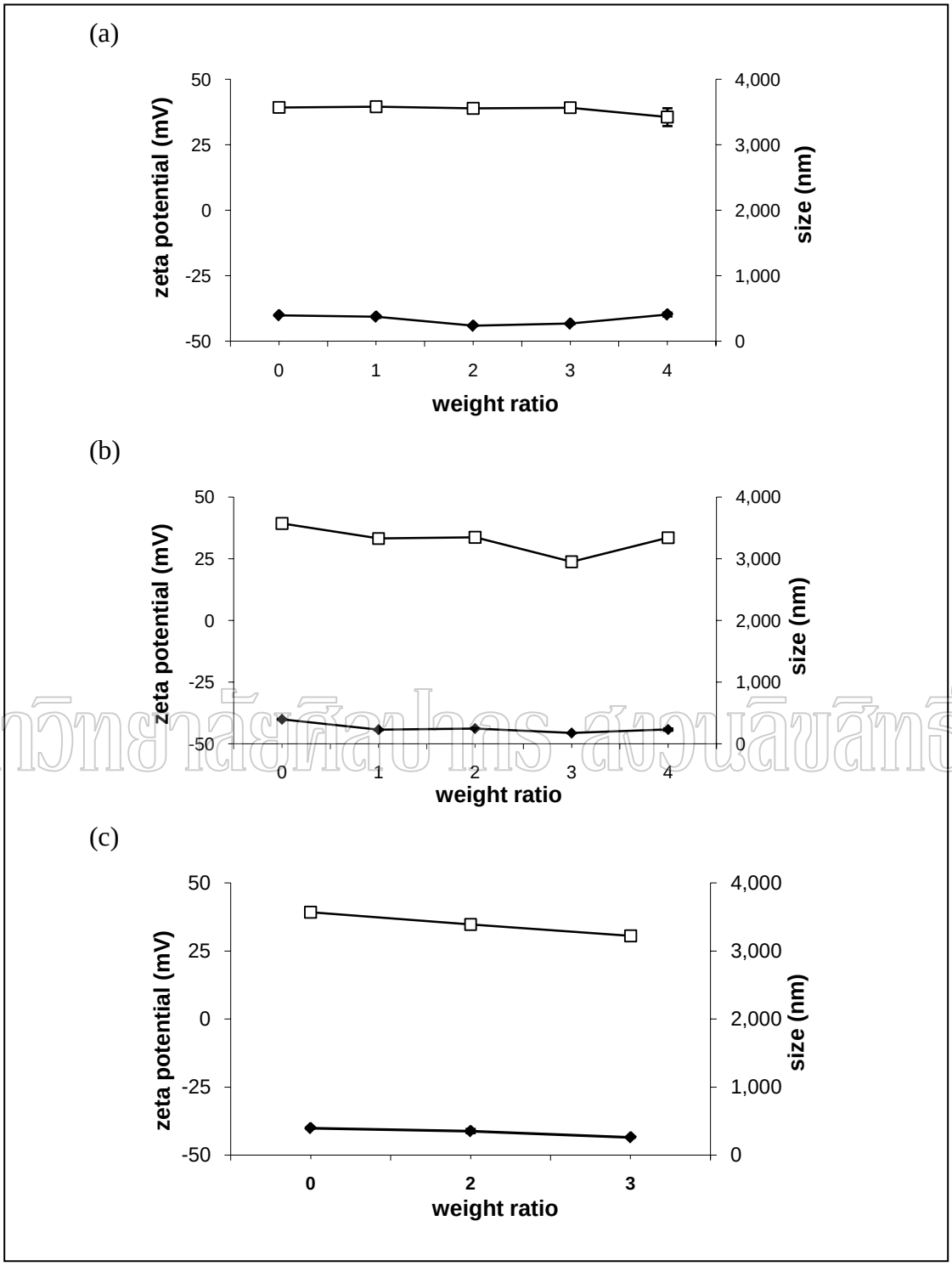


Figure 28 Effect of the weight ratio on particle size and zeta-potential of complexes; (a) CSHCl/DNA/PLA, (b) PLA/DNA/CSHCl, (c) PLA/CSHCl/DNA at pH 6.4

2.2 Transfection efficiency of CSHCl-PLA-DNA complexes

2.2.1 Effect of mixing order

The amount of CSHCl was fixed at 4 µg for all the transfection efficiency experiments. Polyethylenimine (PEI, 25 kDa) was used as a positive control. The transfection efficiency was done in HeLa cells at pH 6.4, the suitable pH for chitosan. The transfection efficiency had a tendency to increase as the weight ratio of PLA/DNA increased (Figure 29). At weight ratio of 1, the transfection efficiencies of all complexes was not significant different from the naked DNA ($P>0.05$). At weight ratio higher than 2, the transfection efficiency of all complexes was higher than for the naked DNA. At a low weight ratio, the transfection efficiency of all complexes was not different in comparing with the naked DNA. This might be resulted from the insufficient amount of positively charged in complexes to transfect cells. All complexes achieved sufficient transfection efficiencies at higher weight ratios (above 1). The reason might be that an increase in the weight of carriers at higher weigh ratios could yield a higher amount of positively charged complexes to transfec cells successfully. The increase in transfection ability with an increase in N/P ratio in arginine-CS/DNA complexes was also demonstrated by Gao et al. (2008 : 244).

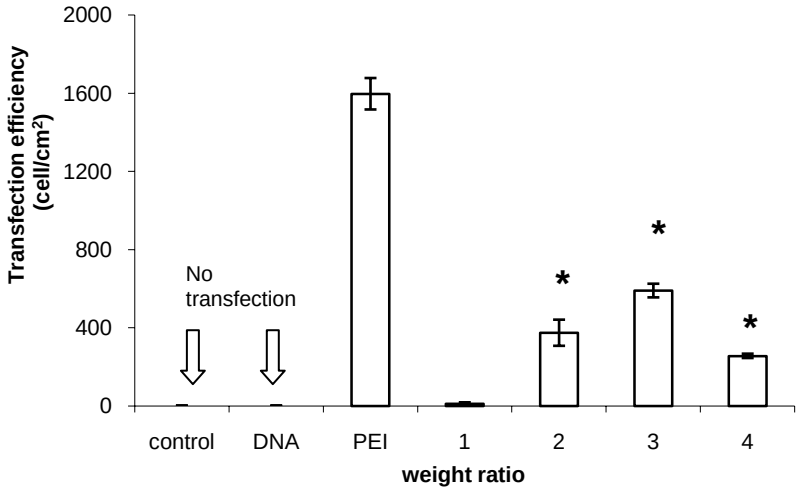


Figure 29 Effect of PLA/DNA complexes on transfection efficiency at pH 6.4 in HeLa cells

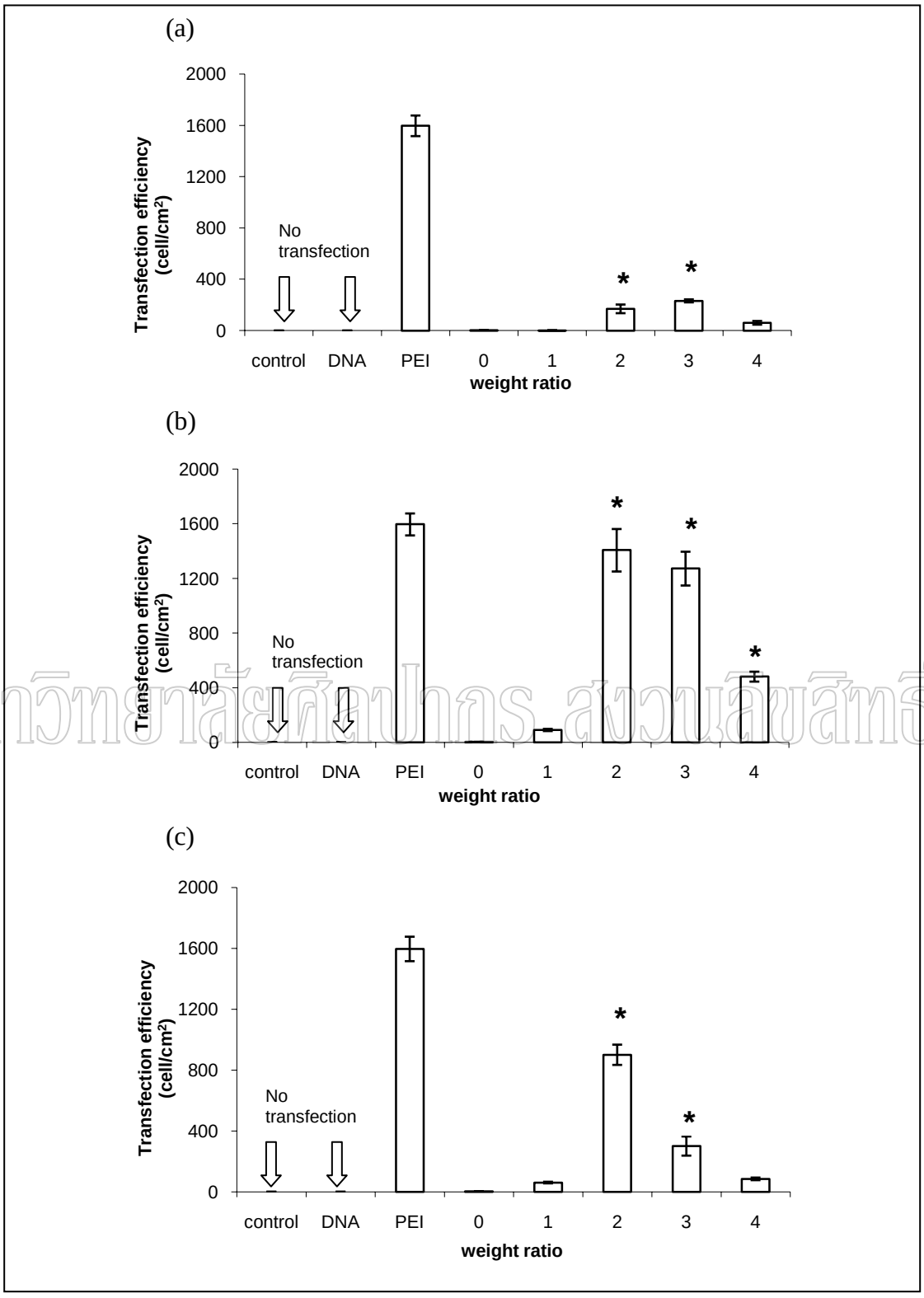


Figure 30 Effect of the weight ratio on transfection efficiency of complexes; (a) CSHCl/DNA/PLA, (b) PLA/DNA/CSHCl and (c) PLA/CSHCl /DNA in HeLa cells at pH 6.4

For PLA/DNA complexes (Figure 29), the highest transfection level was found at the weight ratio of 3 (589.825 ± 34.917 cell/cm^2). The value was significantly lower transfection efficiency than the PEI/DNA complex (1596.491 ± 80.396 cell/cm^2).

For CSHCl/DNA/PLA complexes (Figure 30 (a)), the highest transfection level was found at weight ratio of 3 (231.930 ± 11.737 cell/cm^2). The transfection efficiency of CSHCl/DNA/PLA complexes was significant lower, compared to that of PEI/DNA complex (1596.491 ± 80.396 cell/cm^2) or even that of PLA/DNA complex (589.825 ± 34.917 cell/cm^2).

For PLA/DNA/CSHCl complexes (Figure 30 (b)), the highest transfection level was found at the weight ratio of 2 (1407.368 ± 156.587 cell/cm^2). The transfection efficiency of PLA/DNA/CSHCl complexes was not significant different compared to that of PEI/DNA complex (1596.491 ± 80.396 cell/cm^2). On the other hand, it was significant higher (about 2.39 times) compared to that of PLA/DNA complex.

For PLA/CSHCl/DNA complexes (Figure 30 (c)), the highest transfection level was found at weight ratio of 2 (900.702 ± 66.594 cell/cm^2). The transfection efficiency of PLA/CSHCl/DNA complexes was significant lower compared to that of PEI/DNA complex (1596.491 ± 80.396 cell/cm^2). However, it was significant higher compared to that of PLA/DNA complex.

The maximum transfection efficiency of carriers could be ranked as follows: PLA/DNA/CSHCl > PLA/CSHCl/DNA > PLA/DNA > CSHCl/DNA/PLA.

2.2.2 Effect of serum

The effect of serum on transfection efficiency is shown in Figure 31. The cells transfected in serum containing media (with 10% FBS) had a significantly lower transfection efficiency than those in serum-free media.

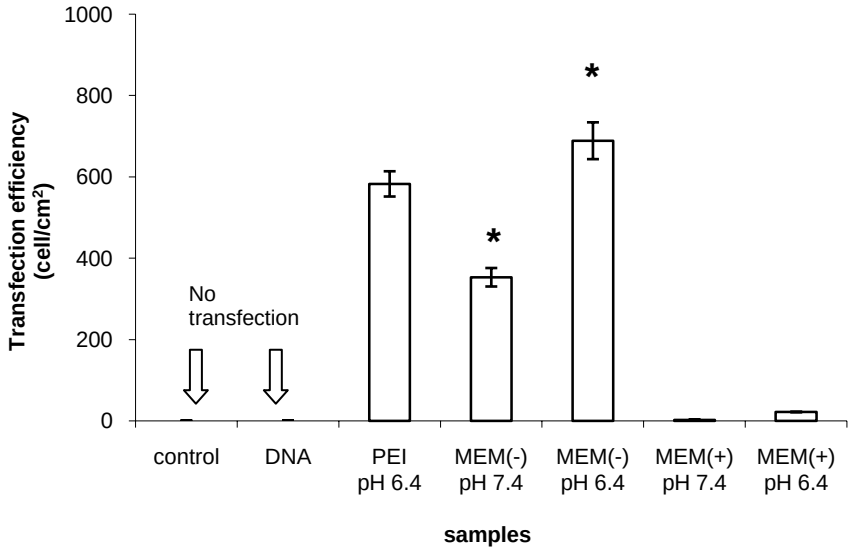


Figure 31 Effect of serum on gene transfection efficiency. HeLa cells were incubated in the absence or presence of 10% serum with PLA MW > 70 kDa/DNA/CSHCl complexes

The efficiency of PLA/DNA/CSHCl complexes to transfect HeLa cells in 10% serum containing media significantly decreased. This result was similar to Choi et al. showed that the level of transfection of arginine-PAMAM (dendritic carrier) was decreased in the presence of serum (Choi et al. 2004 : 455). As demonstrated in Figure 31, PLA/DNA/CSHCl showed a significant increase in potency compared with naked DNA and its efficiency was almost comparable to that of PEI/DNA efficiency in the absence of serum at pH 6.4. However, the expression level remained at a lower level even though the pH decreased in the presence of serum. These would be explained that positively charged carriers share the property of forming complexes with the negatively charged DNA, they also interact with negatively charged serum proteins, resulting in a massive increase in complex size (Eliyahu et al. 2007 : 2342). Additionally, the zeta-potential decreased within seconds of each serum addition.

From these results, it was observed that the transfection mediated by PLA/DNA/CSHCl in HeLa cells is hampered by the presence of serum.

2.3 Cytotoxicity of CSHCl-PLA-DNA complexes

The results of cell viability (IC_{50}) of CSHCl/PLA (MW > 70 kDa) test are shown in Table 16. The results showed that the cytotoxicity of CSHCl/PLA was less than that of PEI and native PLA (IC_{50} = 1.535).

Table 16 Cytotoxicity of polypeptides in HeLa cells at pH 6.4

Samples	IC_{50} (approximately) (µg/ml)
CSHCl/PLA	17.099
PEI	0.927

Although PLA/DNA/CSHCl complexes had high transfection efficiency in HeLa cells, it was interesting to investigate the cytotoxicity of PLA/DNA/CSHCl. The viability of HeLa cells was examined in the presence of complexes between PLA, CSHCl and DNA at various order of mixing samples as studied in the transfection experiment (Figure 32). The average cell viabilities of all complexes were over 80 %.

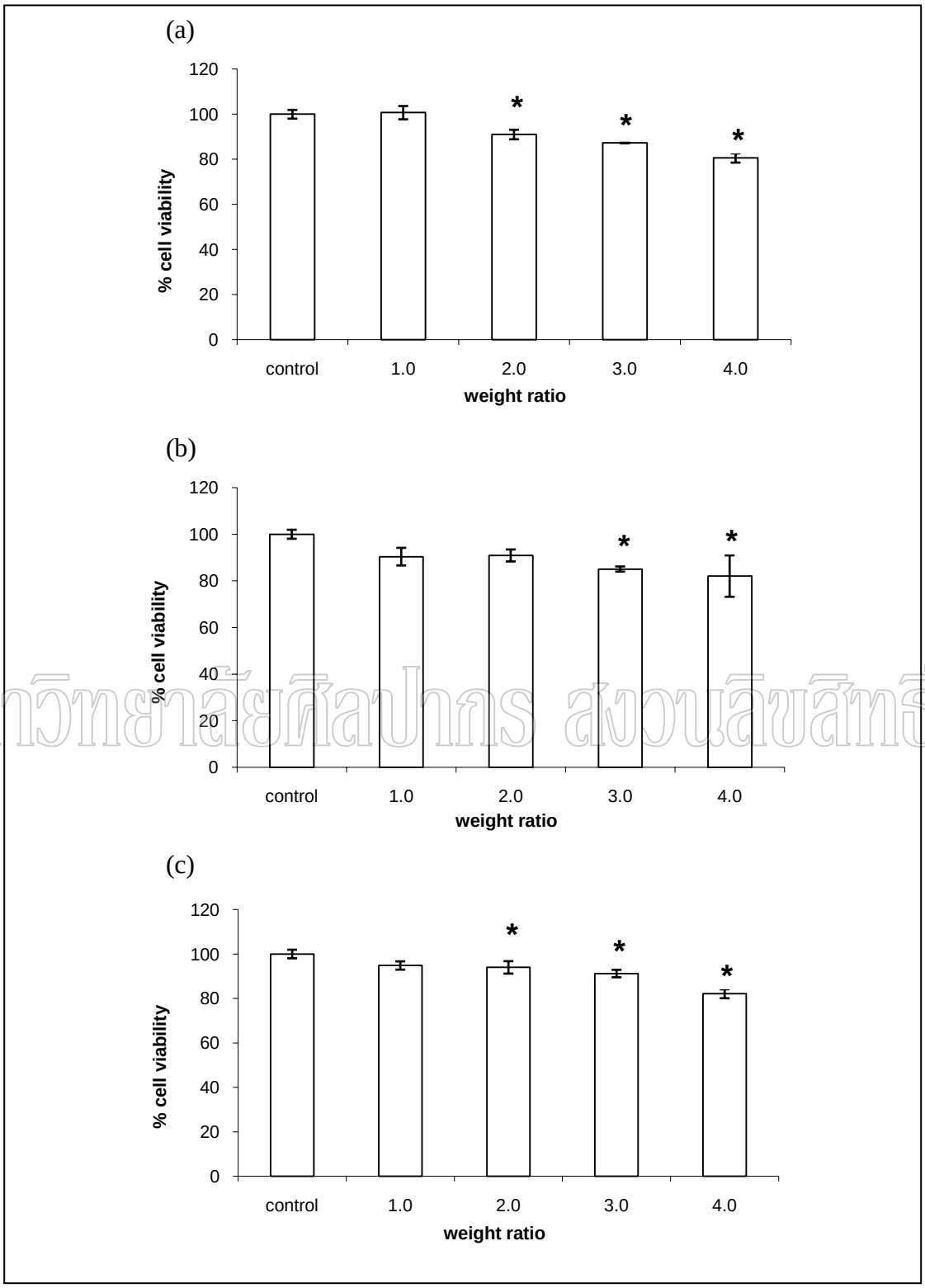


Figure 32 Effect of order of mixing between PLA, CSHCl and DNA on cytotoxicity in HeLa cells at pH 6.4; (a) CSHCl/DNA/PLA, (b) PLA/DNA/CSHCl and (c) PLA/CSHCl /DNA

3. Characterization of anionic liposomes on PLA/DNA complexes

3.1 Physicochemical properties

Physicochemical properties of PLA MW > 70 kDa-coated liposomes/DNA complexes including complex formation, particle size and zeta potential were characterized because these properties might affect the transfection efficiency.

Figure 33 is illustrated gel electrophoresis of PLA-coated liposomes/DNA complexes with various weight ratios. The completely retarded DNA migration from PLA-coated liposomes/DNA complexes was found at the weight ratio above 0.05 in pH 7.4, and at the weight ratio above 0.01 in pH 6.4 (Table 17).

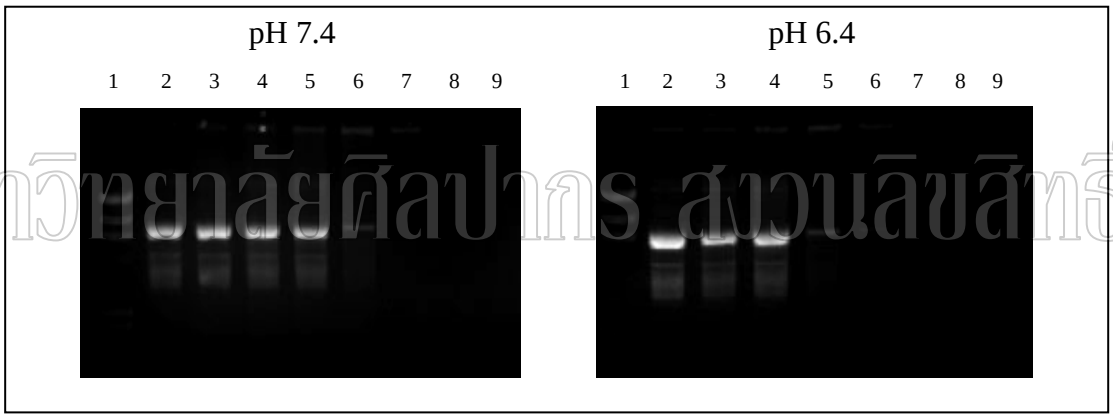


Figure 33 Agarose gel electrophoresis of PLA-coated liposomes/DNA complexes with different weight of PLA. Lane 1, λ*Hin*d III DNA marker; lanes 2-8, weight ratio of 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 0.8, respectively

Table 17 The weight ratio of complete PLA-coated liposomes/DNA complexes formed

Samples	weight ratio of complete complexes formed
pH 7.4	0.1
pH 6.4	0.05

The particle size and zeta potential of PLA-coated liposomes (EPC: NaO=10:2)/DNA complexes were determined. The particle size decreased

with an increasing weight ratio from 1 to 3 (pH 7.4). However at pH 6.4, the particle size of the complexes decreased with an increasing weight ratio from 1 to 8 (Figure 34).

Values of zeta-potential of PLA-coated liposomes indirectly reflect vesicle surface net charge and can therefore be used to evaluate the extent of interaction of the PLA-coated liposomal surface cationic charges with the anionic charges of DNA. The results of zeta-potential were shown in Figure 34. The zeta-potential values trends to increase with increasing the amount of PLA-coated liposomes.

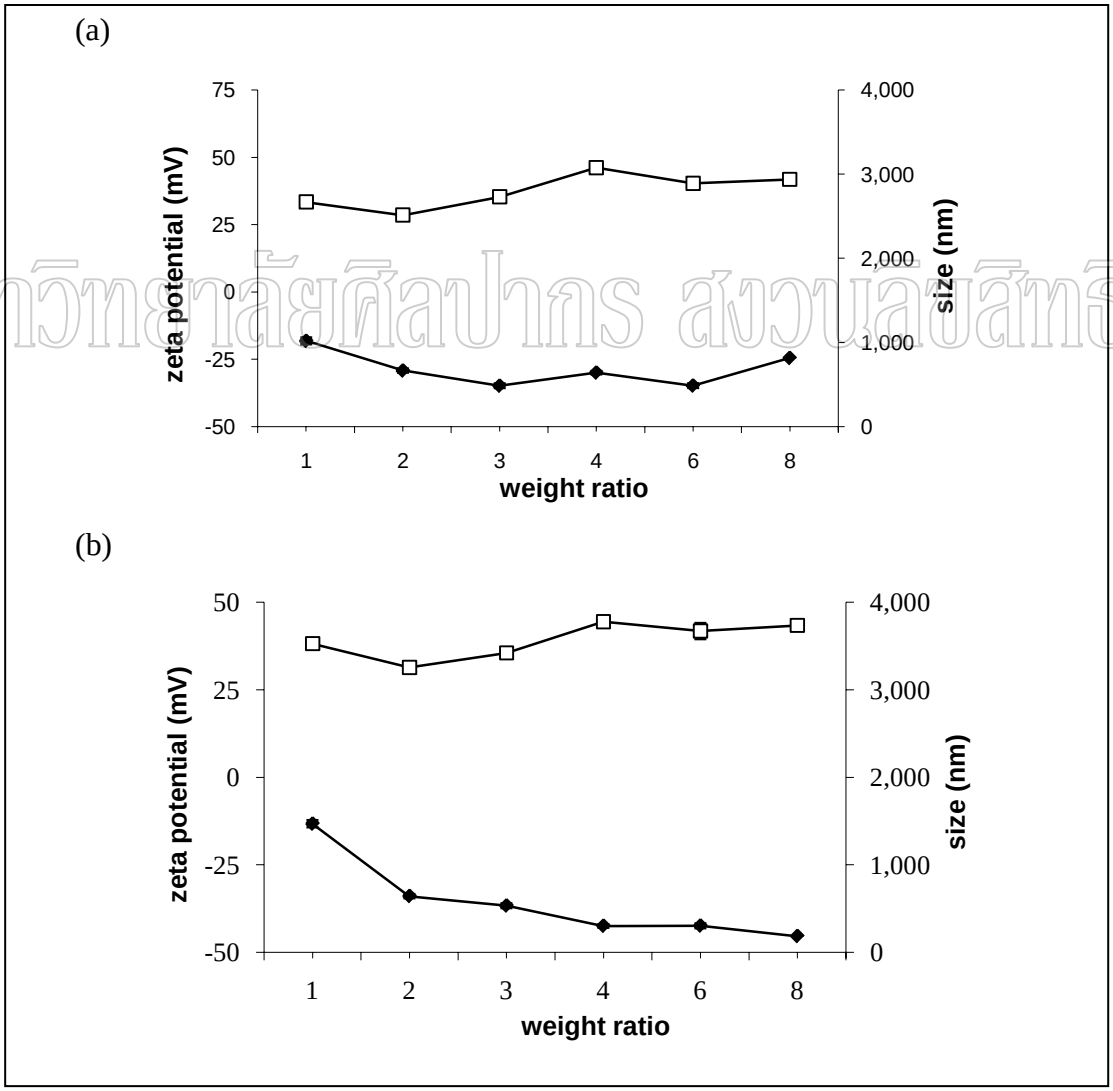


Figure 34 Effect of pH on particle size and zeta-potential of PLA-coated liposomes complexes; (a) pH 7.4 and (b) pH 6.4

3.2 Transfection efficiency of PLA-coated liposomes complexes

In this study, the factors affecting *in vitro* transfection efficiency i.e. type of cells line, weight ratio, pH and the presences of serum were investigated.

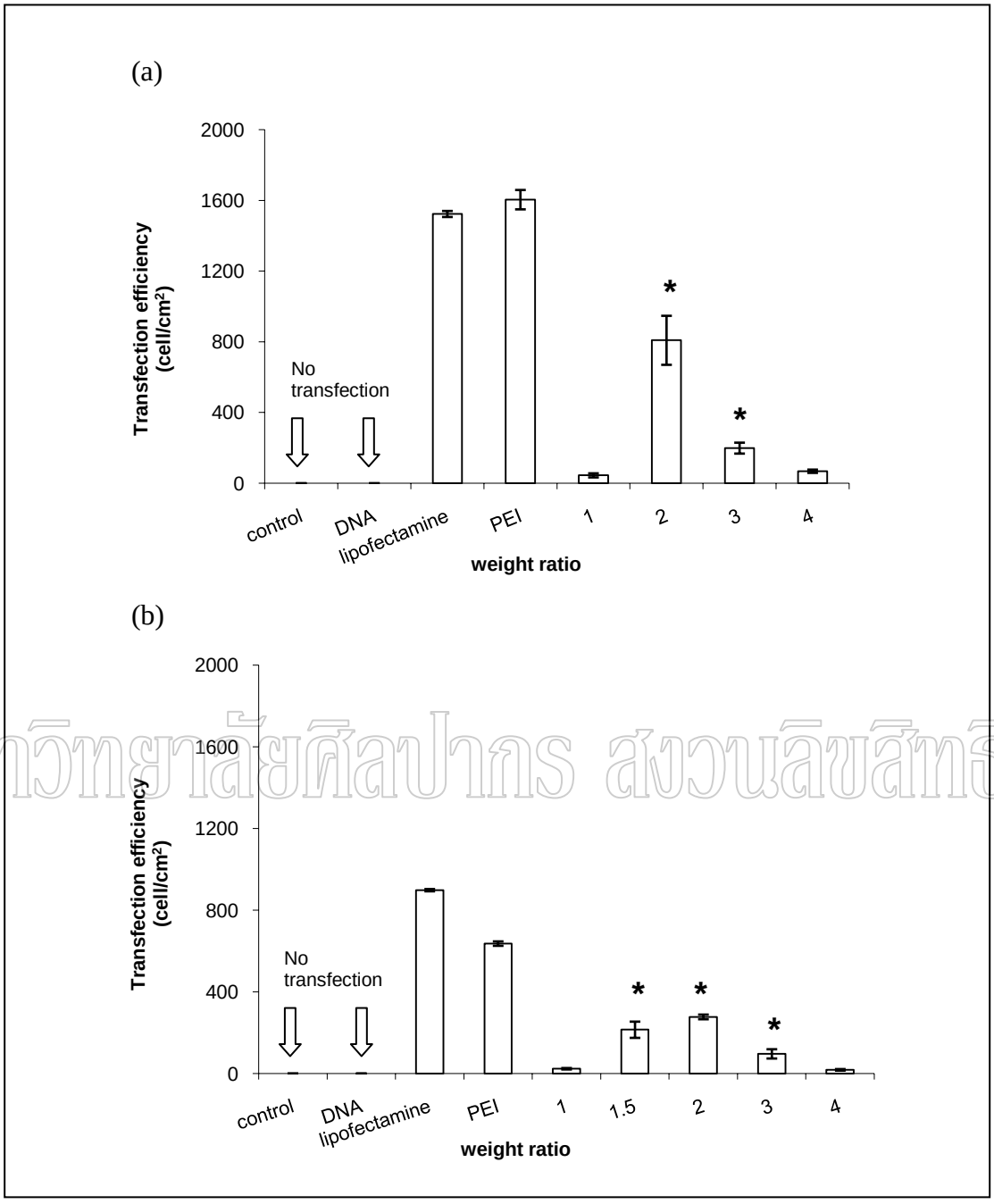
3.2.1 Effect of cells type

3.2.1.1 HeLa cells

Anionic liposomes were prepared by EPC: NaO at amolar ratio of 10:2 and then coated with PLA. The result of transfection efficiency of PLA-coated liposomes was shown in Figure 35 and 36. The gene transfection efficiencies (at pH 7.4) were significantly influenced by the weight ratios of PLA. The transfeciton efficiencies increased to reach the maximum when the weight ratio increase, then decreased by further increment of the ratios. Among PLA tested, PLA-coated liposomes showed the highest transfection efficiency (Figure 35 (a)). The highest transfection efficiency at weight ratio of 2, was 808.947 ± 138.589 cell/cm^2, which was higher than that of the highest transfection efficiency of PLA at weight ratio of 4 (543.860 ± 22.338 cell/cm^2) as shown in Figure 35 (b). The value was significantly higher transfection efficiency than the PLA/DNA complex about 1.49 times.

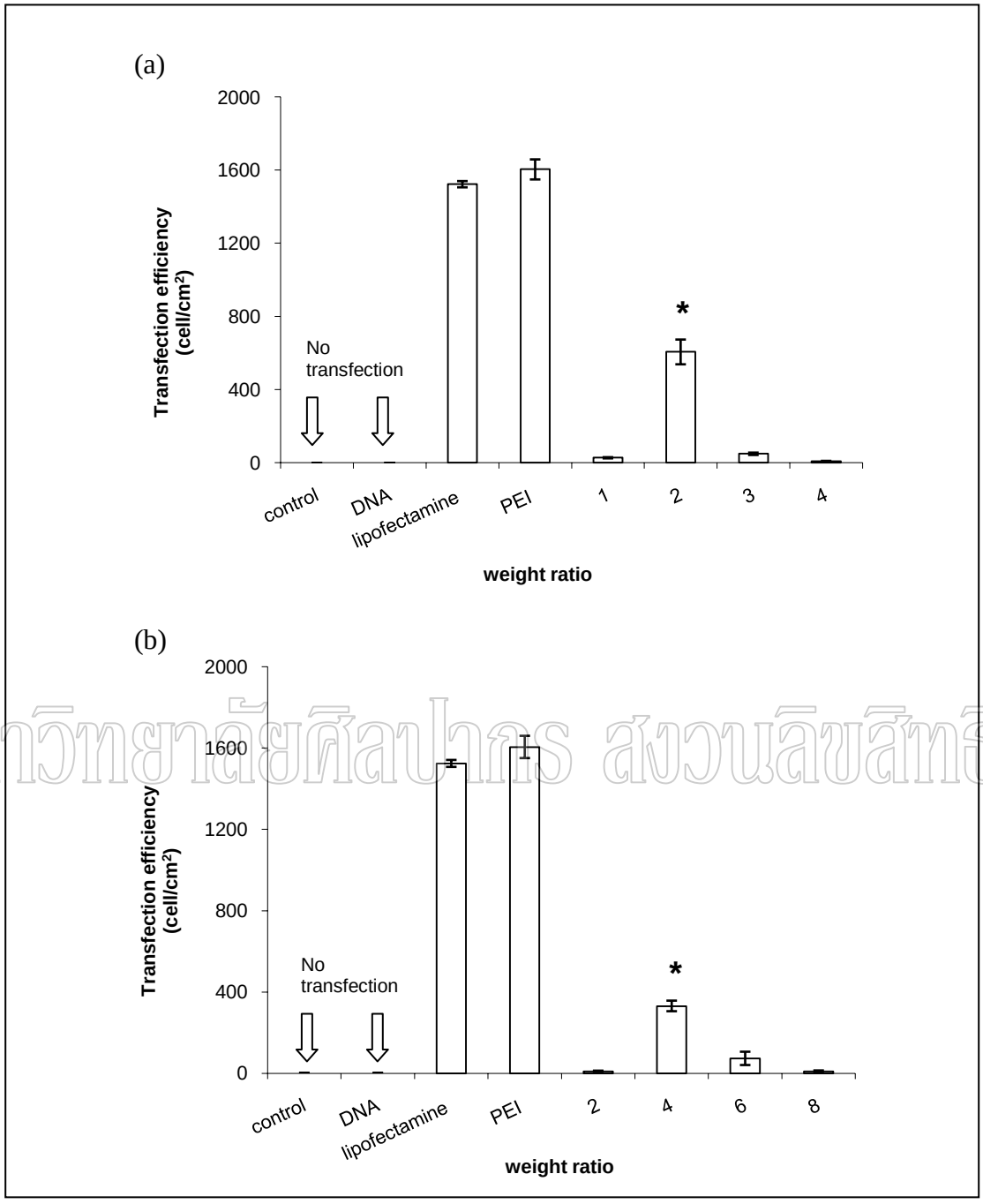
As shown in Figure 36 (a) and (b), the highest transfection efficiency of PLA-coated liposomes at weight ratio of 2, was 606.491 ± 67.681 cells/cm^2 (pH 6.4), which was higher than that of the highest transfection efficiency of PLA at weight ratio of 4 (303.877 ± 25.963 cell/cm^2). The value had significantly higher transfection efficiency than the PLA/DNA complex (about 1.83 times).

The results showed that transfection efficiency of PLA and PLA-coated liposomes complexes at pH 6.4 was significantly lower than at pH 7.4.



Remark : Positive control using pH 7.4

Figure 35 Effect of pH on transfection efficiency of poly-L-arginine (MW > 70 kDa)-coated liposome at pH 7.4 in HeLa; (a) PLA-coated liposome/DNA and (b) PLA/DNA. Values shown are the means ± SD of triplicate experiment (* indicate $p \leq 0.05$).

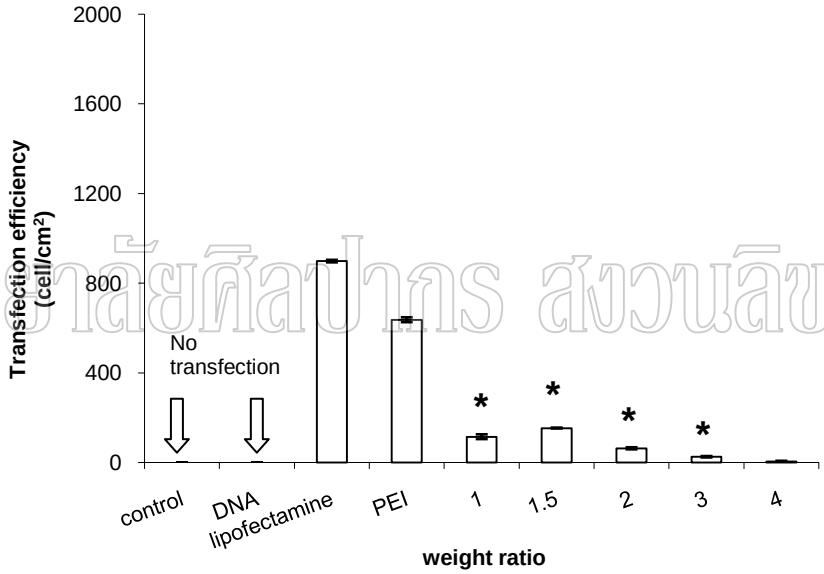


Remark : Positive control using pH 7.4

Figure 36 Effect of pH on transfection efficiency of poly-L-arginine (MW > 70 kDa)-coated liposome at pH 6.4 in HeLa; (a) PLA-coated liposome/DNA and (b) PLA/DNA. Values shown are the means ± SD of triplicate experiment (* indicate $p \leq 0.05$).

3.2.1.2 Huh7 cells

PLA-coated liposomes showed the highest transfection efficiency (Figure 38 (b)) at pH 6.4. The highest transfection efficiency at weight ratio of 1.5, was 304.737 ± 9.517 cell/cm^2, which was higher than that of the transfection efficiency of PLA-coated liposomes at pH 7.4 at weight ratio of 2 (277.544 ± 11.439) as shown in Figure 37, and that of PLA at the weight ratio of 1.5 (153.158 ± 1.823 cell/cm^2) as shown in Figure 38 (a), respectively. It was significantly higher transfection efficiency than the PLA/DNA complex about 1.99 times.



Remark : Positive control using pH 7.4

Figure 37 Effect of PLA /DNA on transfection efficiency at pH 7.4 in Huh7

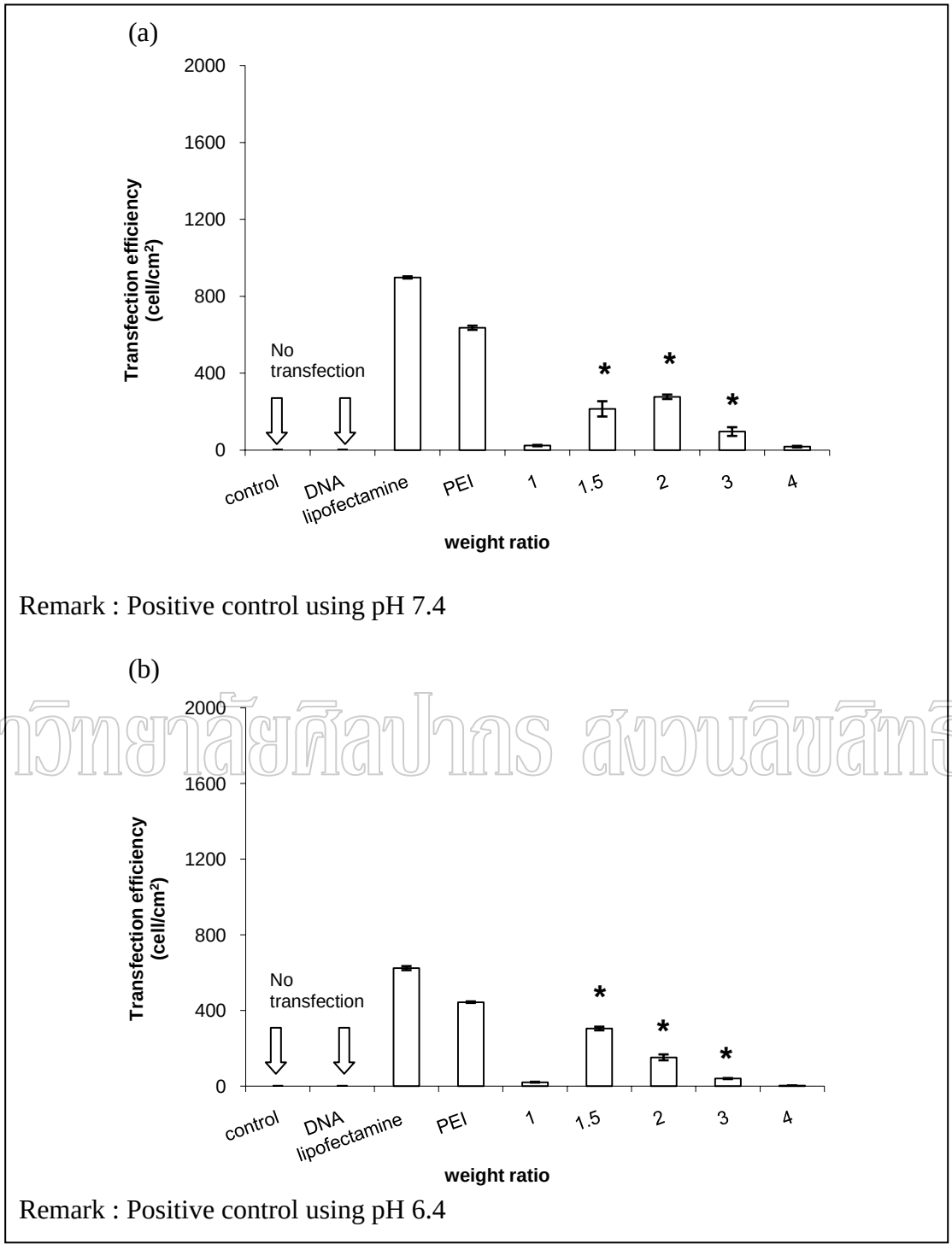
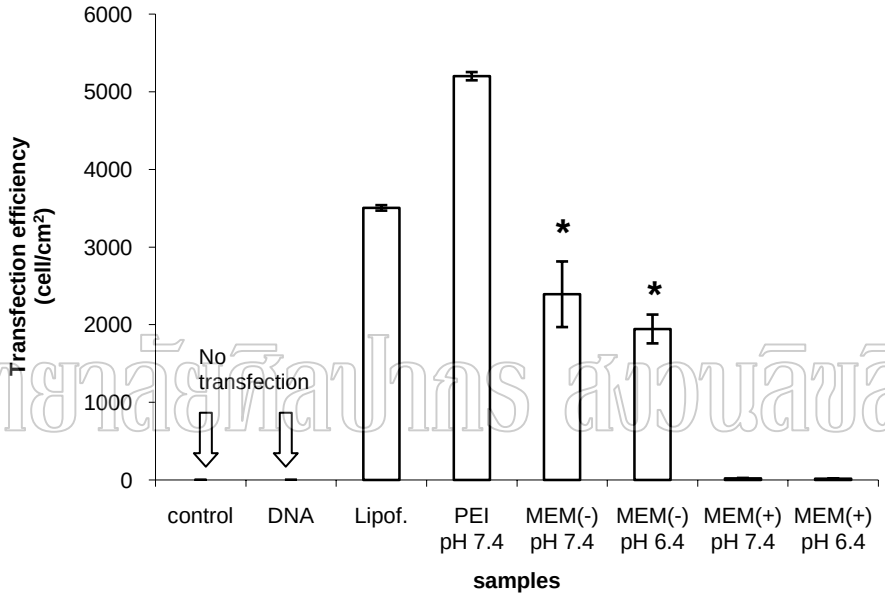


Figure 38 Effect of pH on transfection efficiency of poly-L-arginine (MW > 70 kDa)-coated liposome in Huh7; (a) PLA/DNA pH7.4 and (b) PLA-coated liposome/DNA pH 6.4. Values shown are the means ± SD of tripicated experiment (* indicate $p \leq 0.05$).

3.2.2 Effect of serum

The effect of serum on transfection efficiency of PLA MW > 70 kDa-coated liposomes in the absence or presence of serum was investigated. As shown in Figure 39, the transfection efficiency in cells transfected in serum containing media (10% FBS) was significantly lower than that in serum-free media both in pH 6.4 and 7.4. However, the transfection efficiency at pH 6.4 was no significant difference from that at pH 7.4 in the absence of serum.



Remark: Lipof. = Lipofectamine pH 7.4

Figure 39 Effect of serum on gene transfection efficiency. HeLa cells were incubated in the absence or presence of 10% serum with PLA MW > 70 kDa-coated liposomes/DNA complexes

3.3 Cytotoxicity of PLA-coated liposomes complexes

3.3.1 Effect of cells type

3.3.1.1 HeLa cells

The results showed that the cytotoxicity of PLA-coated liposomes was concentration dependent (Table 18). The cytotoxicity of PLA-coated liposomes increased with decreasing pH from 7.4 to 6.4.

Table 18 Cytotoxicity of PLA-liposomes in HeLa cells

Samples	IC_{50} (approximately) (μg/ml)
pH 7.4	10.676
pH 6.4	5.264

The HeLa cells viability was tested in the presence of PLA-coated liposome/DNA complexes at various weight ratios as studied in the transfection experiment. The results were shown in Figure 40. Approximately 80% of cells were viable after the incubation with PLA-coated liposome/DNA complexes at weight ratio less than 2.5 in HeLa cells at pH 7.4 whereas the average cell viability was less than 80 % at pH 6.4.

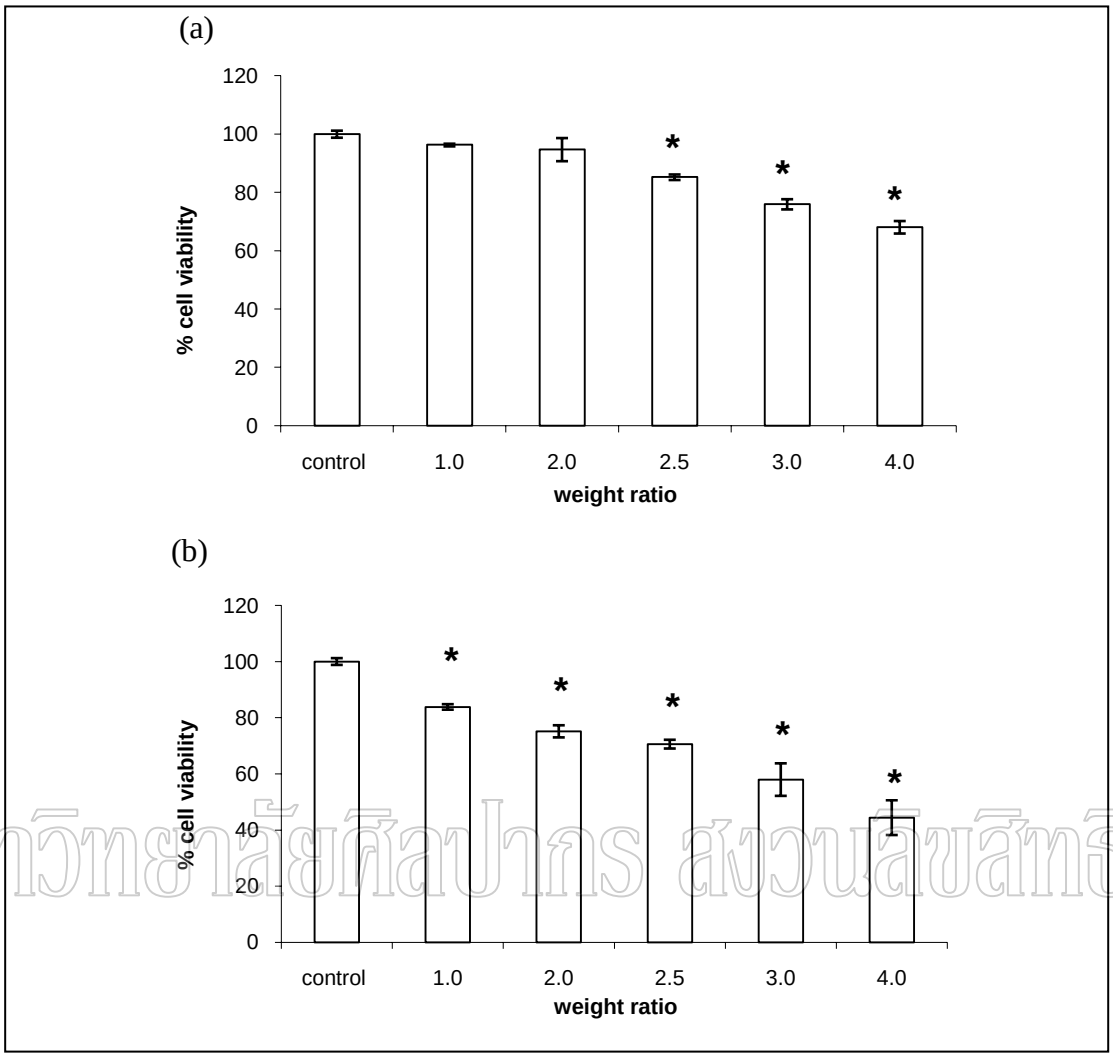


Figure 40 Effect of pH on cytotoxicity of PLA MW > 70 kDa-coated liposomes/DNA complexes in HeLa cells; (a) pH 7.4 and (b) pH 6.4

3.3.1.2 Huh7 cells

The effect of medium pH of PLA-coated liposome on cytotoxicity of Huh 7 cells was shown in Table 19. The result showed that PLA-coated liposome at pH 7.4 was less toxic than pH 6.4. The cell viability of PLA-coated liposome/DNA complexes in Huh 7 cells significant decreased with increasing in weight ratio of PLA-coated liposome/DNA complexes (Figure 41). The average cell viabilities of all complexes were over 75 %.

Table 19 Cytotoxicity of PLA-coated liposome in Huh7 cells

Samples	IC_{50} (approximately) (µg/ml)
pH 7.4	70.288
pH 6.4	54.878

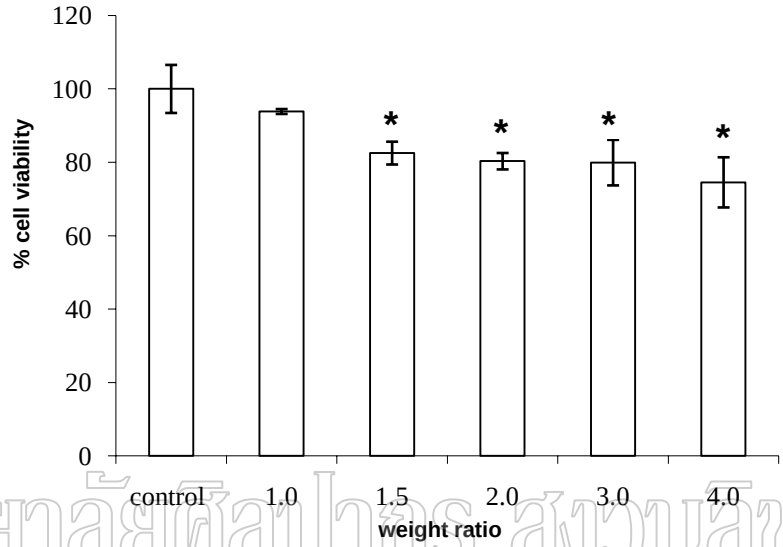


Figure 41 Cytotoxicity of PLA MW > 70 kDa-coated liposomes/DNA complexes in Huh7 cells at pH 7.4

Discussions

Cationic polypeptides have been used as tools for gene carriers. The main reason for the using cationic polypeptides is that cationic polymers vectors provide the flexibility of DNA carrying capacity and simplicity of use. The nature of the basic peptide in efficient protein transduction and in nucleic acid binding capability prompted us to explore the possibility of DNA transfection by these peptides (Kim et al. 2003 : 130). From the study of polypeptide/DNA complexes, the physicochemical properties and the transfection efficiency of the complexes could be resulted from the effect of type and MW of polypeptide. In addition, it has been reported that the biological activities of the transfection reagents highly associate with their physicochemical properties (Pedroso de Lima et al. 2001 : 277-294; Thomas and Klibanov 2003 : 27-34). The variable type and MW of polypeptide, weight ratio and pH of transfection medium influenced the physicochemical properties and transfection efficiency of polypeptide/DNA complexes (Pouton et al. 1998 : 289-299; Ramsay et al. 2000 : 97-107; Choi et al. 2006: 1604-1612; Goparaju, Satishchandran and Gupta 2009 : 162-169). Therefore, the complex formation, particle size and zeta potential of polypeptide/DNA complexes were investigated with the complex solution of different type and MW of polypeptide, weight ratio and pH of transfection medium.

To assess formation of peptide/DNA complexes, gel electrophoretic retardation was employed to measure the electrostatic interaction in the complexes as a function the positive peptide/ negative DNA charge ratios. The results showed that the electrophoretic migration of DNA was retarded with increasing ratio of peptides (positive)/DNA (negative). This result was similar to Kim et al. that no migration of plasmid DNA band was observed at charge ratio 3.0 or higher (Kim et al. 2003 : 131). This absence of migration was probably due to the neutralization of nucleic acid by cationic peptide and/or formation of a large complex between the peptide and the DNA.

The particle size of all polypeptide/DNA complexes is inversely proportional to the charge ratio while their zeta-potential is directly proportional to the charge ratio. The results were similar to Jiang et al. (2007 : 276), Mansouri et al. (2006 : 2062) and Thanou et al. (2002 : 156). The size of carriers/DNA complexes

were in the nanometer range that had a relatively higher intracellular uptake compared to micrometer range (Zauner et al. 2001 : 39-51). It has been previously reported that complexes having a particle size of 100-500 nm have successfully transfected the cells (Maclaughlin et al. 1998: 259-272; Ozgel and Akbuga 2006 : 44-51; Lavertu et al. 2006 : 4815-4824). The average particle size of polypeptide/DNA complexes formulated with various type of polypeptide and MW in these studies was in the range of 200-500 nm, except PLO, their complexes at weight ratio less than 2 were form in size of micrometer (~ 1 μm). The study in DNA, nanoparticles was advantageous in gene transfer because of enhancement of cellular uptake of the carriers/DNA complexes and facilitating its subsequent release from the endo-lysosome pathway (Huang et al. 2005 : 391-406; Sun, Zhang and Zhang 2004 : 533). The particle size of the complexes had a trend showing a slightly increase in the particle size as the MW of PLA was increased (Figure 16). Although it might be expected that the high MW PLA could be interact with DNA and thus condense the DNA more efficiently than low MW PLA. The particle size of the complex with higher MW should be smaller as the weight ratio was increased.

The surface charge of gene delivery systems is known to be one of the major factors influencing their transfection efficiency (Gao et al. 2008 : 243). A positive surface charge of polyplexes is necessary for binding to anionic cell surfaces, which consequently facilitates uptake by the cell (Jiang et al. 2007 : 277; Harush-Frenkel et al. 2007 : 26-32). The zeta-potential of polypeptide/DNA complexes were positive charges after the complete complexes were formed. The complete complexes of these complexes were formed at weight ratio above 0.1 observed by gel electrophoretic retardation experiment. At physiological pH, the amino group of lysine, arginine and ornithine are positively charged and can ionically interact with polyanions, such as DNA, and has been extensively characterized. With all of the polycations, not just polylysine, polyarginine and polyornithine, the procedure for complex formation can influence transfection efficiency (e.g., salt concentration or speed of mixing the cation and anion). Numerous methods have been developed to generate protocols that consistently and repeatedly generate predefined polypeptide/DNA complexes of defined stability and size, such as flash mixing, high-salt conditions followed by dialysis, or high salt and vigorous agitation (Wightman,

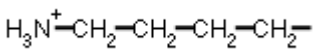
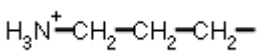
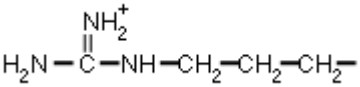
Kircheis and Wagner 2003 : 112). The charge densities of polypeptides are increased when the pH of the polypeptide solution decreases from 7.4 to 6.4. Due to the increased charge density of polypeptide at pH 7.4, a weaker interaction between the DNA and polypeptide is expected.

To investigate the polypeptides mediated gene transfection efficiencies. The PLA/DNA complexes showed higher transfection efficiencies than PLL/DNA and PLO/DNA complexes, respectively. This result might be due to the protonation of side chain. The pK_a of side chain (guanidinium group) of arginine is 12.48, while the side chain (amino group) of lysine and ornithine is 10.53 and 10.76, respectively. Therefore, the pK_a values in the presence of PLA can be increment of protonation of the side chain at pH 7.4 as well as strongly interaction with DNA compare PLL or PLO. In the Henderson-Hasselbalch equation is fulfilled and gives a ratio of protonated form: unprotonated form of an ionizable compound. In this ratio, can be calculated by using equation (4);

$$pH = pK_a + \log \frac{[B]}{[HB^+]} \quad (4)$$

Table 20 shows the protonated form : unprotonated form ratio of arginine, lysine and ornithine. Arginine showed higher protonated form : unprotonated form ratio than ornithine and lysine, respectively. In comparison, the highest transfection efficiency of PLA, PLL and PLO were 644, 346 and 6 cells/cm^2, respectively. In the case of PLO, there was less transfection efficiency than PLL. This might be due to the higher cytotoxicity caused by PLO than that by PLL. Nevertheless, ornithine showed higher protonated form : unprotonated form ratio than lysine.

Table 20 pK_a values of side chain and ratio of protonated (HB^+) : unprotonated (B) form of the arginine, lysine and ornithine

Structure of R	Name	Abbreviations	pK_{a1} (α-CO_2H)	pK_{a2} (α-NH^{3+})	pK_{a3} (R)	Ratio of $[HB^+]$: [B] pH 7.4	Ratio of $[HB^+]$: [B] pH 6.4
	Lysine	K or Lys	2.18	8.95	10.53	$10^{3.10}$	$10^{4.13}$
	Ornithine	O or Orn	1.71	8.69	10.76	$10^{3.36}$	$10^{4.36}$
	Arginine	R or Arg	2.17	9.04	12.48	$10^{5.08}$	$10^{6.08}$

Source : T.M. Graham Solomons, and Graig B. Fryhle, "Amino Acids and Protein," In Organic Chemistry 8th Edition (John Wiley & Sons, Inc., 2004), 1170.

: R. Williams, p*Ka* Values [Online], accessed 19 May 2010. Available from http://research.chem.psu.edu/brpgroup/pKa_compilation.pdf

: Microwave Laboratory Systems, Milestone Inc., Introduction to the analysis of amino acids [Online], accessed 20 May 2010. Available from http://www.milestonesci.com/hydr-fund.php

Haung et al. (2005 : 391-406) and Zhao et al. (2006 : 223-228) reported that highly positive charge density of high MW cationic polymer enhances the cellular uptake and transfection efficiency of their complexes. Kim et al. (2003 : 132) reported that the efficiency of arginine 15 residues is almost sevenfold higher than these of commercial transfection agents (Lipofectin) and arginine 12 and 9 residues in 293T cells. A significant positive dependence of transfection efficiency on the number of arginine residues is also observed. These results were similar to this study, high MW PLA (> 70 kDa) showed higher transfection efficiency than low MW PLA (15-70 kDa and 5-15 kDa), *in vitro* gene transfection assay was performed with HeLa and Huh7 cells using pEGFP-C2 plasmid encoding green fluorescent protein (GFP). PEI complexed with DNA was used as a positive control. In all studies, there were no transfection in control (cells without complexes) and naked DNA.

To investigate the PLA MW > 70 kDa/CSHCl mediated gene transfection efficiencies. Previous studies demonstrated that the gene delivery potential of polymer in mammalian cells depends on several factors such as type of polymer, polymer MW, weight ratio, pH of transfection medium/complex solution and cell type

(Sato, Ishii and Okahata 2001 : 2075-2080; Haung et al. 2005 : 391-406; Zhao et al. 2006 : 223-228). The gene transfection efficiencies of PLA/CSHCl/DNA complexes were influenced by carrier/DNA weight ratio, order of mixing between PLA, CSHCl and DNA, cell types, pH of transfection medium and serum. The transfection efficiency of PLA/CSHCl/DNA complexes was dependent on pH of transfection medium. The transfection efficiency of PLA was independent on pH of culture medium, while CS was dependent on pH of culture medium. The transfection efficiency of CSHCl at the transfection medium pH 6.4 was higher than medium pH 7.4. This result 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; Sato, Ishii and Okahata 2001 : 2077). 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 charged, and can bind with negative charges in cells through electrostatic interaction at below pH 7.0. Weecharangsan et al. showed the maximum transfection efficiency of the CSHCl/DNA complex achieved at an N/P ratio of 12 (CSHCl 6 µg/DNA 1 µg) in CHO-K1 cells at pH 6.5 (Weecharangsan et al. 2008 : 162). Therefore, the preliminary study using CSHCl weight ratio 2, 4 and 8 µg mixed with polypeptide. The CSHCl weight ratio 4 and 8 µg did not show significant different transfection efficiencies (data not shown). Therefore, the amount of CSHCl was fixed at 4 µg for all the transfection efficiency experiments.

The transfection efficiency had a tendency to increase as the weight ratio of PLA/DNA increased. At a low weight ratio, the transfection efficiency of all complexes was not different in comparing with the naked DNA. This might be resulted from the insufficient amount of positively charged in complexes to transfect cells. All complexes achieved sufficient transfection efficiencies at higher weight ratios (above 1). The reason might be that an increase in the weight of carriers at higher weigh ratios could yield a higher amount of positively charged complexes to transfect cells successfully. The increase in transfection ability with an increase in N/P ratio in arginine-CS/DNA complexes was also demonstrated by Gao et al. (2008 : 244).

The order of mixing affected the transfection efficiency. The maximum transfection efficiency of the complexes in HeLa cells could be ranked as follows: PLA/DNA/CSHCl > PLA/CSHCl/DNA > PLA/DNA > CSHCl/DNA/PLA. This might be due to the CSHCl increased the safety of PLA/DNA/CSHCl formulation as well as the binding with DNA of PLA/DNA/CSHCl (binding affinity experiment) compared to that of PLA/DNA complex. Douglas, Piccirillo and Tabrizia reported that CS was less cytotoxic than cationic polypeptide such as PLL (Douglas, Piccirillo and Tabrizia 2006 : 354). Although CSHCl/DNA/PLA formulation increased binding with DNA as compared to that observed with PLA alone, the transfection efficiency of CSHCl/DNA/PLA was less than that of PLA/DNA. It might be explained by the CSHCl bind with DNA before PLA. Therefore, binding of PLA with DNA was interrupted by CSHCl. The results showed that the transfection efficiency of CSHCl/DNA complexes were less than PLA/DNA complexes. Although the exact mechanism still remains unclear, it was considered that the interaction of PLA, DNA and CSHCl might increase the safety of PLA. The transfection efficiency of PLA/DNA/CSHCl was not significantly different compared to that of PEI/DNA complex. In addition, it was significant higher (about 2.39 times) compared to that of PLA/DNA complex.

Our results showed that transfection efficiency of PLA-coated liposomes complexes (EPC: NaO=10:2) at pH 6.4 was significant lower than that of pH 7.4. This low transfection efficiency might be due to the cytotoxicity of these polymers at pH 6.4. PLA-coated liposome showed higher transfection efficiency than PLA alone. The polypeptide-liposomes have been previously referred as promising transfection agents liposome modified with peptide also showed remarkable transfection efficiency such as oligopeptide/cholesterol derivative (DMB-Chol), oligopeptide/ dioleoyl-phosphatidylethanolamine (DOPE), PLL/DC-chol/DOPE liposomes, peptide ligand- EPC lipid, NLS peptide-DOSPA (cationic lipid) and NLS peptide-DOPE (neutral lipid) (Tokunaga, Hazemoto and Yotsuyanagi 2004 : 71-80; Son, Tkach and Patel 2000 : 11-14; Yagi et al. 2007 : 2590-2593; Rea et al. 2009 : 903-912).

Interestingly, higher transfection efficiency was observed for the PLA/DNA/CSHCl than for PLA-coated liposomes/DNA. The higher transfection efficiency of PLA/DNA/CSHCl to higher positive charge in the complex must

increase the interaction of complexes with the cellular surface. The small particle was found in PLA/DNA/CSHCl complexes, whereas the large particle was found in PLA-coated liposome/DNA complexes. In addition, the PLA/DNA/CSHCl may have strong condensation capability than PLA-coated liposome, which would result in stability after passing through the cell membrane. This also would facilitate the higher transfection efficiency of DNA.

To evaluate the effect of serum, the transfection efficiency was carried out on the presence of 10% serum. The PLA/DNACSHCl in the presence of 10% serum was significant lower transfection efficiency (about 31.670 times) than PLA/DNA/CSHCl in the absence of 10% serum at pH 6.4 of transfection medium. This result was similar to PLA-coated liposome showing that the transfection level of PLA-coated liposome was decreased in the presence of serum (about 109.970 times) at pH 7.4 of transfection medium. The poor transfection efficiency of PLA/CS/DNA complexes and PLA-coated liposome/DNA complexes 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 carriers (Nicolazzi et al. 2003 : 1263; Eliyahu et al. 2007 : 2342). Additionally, the zeta-potential decreased within seconds of each serum addition (Mao et al. 2001: 416)

A good gene carrier should have low or no cytotoxicity. The cytotoxicity of cationic polymer was probably caused by polymer aggregation on cell surfaces. This impaired the important membrane function (Jiang et al. 2007 : 278). The complexes of all polypeptide had lower cytotoxicity than the complex of PEI 25 kDa. This might be the result of biodegradable property of polypeptide that leads to lower cytotoxicity (Wong et al. 2006 : 152-158). Significantly, the PLA/CSHCl/DNA complex displayed low cytotoxicity. While most of modifications that have been made to CS to improve transfection resulted in systems with higher toxicity, these results indicated that the presence of PLA does not increase the toxicity of the system, successfully achieving one of the primary goals of this project. The PLA-coated liposome formulation had lower cytotoxicity than PEI. Although the exact mechanism still remains unclear, it is considered that the interaction of liposome with PLA might increase the safety of PLA.

CHAPTER 5
CONCLUSIONS

The cationic polypeptides were successfully used to improve transfection efficiencies of chitosan or liposomes. Three types of cationic polypeptides including poly-L-lysine (PLL), poly-L-arginine (PLA) and poly-L-ornithine (PLO) were selected and mixed with chitosan hydrochloride or anionic liposomes to investigate *in vitro* transfection efficiency and cytotoxicity in human cervical carcinoma cells (HeLa cells) and human hepatoma cells (Huh7 cells). The effect of the type and molecular weight of polypeptides, the weight ratio of carrier/DNA, the pH of transfection medium and serum on transfection efficiency and on cytotoxicity were determined. The results of this study could be concluded as follows:

1. Polypeptides/DNA complexes

1.1 Complex formation of polypeptides with pDNA

Gel retardation illustrated that cationic polypeptides were able to form complex with DNA. The type of cationic polypeptides (PLL, PLA and PLO), MW of PLA (5-15, 15-70 and >70 kDa), and pH (6.4 and 7.4) was not affected. The complete complexes were formed at weight ratio above 0.1.

1.2 Particle size and surface charge of polypeptides/DNA complexes

Cationic polypeptides (PLL, PLA and PLO) were able to form complex with DNA in nanometer (200 – 500 nm). However, PLO/DNA complexes at weight ratio less than 2 were form in size range micrometer size (~ 1 μm). The zeta-potential of these complexes were positively charged after the complete complexes were formed.

1.3 Transfection efficiency

The gene transfection efficiencies were significantly influenced by weight ratio. By increasing weight ratio, transfection efficiencies showed a bell shaped pattern. It can be ranked the maximum transfection efficiency mediated by polypeptides in HeLa cells as PLA > PLL > PLO. MW of PLA, high MW PLA

showed higher transfection efficiency than low MW PLA, with the ranking from high to low of 70 kDa > 15-70 kDa > 5-15 kDa. The pH of culture medium had an influence on transfection efficiency mediated by PLL, not by PLA and PLO in HeLa cells. The transfection efficiency decreased when Huh7 cells were used.

1.4 Cytotoxicity

1.4.1 Cytotoxicity of polypeptides

The cytotoxicity polypeptides increased with increasing the weight ratio. The toxicity to HeLa cells by the polypeptides could be ranked as PLA > PLL > PLO and to Huh7 cells could be ranked as PLO > PLL > PLA.

1.4.2 Cytotoxicity of polypeptides/DNA complexes

The cytotoxicity of polypeptides/DNA complexes were tested in HeLa and Huh7 cells. The results showed that cationic polypeptides led to a significant decrease in cell viability with increasing in the weight ratio of polypeptides/DNA. PLA/DNA and PLL/DNA complexes had a low toxicity to HeLa cells at weight ratio less than 4. Their average cell viability was over 80%. While the cell viability by PLO/DNA complexes was less than 60%. The average cell viability by PLL/DNA and PLA/DNA complexes in Huh7 cells were over 60%, whereas that by PLO/DNA complexes was over 40%. The result revealed the polypeptide/DNA complexes causing higher Huh7 cells cytotoxicity than HeLa cells. The cytotoxicity of polypeptide/DNA complexes could be ranked as PLO/DNA > PLL/DNA ~ PLA/DNA complexes. However, all polypeptides/DNA complexes had lower cytotoxicity than PEI/DNA complexes.

2. CSHCl/PLA/DNA complexes

2.1 Complex formation

The complexes were prepared by adding CSHCl solution (0.01µg), PLA (0 to 50 µg) and DNA (1 µg) as the following order of mixing:

- PLA ⟶ DNA ⟶ CSHCl
- CSHCl ⟶ DNA ⟶ PLA
- PLA ⟶ CSHCl ⟶ DNA

The complete CSHCl/DNA complexes were formed at the weight ratio above 0.1. The complete CSHCl/DNA/PLA, PLA/DNA/CSHCl and

CSHCl/PLA/DNA complexes were formed at the weight ratio above 0.5, 0.5 and 0.1, respectively. The order of mixing did not affect completed complexes formation.

2.2 The binding affinity

The amount of CSHCl was fixed at 4 μg for all the binding affinity experiments. The binding affinity of complexes was studied by ethidium bromide displacement assay. The ranking order of complex binding affinity was PLA/DNA/CSHCl ≈ CSHCl/DNA/PLA > CSHCl/DNA ≈ PLA/DNA.

2.3 Particle size and surface charge

The amount of CSHCl was fixed at 4 μg for all the particle size and surface charge experiments. The particle size of the all complexes was in nanometer size range (< 500 nm). Their zeta-potential was positive in the range of 23 to 40 mV.

2.4 Transfection efficiency

The amount of CSHCl was fixed at 4 μg for all the transfection efficiency experiments. The transfection efficiency had a tendency to increase as the weight ratio of PLA/DNA increased. The order of mixing affected the transfection efficiency. It could be ranked the maximum transfection efficiency of the complexes in HeLa cells as follows: PLA/DNA/CSHCl > PLA/CSHCl/DNA > PLA/DNA > CSHCl/DNA/PLA. The cells transfected in serum containing media (with 10% FBS) were significantly lower efficient than those in serum-free media. The cells transfected at pH 7.4 were significantly lower efficient than at pH 6.4 in media at both absence and presence of serum.

2.5 Cytotoxicity

The viability of HeLa cells was examined in the presence of complexes between PLA, CSHCl and DNA at various orders of mixing samples as studied in the transfection experiment. All complexes caused the average cell viability over 80 %.

3. Polypeptide-coated liposome/DNA complexes

3.1 Complex formation

Gel retardation illustrated that PLA-coated liposome were able to form complex with DNA. The complete complexes were formed at the weight ratio above 0.05 and 0.01 for pH 7.4 and pH 6.4, respectively.

3.2 Particle size and surface charge

The particle size of all complexes was nanosize and dependent on the pH (pH 7.4 > 6.4). The zeta-potential was positive in the range of 23 to 40 mV.

3.3 Transfection efficiency

The transfection efficiency was significantly influenced by cell types, weight ratio, pH of transfection medium and serum. The transfection efficiency in HeLa cells was higher than that in Huh7 cells. By increasing weight ratio, transfection efficiencies showed a bell shaped pattern. The cells transfected in serum containing media (with 10% FBS) were significantly lower efficient than those in serum-free media. The cells transfected at pH 6.4 were significantly lower efficient than at pH 7.4 in media at both absence and presence of serum. The transfection efficiency in HeLa cells of PLA-coated liposomes/DNA complex was about 1.83 and 1.49 times higher than the PLA/DNA complexes at pH 7.4 and 6.4, respectively.

3.4 Cytotoxicity

3.4.1 Cytotoxicity of PLA-coated liposomes

The cytotoxicity of PLA-coated liposomes in HeLa cells and Huh7 cells depended on pH of the medium. The result showed that the toxicity of PLA-coated liposome at pH 7.4 was less than that at pH 6.4.

3.4.2 Cytotoxicity of PLA-coated liposomes/DNA complexes

The cytotoxicity of PLA-coated liposomes/DNA complexes were tested in both HeLa and Huh7 cells. The results showed that PLA-coated liposomes/DNA complexes significant decreased in cell viability with increasing in weight ratio of polypeptides/DNA complexes. The average cell viability of all complexes was over 75 %.

BIBLIOGRAPHY

Akita, Hidetaka et al. "Pharmacokinetics of gene delivery in cells." In Non-viral gene therapy : gene design and delivery, 136. Edited by K. Taira, K. Kataoka, and T. Niidome. Tokyo, Japan : Springer-Verlag, 2005.

Bally, Marcel B. et al. "Biological barriers to cellular delivery of lipid-based DNA carriers." Advanced Drug Delivery Reviews 38 (1999) : 291-315.

Brooks, G. Gene therapy the use of DNA as a drug. UK : Pharmaceutical Press, 2002.

Bouragaize, D., T.R. Jewell, and R. G. Buiser. "Genetic disease and gene therapy." In Biotechnology, 224-230. California, USA: Addison Wesley Longman, Inc., 2000.

Byrnes, A. P. et al. "Adenovirus gene transfer causes inflammation in the brain." Neuroscience 66 (1995) : 1015-1024.

Choi, Hong Seok et al. "An insight into the gene delivery mechanism of the arginine peptide system: Role of the peptide/DNA complex size." Biochimica et Biophysica Acta 1760 (2006) : 1604-1612.

Choi, Joon Sig et al. "Enhanced transfection efficiency of PAMAM dendrimer by surface modification with L-arginine." Journal of Controlled Release 99 (2004) : 445-456.

Choi, Joon Sig et al. "New cationic liposomes for gene transfer into mammalian cells with high efficiency and low toxicity." Bioconjugate Chem. 12 (2001) : 108-113.

Dokka, Sujatha, and Yon Rojanasakul. "Physical methods of DNA delivery." In Polymeric gene delivery: principles and applications, 54-55. Edited by M. M. Amiji, Florida : CRC Press LLC, 2005.

Douglas, Kimberly L., Ciriaco A. Piccirillo, and Maryam Tabrizian. "Effects of alginate inclusion on the vector properties of chitosan-based nanoparticles." Journal of Controlled Release 115 (2006) : 354-361.

Eliyahu, H. et al. "Characterization and in vivo performance of dextran–spermine polyplexes and DOTAP/cholesterol lipoplexes administered locally and systemically." Biomaterials 28 (2007) : 2339-2349.

Espinos, E. et al. "Efficient non-viral DNA-mediated gene transfer to human primary myoblasts using electroporation." Neuromuscular Disorders 11 (2001) : 341–349.

Evans, C. H. et al. "Gene therapeutic approach hes-transfer *in vivo*." Advanced Drug Delivery Reviews 58 (2006) : 243-258.

Farhood, Hassan, Natalya Serbina, and Leaf Huang. "The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer." Biochimica et Biophysica Acta, 1235 (1995) : 289-295.

Gao, Yu et al. "Arginine-chitosan/DNA self-assemble nanoparticles for gene delivery: *In vitro* characteristics and transfection efficiency." International Journal of Pharmaceutics 359 (2008) : 241-246.

Goparaju, Geetha N., C. Satishchandran, and Pardeep K. Gupta. "The effect of the structure of small cationic peptides on the characteristics of peptide-DNA complexes." International Journal of Pharmaceutics 369 (2009) : 162-169.

Harush-Frenkle, O. et al. "Targeting of nanoparticles to the clathrin-mediated endocytic pathway." Biochemical Biophysical Research Communications 1 (2007) : 26-32.

Haung, M. et al. "Transfection efficiency of chitosan vectors: Effect of polymer molecular weight and degree of deacetylation." Journal of Controlled Release 106 (2005) : 391-406.

Honma, K. et al. "Atelocollagen-based gene transfer in cells allows high-throughput screening of gene functions." Biochemical and Biophysical Research Communications 289 (2001) : 1075-1081.

Hui et al. "The role of helper lipids in cationic liposome-mediated gene transfer." Biophysical Journal 71 (1996) : 590-599.

Jeong, J. H., S. W. Kim, and T. G. Park. "Molecular design of functional polymers for gene therapy." Prog. Polym. Sci. 32 (2007) : 1245.

Jiang, H. L. et al. "Chitosan-graft-polyethylenimine as a gene carrier." Journal of Controlled Release 117 (2007) : 273-280.

Kaul, Goldie, and Mansoor Amiji. “Protein Nanoparticles for gene delivery.” In Polymeric gene delivery: principles and applications, 433. Edited by M. M. Amiji, Florida : CRC Press LLC, 2005.

Kean, T., S. Roth, and M. Thanou. “Trimethylated chitosans as non-viral gene delivery vectors: Cytotoxicity and transfection efficiency.” Journal of Controlled Release 103 (2005) : 643-653.

Kim, H. H. et al. “Basic peptide system for efficient delivery of foreign genes.” Biochimica et Biophysica Acta 1640 (2003) : 129-136.

Kircheis, R., L. Wightman, and E. Wagner. “Design and gene delivery activity of modified polyethylenimines.” Advanced Drug Delivery Reviews 53 (2001) : 341-358.

Kwoh, D. Y. et al. “Stabilization of poly-L-lysine/DNA polyplexes for in vivo gene delivery to the liver.” Biochimica et Biophysica Acta 1444 (1999) : 171-190.

Latchman, D. S. “Gene delivery and gene therapy with herpes simplex virus-based vectors.” Gene 264 (2001) : 1-9.

Lavertu, M. et al. “High efficiency gene transfer using chitosan/DNA nanoparticles with specific combinations of molecular weight and degree of deacethylation.” Biomaterials 27 (2006) : 4815-4824.

Lee, K.Y. et al. “Complex formation between plasmid DNA and self-aggregates of deoxycholic acid-modified chitosan.” Polymer 46 (2005) : 8107-8112.

Lee, Minhyung, and Sung Wan Kim. “Poly(L-Lysine) and copolymers for gene delivery.” In Polymeric gene delivery : principles and applications, 81. Edited by M. M. Amiji. Florida : CRC Press LLC, 2005.

Lee, Y. et al. “Enhancement of the transfection efficiency of poly(ethylenimine) by Guanidylation.” Bull. Korean Chem. Soc. 29 (2008) : 666-668.

Lin, C. et al. “Electroporation for direct spinal gene transfer in rats.” Neuroscience Letters 317 (2002) : 1-4.

Mahato, Ram I., and Sung Wan Kim. “Water soluble lipopolymers for gene delivery.” In Polymeric gene delivery : principles and applications, 175-185. Edited by M. M. Amiji. Florida : CRC Press LLC, 2005.

Maitani, Y. et al. "Cationic liposome (DC-Chol/DOPE =1:2) and a modified ethanol injection method to prepare liposomes, increased gene expression." International Journal of Pharmaceutices 342 (2007) : 33-39.

MacLaughlin, F. et al. "Chitosan and depolymerized chitosan oligomers as condensing carriers for *in vivo* plasmid delivery." Journal of Controlled Release 56 (1998) : 259-272.

Mann, A. et al. "Peptides in DNA delivery: current insights and future directions." Drug Discovery Today 13 (2008) : 152-160.

Mansouri, S. et al. "Chitosan-DNA nanoparticles as non-viral vectors in gene therapy: strategies to improve transfection efficacy." European Journal of Pharmaceutics and Biopharmaceutics 57 (2004) : 1-8.

Mao, H. et al. "Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency." Journal of Controlled Release 79 (2001) : 399-421.

Mao, H. et al. "The depolymerization of chitosan : effects on physicochemical and biological properties." International Journal of Pharmaceutics 281 (2004) : 45-54.

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

Mehier-Humbert, S. and R. H. Guy. "Physical methods for gene transfer: Improving the kinetics of gene delivery into cells." Advanced Drug Delivery Reviews 57 (2005) : 733-753.

Microwave Laboratory Systems, Milestone Inc., UK. Introduction to the analysis of amino acids [Online]. Accessed 20 May 2010. Available from http://www. milestonesci.com/hydr-fund.php

Mochizuki, H., T. Yasuda, and M. M. Mouradian. "Advances in Gene Therapy for Movement Disorders." The American Society for Experimental NeuroTherapeutics 5 (2008) : 260-269.

Mumper, R. J. et al. "Novel polymeric condensing carriers for gene delivery." Proceeding of International Symposium of Controlled Release BioactiveMaterial 22 (1995) : 178-179.

Nicolazzi, C. et al. "Cationic lipid for transfection." Current Medicinal Chemistry 10 (2003) : 1263-1277.

Nishikawa, Makiya, Yoshinobu Takakura, and Mitsuru Hashida. "Pharmacokinetics of polymer-plasmid DNA complex." In Polymeric gene delivery : principles and applications, 71. Edited by M. M. Amiji. Florida : CRC Press LLC, 2005.

Nunthanid, J. et al. "Characterization of chitosan acetate as a binder for sustained release tablets." Journal of Controlled Release 99 (2004) : 15-26.

Ozgel, G., and J. Akbuga. "In vitro characterization and transfection of IL-2 gene complexes." International Journal of Pharmaceutics 315 (2006) : 44-51.

Park, Y. K. et al. "Galactosylated chitosan-graft-dextran as hepatocyte-targeting DNA carrier." Journal of Controlled Release 69 (2000) : 97-108.

Patil, S. D., D. G. Rhodes, and D. J. Burgess. "Anionic liposomal delivery system for DNA transfection." AAPS PharmSciTech. 6 (2004) : 1–10.

Philipp, Alexander, and Ernst Wagner. "Receptor-targeted polyoplexes for DNA and siRNA delivery." In Gene and Cell Therapy, 341-355. Edited by N. S. Templeton. New York : CRC Press 2009.

Pouton, C.W., and L. W. Seymour. "Key issues in non-viral gene delivery." Advanced Drug Delivery Reviews 46 (2001) : 187-203.

Pouton, C.W. et al. "Polycation-DNA complexes for gene delivery: a comparison of the biopharmaceutical properties of cationic polypeptides and cationic lipids." Journal of Controlled Release 53 (1998) : 289-299.

Pedroso de Lima, M. C. et al. "Cationic lipid-DNA complexes in gene delivery: from biophysics to biological applications." Advanced Drug Delivery Reviews 47 (2001) : 277-294.

Ramsay, E. et al. "Examination of the biophysical interaction between plasmid DNA and the polycations, polylysine and polyornithine, as a basis for their differential gene transfection in-vitro." International Journal of Pharmaceutics 210 (2000) : 97-107.

Rea, J. C. et al. "Self-assembling peptide-lipoplexes for substrate-mediated gene delivery." Acta Biometerialia 5 (2009) : 903-912.

Rinaudo, M. "Chitin and chitosan: Properties and applications." Progress in Polymer Science 31 (2006) : 603-632.

Saccardo, P., A. Villaverde, and N. Gonzalex-Montalban. "Peptide-mediated DNA condensation for non-viral gene therapy." Biotechnology Advances 27 (2009) : 432-438.

Sato, T., T. Ishii, and Y. Okahata. "*In vitro* gene delivery mediated by chitosan. Effect of pH, serum and molecular mass of chitosan on the transfection efficiency." Biomaterials 22 (2001) : 2075-2080.

Solomons, T.M. Graham, and Graig B. Fryhle. "Amino Acids and Protein." In Organic Chemistry 8th Edition, 1170. John Wiley & Sons, Inc., 2004.

Son, K. K., D. Tkach, and D. H. Patel, "Zeta potential of transfection complexes formed in serum-free medium can predict in vitro gene transfer efficiency of transfection reagent." Biochimica et Biophysica Acta 1468 (2000) : 11-14.

Srinivasan, C., and D. J. Burgess. "Optimization and characterization of anionic lipoplexes for gene delivery." Journal of Controlled Release 136 (2009) : 62-70.

Sullivan, Sean M. "Introduction to gene therapy and guidelines to pharmaceutical development." In Pharmaceutical Gene Delivery Systems, Volume 131. 1-2. Edited by A. Rolland, and S. Sullivan. USA: Marcel Dekker, 2003.

Sun, X., H. W. Zhang, and Z. R. Zhang. "Transfection efficiency of pORF lacZ plasmid lipopolyplex to hepatocytes and hepatoma cells." World Journal Gastroenterol. 10 (2004) : 531-534.

Svintradze, D. V., and G. M. Mrevlishvili. "Fiber molecular model of atelocollagen-small interfering RNA (siRNA) complex." International Journal of Biological Macromolecules 37 (2005) : 283-286.

Thanou, M. et al. "Quaternized chitosan oligomers as novel gene delivery vectors in epithelial cell lines." Biomaterials 23 (2002) : 153-159.

Thomas, M. and A. M. Klibanov. "Non-viral gene therapy: polycation-mediated DNA delivery." Applied Microbiology and Biotechnology 62 (2003) : 27-34.

Tjelle, T. E. et al. "A novel electroporation device for gene delivery in large animals and humans." Vaccine 24 (2006) : 4667-4670.

Tokunaga, M., N. Hazemoto, and T. Yotsuyanage. “Effect of oligopeptides on gene expression: comparison of DNA/peptide and DNA/peptide/liposome complex.” International Journal of Pharmaceutics 269 (2004) : 71-80.

Torchilin, V. P. “Structure and design of polymeric surfactant-based drug delivery system.” Journal of Controlled Release 73 (2001) : 137-172.

Torchilin, V. P., and V. Weissig. Liposomes : A Practical Approach (New York: Oxford University Press 2003) : 299.

Uchegbu et al. “Polymers and dendrimers for gene delivery in gene therapy.” In Gene and cell therapy, 321-340. Edited by N. S. Templeton. America: CRC Press LLC 2009.

Wagner, E. “Effects of membrane-active agents in gene delivery.” Journal of Controlled Release 53 (1998) : 155-158.

Wang, J. et al. “Evaluation of collagen and methylated collagen as gene carriers.” International Journal of Pharmaceutics 279 (2004) : 115-126.

Weecharangsan, W. et al. “Evaluation of chitosan salts as non-viral gene vector in CHO-K1 cells.” International Journal of Pharmaceutics 348 (2008) : 161-168.

Wightman, Lionel, Ralf Kircheis, and Ernst Wagner. “Polymer-based gene delivery systems.” In Pharmaceutical gene delivery systems, 109-136. Edited by A. Rolland, and S. M. Sullivan. New York : Marcel Dekker, Inc., 2003.

Williams, R. pK_a Values [Online]. Accessed 19 May 2010. Available from http://research.chem.psu.edu/brpgroup/pKa_compilation.pdf

Wong, K. et al. “PEI-g-chitosan, a novel gene delivery system with transfection efficiency comparable to polyethyenimine *in vitro* and after liver administration *in vivo*.” Biocnojugate Chem 17 (2006) : 152-158.

Wong, S.Y., J. M. Pelet, and D. Putnam. “Polymer system for gene delivery-Past, present and future.” Progress in Polymer Science 32 (2007) : 799-837.

Wunderbaldinger, P., A. B. Jr, and R. Weissleder. “New approaches for imaging in gene therapy.” European Journal of Radiology 34 (2000) : 156-165.

Yagi, N. et al. “Synthesis and evaluation of a novel lipid-peptide conjugate for functionalized liposome.” Bioorganic & Medicinal Chemistry Letters 17 (2007) : 2590-2593.

Yamazaki, Y. et al. "Polycation liposomes, a novel nonviral gene transfer system, constructed from cetylated polyethylenimine." Gene Therapy 7 (2000) : 1148-1155.

Zabner, J. et al. "Cellular and molecular barriers to gene transfer by a cationic liposome." Journal Biol. Chem. 270 (1995) : 18997-19007.

Zauner, W. et al. "In vitro uptake of polystyrene microspheres: effect of particle size, cell line and cell density." Journal of Controlled Release 71 (2001) : 39-51.

Zhang, S. et al. "Cationic compounds used in lipoplexes and polyplexes for gene delivery." Journal of Controlled Release 100 (2004) : 165-180.

Zhao, X. et al. "Transfection of primary chondrocytes using chitosan-pEGFP nanoparticles." Journal of Controlled Release 112 (2006) : 223-228.

Zhou, X. and L. Huang. "DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action." Biochimica et Biophysica Acta 1189 (1994) : 195-203.

APPENDIX

APPENDIX A

Calculation of liposomes/DNA ratio (weight ratio)

Calculation of anionic liposomes (EPC:NaO = 10:2, molar ratio) : 1mg/mL polypeptide = 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 2 mM NaO

pipette 20 mM of NaO 600 μL = 20 mmole/1000 mL × 0.6 mL

= 0.012 mmole

= 12 μmole (Add 6 mL of Tris buffer)

∴ concentration of NaO in Tris buffer = 12 μmole/6 mL = 2 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 600 μL;

20 mmole/1000 mL × 0.6 mL = 0.012 mmole

= 0.012×10^{-3} mole × 304.4 g/mole

= 3.6528 mg

EPC + NaO (total lipid) = 50.0328 mg/6 mL

= 8.3388 mg/mL (approx. 8 mg/mL)

Because the ratio of $\frac{4mg/0.5mL(Lipid)}{4mg/4mL(Peptide)}$ = 1:1 (w/w) or 0.5:4 (v/v)

∵ Totol volume of lipid + polypeptide = 4.5 mL

(but, total volume of formulation is 5 mL)

∴ Add 0.5 mL of Tris buffer pH 7.1

Concentration of polypeptide (cationic polymer) = 4 mg/5 mL

= 0.8 mg/mL (or 0.8 μg/μL)

Pipette suspension of polypeptide-coated liposomes 1.25, 2.5, 3.125, 3.75 and 5 μL, which has the content of polymer = 1, 2, 2.5, 3 and 4 μg, respectively and form complexes with DNA 1 μg

∴ Polypeptide/DNA ratio (weight ratio) = 1, 2, 2.5, 3 and 4 μg respectively

APPENDIX B

The particle size and zeta-potential of carriers/DNA complexes

Table 21 The particle size and zeta-potential of the PLL MW 30-70 kDa /DNA complexes at pH 7.4

Weight ratio	Size (nm)	AVG	SD	Zeta-potential (mV)	AVG	SD
2	317 383 312	337.300	1.266	20.7 19.1 21.6	20.467	39.212
3	353 384 396	377.567	0.981	28.4 26.7 26.7	27.267	22.216
4	380 378 334	364.067	0.493	27.3 27.2 28.1	27.533	25.705
5	238 262 273	257.633	0.666	24.6 25.0 25.9	25.167	18.118
6	375 392 341	369.400	2.261	33.5 37.6 37.2	36.100	26.055

Table 22 The particle size and zeta-potential of the PLA MW 5-15 kDa /DNA complexes at pH 7.4

Weight ratio	Size (nm)	AVG	SD	Zeta-potential (mV)	AVG	SD
1	551 579 602	577.633	25.586	20.1 18.9 21.3	20.100	1.200
1.5	371 361 327	353.067	23.344	21.1 21.8 22.0	21.633	0.473
2	291 330 290	303.433	22.670	25.5 24.8 25.6	25.300	0.436
2.5	227 237 236	232.867	5.532	32.6 32.2 32.2	32.333	0.231
3	352 339 313	334.767	19.629	29.9 30.6 30.2	30.233	0.351

Table 23 The particle size and zeta-potential of the PLA MW 15-70 kDa /DNA complexes at pH 7.4

Weight ratio	Size (nm)	AVG	SD	Zeta-potential (mV)	AVG	SD
1	273 274 254	266.700	11.459	27.7 28.6 25.2	27.167	1.762
1.5	346 322 414	360.967	47.721	25.8 23.5 23.6	24.300	1.300
2	328 355 457	379.967	67.966	28.1 30.0 27.1	28.400	1.473
2.5	373 381 330	361.367	27.729	28.0 28.8 27.2	28.000	0.800
3	597 475 361	477.433	118.017	35.6 30.8 27.4	31.267	4.120

Table 24 The particle size and zeta-potential of the PLA MW >70 kDa /DNA complexes at pH 7.4

Weight ratio	size (nm)	AVG	SD	Zeta-potential (mV)	AVG	SD
1	752 845 768	788.233	49.849	25.6 26.8 26.6	26.333	0.643
1.5	256 286 261	267.533	15.954	22.0 21.0 20.7	21.233	0.681
2	323 311 318	317.567	5.934	19.8 20.5 20.3	20.200	0.361
2.5	299 269 361	309.733	46.700	21.1 17.5 20.6	19.733	1.950
3	330 345 318	331.000	13.727	30.4 27.7 22.6	26.900	3.961

Table 25 The particle size and zeta-potential of the PLO MW 30-70 kDa /DNA complexes at pH 7.4

Weight ratio	Size (nm)	AVG	SD	Zeta-potential (mV)	AVG	SD
1	1096 1199 1046	1113.667	78.015	23.9 21.1 23.0	22.667	1.429
2	346 329 346	340.400	9.787	24.6 23.3 23.6	23.833	0.681
3	528 576 560	554.800	24.157	19.6 18.5 21.2	19.767	1.358
4	165 127 112	134.967	27.444	42.4 35.7 39.3	39.133	3.353

Table 26 The particle size and zeta-potential of the PLA MW >70 kDa-coated liposome /DNA complexes at pH 7.4

Weight ratio	Size (nm)	AVG	SD	Zeta-potential (mV)	AVG	SD
1	1214 866 980	1019.833	177.600	32.3 35.3 32.6	33.400	1.652
2	653 667 681	666.967	14.302	29.8 27.2 28.6	28.533	1.301
3	464 518 477	486.667	28.217	35.0 34.8 36.2	35.333	0.757
4	551 683 694	642.433	79.780	41.8 48.4 48.3	46.167	3.782
6	472 497 496	488.333	14.324	38.8 41.8 40.4	40.333	1.501
8	842 868 740	816.633	67.391	41.6 42.3 41.5	41.800	0.436

Table 27 The particle size and zeta-potential of the PLL MW 30-70 kDa /DNA complexes at pH 6.4

Weight ratio	Size (nm)	AVG	SD	Zeta-potential (mV)	AVG	SD
2	619 527 479	541.600	71.229	58.4 68.2 68.0	64.867	5.601
3	428 499 509	478.767	44.358	59.0 58.3 55.6	57.633	1.795
4	429 361 419	402.867	36.800	43.2 46.4 41.4	43.667	2.532
5	504 522 566	530.400	32.077	57.9 67.3 53.2	59.467	7.179
6	368 338 382	362.833	22.466	53.1 61.2 47.4	53.900	6.935

Table 28 The particle size and zeta-potential of the PLA MW 15-70 kDa /DNA complexes at pH 6.4

Weight ratio	Size (nm)	AVG	SD	Zeta-potential (mV)	AVG	SD
1	322 270 256	282.667	34.491	31.3 35.7 38.1	35.033	3.449
1.5	279 289 291	286.467	6.529	27.5 27.4 27.4	27.433	0.058
2	252 265 240	252.667	12.402	36.7 39.2 39.2	38.367	1.443
2.5	365 333 331	342.833	18.802	38.8 40.1 40.5	39.800	0.889
3	403 310 308	340.300	54.317	36.5 40.3 41.1	39.300	2.458

Table 29 The particle size and zeta-potential of the PLO MW 30-70 kDa /DNA complexes at pH 6.4

Weight ratio	Size (nm)	AVG	SD	Zeta-potential (mV)	AVG	SD
1	466 517 441	474.767	38.705	57.3 60.3 35.2	50.933	13.708
2	505 520 478	500.900	20.889	51.9 54.1 53.1	53.033	1.102
3	296 299 255	282.967	24.705	23.9 21.4 25.5	23.600	2.066
4	793 866 600	752.800	137.622	52.5 59.9 64.4	58.933	6.009

Table 30 The particle size and zeta-potential of the CSHCl/DNA/PLA MW >70 kDa complexes at pH 6.4

Weight ratio	Size (nm)	AVG	SD	Zeta-potential (mV)	AVG	SD
0	395 405 395	398.200	5.976	38.0 40.5 39.4	39.300	1.253
1	388 388 356	377.033	18.389	38.2 40.2 40.3	39.567	1.185
2	234 240 240	237.667	3.350	39.7 39.7 37.5	38.967	1.270
3	267 262 280	269.467	9.145	38.3 40.2 39.1	39.200	0.954
4	381 407 441	409.500	29.785	37.1 31.7 38.0	35.600	3.407

Table 31 The particle size and zeta-potential of the PLA MW >70 kDa/ DNA/CSHCl complexes at pH 6.4

Weight ratio	Size (nm)	AVG	SD	Zeta-potential (mV)	AVG	SD
0	395 405 395	398.200	5.976	38.0 40.5 39.4	39.300	1.253
1	238 230 221	229.467	8.252	35.2 33.7 30.7	33.200	2.291
2	243 255 247	248.300	6.219	34.2 30.6 36.3	33.700	2.883
3	177 171 177	175.167	3.444	23.5 22.7 25.2	23.800	1.277
4	254 221 222	231.967	18.914	33.8 33.0 33.8	33.533	0.462

Table 32 The particle size and zeta-potential of the PLA MW >70 kDa/ CSHCl/DNA complexes at pH 6.4

Weight ratio	Size (nm)	AVG	SD	Zeta-potential (mV)	AVG	SD
0	395 405 395	398.200	5.976	38.0 40.5 39.4	39.300	1.253
2	318 378 364	353.167	31.533	32.9 35.4 36.0	34.767	1.644
3	269 262 255	261.900	7.251	29.2 32.2 30.3	30.567	1.518

Table 33 The particle size and zeta-potential of the PLA MW >70 kDa-coated liposomes/DNA complexes at pH 6.4

Weight ratio	Size (nm)	AVG	SD	Zeta-potential (mV)	AVG	SD
1	1263 1448 1699	1470.000	218.831	38.1 36.9 39.6	38.200	1.353
2	643 662 621	641.900	20.491	31.4 31.9 30.8	31.367	0.551
3	536 510 552	532.700	21.564	33.7 36.0 36.9	35.533	1.650
4	313 260 330	300.933	36.141	41.1 46.0 46.4	44.500	2.951
6	311 299 299	303.033	6.900	40.6 43.2 41.6	41.800	1.311
8	187 173 191	183.700	9.829	44.3 43.2 42.8	43.433	0.777

APPENDIX C

Relative fluorescent intensity of CSHCl-DNA-PLA MW > 70 kDa with various the order of mixing

Table 34 Relative fluorescent intensity of the CSHCl/DNA complexes at various weight of CSHCl at pH 6.4

Complex	Weight ratio						
	0	0.1	1	4	5	10	50
CSHCl/DNA	100.000	96.232	85.357	57.880	46.228	31.822	28.313
	100.000	96.017	84.442	56.155	38.867	31.390	28.781
	100.000	94.149	81.572	55.872	41.164	31.064	28.386
AVG	100.000	95.466	83.791	56.636	42.086	31.425	28.493
SD	0.000	1.146	1.975	1.087	3.766	0.380	0.252

Table 35 Relative fluorescent intensity of the PLA MW>70 kDa/DNA complexes at various weight of PLA at pH 6.4

Complex	Weight ratio						
	0	0.1	1	4	5	10	50
PLA MW > 70 kDa/DNA	100.000	96.277	82.346	68.134	33.828	20.342	19.868
	100.000	92.621	76.015	59.124	27.720	20.471	20.054
	100.000	93.307	77.833	57.737	26.739	19.974	19.624
AVG	100.000	94.069	78.731	61.665	29.429	20.262	19.849
SD	0.000	1.943	3.259	5.645	3.841	0.258	0.216

Table 36 Relative fluorescent intensity of the CSHCl (4 µg)/DNA (1 µg)/PLA MW >70 kDa complexes at various weight of PLA at pH 6.4

Complex	PLA (µg)						
	0	0.1	1	4	5	10	50
CSHCl/DNA/PLA MW > 70 kDa	100.000	59.884	47.044	36.297	24.428	19.708	19.065
	100.000	57.855	41.133	30.858	22.685	19.393	18.889
	100.000	59.883	43.119	32.171	23.450	19.915	19.446
AVG	100.000	59.207	43.765	33.109	23.521	19.672	19.133
SD	0.000	1.171	3.008	2.838	0.874	0.263	0.284

Table 37 Relative fluorescent intensity of the PLA MW >70 kDa /DNA (1 µg)/CSHCl (4 µg) complexes at various weight of PLA at pH 6.4

Complex	PLA (µg)						
	0	0.1	1	4	5	10	50
PLA MW > 70 kDa/DNA/CSHCl	100.000	60.431	54.742	45.481	28.358	20.771	19.554
	100.000	59.921	52.033	44.972	28.009	20.697	20.211
	100.000	60.271	46.274	47.471	28.436	20.803	19.849
AVG	100.000	60.207	51.016	45.975	28.268	20.757	19.871
SD	0.000	0.261	4.324	1.321	0.227	0.054	0.329

APPENDIX D

In vitro transfection efficiency

Preliminary study

1. Effect of polypeptides

1.1 HeLa cells

Table 38 Transfection efficiency of PLL MW 30-70 kDa medium without serum at 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	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PEI	1020	536.842	1203	633.158	1377	724.737	727.368	95.553
0.1	0	0.000	67	35.263	75	39.474	38.070	2.431
0.5	3	1.579	331	174.210	235	123.684	190.000	75.460
1	26	13.684	333	175.263	357	187.895	215.790	59.590
2	80	42.105	710	373.684	452	237.895	283.333	78.247
4	368	193.684	652	343.158	530	278.947	346.491	69.271
6	33	17.368	180	94.737	150	78.947	94.737	15.789

Table 39 Transfection efficiency of PLA MW 15-70 kDa medium without serum at 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	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PEI	2776	1461.053	2985	1571.053	2629	1383.684	1471.930	94.157
1.5	115	60.526	144	75.789	130	68.421	68.246	7.633
2	1314	691.579	1122	590.526	1218	641.053	641.053	50.526
2.5	1172	616.842	1276	671.579	1224	644.211	644.211	27.368
3	1196	629.474	1026	540.000	1111	584.737	584.737	44.737
4	504	265.263	360	189.474	432	227.368	227.368	37.895

Table 40 Transfection efficiency of PLO MW 30-70 kDa medium without serum at 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	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PEI	2881	1516.316	2962	1558.947	2923	724.737	1537.895	21.321
0.1	1	0.526	0	0.000	1	0.526	0.351	0.304
0.5	4	2.105	7	3.684	6	3.158	2.982	0.804
1	10	5.263	15	7.895	12	6.316	6.491	1.325
2	1	0.526	3	1.579	2	1.053	1.053	0.526
4	0	0.000	0	0.000	0	0.000	0.000	0.000
6	0	0.000	0	0.000	0	0.000	0.000	0.000

1.2 Huh7 cells

Table 41 Transfection efficiency of PLL MW 30-70 kDa medium without serum at 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	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PEI	1203	633.158	1141	600.526	1172	616.842	616.842	16.316
0.1	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	0	0.000	0	0.000	0	0.000	0.000	0.000
1	16	8.421	18	9.474	17	8.947	8.947	0.526
2	166	87.368	184	96.842	175	92.105	92.105	4.737
4	53	27.895	30	15.789	42	22.105	21.930	6.055
6	0	0.000	0	0.000	0	0.000	0.000	0.000

Table 42 Transfection efficiency of PLA MW 15-70 kDa medium without serum at 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	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PEI	375	197.368	358	188.421	381	200.526	195.439	6.279
0.1	114	60.000	91	47.895	128	67.368	58.421	9.832
0.5	240	126.316	252	132.632	272	143.158	134.035	8.508
1	198	104.211	148	77.895	142	74.737	85.614	16.182
2	60	31.579	68	35.789	78	41.053	36.140	4.747
4	22	11.579	22	11.579	14	7.368	10.175	2.431
6	3	1.579	2	1.053	5	2.632	1.754	0.804

Table 43 Transfection efficiency of PLO MW 30-70 kDa medium without serum at 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	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PEI	1150	605.263	1040	547.368	1132	595.789	582.807	31.054
1	9	4.737	12	6.316	10	5.263	5.439	0.804
2	12	6.316	16	8.421	21	11.053	8.596	2.373
3	8	4.211	9	4.737	6	3.158	4.035	0.804
4	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

2. Effect of pH

1.1 HeLa cells

Table 44 Transfection efficiency of PLL MW 30-70 kDa medium without serum at pH 6.4

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	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PEI	1020	536.842	987	519.474	1005	528.947	528.421	8.696
0.1	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	3	1.579	0	0.000	2	1.053	0.877	0.804
1	26	13.684	11	5.789	18	9.474	9.649	3.950
2	80	42.105	85	44.737	83	43.684	43.509	1.325
4	368	193.684	382	201.053	375	197.368	197.368	3.684
6	33	17.368	90	47.368	61	32.105	32.281	15.001

Table 45 Transfection efficiency of PLA MW 15-70 kDa medium without serum at pH 6.4

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	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PEI	2688	1414.737	2823	1485.789	2763	1454.211	1451.579	35.599
1.5	569	299.474	558	293.684	562	295.789	296.316	2.930
2	1223	643.684	1229	646.842	1220	642.105	644.211	2.412
2.5	675	355.263	766	403.158	752	395.789	384.737	25.789
3	153	80.526	136	71.579	140	73.684	75.263	4.678
4	32	16.842	25	13.158	27	14.211	14.737	1.898

1.2 Huh7 cells

Table 46 Transfection efficiency of PLL MW 30-70 kDa medium without serum at pH 6.4

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	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PEI	455	239.474	512	269.474	485	255.263	254.737	15.007
0.1	0	0.000	0	0.000	0	0.000	0.000	0.000
0.5	0	0.000	0	0.000	0	0.000	0.000	0.000
1	4	2.105	2	1.053	3	1.579	1.579	0.526
2	59	31.053	71	37.368	64	33.684	34.035	3.172
4	9	4.737	4	2.105	5	2.632	3.158	1.393
6	0	0.000	0	0.000	0	0.000	0.000	0.000

***In vitro* transfection study**

1. Effect of molecular weight of PLA

Table 47 Transfection efficiency of PLA MW 5-15 kDa medium without serum in HeLa cells at pH 6.4

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	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PEI	6000	3157.895	5200	2736.842	6000	3157.895	3017.544	243.095
1	0	0.000	0	0.000	0	0.000	0.000	0.000
2	0	0.000	0	0.000	0	0.000	0.000	0.000
2.5	8	4.211	18	9.474	18	9.474	7.719	3.039
3	38	20.000	14	7.368	50	26.316	17.895	9.648
4	14	7.368	10	5.263	20	10.526	7.719	2.649
6	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 48 Transfection efficiency of PLA MW 15-70 kDa medium without serum in HeLa cells at pH 6.4

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	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PEI	6000	3157.895	5200	2736.842	6000	3157.895	3017.544	243.095
1	252	132.632	270	142.105	266	140.000	138.246	4.975
2	2152	1132.632	2292	1206.316	2216	1166.316	1168.421	36.887
2.5	1634	860.000	1470	773.684	1878	988.421	874.035	108.054
3	342	180.000	216	113.684	200	105.263	132.982	40.936
4	128	67.368	148	77.895	148	77.895	74.386	6.077
6	4	2.105	4	2.105	12	6.316	3.509	2.431
8	0	0.000	0	0.000	0	0.000	0.000	0.000

Table 49 Transfection efficiency of PLA MW >70 kDa medium without serum in HeLa cells at pH 6.4

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	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PEI	6000	3157.895	5200	2736.842	6000	3157.895	3017.544	243.095
1	62	32.632	140	73.684	78	41.053	49.123	21.684
2	1932	1016.842	2874	1512.632	2266	1192.632	1240.702	251.366
2.5	3206	1687.368	3982	2095.789	3476	1829.474	1870.877	207.335
3	1912	1006.316	2106	1108.421	1964	1033.684	1049.474	52.852
4	666	350.526	530	278.947	894	470.526	366.667	96.804
6	94	49.474	238	125.263	164	86.316	87.018	37.900
8	28	14.737	30	15.789	11	5.789	12.105	5.495

2. Effect of CSHCl

2.1 Effect of order of mixing

Table 50 Transfection efficiency of CSHCl (4 µg)/DNA (1 µg)/PLA MW >70 kDa (various weight) medium without serum in HeLa cells at pH 6.4

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	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PEI	3200	1684.211	3000	1578.947	2900	1526.316	1596.491	80.396
0	5	2.632	8	4.211	5	2.632	3.158	0.912
1	1	0.526	2	1.053	5	2.632	1.404	1.096
2	396	208.421	288	151.579	282	148.421	169.474	33.766
3	432	227.368	424	223.158	466	245.263	231.930	11.737
4	132	69.474	130	68.421	84	44.211	60.702	14.292

Table 51 Transfection efficiency of PLA MW >70 kDa (various weight)/DNA (1 µg) /CSHCl (4 µg) medium without serum in HeLa cells at pH 6.4

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	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PEI	3200	1684.211	3000	1578.947	2900	1526.316	1596.491	80.396
0	5	2.632	8	4.211	5	2.632	3.158	0.912
1	156	82.105	184	96.842	180	94.737	91.228	7.970
2	2390	1257.895	2652	1395.789	2980	1568.421	1407.368	155.587
3	2274	1196.842	2292	1206.316	2690	1415.789	1272.982	123.765
4	986	518.947	848	446.316	912	480.000	481.754	36.348

Table 52 Transfection efficiency of PLA MW >70 kDa (various weight)/CSHCl (4 µg)/DNA (1 µg) medium without serum in HeLa cells at pH 6.4

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	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PEI	3200	1684.211	3000	1578.947	2900	1526.316	1596.491	80.396
0	5	2.632	8	4.211	5	2.632	3.158	0.912
1	104	54.737	119	62.632	123	64.737	60.702	5.272
2	1844	970.526	1698	893.684	1592	837.895	900.702	66.594
3	538	283.158	472	248.421	704	370.526	300.702	62.915
4	144	75.789	176	92.632	164	86.316	84.912	8.508

2.2 Effect of serum

Table 53 Transfection efficiency of PLA MW >70 kDa (2 µg)/DNA (1 µg)/CSHCl (4 µg) medium with and without serum in HeLa cells at pH 6.4 and 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	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PEI pH 6.4	1150	605.263	1040	547.368	1132	595.789	582.807	31.054
MEM(-) pH 7.4	633	333.158	662	348.421	718	377.895	353.158	22.741
MEM(-) pH 6.4	1229	646.842	1298	683.158	1400	736.842	688.947	45.278
MEM(+) pH 7.4	5	2.632	3	1.579	6	3.158	2.456	0.804
MEM(+) pH 6.4	42	22.105	42	22.105	40	21.053	21.754	0.608

3. Effect of anionic liposomes

3.1 Effect of EPC:NaO at the molar ratio of 10:2

3.1.1 HeLa cells

Table 54 Transfection efficiency of PLA MW >70 kDa-coated liposome in medium without serum at 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
pDNA	0	0.000	0	0.000	0	0.000	0.000	0.000
lipofectamine	2869	1510.000	2931	1542.632	2884	1517.895	1523.509	17.025
PEI	3167	1666.842	2973	1564.737	3005	1581.579	1604.386	54.740
1	73	38.421	111	58.421	73	38.421	45.088	11.547
2	1390	731.579	1380	726.316	1841	968.947	808.947	138.589
3	384	202.105	317	166.842	434	228.421	199.123	30.898
4	150	78.947	122	64.211	118	62.105	68.421	9.177

Table 55 Transfection efficiency of PLA MW >70 kDa-coated liposome in medium without serum at pH 6.4

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
pDNA	0	0.000	0	0.000	0	0.000	0.000	0.000
lipofectamine	2869	1510.000	2931	1542.632	2884	1517.895	1523.509	17.025
PEI	3167	1666.842	2973	1564.737	3005	1581.579	1604.386	54.740
1	52	27.368	47	24.737	59	31.053	27.719	3.172
2	1005	528.947	1242	653.684	1210	636.842	606.491	67.681
3	86	45.263	88	46.316	108	56.842	49.474	6.403
4	17	8.947	5	2.632	18	9.474	7.018	3.807

3.1.2 Huh7 cells

Table 56 Transfection efficiency of PLA MW >70 kDa-coated liposome in medium without serum at 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
pDNA	0	0.000	0	0.000	0	0.000	0.000	0.000
lipofectamine	1718	904.211	1698	893.684	1703	986.316	898.070	5.478
PEI	1224	644.211	1186	624.211	1218	641.053	636.491	10.752
1	52	27.368	39	20.526	45	23.860	23.860	3.424
1.5	488	256.842	338	177.895	398	209.474	214.737	39.736
2	552	290.526	519	273.158	511	268.947	277.544	11.439
3	220	115.789	136	71.579	195	102.632	96.667	22.701
4	41	21.579	27	14.211	37	19.474	18.421	3.795

Table 57 Transfection efficiency of PLA MW >70 kDa-coated liposome in medium without serum at pH 6.4

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
pDNA	0	0.000	0	0.000	0	0.000	0.000	0.000
lipofectamine	1164	612.632	1188	625.263	1204	633.684	623.860	10.596
PEI	840	442.105	839	441.579	852	448.421	444.035	3.807
1	44	23.158	36	18.947	39	20.526	20.877	2.127
1.5	562	295.789	598	314.737	577	303.684	304.737	9.517
2	319	167.895	260	136.842	289	152.105	152.281	15.527
3	71	37.368	81	42.632	76	40.000	40.000	2.632
4	4	2.105	6	3.158	6	3.158	2.807	0.608

3.2 Effect of serum

Table 58 Transfection efficiency of PLA MW >70 kDa-coated liposome in medium with and without serum on HeLa cells at pH 6.4 and 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	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
pDNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Lipofec.* pH 7.4	6702	3527.368	6693	3522.632	6582	3464.211	3504.737	35.177
PEI pH 7.4	9970	5247.368	9772	5143.158	9904	5212.632	5201.053	53.061
MEM(-) pH 7.4	5252	2764.211	3672	1932.632	4712	2480.000	2392.281	422.672
MEM(-) pH 6.4	3940	2073.684	3292	1732.632	3856	2029.474	1945.263	185.466
MEM(+) pH 7.4	52	27.368	36	18.947	36	18.947	21.754	4.862
MEM(+) pH 6.4	28	14.737	30	15.789	36	16.491	16.491	2.191

*lipofectamine

APPENDIX E

Cytotoxicity of carriers and pDNA/carriers complexes

1. Cytotoxicity of carriers on HeLa cells

Table 59 Cytotoxicity of PLL MW 30-70 kDa at pH 7.4

n	weight ratio						
	control	0.001	0.010	0.100	1.000	10.000	100.000
1	99.977	99.422	99.076	98.798	92.212	65.172	15.322
2	98.729	99.214	98.937	98.521	92.489	65.727	14.906
3	101.294	100.393	98.452	97.828	92.073	70.788	14.768
AVG	100.000	99.676	98.821	98.382	92.258	67.229	14.999
SD	1.283	0.629	0.328	0.500	0.212	3.095	0.289

Table 60 Cytotoxicity of PLA MW 5-15 kDa at pH 7.4

n	weight ratio					
	control	0.010	0.100	1.000	10.000	100.000
1	99.569	99.225	104.393	101.292	90.009	20.758
2	101.378	107.494	104.996	97.416	89.836	21.361
3	99.053	111.542	92.679	97.071	90.009	21.447
AVG	100.000	106.087	100.689	98.593	89.951	21.189
SD	1.221	6.278	6.944	2.344	0.099	0.375

Table 61 Cytotoxicity of PLO MW 30-70 kDa at pH 7.4

n	weight ratio						
	control	0.001	0.010	0.100	1.000	10.000	100.000
1	99.656	95.473	97.192	96.103	95.186	91.404	15.702
2	100.172	96.103	96.676	96.160	93.696	93.582	17.192
3	100.172	92.951	92.951	94.269	92.092	96.046	15.186
AVG	100.000	94.842	95.606	95.511	93.658	93.677	16.027
SD	0.298	1.668	2.314	1.076	1.548	2.322	1.042

Table 62 Cytotoxicity of PEI at pH 7.4

n	weight ratio					
	control	0.010	0.100	1.000	10.000	100.000
1	102.331	83.263	77.063	68.438	34.079	13.660
2	96.923	73.240	77.809	71.655	33.800	13.007
3	100.746	74.592	73.007	67.273	35.711	12.587
AVG	100.000	77.032	75.960	69.122	34.530	13.085
SD	2.780	5.439	2.584	2.270	1.032	0.540

Table 63 Cytotoxicity of PLA MW >70 kDa-coated liposomes at pH 7.4

n	weight ratio				
	control	0.100	1.000	10.000	100.000
1	101.352	98.817	98.744	47.090	17.242
2	99.034	98.092	93.745	54.262	16.373
3	99.614	96.933	95.629	46.873	18.256
AVG	100.000	97.947	96.040	49.408	17.291
SD	1.206	0.950	2.525	4.205	0.943

Table 64 Cytotoxicity of PLL MW 30-70 kDa at pH 6.4

n	weight ratio						
	control	0.001	0.010	0.100	1.000	10.000	100.000
1	99.977	100.324	100.740	100.809	97.412	67.252	14.698
2	98.729	103.651	102.958	96.302	98.174	65.172	15.530
3	101.294	98.590	103.028	100.324	97.966	68.569	15.530
AVG	100.000	100.855	102.242	99.145	97.851	66.998	15.253
SD	1.283	2.572	1.301	2.474	0.394	1.713	0.480

Table 65 Cytotoxicity of PLA MW 15-70 kDa at pH 6.4

n	weight ratio					
	control	0.010	0.100	1.000	10.000	100.000
1	101.563	81.749	79.027	73.280	27.943	10.323
2	98.576	76.531	77.666	75.662	26.771	10.058
3	99.861	77.136	76.985	72.977	26.506	9.831
AVG	100.000	78.472	77.893	73.973	27.073	10.071
SD	1.498	2.854	1.040	1.470	0.765	0.246

Table 66 Cytotoxicity of PLO MW 30-70 kDa at pH 6.4

n	weight ratio					
	control	0.010	0.100	1.000	10.000	100.000
1	100.237	87.805	80.062	63.083	17.547	14.634
2	96.188	94.909	80.843	62.799	17.405	14.847
3	103.576	81.056	84.679	60.455	17.192	14.705
AVG	100.000	87.923	81.861	62.112	17.381	14.729
SD	3.700	6.927	2.471	1.443	0.179	0.109

Table 67 Cytotoxicity of PEI at pH 6.4

n	weight ratio					
	control	0.010	0.100	1.000	10.000	100.000
1	102.331	72.075	69.790	66.061	24.848	15.851
2	96.923	66.900	67.179	66.154	26.434	16.037
3	100.746	71.189	69.697	70.816	24.196	15.291
AVG	100.000	70.054	68.889	67.677	25.159	15.726
SD	2.780	2.768	1.481	2.719	1.151	0.388

Table 68 Cytotoxicity of PLA MW >70 kDa-CSHCl at pH 6.4

n	weight ratio					
	control	0.100	1.000	10.000	100.000	1000.000
1	100.237	92.138	87.023	60.952	20.104	14.279
2	96.188	88.942	87.237	62.586	17.831	14.634
3	103.576	91.215	91.144	64.859	20.317	14.279
AVG	100.000	90.765	88.468	62.799	19.417	14.397
SD	3.700	1.645	2.320	1.962	1.378	0.205

Table 69 Cytotoxicity of PLA MW >70 kDa-coated liposomes at pH 6.4

n	weight ratio				
	control	0.100	1.000	10.000	100.000
1	101.352	92.659	86.718	30.935	15.793
2	99.034	89.254	82.444	29.993	16.735
3	99.614	91.717	86.356	31.949	18.546
AVG	100.000	91.210	85.173	30.959	17.025
SD	1.206	1.758	2.370	0.978	1.399

2. Cytotoxicity of carriers on Huh7 cells

Table 70 Cytotoxicity of PLL MW 30-70 kDa at pH 7.4

n	weight ratio						
	control	0.001	0.010	0.100	1.000	10.000	100.000
1	99.455	99.339	91.634	85.564	84.047	66.887	38.054
2	106.109	96.770	95.837	85.214	88.016	65.837	33.152
3	94.436	98.872	85.914	88.249	87.782	68.171	33.268
AVG	100.000	98.327	91.128	86.342	86.615	66.965	34.825
SD	5.856	1.368	4.980	1.660	2.227	1.169	2.798

Table 71 Cytotoxicity of PLA MW 15-70 kDa at pH 7.4

n	weight ratio					
	control	0.010	0.100	1.000	10.000	100.000
1	101.068	85.231	86.121	83.986	76.335	56.050
2	101.068	94.484	89.146	82.918	90.925	58.185
3	97.865	88.790	88.256	75.267	67.260	52.847
AVG	100.000	89.502	87.841	80.724	78.173	55.694
SD	1.849	4.667	1.555	4.756	11.939	2.687

Table 72 Cytotoxicity of PLO MW 30-70 kDa at pH 7.4

n	weight ratio					
	control	0.010	0.100	1.000	10.000	100.000
1	101.068	86.477	76.690	78.114	47.865	45.552
2	101.068	84.164	79.181	70.819	48.221	41.281
3	97.865	82.562	85.053	81.851	47.331	44.840
AVG	100.000	84.401	80.308	76.928	47.805	43.891
SD	1.849	1.968	4.294	5.611	0.448	2.288

Table 73 Cytotoxicity of PLA MW >70 kDa-coated liposomes at pH 7.4

n	weight ratio				
	control	0.100	1.000	10.000	100.000
1	101.420	96.307	95.739	55.398	50.568
2	100.000	94.318	94.318	55.966	50.852
3	98.580	97.443	93.182	51.989	49.716
AVG	100.000	96.023	94.413	54.451	50.379
SD	1.420	1.582	1.281	2.151	0.591

Table 74 Cytotoxicity of PLL MW 30-70 kDa at pH 6.4

n	weight ratio						
	control	0.001	0.010	0.100	1.000	10.000	100.000
1	103.906	73.750	74.219	70.313	69.531	65.313	33.438
2	89.688	69.688	70.156	69.219	66.250	60.156	35.781
3	106.406	69.219	78.125	71.875	66.406	64.688	41.250
AVG	100.000	70.885	74.167	70.469	67.396	63.385	36.823
SD	9.018	2.492	3.985	1.335	1.851	2.814	4.009

Table 75 Cytotoxicity of PLA MW 15-70 kDa at pH 6.4

n	weight ratio					
	control	0.010	0.100	1.000	10.000	100.000
1	104.412	38.912	35.032	32.522	23.849	22.024
2	95.169	39.140	31.609	32.636	23.963	22.594
3	100.418	36.744	37.086	31.837	23.393	22.024
AVG	100.000	38.266	34.576	32.332	23.735	22.214
SD	4.636	1.323	2.767	0.432	0.302	0.329

Table 76 Cytotoxicity of PLO MW 30-70 kDa at pH 6.4

n	weight ratio					
	control	0.010	0.100	1.000	10.000	100.000
1	104.412	33.435	33.777	32.065	22.708	19.741
2	95.169	32.636	35.261	31.267	22.822	21.111
3	100.418	33.891	28.870	30.924	22.822	18.943
AVG	100.000	33.321	32.636	31.419	22.784	19.932
SD	4.636	0.635	3.344	0.586	0.066	1.097

Table 77 Cytotoxicity of PLA MW >70 kDa-coated liposomes at pH 6.4

n	weight ratio				
	control	0.100	1.000	10.000	100.000
1	101.420	96.307	88.068	51.989	48.864
2	100.000	90.341	90.909	52.841	45.739
3	98.580	98.580	97.727	58.807	48.011
AVG	100.000	95.076	92.235	54.545	47.538
SD	1.420	4.255	4.964	3.715	1.615

Calculation of toxicity value of carriers

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

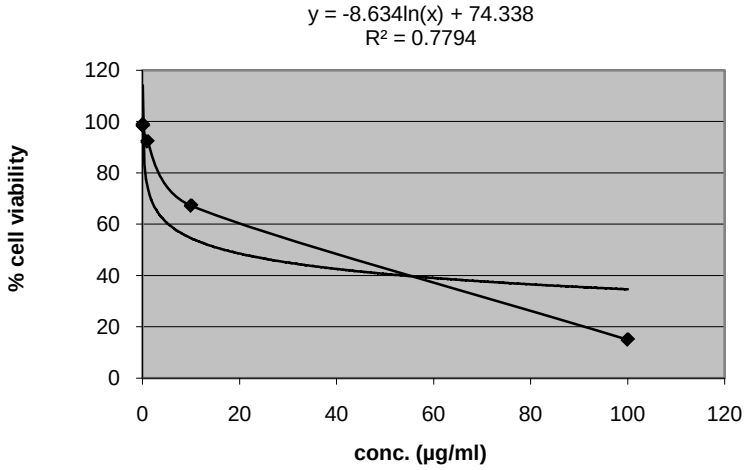
$$y = -8.63\ln(x) + 74.33 \qquad (y = 50)$$

$$\ln(x) = (50 - 74.33)/-8.63$$

$$x = e^{(50 - 74.33)/-8.63}$$

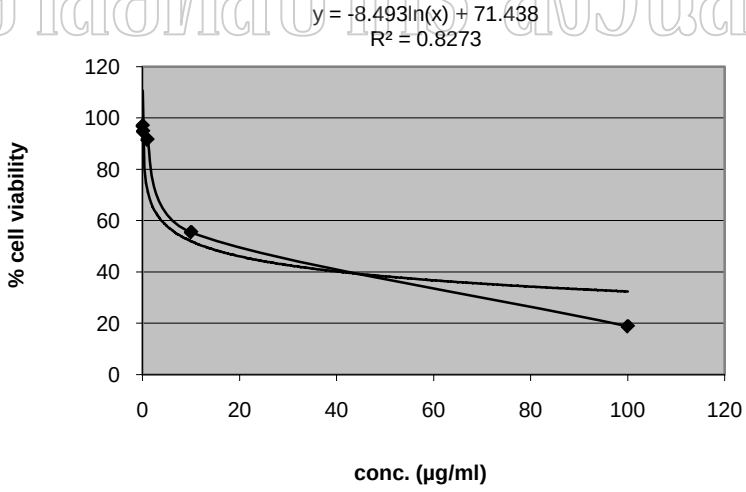
$$x = 16.759\ \mu g/ml$$

$\therefore IC_{50}$ of PLL MW 30-70 kDa = 16.759 µg/ml



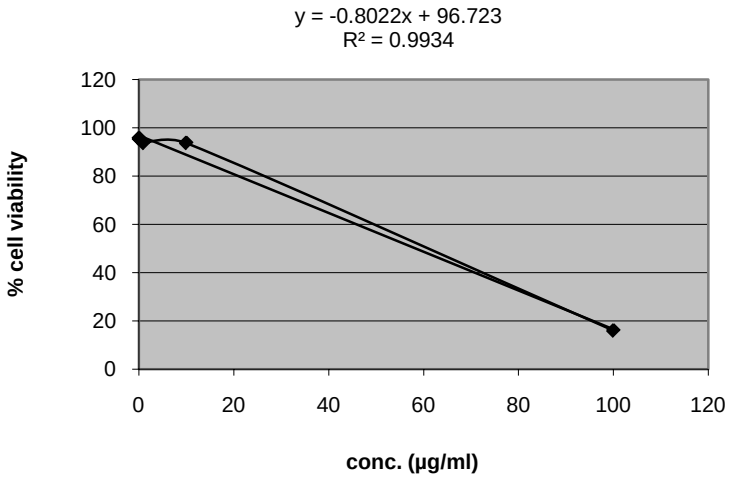
IC_{50} = 16.759 µg/ml

Figure 42 IC_{50} data of PLL MW 30-70 kDa in HeLa cells at pH 7.4



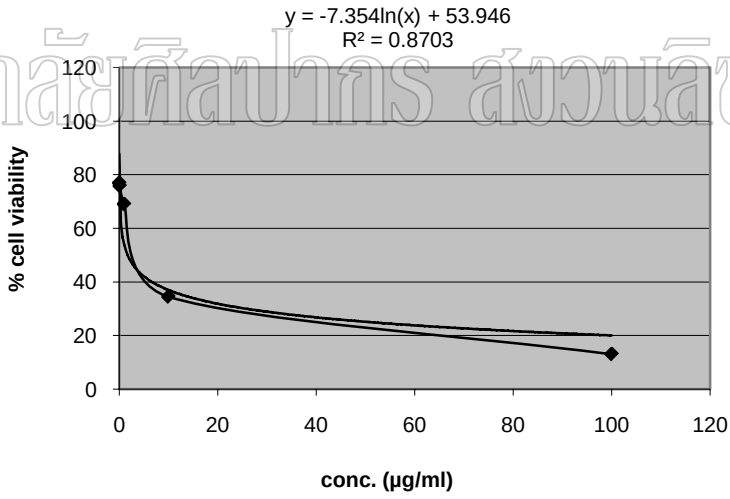
IC_{50} = 12.480 µg/ml

Figure 43 IC_{50} data of PLA MW 15-70 kDa in HeLa cells at pH 7.4



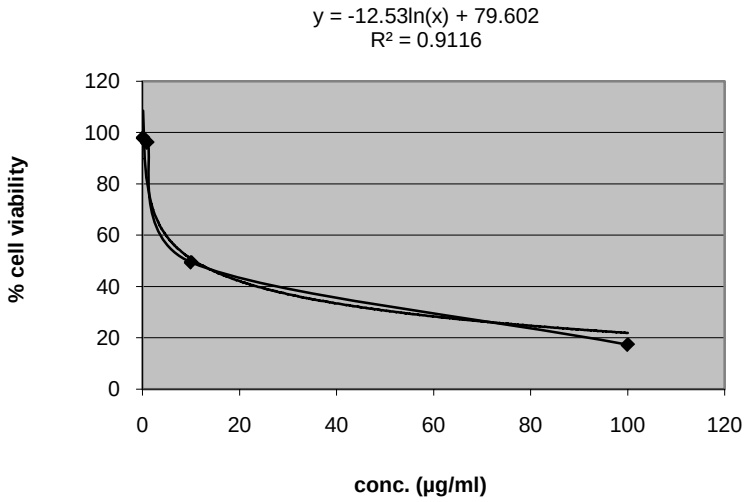
IC_{50} = 58.118 µg/ml

Figure 44 IC_{50} data of PLO MW 30-70 kDa in HeLa cells at pH 7.4



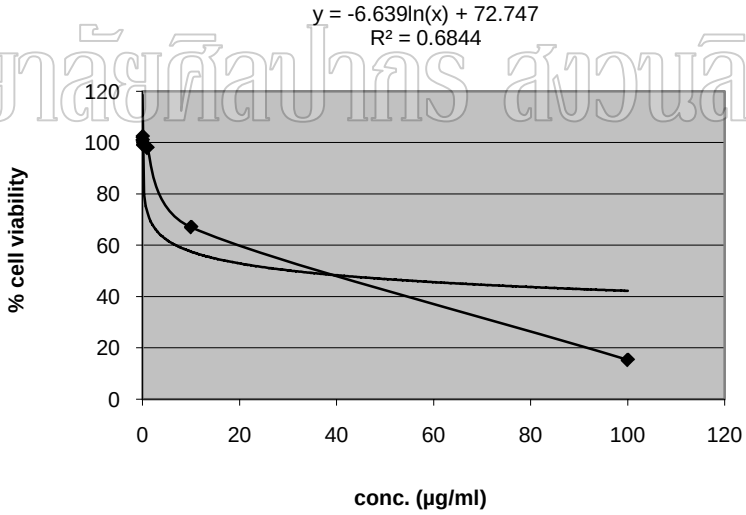
IC50 = 1.709 µg/ml

Figure 45 IC_{50} data of PEI 25 kDa in HeLa cells at pH 7.4



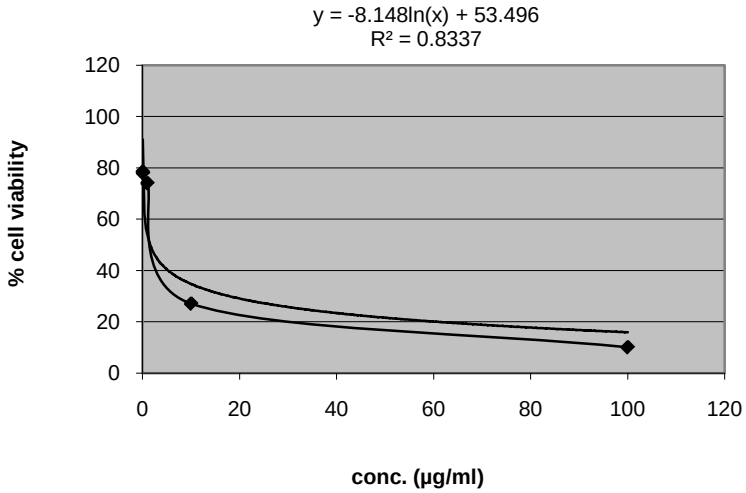
IC_{50} = 10.676 µg/ml

Figure 46 IC_{50} data of PLA MW >70 kDa-coated liposomes in HeLa cells at pH 7.4



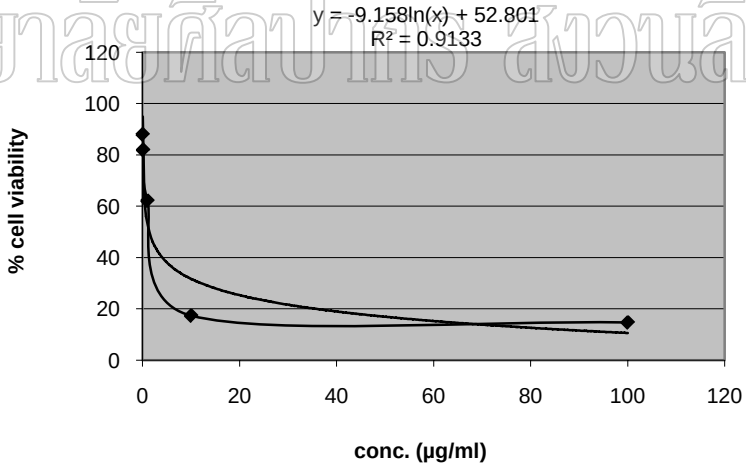
IC_{50} = 30.872 µg/ml

Figure 47 IC_{50} data of PLL MW 30-70 kDa in HeLa cells at pH 6.4



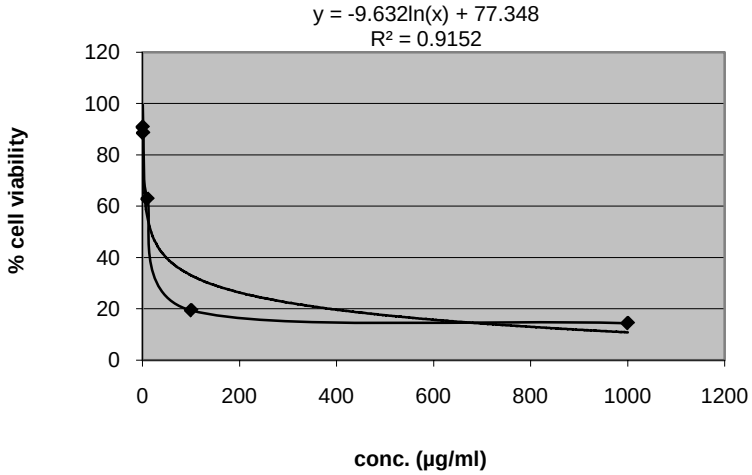
IC_{50} = 1.535 µg/ml

Figure 48 IC_{50} data of PLA MW 15-70 kDa in HeLa cells at pH 6.4



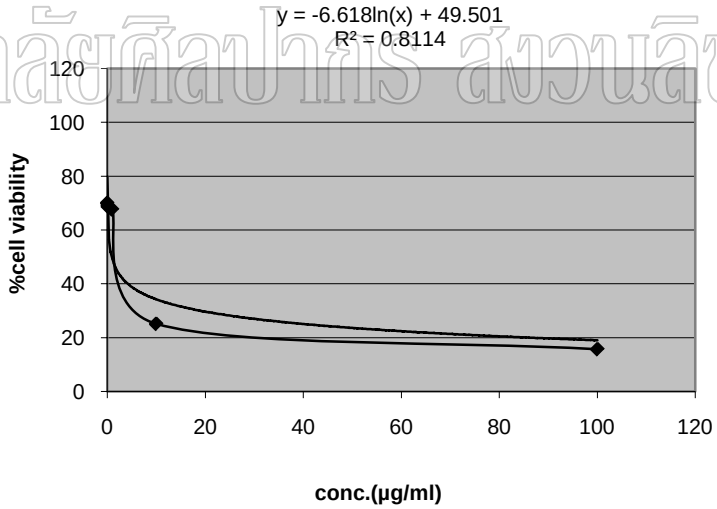
IC_{50} = 1.358 µg/ml

Figure 49 IC_{50} data of PLO MW 30-70 kDa in HeLa cells at pH 6.4



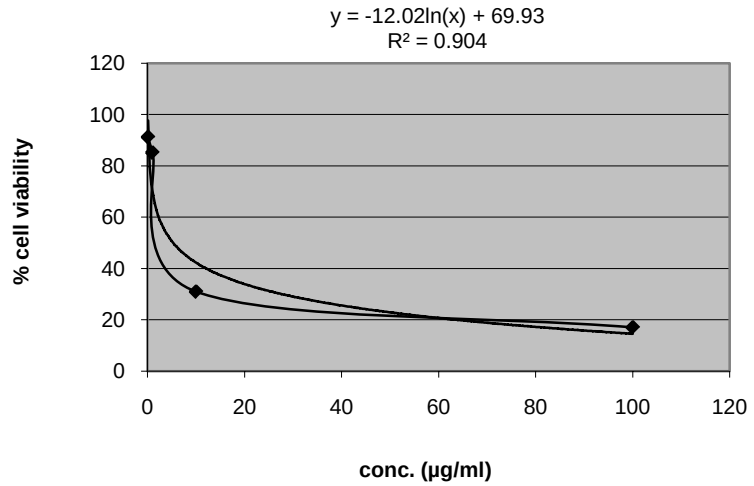
IC_{50} = 17.099 µg/ml

Figure 50 IC_{50} data of PLA MW >70 kDa-CSHCl in HeLa cells at pH 6.4



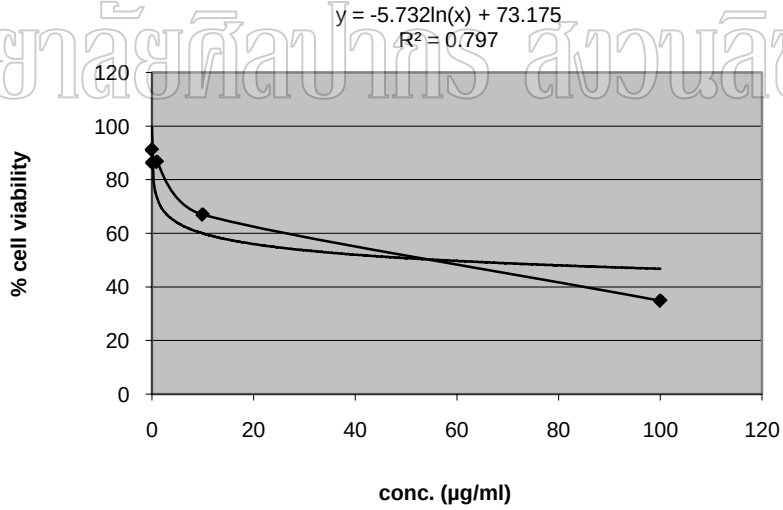
IC50 = 0.927 µg/ml

Figure 51 IC_{50} data of PEI 25 kDa in HeLa cells at pH 6.4



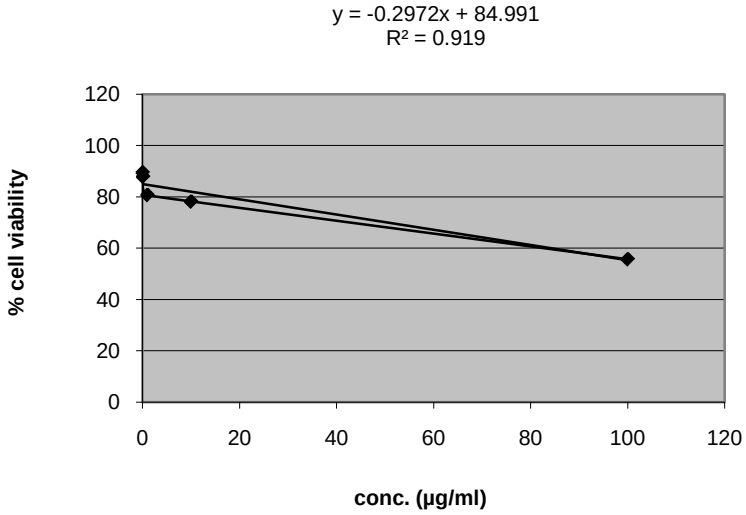
IC_{50} = 5.264 µg/ml

Figure 52 IC_{50} data of PLA MW >70 kDa-coated liposomes in HeLa cells at pH 6.4



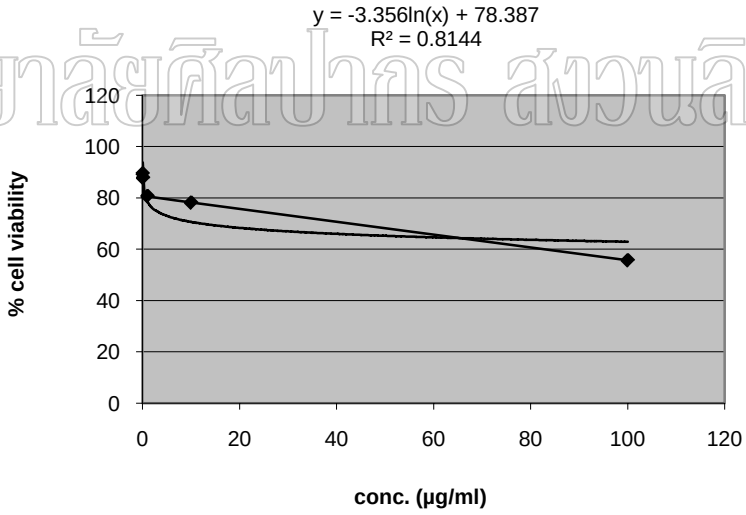
IC_{50} = 57.002 µg/ml

Figure 53 IC_{50} data of PLL MW 30-70 kDa in Huh7 cells at pH 7.4



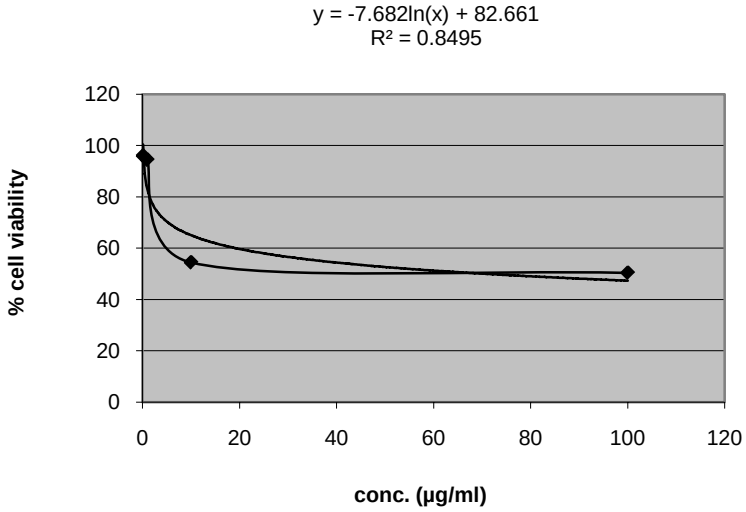
IC_{50} = 117.811 µg/ml

Figure 54 IC_{50} data of PLA MW 15-70 kDa in Huh7 cells at pH 7.4



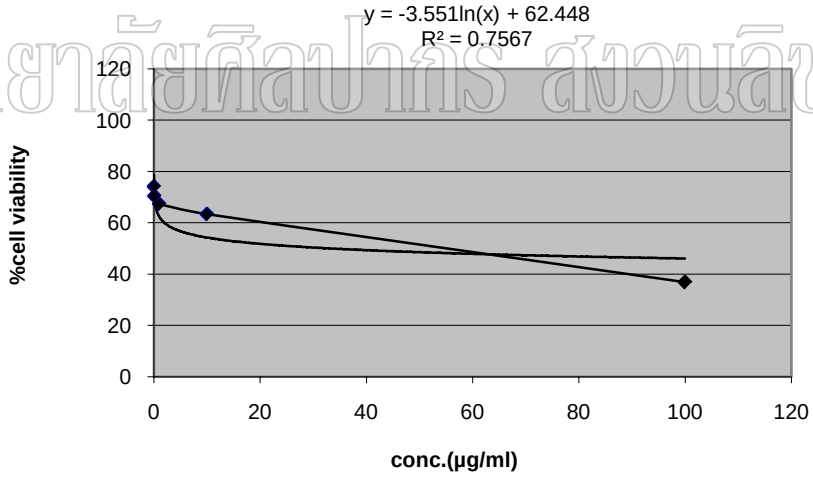
IC_{50} = 29.351 µg/ml

Figure 55 IC_{50} data of PLO MW 30-70 kDa in Huh7 cells at pH 7.4



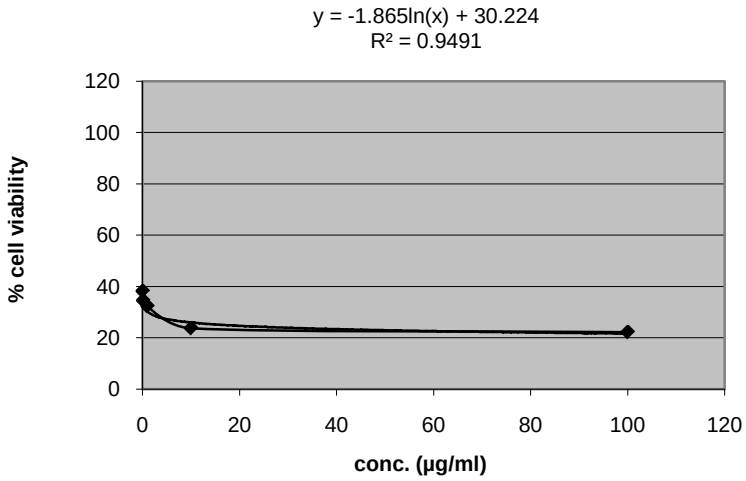
IC_{50} = 70.288 µg/ml

Figure 56 IC_{50} data of PLA MW >70 kDa-coated liposome in Huh7 cells at pH 7.4



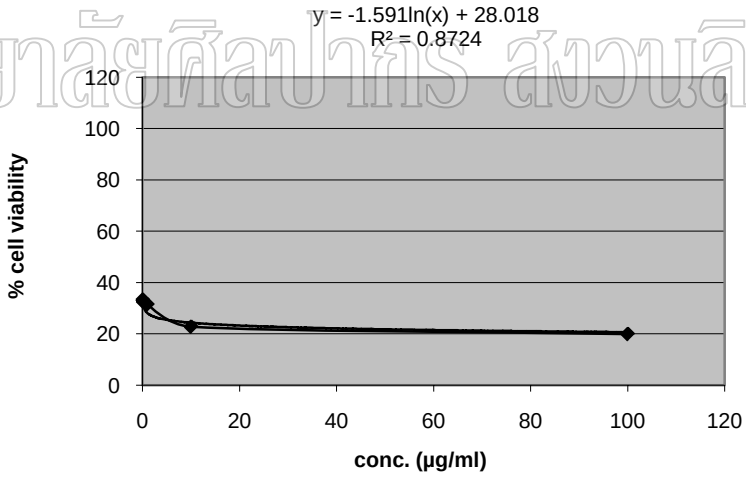
IC_{50} = 33.288 µg/ml

Figure 57 IC_{50} data of PLL MW 30-70 kDa in Huh7 cells at pH 6.4



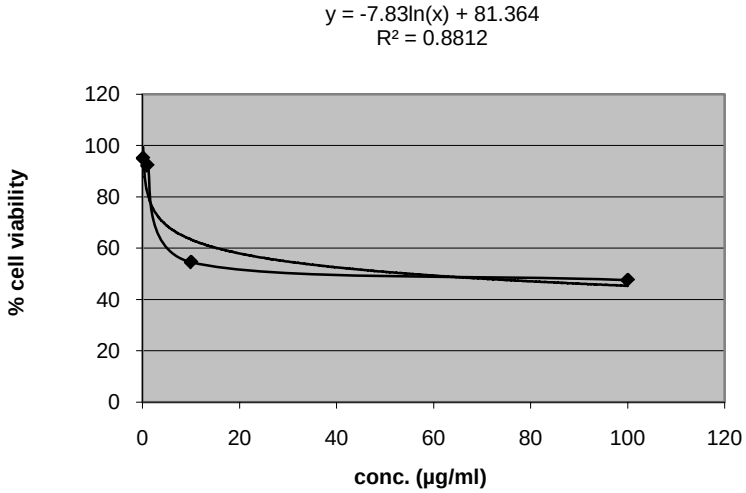
IC_{50} < 0.01 µg/ml

Figure 58 IC_{50} data of PLA MW 15-70 kDa in Huh7 cells at pH 6.4



IC_{50} < 0.01 µg/ml

Figure 59 IC_{50} data of PLO MW 30-70 kDa in Huh7 cells at pH 6.4



IC_{50} = 54.878 µg/ml

Figure 60 IC_{50} data of PLA MW >70 kDa-coated liposome in Huh7 cells at pH 6.4

3. Cytotoxicity of carriers/DNA on HeLa cells

Table 78 Cytotoxicity of PLL MW 30-70 kDa/DNA complexes at pH 7.4

n	weight ratio						
	control	0.1	0.5	1.0	2.0	4.0	6.0
1	97.445	85.597	86.103	87.181	87.371	80.401	59.683
2	97.825	86.737	85.787	86.864	90.539	84.203	64.055
3	104.731	87.751	86.294	86.610	89.778	82.429	68.110
AVG	100.000	86.695	86.061	86.885	89.229	82.344	63.949
SD	4.101	1.078	0.256	0.286	1.654	1.902	4.214

Table 79 Cytotoxicity of PLA MW 5-15 kDa/DNA complexes at pH 7.4

n	weight ratio							
	control	1.0	2.0	2.5	3.0	4.0	6.0	8.0
1	99.569	99.397	97.158	88.372	87.855	78.381	68.562	27.562
2	101.378	101.034	100.775	91.817	84.927	88.544	61.326	31.611
3	99.053	96.296	100.431	91.128	87.941	86.219	74.332	26.960
AVG	100.000	98.909	99.454	90.439	86.908	84.381	68.073	28.711
SD	1.221	2.406	1.997	1.823	1.716	5.325	6.517	2.529

Table 80 Cytotoxicity of PLA MW 15-70 kDa/DNA complexes at pH 7.4

N	weight ratio							
	control	1.0	2.0	2.5	3.0	4.0	6.0	8.0
1	99.569	96.296	100.947	93.109	86.822	83.979	65.547	59.001
2	101.378	102.153	98.191	91.042	78.381	78.984	63.997	49.440
3	99.053	93.885	91.731	85.874	89.492	82.429	64.858	45.650
AVG	100.000	97.445	96.957	90.009	84.898	81.797	64.800	51.364
SD	1.221	4.252	4.731	3.727	5.800	2.557	0.777	6.880

Table 81 Cytotoxicity of PLA MW >70 kDa/DNA complexes at pH 7.4

n	weight ratio							
	control	1.0	2.0	2.5	3.0	4.0	6.0	8.0
1	99.569	99.483	97.158	95.090	99.139	82.946	71.059	50.732
2	101.378	105.512	98.622	100.172	96.727	97.674	74.505	47.028
3	99.053	98.105	101.981	101.206	93.798	85.616	66.494	45.909
AVG	100.000	101.034	99.254	98.823	96.555	88.745	70.686	47.890
SD	1.221	3.940	2.473	3.273	2.674	7.847	4.018	2.524

Table 82 Cytotoxicity of PLO MW 30-70 kDa/DNA complexes at pH 7.4

n	weight ratio					
	control	1.0	2.0	4.0	8.0	12.0
1	100.798	68.016	60.179	58.684	18.784	11.306
2	98.046	67.717	64.726	54.975	17.587	9.751
3	101.157	69.332	64.487	59.521	21.834	11.127
AVG	100.000	68.355	63.131	57.727	19.402	10.728
SD	1.702	0.859	2.559	2.420	2.190	0.851

Table 83 Cytotoxicity of PEI 25 kDa/DNA complexes at pH 7.4

n	weight ratio					
	control	1.0	2.0	2.5	3.0	4.0
1	98.103	70.299	66.415	57.608	44.917	18.844
2	102.256	70.145	66.722	57.762	42.341	15.998
3	99.641	71.029	66.876	61.184	43.302	16.806
AVG	100.000	70.491	66.671	58.851	43.520	17.216
SD	2.100	0.473	0.235	2.022	1.302	1.467

Table 84 Cytotoxicity of PLA MW >70 kDa-coated liposomes/DNA complexes at pH 7.4

n	weight ratio					
	control	1.0	2.0	2.5	3.0	4.0
1	101.352	95.774	97.585	85.342	74.330	66.361
2	99.034	96.643	90.196	86.139	75.779	67.447
3	99.614	96.426	96.354	84.255	77.807	70.490
AVG	100.000	96.281	94.711	85.245	75.972	68.099
SD	1.206	0.452	3.959	0.946	1.747	2.141

Table 85 Cytotoxicity of PLL MW 30-70 kDa/DNA complexes at pH 6.4

n	weight ratio						
	control	0.1	0.5	1.0	2.0	4.0	6.0
1	97.445	91.489	88.448	87.181	86.610	72.482	60.887
2	97.825	91.932	91.045	87.434	83.949	73.875	63.041
3	104.731	91.426	88.891	88.891	86.103	73.559	63.992
AVG	100.000	91.616	89.461	87.835	85.554	73.305	62.640
SD	4.101	0.276	1.390	0.923	1.413	0.731	1.591

Table 86 Cytotoxicity of PLA MW 15-70 kDa/DNA complexes at pH 6.4

n	weight ratio							
	control	1.0	2.0	2.5	3.0	4.0	6.0	8.0
1	98.643	95.400	80.543	79.940	78.054	71.644	63.801	33.937
2	101.508	97.059	78.959	75.716	81.146	75.867	63.122	34.389
3	99.849	96.305	80.090	78.959	75.038	75.415	66.139	32.428
AVG	100.000	96.254	79.864	78.205	78.079	74.309	64.354	33.585
SD	1.439	0.831	0.816	2.210	3.054	2.319	1.583	1.027

Table 87 Cytotoxicity of PLO MW 30-70 kDa/DNA complexes at pH 6.4

n	weight ratio					
	control	1.0	2.0	4.0	8.0	12.0
1	100.798	62.572	57.428	45.105	20.877	12.383
2	98.046	62.931	54.497	43.908	22.971	13.280
3	101.157	69.093	58.744	43.549	22.433	12.622
AVG	100.000	64.865	56.889	44.187	22.094	12.762
SD	1.702	3.665	2.174	0.814	1.087	0.465

Table 88 Cytotoxicity of PEI 25 kDa/DNA complexes at pH 6.4

n	weight ratio					
	control	1.0	2.0	2.5	3.0	4.0
1	98.103	75.490	73.337	58.762	36.841	19.728
2	102.256	75.413	74.567	57.147	36.841	19.651
3	99.641	70.837	73.721	57.339	36.726	20.382
AVG	100.000	73.914	73.875	57.749	36.803	19.921
SD	2.100	2.665	0.630	0.882	0.067	0.401

Table 89 Cytotoxicity of PLA MW >70 kDa-coated liposomes/DNA complexes at pH 6.4

n	weight ratio				
	control	1.0	2.0	3.0	4.0
1	102.112	97.378	89.002	87.109	79.607
2	98.325	101.748	93.227	87.327	79.243
3	99.563	102.986	90.823	87.254	82.957
AVG	100.000	100.704	91.017	87.230	80.602
SD	1.931	2.946	2.119	0.111	2.048

Table 90 Cytotoxicity of CSHCl/DNA/PLA MW >70 kDa complexes at pH 6.4

n	weight ratio				
	control	1.0	2.0	3.0	4.0
1	102.112	94.756	90.240	84.195	86.526
2	98.325	88.201	93.736	86.307	87.837
3	99.563	88.201	88.711	84.705	71.814
AVG	100.000	90.386	90.896	85.069	82.059
SD	1.931	3.785	2.576	1.102	8.897

Table 91 Cytotoxicity of PLA MW >70 kDa/DNA/CSHCl complexes at pH 6.4

n	weight ratio				
	control	1.0	2.0	3.0	4.0
1	102.112	95.047	94.683	90.095	82.374
2	98.325	96.577	96.358	90.386	79.971
3	99.563	92.862	90.896	93.154	83.977
AVG	100.000	94.829	93.979	91.211	82.107
SD	1.931	1.867	2.798	1.688	2.016

Table 92 Cytotoxicity of PLA MW >70 kDa/CSHCl/DNA complexes at pH 6.4

n	weight ratio					
	control	1.0	2.0	2.5	3.0	4.0
1	101.352	84.907	77.518	72.084	64.260	37.237
2	99.034	83.023	74.547	68.969	56.653	47.670
3	99.614	83.531	73.316	70.635	52.886	48.249
AVG	100.000	83.820	75.127	70.563	57.933	44.385
SD	1.206	0.975	2.160	1.559	5.794	6.197

4. Cytotoxicity of carriers/DNA in Huh7 cells

Table 93 Cytotoxicity of PLL MW 30-70 kDa/DNA complexes at pH 7.4

n	weight ratio						
	control	0.1	0.5	1.0	2.0	4.0	6.0
1	101.589	100.306	101.406	100.856	93.337	74.083	69.315
2	99.389	102.323	97.738	101.956	92.237	79.034	67.848
3	99.022	100.306	100.306	102.873	93.154	74.267	68.949
AVG	100.000	100.978	99.817	101.895	92.910	75.795	68.704
SD	1.388	1.165	1.882	1.010	0.589	2.807	0.763

Table 94 Cytotoxicity of PLA MW 15-70 kDa/DNA complexes at pH 7.4

n	weight ratio					
	control	1.0	1.5	2.0	3.0	4.0
1	102.624	91.726	88.698	87.487	76.287	70.535
2	98.385	94.147	95.358	84.460	73.562	70.535
3	98.991	90.817	88.698	77.195	78.406	69.627
AVG	100.000	92.230	90.918	83.047	76.085	70.232
SD	2.292	1.721	3.845	5.290	2.428	0.524

Table 95 Cytotoxicity of PLO MW 30-70 kDa/DNA complexes at pH 7.4

n	weight ratio					
	control	1.0	2.0	4.0	8.0	12.0
1	103.248	77.014	78.139	41.224	34.291	32.979
2	99.126	79.263	80.387	48.532	33.916	33.729
3	97.626	77.764	77.014	44.597	33.167	30.731
AVG	100.000	78.014	78.513	44.785	33.791	32.480
SD	2.911	1.145	1.717	3.658	0.572	1.560

Table 96 Cytotoxicity of PLA MW >70 kDa-coated liposomes/DNA complexes at pH 7.4

n	weight ratio					
	control	1.0	1.5	2.0	3.0	4.0
1	97.118	94.236	84.726	82.133	72.911	73.487
2	95.389	94.236	83.862	80.980	84.726	68.300
3	107.493	93.084	78.963	77.810	82.133	81.844
AVG	100.000	93.852	82.517	80.307	79.923	74.544
SD	6.546	0.666	3.108	2.238	6.210	6.834

Table 97 Cytotoxicity of PLL MW 30-70 kDa/DNA complexes at pH 6.4

n	weight ratio						
	control	0.1	0.5	1.0	2.0	4.0	6.0
1	101.589	59.963	56.846	54.829	52.445	54.095	45.844
2	99.389	58.863	57.763	56.846	53.178	52.078	50.795
3	99.022	60.330	61.064	60.147	53.362	46.577	50.795
AVG	100.000	59.719	58.557	57.274	52.995	50.917	49.144
SD	1.388	0.763	2.218	2.685	0.485	3.891	2.859

Table 98 Cytotoxicity of PLA MW 15-70 kDa/DNA complexes at pH 6.4

n	weight ratio					
	control	1.0	1.5	2.0	3.0	4.0
1	102.624	97.780	97.477	92.331	77.800	64.178
2	98.385	90.817	97.477	90.817	80.525	72.654
3	98.991	102.624	97.780	85.974	80.222	69.627
AVG	100.000	97.074	97.578	89.707	79.516	68.819
SD	2.292	5.935	0.175	3.321	1.493	4.295

Table 99 Cytotoxicity of PLO MW 30-70 kDa/DNA complexes at pH 6.4

n	weight ratio					
	control	1.0	2.0	4.0	8.0	12.0
1	103.248	52.842	50.593	43.285	38.788	29.606
2	99.126	52.280	45.534	40.287	37.102	33.542
3	97.626	51.530	50.406	46.096	39.163	30.169
AVG	100.000	52.217	48.844	43.223	38.351	31.106
SD	2.911	0.658	2.868	2.905	1.098	2.128

Table 100 List of abbreviations

Symbol	Definition
°C	degree Celsius
>	more than
<	less than
%	percent
mV	milivolt
MW	molecular weight
pK_a	minus logarithm base 10 of K_a, -log K_a
DNA	deoxyribonucleic acid
pDNA	plasmid of deoxyribonucleic acid
w/w	weight by weight
cm^2	square centimeter
g	gram
nm	nanometer
nm	nanometer
min	minute
h	hours
IC50	Inhibition Concentration Fifty
NaCl	sodium chloride
PBS	phosphate-buffered saline
CSHCl	chitosan hydrochloride
PLL	poly-L-lysine
PLA	poly-L-arginine
PLO	poly-L-ornithine
pH	The negative logarithm of the hydrogen ion concentration
qs. to	add to

Table 100 (continue)

Symbol	Definition
R^2	coefficient of determination
rpm	revolution per minute
SD	standard deviation
AVG	Average
UV	ultraviolet
et al.	and others
etc.	for example, such as
v/v	volume by volume
mg	milligram
µg	microgram
mL	milliliter
µL	microliter

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Presentation

1. Jintana Tragulpakseerojn**,** Auayporn Apirakaramwong, Praneet Opanasopit, Tanasait Ngawhirunpat, Theerasak Rojanarata and Uracha Ruktanonchai "Self-assembling poly-l-arginine/chitosan/DNA complexes as a novel gene carrier " The 26th Annual Research Conference in Pharmaceutical Sciences, 4 December 2009, Bangkok, Thailand, Poster presentation.
   (Poster Presentation Aword 2009, Pharmaceutical Technology)

2. Jintana Tragulpakseerojn**,** Auayporn Apirakaramwong, Praneet Opanasopit, and Uracha Ruktanonchai "Evaluation of cationic polypeptides and chitosan for gene delivery" The 3rd Annual Research Conference in Silpakorn Research, 28-29 January 2009, Nakornpathom, Thailand, Poster presentation.
3. Jintana Tragulpakseerojn**,** Auayporn Apirakaramwong, Praneet Opanasopit, Tanasait Ngawhirunpat, Theerasak Rojanarata and Uracha Ruktanonchai "Liposomes coated with cationic polymers and its potential use in gene delivery" The 1st Current Drug Development International Conference, 6-8 May 2010, Phuket, Thailand, Poster presentation.