ENHANCED GENE DELIVERY MEDIATED BY INCORPORATION OF RECOMBINANT FUSION PROTEINS: LISTERIOLYSIN O AND PEPTIDES DERIVED FROM PROTAMINE

by

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To my Lord, my God

And

To my parents

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LIST OF ABBREVIATIONS

APC	Antigen-presenting cells
BCA	Bicinchoninic acid
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CHEMS	Cholesteryl hemisuccinate
CTL	Cytotoxic T lymphocytes
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
FBS	Fetal bovine serum
h	Hour(s)
HBG	HEPES-buffered glucose
HRP	Horse radish peroxidase
IFN-γ	Interferon gamma
IPTG	Isopropyl β-D-thiogalactopyranoside
LLO	Listeriolysin O
LLO-PN	Listeriolysin O and single fragment of protamine fusion protein
LLO-PNPN	Listeriolysin O and two fragments of protemine fusion protein
Min	Minute(s)
OVA	Ovalbumin
PBS	Phosphate-buffered saline

PBST	Phosphate-buffered saline with 0.05% Tween
PEI	Polyethylenimine
PE	Phosphatidylethanolamine
PN-LLO	Fragment of protamine and listeriolysin O fusion protein
RBC	Red blood cell
RES	Reticuloendothelial system
RLU	Relative luminescence units
SFU	Spot-forming unit
SLIM	Site-directed, ligase-independent mutagenesis

ABSTRACT

One of the important requirements for non-viral gene delivery systems is to achieve high levels of transfection efficiency by overcoming extracellular and intracellular barriers that exogenous genes need to pass through for gene expression. The neutralization and condensation of large, anionic DNA molecules with cationic carriers improve the cellular binding and uptake as well as the protection of DNA from enzymatic degradation in the extracellular environment. Most of the non-viral vectors are internalized by cells in endocytic compartments and ultimately degraded by hydrolytic enzymes along the endolysosomal pathway. Therefore, promoting endosomal escape of most non-viral vectors confers a significant improvement in transfection efficiency. In order to overcome several important biological barriers, genetically engineered fusion proteins containing multiple functional domains have been recently studied as efficient non-viral gene carriers. In this thesis, we focused on listeriolysin O (LLO) and protamine fusion proteins as one example of recombinant fusion protein-based non-viral multifunctional carriers. Our laboratory has previously demonstrated that LLO, the endosomolytic pore-forming protein from *Listeria monocytogenes*, increased cytosolic delivery of antigen or DNA by promoting its endosomal escape. In this study, we designed genetically engineered fusion proteins as non-viral vectors that incorporate LLO at the N-terminus and a fragment of the DNA-condensing polypeptide protamine (PN) at the C-terminus, a strategy that makes it possible to produce large amounts of

homogeneous fusion protein using relatively simple methods. Along with successful expression and purification of LLO-PN and LLO-PNPN fusion proteins, we demonstrated dramatic enhancement of gene delivery efficiency of protamine/DNA complexes with LLO-PN and further improvement with LLO-PNPN both *in vitro* and *in vivo*. In addition, the association of anionic liposomes composed of PE and CHEMS with LLO-PNPN/protamine/DNA complexes, yielding a net negative surface charge to reduce potential interactions with plasma proteins, showed improved *in vitro* transfection efficiency in the presence of serum and *in vivo* gene expression in spleens and lungs of mice following intravenous administration. The cleavage of a cathepsin D-cleavable linker introduced between LLO and PN was not observed in *in vitro* studies and the linker did not affect the transfection efficiency of protamine/DNA complexes. The applicability of this gene delivery system in DNA vaccine was also demonstrated, though limited, generating weak immune responses in mice. Taken together, this study suggests that incorporation of a recombinant LLO-protamine fusion protein in a non-viral vector is a promising strategy that possesses the potential to improve transfection efficiency of non-viral gene delivery systems.

CHAPTER I

BACKGROUND

SUMMARY

Non-viral gene delivery vectors have been studied as attractive alternatives with advantages over viral vectors, such as low immunogenicity and ease of production. However, relatively poor transfection efficiency has been a major drawback of non-viral vectors, which is mainly due to multiple biological barriers, including the extracellular environment, plasma membrane, endosomes, cytosolic environment and nuclear membrane, all of which exogenous genes should pass through for expression. Therefore, in order to improve the efficiency of non-viral gene delivery vectors, studies have been extensively performed to develop non-viral vectors that survive in the extracellular environment, bind efficiently and often specifically to cells, be internalized into cells, and overcome intracellular barriers. The objective of this study is to design a non-viral gene delivery vector that overcomes several major biological barriers for the improvement of gene delivery. Genetically engineered fusion protein with multiple functional domains is one of the more recent strategies for incorporating multiple components into a single vector in order to overcome multiple barriers. In this thesis, we designed and characterized genetically engineered LLO and protamine fusion proteins as an example of multifunctional non-viral gene delivery carriers.

INTRODUCTION

The concept of gene therapy was initially developed more than three decades ago, and the completion of the human genome project has provided more possibilities to gene therapy, which has accelerated the understanding of genetic diseases [1-3]. Since then, gene therapy has become an attractive strategy to treat or prevent a wide range of diseases by administering therapeutic genes for gain or loss of gene function, and for specific antigen expression for DNA-based vaccination [2, 4, 5]. Along with intensive research *in vitro* and in *in vivo* models, to date about 1644 gene therapy clinical trials have been performed addressing diverse indications such as cancer, cardiovascular diseases, monogenic diseases, infectious diseases, etc [6]. Despite its great potential and intensive research with numerous candidates in advanced stages of human clinical trials, human gene therapy is still limited in its clinical utility. Thus far, only one antisense oligonucleotide product has been approved by the FDA for clinical use in the U.S., which is Vitravene administered locally by intravitreal injection for the treatment of retinitis caused by cytomegalovirus in patients with acquired immunodeficiency syndrome (AIDS) [7]. For successful gene therapy, the primary challenge is the development of an efficient gene delivery system that can deliver a gene to target cells with sufficient gene expression and minimal side effects [5, 8].

GENE DELIVERY VECTORS

Naked DNA can be locally delivered into certain organs such as muscle or liver by direct administration, but the systemic administration of naked DNA generally results in a relatively low level of gene expression in all major organs, with the exception of hydrodynamic injection [2, 9-12]. The half-life of naked plasmid DNA in blood is less than 10 min due to its rapid degradation by intra- and extracellular nucleases, and the large size and highly anionic charge of DNA make its cellular uptake difficult [13, 14]. To generate a therapeutic effect, DNA has to be protected in such a way as to be able to reach the target tissue, cross the cell membrane and be delivered to the intracellular environment to be expressed. Therefore, in order for the improvement of DNA delivery, carrier-mediated gene delivery systems have been extensively studied and can be roughly divided into two groups: viral vectors and non-viral vectors.

With the natural ability of viruses to deliver their genes into host cells, viral vectors such as those based on adenovirus, retroviruses, and adeno-associated virus have been intensively studied as efficient gene delivery systems with the advantage of high transfection efficiency both *in vitro* and *in vivo* [1, 15, 16]. With this advantage, about 70% of human gene therapy clinical trials have used viral vectors [6]. However, major safety concerns with using viral vectors include adverse reactions due to the high immunogenicity of the carrier itself, insertional mutagenesis, and potential for oncogenic transformation. Although gene therapy of X-linked severe combined immunodeficiency disease (SCID-X1) has been successful with the restoration of immune function by retroviral vector-mediated gene replacement of the deficient gene, four out of twenty treated children developed leukemia due to the insertional oncogenesis by the retroviral

vector [17, 18]. Also, the death of one patient treated for ornithine transcarbamylase deficiency, resulting from a systemic inflammatory response syndrome, was reported following adenoviral vector-mediated gene therapy [19]. Furthermore, the limitation in the size of DNA that the virus can carry is one of the restrictions of viral vectors [15].

Non-viral vectors have advantages including low immunogenicity and ease of production [15, 20, 21]. Therefore, non-viral vectors, such as those based on lipids, polymers, proteins and peptides, have been studied as attractive alternatives to viral vectors, which can circumvent the problems of viral vectors [22-24]. However, relatively poor transfection efficiency has been a major drawback of non-viral vectors, which is mainly due to multiple biological barriers including the extracellular environment, plasma membrane, endosomes, cytosol and nuclear membrane, all of which exogenous genes must pass through for successful modification of cellular phenotype (Figure 1-1) [1, 25].

BIOLOGICAL BARRIERS TO GENE DELIVERY USING NON-VIRAL VECTORS

Extracellular environment

Once administered to the systemic circulation, non-viral vectors are exposed to various components in blood, such as blood cells, proteins and enzymes, which can cause destabilization of gene delivery vectors and degradation of DNA [1, 26]. In addition, vectors can electrostatically interact with oppositely charged blood components, form large aggregates, potentially induce unwanted immune responses, and be cleared from blood circulation by the reticuloendothelial system (RES) before reaching the target cells [27, 28]. Following local administration, non-specific interactions with extracellular matrix components or high interstitial fluid pressures can also be a barrier for non-viral vectors to be transported to tissues through the interstitial space [1, 29].

Plasma membrane

After encountering target cells, the vectors need to bind to the plasma membrane and be internalized into the cells. Since the plasma membrane allows the passive diffusion of only small nonpolar molecules, large polyanionic DNA hardly passes through plasma membrane without some sort of gene delivery carrier [25, 26]. By appropriately designing a carrier, interaction and binding of the plasma membrane with the gene delivery vector can be promoted by, for example, electrostatic interactions between cationic vector and anionic cell surface components such as heparan sulfate proteoglycan and integrins, or alternatively by specific interactions between ligand of vector and cell-surface receptor [25, 30]. Following binding to plasma membrane

through interactions, most non-viral vectors are internalized into cells by endocytosis, such as clathrin-mediated endocytosis, caveolar-mediated endocytosis, and macropinocytosis [14, 20, 25, 30].

Endosomes

Following entry into cells, non-viral vectors should escape several intracellular barriers in order to deliver the DNA to be expressed. Most non-viral vectors are internalized into endosomal compartments, which begin as early endosomes that are slightly acidic (pH ~ 6) due to ATP-dependent proton pumps. The early endosome becomes more acidified and matures to a late endosome (pH 5 - 6), then merges with the lysosome (pH 4 - 5); degradation of internalized cargo occurs throughout this pathway due to the presence of endosomal and lysosomal hydrolytic enzymes [25, 30]. In order for non-viral vectors to reach the cytosol and ultimately the nucleus after internalization, the vectors should escape the endosomal compartments before degradation. Therefore, a strategy for promoting endosomal escape is necessary for efficient non-viral vector-mediated gene delivery.

Cytosolic environment

The cytoplasm contains many organelles, proteins and filamentous structures that hinder the free diffusion of large molecules. The mobility of DNA larger than 2000 bp is severely restricted in the cytosol [31]. It has also been reported that transfection efficiency was decreased from 56% after microinjection of DNA near the nucleus to 8% after microinjection far from the nucleus [32]. In addition to the immobility of DNA, dissociation of vector that allows the release of DNA is considered as one of the major

barriers. DNA should be unpackaged from the vector for efficient transcription, but an optimum degree of dissociation is required since rapid dissociation of the vector results in degradation of DNA by cytosolic nucleases [25].

Nuclear membrane

The nuclear membrane consists of intact double membranes that are penetrated by 3000-4000 nuclear pore complexes (NPCs). The NPC allows passive diffusion of small molecules that are less than ~9 nm in diameter, proteins less than ~60 kDa and DNA up to about 500 bp. Larger molecules, including ribosomal subunits and RNA, are transported in and out of the nucleus by active transport mechanisms. This selective and energy-dependent nuclear transport is mediated by nuclear localization signals (NLSs) present in proteins destined for the nucleus, which are recognized by nuclear import proteins (importin α and β). The NLS/importin complex docks onto the NPC and is actively transported through NPC into nucleus [33-35].

Nuclear import of exogenous DNA, which is usually larger than diffusion size limit, is inefficient and has been considered as one of the major limiting steps for non-viral vector-mediated gene delivery [25]. Early studies reported that DNA microinjected into the cytoplasm showed very little gene expression, whereas nuclear injection of DNA led to high expression [36]. It has been generally known that exogenous DNA can easily reach the nuclei in proliferating cells as the nuclear membrane is temporarily broken down during mitosis. However, most cells in our body, especially fully differentiated cells, do not divide or divide slowly; in such cases nuclear import of DNA is a significant barrier for non-viral gene delivery [34, 37].

STRATEGIES TO OVERCOMING THE BIOLOGICAL BARRIERS

In order to overcome each barrier for efficient gene delivery, many strategies have been studied.

Protection from extracellular environment

In order to protect DNA from enzymatic degradation before reaching target cells, cationic polymers and lipids have been typically used to condense the anionic DNA molecules.

Cationic polymers, such as polylysine, polyethylenimine, chitosan, dendrimer, etc. have some advantages such as efficient condensation of DNA to form relatively small and stable polymer/DNA complexes, and ease of manipulation [8, 15, 22]. Polyethylenimine (PEI) is one of the most studied polymers for gene delivery and has shown to mediate efficient transfection efficiency, with its intrinsic buffering effect facilitating endosomal escape [15]. Major drawbacks of PEI include its non-biodegradability and toxicity, while the toxicity and transfection efficiency of PEI are both directly proportional to its molecular weight [8]. Most polymers, which consist of –C-C- bond or amide bond backbones, are not readily degraded in physiological solutions, causing accumulation in cells or tissue and cytotoxicity [8, 22]. To address this problem, several biodegradable polymers such as polyaminoester, acid-labile PEI, or reversibly crosslinked low molecular weight polymers have been investigated as potential gene delivery vectors [22, 38, 39].

Protamine, an arginine-rich cationic polypeptide, is also commonly used as a non-viral gene delivery vector. Protamine is different from synthetic polymers such as polylysine or PEI in that it is a naturally occurring sperm chromatin component, and

relatively well characterized and homogenous [40, 41]. During mammalian spermatogenesis, protamine replaces the histone subunits of nucleosomes, binds with DNA to form compact structures 50 – 100 nm in diameter, and delivers the DNA to the egg nucleus after fertilization [42]. With these characteristics, as a non-viral gene delivery vector protamine efficiently condenses DNA into small complexes by electrostatic interactions and protects DNA from enzymatic degradation [41]. In addition, it is suggested that protamine contains nuclear localization signals to improve nuclear import of DNA from the cytoplasm [43, 44]. Furthermore, protamine sulfate is a USP compound and approved for human use by the U.S. FDA as a drug to reverse heparin-induced anticoagulation. Protamine sulfate has also been used with insulin to increase the duration of insulin action. The clinical application of protamine demonstrates the safety of protamine for human use with minimal toxicity and immunogenicity [43].

Since the first successful DNA transfection with cationic lipid by Felgner *et al*. in 1987, various gene delivery studies using cationic lipids, including clinical trials, have been extensively performed [45, 46]. Lipids are generally well tolerated in terms of biocompatibility; cationic lipids/DNA complexes (lipoplexes), which protect DNA from nuclease degradation and bind to the negatively charged plasma membrane resulting in greater cellular uptake, have demonstrated relatively high transfection efficiencies. However, *in vivo* stability and toxicity of cationic lipoplexes are of tremendous concern [1, 2, 8, 24]. As with other cationic vectors, the cationic surface of lipoplexes rapidly interacts with plasma proteins and forms large aggregates. These interactions alter the stability of lipoplexes and lead to their rapid elimination from the systemic circulation by

the RES, which results in inflammatory adverse reactions and limited gene expression in lung and liver. Various strategies have been developed to overcome these problems, as discussed below.

Anionic lipids are an effective alternative to cationic lipids to reduce the nonspecific interactions with negatively charged plasma proteins and prolong the circulation time [15, 24]. In addition, low toxicity of anionic liposome has been demonstrated after pulmonary administration in mice [47]. Patil *et al*. have also reported that the transfection efficiency of anionic lipoplexes was comparable to that of cationic lipoplexes, while their relative toxicity was lower [48]. Inefficient condensation of DNA molecules with anionic liposomes, which is one of the shortcomings of this strategy, has been overcome by pre-condensation of DNA with polycations such as polylysine or protamine [24, 49]. Furthermore, it has been shown that DNA is better protected and has greater transfection efficiency when pre-condensed with polycation and coated with cationic or anionic liposomes [8, 43].

Methods for associating DNA with liposomes have also been studied as strategies to overcome cationic lipoplex-mediated problems [24]. Most cationic lipoplexes are prepared by simple mixing of DNA and cationic liposome, which results in heterogeneous complexes in terms of size and morphology [8]. Multiple methods have been reported for DNA encapsulation into liposomes such as direct hydration of lipid film with DNA-containing solutions and sonication, multiple cycles of freezing/thawing of liposomes with DNA solutions, ethanol-exchange dialysis, etc [50-52]. The liposomes encapsulating DNA have some advantages such as ease of size control and surface modification with targeting ligand.

Polyethylene glycol (PEG) has been incorporated on the surface of gene delivery vectors to reduce non-specific binding with plasma proteins and prolong the circulation time which allows for a greater probability of reaching the target site [37]. Moreover, the presence of PEG strands can provide sites for conjugation of targeting ligands.

Cell binding and internalization

The charge neutralization and condensation of large, anionic DNA with cationic carriers improve cell binding and uptake of DNA as well as protection from degradation [14, 15]. Conjugation of target-specific ligands to gene delivery vectors also reduces nonspecific binding and therefore nonspecific internalization and risk of side effects, while achieving preferential delivery of DNA to target sites [25, 53]. Several different ligands for cell-specific or overexpressed receptors have been studied, including the asialoglycoprotein, insulin, folate, transferrin, and epidermal growth factor receptors, etc [49, 54-58].

Endosomal escape

After internalization of non-viral vectors by endocytosis, the endosomes containing vectors fuse with lysosomes resulting in degradation of vectors and poor transfection efficiency [2, 14, 15]. Since endosomal escape is required for efficient non-viral gene delivery, a significant number of studies have investigated different strategies for their efficiency.

The pH-sensitive liposome, which generally consists of unsaturated phosphatidylethanolamine (PE) such as DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) and acidic stabilizer lipid such as CHEMS (cholesteryl

hemisuccinate), helps the release of DNA into the cytoplasm [59, 60]. At neutral pH and above, the stabilizer lipid has the net negative charge allowing the formation of a typical bilayer structure. However, in an acidic environment such as endosomal compartments, the stabilizer lipids undergo protonation, losing its bilayer-supporting capacity, which results in PE forming an inverted hexagonal structure. This leads to the disruption of endosomal membrane and cytoplasmic delivery of encapsulated DNA. Increased transfection efficiency has been reported using pH-sensitive liposome [61, 62].

Endosomal release can also be mediated by polymer or peptide with endosomal buffering capacity [23]. For example, PEI or histidine-rich peptide has amine groups which can be protonated and induce high chloride ion influx into the endosome, causing osmotic swelling of the endosome and membrane disruption [63, 64]. Although the transfection efficiency can be improved using PEI or polyhistidine peptide, it is not clear that they can achieve the same buffering capacity *in vivo* [37]. The addition of a lysosomotropic agent such as chloroquine to transfection samples provided some increase in gene expression by preventing DNA degradation, although this approach has limited *in vivo* utility [25, 63].

The fusogenic peptides such as melittin, hemagglutinin 2, GALA and KALA have also been used for endosomal escape [23, 65]. These peptides have been shown *in* vitro to undergo conformational change to α-helical structure at pH values similar to those of endosomes, leading to membrane destabilization. The ability of these peptides to enhance gene transfer was demonstrated by conjugation to cationic lipids or polymers. However, their endosomolytic ability can be influenced upon modification by covalent linkage, and synthesis and purification of these peptides are often complicated [37].

The other approach for endosomal escape is to use pore-forming proteins of intracellular bacteria, such as listeriolysin O (LLO) form *Listeria monocytogenes* [66]. LLO can breach endosomal membranes and deliver the whole bacterial particle, which is much larger than the size of most of the currently studied nano-scale gene delivery vectors [67]. Since the optimal pH for LLO function is 5.5 – 5.9 and its activity gets attenuated at neutral pH, cytosolic delivery of macromolecules with LLO can be achieved without severe cytotoxicity, including that resulting from permeabilization of plasma membrane [68]. LLO has also been shown to be degraded relatively rapidly upon reaching the cytosol by the proteasome, further limiting potential damage to cells. Moreover, LLO is a member of the sulfhydryl-activated cytolysin family, and the hemolytic activity is abolished upon modification of the unique cysteine residue by oxidation of the sulfhydryl group [66]. This interesting feature allows one to regulate the hemolytic activity of LLO as well as to reversibly conjugate LLO with lipids or polymers without affecting its pore-forming ability. The enhanced gene delivery capability in different cell lines was reported using LLO reversibly conjugated with protamine or polyethylenimine by disulfide bond [69, 70]. Furthermore, LLO can be efficiently incorporated into liposomes by multiple cycles of freezing/thawing and sonication, and it has been reported that LLO-containing pH-sensitive anionic liposomes complexed with protamine/DNA complexes improved *in vitro* and *in vivo* reporter gene transfection [71, 72]. With LLO-containing pH-sensitive liposomes, strong enhancement in cellular immune response upon cytosolic delivery of a model antigen has also been demonstrated in mice [72, 73]. Theses previous studies suggest that LLO is a promising agent to

overcome the endosomal membrane barrier for increased non-viral gene delivery efficiency.

Cytosolic trafficking

Intracellular vesicles and macromolecules are transported by molecular motors along cytoskeletal filaments [74]. Dynein is one of the molecular motors that mediate retrograde transport toward the nucleus along microtubules. Several viruses, such as adenovirus and herpes simplex virus, are known to use dynein to facilitate transport of viral proteins and nucleic acids toward the host nuclear envelope. Similar strategies have been tried to actively transport non-viral vectors within the cytoplasm; for instance, via modification of a gene carrier with dynein-binding peptide or synthetic motor protein [75]. Some nuclear localization signals have also been reported as facilitating transport of DNA along microtubules toward the nucleus [76]. However, appropriate methods for efficient transport and its effect on gene transfection require further investigation.

It is not clearly known where and how DNA gets released from its vectors, although it is generally agreed that intracellular release of DNA is necessary for gene expression [25]. High molecular weight peptides or polymers efficiently bind and condense DNA, but interfere with decondensation of the vectors inside cells, thus producing toxicity. One of the strategies for efficient release of DNA is to design carriers containing multiple disulfide bonds that are reducible in the intracellular environment, in effect promoting biochemical fragmentation of the polymer after cellular uptake and hence more efficient decondensation of vectors. These reversibly cross-linked small molecular weight polymers have shown enhanced transfection efficiencies with minimal toxicity [38, 77, 78].

Nuclear import

The nuclear import of exogenous genes can be facilitated by nuclear localization signal-containing peptides [33-35, 79]. The most commonly studied NLS peptide is SV40 large T-antigen, and several others such as hnRNP A1 protein M9, HIV-1 Rev protein and polyoma virus capsid protein VP1, have also been used for gene delivery. NLS peptides have been incorporated into gene delivery vectors by different methods, including, electrostatic interactions with DNA, covalent association with DNA or carrier, and site-specific association with DNA via peptide nucleic acid. Other than peptides, DNA nuclear targeting sequences that bind to transcription factors, such as SV40 enhancer region or NF-κB binding site, have been inserted into DNA. Despite an increasing number of studies concerning NLS-mediated gene delivery, their success in improving gene delivery has varied [80-85]. Some papers have reported increased gene expression, while others have found no significant enhancement in gene expression or nuclear import of the gene. Also, some successful studies have not fully verified the specific role of NLS in the gene delivery system and the success has been mostly restricted to *in vitro* studies.

GENETICALLY ENGINEERED FUSION PROTEIN AS A NON-VIRAL VECTOR

In order to achieve efficient gene delivery, it is not enough for a non-viral vector to contain a single functional component to overcome only one barrier. Non-viral vectors should contain various functional components which can overcome multiple barriers for significantly improved transfection efficiency. With this understanding, multifunctional non-viral vectors containing several functional components have been increasingly studied. In most cases, each functional component is assembled by chemical conjugation; for example, conjugation of a receptor-targeting antibody and/or a membrane fusogenic peptide to liposomes or polymers [54-57, 86-90]. While those non-viral vectors have shown improved gene delivery, there are also some concerns with chemical conjugation: the processes of chemical conjugation of several components and purification of final products are relatively complicated since conjugation strategies are dictated by the available functional group of each component, and there are always mixtures of unreacted components or byproducts after conjugation reactions.

Instead of chemical conjugation, one of the more recent methods for incorporating multiple components into a single vector utilizes genetically engineered fusion proteins containing multiple functional motifs [91-95]. Genetically engineered fusion proteins as non-viral vectors have advantages over chemical synthesis-based vectors, both in terms of the relatively straightforward preparation of large amounts of homogenous fusion proteins and production of various fusion proteins with different functional groups relatively easily. Examples of gene delivery using recombinant fusion proteins include: Her-NLS fusion protein consisting of heregulin-α1 for targeting epidermal growth factor

receptors and nuclear localization sequence [95]; designer biomimetic vectors (DMV) or multifunctional DNA carriers (MDC) containing multiple peptide subunits in a single molecule such as adenovirus μ peptide or protamine for DNA condensation, a targeting peptide, influenza virus fusogenic peptide or diphtheria toxin endosome-translocation domain for endosomal disruption and nuclear localization signal from human immunodeficiency virus type 1 [93, 94]. These studies have demonstrated the functionality of each domain of fusion proteins and reported improved DNA delivery efficiency using genetically engineered vectors with functional components *in vitro*. However, studies reporting enhanced *in vivo* DNA expression are very limited, with the exception of *in vivo* delivery of siRNA with antibody-protamine fusion proteins [91, 96]. Therefore, there is a lot of room for improving non-viral gene delivery systems using genetically engineered fusion proteins by designing efficient fusion proteins with various functional domains and incorporating into non-viral gene delivery system targeting various gene therapy.

RESEARCH OBJECTIVES

In order to improve their efficiency, non-viral gene delivery vectors should survive in the extracellular environment, bind to and be internalized by cells, and overcome intracellular barriers such as membranes and degradative enzymes. The objective of this study is to design a non-viral gene delivery vector using a genetically engineered fusion protein that overcomes several major biological barriers for improvement of gene delivery. Previously we reported increased *in vitro* gene expression using LLO that was chemically conjugated to either protamine or polyethylenimine [69, 72]. Here, it is hypothesized that a genetically engineered fusion protein consisting of LLO and a polypeptide fragment of human protamine can be incorporated into a non-viral gene delivery system in order to improve DNA delivery.

As a gene delivery carrier, we designed bifunctional recombinant fusion proteins that incorporate listeriolysin O (LLO), the endosomolytic pore-forming protein from *Listeria monocytogenes*, at the N-terminus and a DNA-condensing peptide fragment of protamine (PN) at the C-terminus. In order to further optimize this carrier, a cathepsin D cleavable linker RGFFP (cathepsin D recognition sequence of the B-chain of insulin) was introduced between LLO and protamine fragment of the fusion proteins [97]. Cathepsin D is an aspartyl endopeptidase in endosomes and lysosomes that cleaves peptide bonds formed by hydrophobic amino acids, especially aromatic amino acids such as phenylalanine [98, 99]. The C-terminal domain 4 of LLO is involved in the membrane binding step of LLO's pore formation [100]. We therefore hypothesized that if LLO is dissociated from PN/DNA complexes in the endosome by cleavage of the cathepsin D

substrate linker, it would allow more efficient release and endosomal escape of DNA and result in optimized gene expression.

The fundamental concept of the non-viral gene delivery system in this study is shown in Figure 1-2. In this gene delivery system, anionic liposomes are also incorporated in order to reduce potential non-specific interaction with negatively charged plasma proteins. The main goals of this study are to: (i) successfully express and purify LLO-PN fusion proteins and demonstrate the effect of LLO-PN fusion proteins on the transfection efficiency of protamine/DNA complexes in cultured cells; (ii) in order to further optimize the gene delivery vector, examine the cleavability of cathepsin D cleavable linker in LLO-PN fusion proteins and its effect on transfection efficiency, and characterize the physical properties of LLO-PN/protamine/DNA complexes; (iii) in order to observe the *in vivo* efficiency of the non-viral vector, demonstrate the luciferase gene delivery efficiency of the non-viral vector in mice, and examine the potential for application in a DNA vaccine of this vector by measuring immune responses in mice.

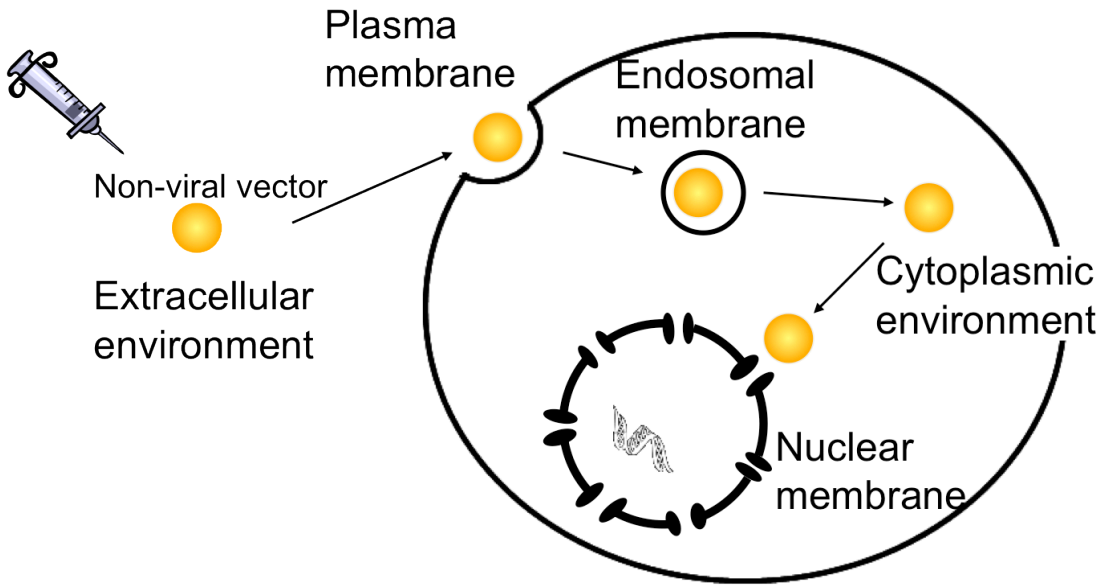

Figure 1-1. Biological barriers to gene delivery using non-viral vectors.

For efficient gene delivery, exogenous genes must pass through multiple biological barriers, including the extracellular environment, plasma membrane, endosomes, cytosol and nuclear membrane.

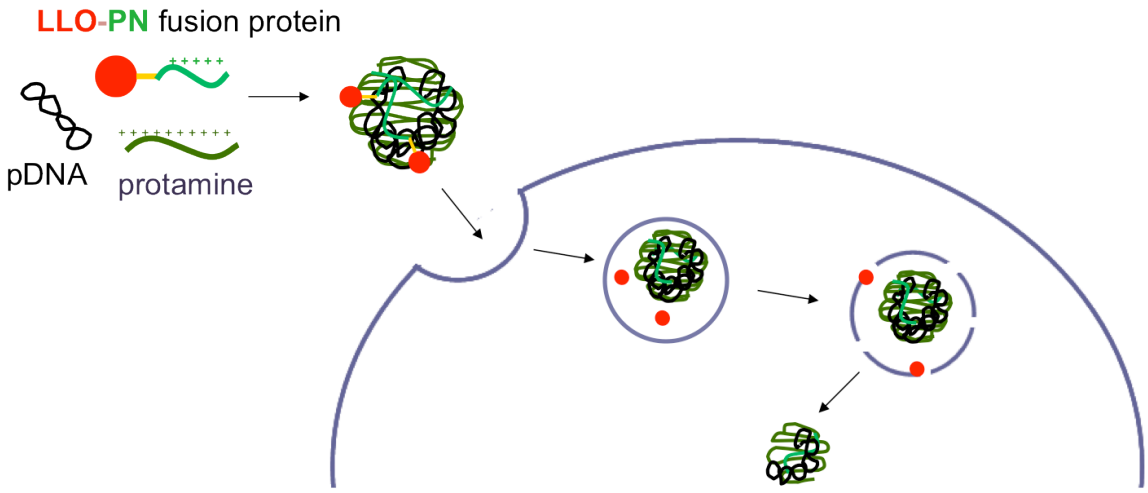

Figure 1-2. Scheme of plasmid DNA delivery using LLO-PN recombinant fusion protein.

Plasmid DNA condensed with full-length protamine and LLO-PN fusion protein can be protected from enzymatic degradation and be internalized into cells. Following endocytosis, LLO of the LLO-PN fusion protein makes pores in endosomal membranes, promoting endosomal escape of the vector; enhanced cytosolic delivery results in improved transfection efficiency.

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CHAPTER II

ENHANCED GENE DELIVERY WITH LLO-PROTAMINE FUSION PROTEINS

SUMMARY

In order to improve the efficiency of non-viral vector-mediated gene delivery, it is essential that the system contain several functional components to overcome important extracellular and intracellular barriers. In this study, we designed recombinant fusion proteins that can bind to DNA and facilitate endosomal escape. The recombinant fusion proteins are composed of listeriolysin O (LLO), the endosomolytic pore-forming protein from *Listeria monocytogenes* at the N-terminus, and a polypeptide fragment of the DNA binding protein protamine (PN) at the C-terminus. The recombinant fusion proteins, LLO-PN with one protamine fragment and LLO-PNPN with two protamine fragments in tandem were successfully expressed and purified, and determined to be as active as wild type LLO at relatively high concentrations. Through the incorporation of LLO-PN fusion protein in protamine/DNA complexes, the luciferase gene transfection efficiency was enhanced about two to three orders of magnitude compared to that with protamine/DNA complexes without fusion protein in the murine macrophage-like cell line P388D1 without severe toxicity. With LLO-PNPN fusion protein, the gene expression of protamine/DNA was three- to four-fold more enhanced compared to that with LLO-PN, and in the presence of serum the luciferase gene expression of complexes with fusion

proteins was higher than that without fusion protein. These results demonstrate that incorporation of a recombinant LLO-protamine fusion protein in DNA delivery systems is a promising strategy to improve non-viral vector-mediated gene delivery.

INTRODUCTION

Non-viral gene delivery vectors, including lipids, polymers, proteins and peptides, have been studied as attractive alternatives over viral vectors, with advantages such as low immunogenicity and ease of production [1-3]. However, relatively poor transfection efficiency has been a major drawback of non-viral vectors, which is mainly due to multiple biological barriers including the extracellular environment, plasma membrane, endosomes, cytosol and nuclear membrane, all of which exogenous genes must pass through for successful modification of cellular phenotype [4-6]. Therefore, the success of non-viral vector-mediated gene delivery depends on the development of delivery vectors that can survive in the extracellular environment, bind to and be internalized into cells, and efficiently overcome intracellular barriers.

In order to improve transfection efficiency, functional components have been incorporated into vectors that allow DNA binding and condensation, cellular targeting, endosomal escape or nuclear import [7, 8]. In most cases, each component is assembled by chemical conjugation; for example, conjugation of a receptor-targeting antibody and/or a membrane fusogenic peptide to liposomes or polymers [9-14]. Instead of chemical conjugation, one of the more recent methods for incorporating multiple components into a single vector utilizes genetically engineered fusion proteins containing multiple functional motifs, which has advantages over chemical conjugation both in terms of the relatively straightforward preparation of large amounts of homogenous fusion proteins and production of various fusion proteins with different functional groups relatively easily. Some studies have reported improved DNA delivery efficiency using

genetically engineered vectors with functional components *in vitro*, but reports about enhanced *in vivo* gene expression are very limited [15-19].

In this study, we report dramatic enhancement of gene delivery efficiency both *in vitro* and *in vivo* using genetically engineered fusion proteins in a non-viral vector. We designed bifunctional recombinant fusion proteins that incorporated listeriolysin O (LLO), the endosomolytic pore-forming protein from *Listeria monocytogenes*, at the N-terminus, and a DNA condensing peptide fragment of protamine (PN) at the C-terminus. Protamine is a positively charged sperm chromatin component that electrostatically binds to and condenses DNA [20, 21]. The condensation of large, anionic DNA molecules with cationic protamine improves the cellular binding and uptake as well as the protection of DNA from enzymatic degradation in biological environments. Following entry into cells, most of the non-viral vectors are internalized in endocytic compartments and ultimately degraded by hydrolytic enzymes along the endolysosomal pathway [7, 22]. Therefore, promoting endosomal escape of most non-viral vectors confers a significant improvement in transfection efficiency. LLO has several properties that make it an attractive delivery vehicle for macromolecules: (i) LLO can breach the endosomal membrane and promote the cytosolic delivery of whole Listeria bacteria which are much larger than the size of most, if not all, currently studied nano-scale gene delivery vectors; (ii) LLO is most active at the pH of the endosome (5.5-5.9) and attenuated at the neutral pH of the cytosol; (iii) LLO has been shown to be degraded relatively rapidly upon reaching the cytosol, further limiting potential damage to cells [23-25]. Thus, cytosolic delivery of macromolecules with LLO can in principle be achieved with relatively limited

cytotoxicity, especially that which might result from permeabilization of membranes in a pH-neutral environment such as the plasma membrane, Golgi, etc.

Previously we reported increased *in vitro* gene expression using LLO that was chemically conjugated to either protamine or polyethylenimine [26, 27]. Here, we hypothesized that a genetically engineered fusion protein consisting of LLO and a polypeptide fragment of human protamine can be incorporated into a non-viral gene delivery system in order to improve DNA delivery. We designed and expressed fusion proteins with one protamine fragment (LLO-PN) and two protamine fragments (LLO-PNPN), and tested transfection efficiencies of a non-viral gene delivery system containing fusion proteins and protamine in cultured cells.

EXPERIMENTAL PROCEDURES

Construction of LLO-PN and LLO-PNPN fusion proteins

The DNA encoding cathepsin D recognition sequence (RGFFP) [28] and Arg-8 to Ser-29 of human protamine (RSQSRSRYYRQRQRSRRRRRRS) [29], made by annealing two complementary oligonucleotides (IDT) (Figure 2-1B), was inserted into 3'-end of the LLO DNA in the bacterial expression vector pET29b at restriction sites BglII and NotI to ultimately produce a C-terminal protamine followed by a 6×His tag (Figure 2-1A).

In order to insert linkers (GGGGSGGGGS) [30] before and after cathepsin D recognition sequence, a site-directed, ligase-independent mutagenesis (SLIM) method, which involves DNA amplification, denaturation and hybridization resulting in the formation of complementary 5' and 3' single-stranded overhangs, was used as previously described with modifications (Figure 2-2A) [31, 32]. Briefly, four primers were used for amplification: two gene-specific sequences which recognize template, F_S and R_S; two tailed primers F_T and R_T which have identical gene-specific sequences to F_S and R_S, respectively, but also have sequences to be inserted, complementary between F_T and R_T (Figure 2-2B). For amplification, each set of primers (10 pmol each), F_T and R_S or F_S and R_T, was mixed with 100 ng of plasmid template (LLOCDPN/pET29b) in 25 μL solution containing 2.5 μL of 10× PfuUltra HF reaction buffer, 200 μM each dNTP, 1.25U PfuUltra Hotstart DNA polymerase (Stratagene). The reactions were started by heating at 98°C for 1 min, and then PCR reactions were subjected to 25 cycles of 95°C for 30 s, 50°C for 1 min and 72°C for 7 min (1 min per kb), with a final 10 min extension step at 72°C. Plasmid template was then digested by incubation with DpnI (New

England Biolabs) at 37°C for 1 h. Two DpnI digested PCR products were mixed for denaturation and hybridization. The reaction contained 10 μL of each PCR product, 10 μL of 5 × H-Buffer (700 mM NaCl, 125 mM Tris pH 9.0 and 100 mM EDTA, pH 8.0) and water to a final volume of 50 μL. The denaturation was performed at 99°C for 3 min, followed by 3 cycles of 65°C for 5 min and 30°C for 30 min for hybridization. After hybridization, the double-stranded products with complementary overhangs spontaneously circularized, and the circular DNA molecules containing desired sequences were transformed into One Shot® Mach1™-T1 chemically competent *E.coli* (Invitrogen) and gave rise to kanamycin-resistant colonies. The plasmid DNA containing LLO-PN was isolated using a miniprep kit (Qiagen) and the sequence of the resultant construct was verified by DNA sequencing at the University of Michigan Sequencing Core.

In order to construct DNA encoding LLO-PNPN, another oligonucleotide encoding an identical human protamine fragment with restriction sites NotI and XhoI was inserted into the 3'-end of the first protamine DNA (Figure 2-3). The resultant expression construct was transformed into One Shot® Mach1™-T1 chemically competent *E.coli* (Invitrogen), and colonies were grown in LB media. Plasmid DNA was isolated using a miniprep kit (Qiagen) and the fidelity of resultant construct to original design was verified by DNA sequencing.

Expression and purification of fusion proteins

The expression construct containing LLO-PN or LLO-PNPN was transformed into *E.coli* strain BL21(DE3) RIPL (Stratagene). Starting cultures from single colonies

were grown in 50 mL LB media at 37°C overnight with 30 μg/mL kanamycin and 25 μg/mL chloramphenicol. The starting culture was diluted 1:50 into 2 L LB media with 30 μg/mL kanamycin, and incubated at 37°C until the absorbance at 600 nm reached ~ 0.7. The culture was induced at 30°C for 6 h with 1 mM IPTG, and then centrifuged at 6,000 × g for 10 min at 4°C and the bacterial cell pellet was frozen at -80°C until purification. The bacterial pellet was resuspended in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, 200 μM PMSF) and lysed using a French press (Thermo Spectronic). The lysate was centrifuged at 10,000 × g for 40 min and the supernatant was incubated with Ni^{2+}-NTA agarose (Qiagen) for 2 h. The Ni^{2+}-NTA agarose was washed with a total of 400 mL wash buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole) and eluted with wash buffer containing 250 mM imidazole. The fusion proteins were added to PD-10 desalting column (GE Healthcare Life Sciences) for buffer exchange (50 mM sodium phosphate, 300 mM NaCl), and stored in 40% glycerol at -80°C. The expression of the fusion protein was confirmed by SDS-PAGE with Simply Blue (Invitrogen) staining, and protein concentration was determined by a bicinchoninic acid (BCA) protein assay using bovine serum albumin as a standard (Pierce).

Recombinant wild type LLO in the bacterial expression vector pET29b was also expressed in *E.coli* strain BL21(DE3) with a C-terminal six-histidine tag, and purified using Ni^{2+}-NTA agarose. The purity of LLO was determined by SDS-PAGE, concentrations were measured by BCA assay.

Hemolysis assay

The membrane pore-forming activity of LLO-PN or LLO-PNPN was assessed using an *in vitro* red blood cell (RBC) hemolysis assay as previously described [33]. Briefly, RBCs were washed three times with phosphate-buffered saline (PBS, pH 7.4) and resuspended at a concentration of 2×10^8 cells/mL in MBSE (10 mM MES pH 5.5 containing 140 mM NaCl and 1 mM EDTA) with 2 mM DTT. To 100 μL of RBCs, 0 – 100 ng of fusion protein in 100 μL of HEPES-buffered glucose (HBG: 280 mM glucose, 10 mM HEPES, pH 8.4) were added and incubated for 15 min at 37°C. The released hemoglobin from lysed RBCs was measured by absorbance at 450 nm.

Preparation of plasmid DNA for transfection studies

The plasmid DNA pNGVL3 encoding firefly luciferase and green fluorescent protein (GFP) under the control of the cytomegalovirus promoter was a gift from Dr. Gary Nabel (Vaccine Research Center, National Institutes of Health, MD, USA). The plasmid DNA was isolated and purified from *E.coli* using Qiagen Giga Endofree Plasmid Purification kits (Qiagen). Concentrations of plasmid DNA were spectrophotometrically determined using absorbance at 260 nm, and the ratio of absorbance at 260 nm to 280 nm was consistently over 1.8.

Preparation of fusion protein/protamine/DNA complexes

To prepare LLO-PN/protamine/DNA or LLO-PNPN/protamine/DNA complexes, various amounts (0 – 0.6 mole% of protamine) of LLO-PN or LLO-PNPN were mixed with DNA in HBG and incubated for 20 min at room temperature, and then an equal volume of protamine (Salmine, Sigma-Aldrich) in HBG was added at a weight ratio of

1.2 (which corresponds to a positive/negative charge (+/-) ratio of 1.6), and the complexes were further incubated for 20 min. The final concentration of DNA in the complexes was 150 μg/mL.

Transfection studies

The murine macrophage-like cell line P388D1 (ATCC) were plated in 24-well plates at 1.5×10^5 cells per well and incubated in RPMI-1640 containing 10% FBS, antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) and 1 mM sodium pyruvate for 24 h before transfection. Cells were grown at 37°C in a 5% CO_2 humidified atmosphere and were typically ~70% confluent at the time of transfection. For transfection studies, 300 μL of the transfection sample containing 2 μg of DNA in serum-free or 10% serum-containing RPMI 1640 was added dropwise into each well. All experiments were performed using triplicate samples. After a 4 h incubation with cells at 37°C, transfection samples were replaced with fresh complete medium and cells were further incubated for 24 h. Thereafter, the medium was removed and the cells were washed once with PBS. The luciferase gene expression in cells was measured using a luciferase assay kit according to the manufacturer's protocol (Promega). Briefly, cells in each well were lysed with 100 μL of Cell Culture Lysis Buffer (Promega), and lysed cells were transferred to a microcentrifuge tube, vortexed for 10 seconds, and centrifuged at 12,000 × g for 1 min. Then, 20 μL of supernatant was assayed for its luciferase activity with 100 μL of luciferase substrate (Promega) using a BioTek Synergy HT plate reader at 25 °C in luminescence mode. The luciferase activity was expressed as relative luminescence units (RLU) normalized by total cellular protein as determined by BCA assay. Cell viability

was reported as the percentage of cellular protein recovery, determined by BCA assay, after transfection compared to untransfected control cells without DNA.

Statistical analysis

Data were compared by one-way ANOVA and Turkey's post-test, or by two-way ANOVA and Bonferroni post-test. A p-value less than 0.05 was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

RESULTS

Cloning, expression and purification of LLO-protamine fusion proteins

The DNA encoding the human protamine fragment (Arg-8 to Ser-29) was inserted singly or in tandem into the 3'-end of the LLO DNA and subcloned into the bacterial expression vector pET29b, and the sequences were verified by DNA sequencing. His-tagged LLO-PN or LLO-PNPN fusion proteins were expressed in *E. coli* strain BL21(DE3) RIPL, and purified using Ni^{2+}-NTA agarose with a typical yield of 5 mg/L for LLO-PN and 2.5 mg/L for LLO-PNPN. The successful expression and high purity (> 95%) of LLO-PN (molecular weight, 63 kDa) and LLO-PNPN (molecular weight, 66 kDa) fusion proteins were confirmed by SDS-PAGE (Figure 2-4).

Hemolytic activity

The membrane pore-forming activities of LLO-PN and LLO-PNPN fusion proteins were examined by an *in vitro* RBC hemolysis assay. The hemolytic activities of fusion proteins were somewhat reduced compared to that of wild type LLO at low concentrations, but similar at relatively high concentrations (≥ 0.5 ug/mL) and equal at the highest concentration tested (Figure 2-5). The relatively attenuated activities of the fusion proteins at the lower concentrations can be explained by the position of protamine fragment, C-terminus of LLO, in the fusion proteins because the C-terminus of LLO has been implicated in binding to cholesterol-containing membranes for pore formation [23]. The N-terminus protamine fragment-LLO fusion protein showed equal hemolytic activity at a range of concentrations (data shown in Chapter IV).

Effect of LLO-PN fusion protein on transfection efficiency of protamine/DNA complexes

In order to investigate the effect of the LLO-PN fusion proteins on the transfection efficiency of protamine/DNA complexes, 0% - 0.6% of LLO-PN was added to the protamine/DNA complexes, keeping the ratio of protamine to DNA constant at 1.2 (w/w) and 1.6 (+/-). The murine macrophage-like cell line P388D1 was used to test the *in vitro* transfection efficiency of the complexes. The luciferase gene expression with LLO-PN was about twenty to four hundreds fold higher than that of protamine/DNA complexes without fusion proteins under serum-free conditions (Figure 2-6). With increasing amounts of LLO-PN higher than 0.15% of protamine we observed a concomitant increase in cytotoxicity, as indicated by the reduced recovery of total cellular protein after transfection. The effect of changing the ratio of full-length protamine to DNA on gene delivery was also examined with various amounts of protamine (0.1 - 2.4 protamine/DNA (w/w)) and a fixed amount of LLO-PN (0.15%) (Figure 2-7). The addition of LLO-PN fusion protein to pre-condensed protamine/DNA complexes (ratio of protamine/DNA $\leq$ 0.8 (w/w)) enhanced its luciferase gene expression 15- to 180-fold. With even higher ratios of 1.2 and 2.4, which can condense DNA and result in complete retardation in agarose gel retardation assay [26], luciferase gene expression was further improved. The luciferase gene expression at the 2.4 protamine/DNA ratio slightly decreased compared to that with the 1.2/1 weight ratio. To determine the dose-dependency of the complexes, LLO-PN/protamine/DNA complexes were serially diluted from 2 μg to 0.0625 μg DNA/well and transfected into P388D1 cells. Luciferase gene expression was dose-dependently increased, and beyond 0.5 μg

DNA/well the rate of increase was attenuated. Since biological fluid is inevitable *in vivo*, in order to investigate whether the presence of serum affects the transfection efficiency of the complexes, we compared the luciferase gene expression in media with and without 10% serum. As shown in Figure 2-9, in the presence of serum the transfection efficiency of LLO-PN/protamine/DNA was slightly decreased, but still significantly higher than that of protamine/DNA without LLO-PN.

Improved transfection efficiency with LLO-PNPN fusion proteins

In order to investigate the effect of positive charge density of protamine in fusion proteins on DNA delivery, we examined the luciferase gene expression with wild type LLO without protamine fragment. The addition of wild type LLO to protamine/DNA complexes did not produce a dramatic enhancement of *in vitro* transfection efficiency, which was about 10-fold lower than that with LLO-PN/protamine/DNA (Figure 2-10A). We also prepared fusion proteins with one protamine fragment (LLO-PN) and two protamine fragments (LLO-PNPN), and compared the transfection efficiency of protamine/DNA complexes with each fusion protein. While both LLO-PN and LLO-PNPN markedly enhanced the luciferase gene expression of protamine/DNA complexes, the gene expression with LLO-PNPN was three to four-fold more enhanced, compared to that with LLO-PN (Figure 2-10B). In order to further investigate the effect of LLO-PNPN on luciferase gene expression in P388D1 cells, 0% - 0.6% of LLO-PNPN fusion protein was added to the protamine/DNA complexes, keeping the ratio of protamine to DNA constant at 1.2 (w/w) and 1.6 (+/-). The luciferase gene expression with LLO-PNPN was two to four orders of magnitude higher than that of protamine/DNA complexes without fusion protein, and in the presence of 10% serum the transfection

efficiency was significantly higher as well (Figure 2-11). The cytotoxicity was observed with increasing amounts of LLO-PNPN beyond 0.3% which was denoted by the reduced recovery of total cellular protein amount after transfection.

DISCUSSION

Recognizing the importance of a non-viral gene delivery system that contains multiple functional components for the efficient transport of DNA through multiple biological barriers, genetically engineered fusion proteins that consist of diverse functional motifs have recently been studied as potentially effective and relatively safe non-viral vectors. In this report, we designed and purified fusion proteins containing two functional components, LLO and a fragment of human protamine, which can bind to DNA and facilitate its endosomal escape, resulting in an enhanced transfection efficiency of protamine/DNA complexes in cultured cells.

Initially, we designed fusion proteins consisting of full-length protamine at the N-terminus of LLO; these fusion proteins failed to be expressed in our bacterial expression system or in baculovirus expression system in insect cells. One possibility is that the relatively high density of positively charged arginines in the N-terminus of the protamine-LLO fusion protein resulted in aberrant intracellular localization, misfolding or toxic effects. The effects of excessive positively charged amino acids of proteins on their decreased expression or delayed translocation across membrane have been reported [34, 35]. Li *et al*. also reported incorrect folding of an ErbB2-specific single chain antibody fragment and full-length protamine fusion protein [29]. With these concerns, we designed fusion proteins composed of LLO and a truncated form of protamine (PN) at the C-terminus. Both LLO-PN and LLO-PNPN fusion proteins were successfully expressed with relatively high yields, 5 mg/L culture and 2.5 mg/L culture, respectively, while N-terminal protamine fragment-LLO fusion proteins showed decreased expression with a relatively low yield of 0.5 mg/L.

In this study, plasmid DNA was mixed with LLO-PN fusion proteins and then condensed with full-length protamine. It is generally recognized that DNA condensation by positively charged polycations protects DNA from degradation and improves uptake. The degree of condensation by polycations depends on the charge ratio or weight ratio of polycation to DNA. According to Saito *et al*, the electrophoretic retardation of plasmid DNA was proportional to the amount of protamine in agarose gel retardation assays, and complete retardation was observed at 0.8/1 (w/w) protamine/DNA ratio, which suggests the required amount of protamine for DNA condensation [26]. Also, it was reported that the addition of protamine to Her-NLS fusion protein and DNA complexes (DNA:Her-NLS:protamine 1:8:2 charge ratio) showed improved protection of DNA from DNase degradation and transfection efficiency [15].

The LLO-PN fusion proteins used in the current studies can bind to DNA through the positive charges of the protamine fragment, but the amount added was insufficient for condensing the DNA because only relatively small amounts of fusion proteins were needed for the improvement of transfection with minimal toxicity. We therefore added full-length protamine to the fusion protein/DNA complexes to promote further condensation. In Figure 2-7, the transfection efficiencies increased with increasing ratios of protamine to DNA at a fixed amount of LLO-PN, even at 0.1 to 0.6 w/w ratios, which are lower than that required for full DNA condensation, suggesting the efficiency and importance of endosomal escape with LLO-PN fusion proteins. The efficient condensation of DNA with protamine at a 1.2/1 ratio further enhanced the luciferase gene expression. The slight decrease in the transfection efficiency at the ratio of 2.4 may be at least partly explained by the possibility that the association of LLO-PN fusion proteins

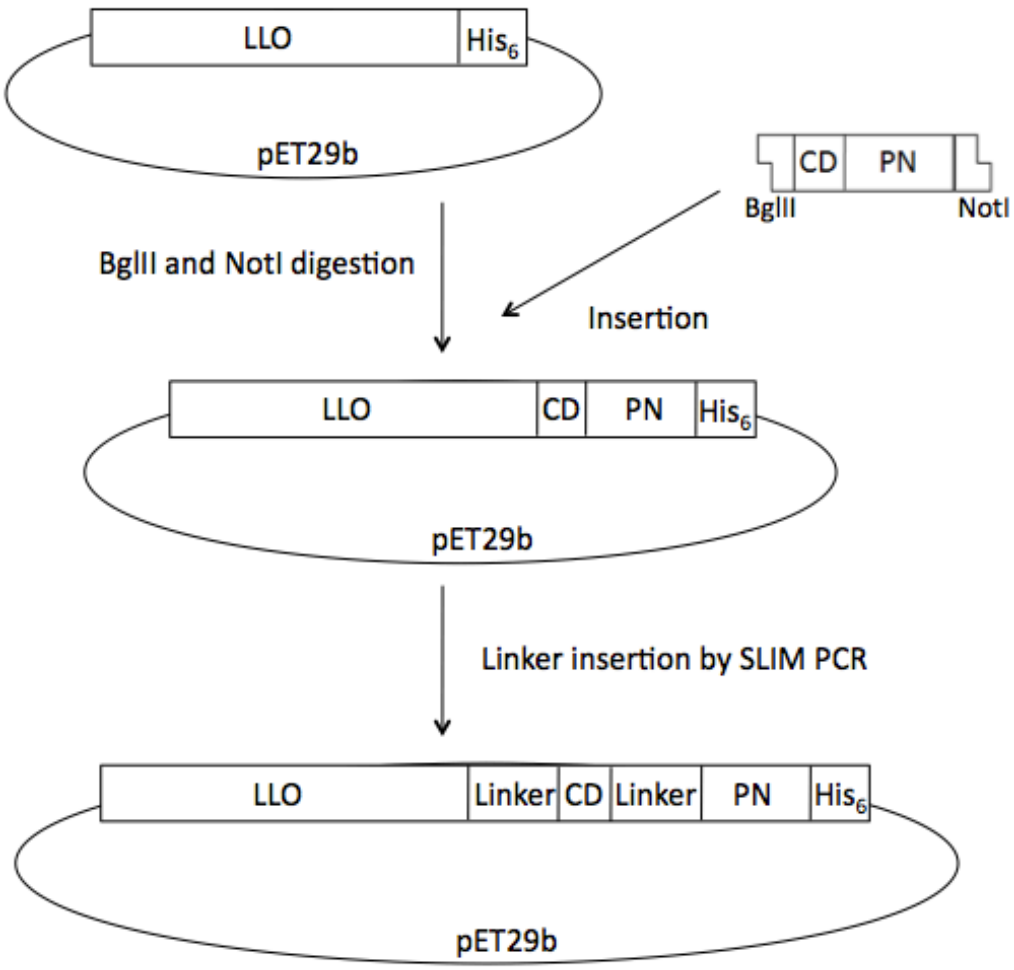
with protamine/DNA complexes decreases in the presence of high amounts of positively charged full-length protamine, since protamine can bind to DNA more tightly than LLO-PN fusion proteins containing a bulky LLO moiety that could interfere with the binding of LLO-PN. This result is consistent with the transfection study of LLO-s-s-protamine/protamine/DNA complexes which showed decreased gene expression beyond the ratio of 0.8 [26].

In addition to the condensation of DNA, in order to optimally deliver DNA to the cytosol, the fusion proteins and DNA should be internalized together by the cells. With the hypothesis that the number and/or density of positive charges in the fusion proteins affect the stability of their interaction with the complexes as well as the efficiency of LLO-mediated endosomal release of DNA, we designed and tested fusion proteins with one protamine fragment, LLO-PN (22 amino acids, 12 arginines) and two protamine fragments, LLO-PNPN (44 amino acids total, 24 arginines). With LLO-PNPN, the luciferase gene expression in P388D1 cells was three to four-fold more enhanced than that with LLO-PN (Figure 2-10B). The addition of wild type LLO without protamine fragment to protamine/DNA complexes did not produce a dramatic enhancement of *in vitro* transfection efficiency compared to that with fusion proteins, which was about 10-fold lower than that with LLO-PN/protamine/DNA (Figure 2-10A). These transfection results support our hypothesis, suggesting that with 12 arginines, LLO-PN can interact with DNA and improve transfection efficiency, but with 24 arginines, the increase in affinity of LLO-PNPN for DNA is sufficient to see an increase in transfection efficiency. *In vivo* luciferase gene expression results, shown in Chapter III, further support our hypothesis.

Previously, we observed an increase in transfection efficiency using LLO disulfide bonded with protamine (LLO-s-s-protamine) or 25 kDa polyethylenimine (LLO-s-s-PEI) [26, 27]. The highest transfection efficiencies were achieved in those studies when 1.2% of LLO-s-s-protamine was incorporated in protamine/DNA complexes, or 1% of LLO-s-s-PEI was used for PEI/DNA complexes, respectively. In this study, only 0.15% of LLO-PNPN was needed for a dramatic increase in luciferase gene expression, which is approximately ten-fold less than the amount that was required with the disulfide conjugates in the previous studies to achieve roughly equivalent transfection levels. LLO has a unique cysteine at amino acid position 484 (C484); oxidation of the sulfhydryl group with a sufficiently bulky moiety abolishes the activity of LLO [25]. The attachment of protamine or PEI via disulfide bond using the sulfhydryl of C484 also reversibly inactivates LLO, and upon reduction of this disulfide inside cells LLO's hemolytic activity is restored. While conjugation of a polycation via a disulfide may be a reasonable strategy for regulating LLO's activity, potential variations in reduction processes between different cell types may result in incomplete reactivation of LLO or differences in the intracellular locale of LLO reactivation [36, 37]. In either case, a relatively higher quantity of LLO-s-s-polycation may therefore be needed to see an improvement in transfection efficiency. However, the LLO-PN fusion proteins have hemolytic activity by themselves and do not require reduction for the restoration of LLO activity. Thus, relatively small amounts of fusion proteins (0.015 - 0.6 %) were sufficient for the improvement of transfection efficiency with minimal toxicity, although some cytotoxicity was observed at higher ranges of doses (Figure 2-6 & 2-11). The 0.15% of LLO-PNPN corresponds to approximately two LLO-PNPN molecules per 7kbp plasmid

DNA. The family of cholesterol-dependent pore-forming cytolysins to which LLO belongs generally requires 33 – 50 monomers per pore [23]. Perales *et al.*, have calculated that each polycation/DNA complex having an average diameter of 50 – 200 nm contains from 5 to 20 plasmid DNA molecules [38]. If each LLO-PNPN/protamine/DNA complex with an average diameter of 150 – 200 nm contains 15 – 20 plasmid DNA molecules, and approximately 30 – 40 LLO-PNPN molecules are in each protamine/DNA complex, then 0.15% LLO-PNPN is theoretically enough for pore formation in endosomal membranes as well.

In conclusion, we have demonstrated that the incorporation of a recombinant LLO-protamine fusion protein in protamine/DNA complexes dramatically enhances their gene transfection efficiency in cultured cells. The enhanced transfection efficiency with the fusion proteins in the presence of 10% serum suggests the potential ability of this gene delivery vector to transfer DNA in *in vivo* systems.



CD: Cathepsin D cleavable linker, RGFFP

PN: Human protamine fragment, RSQSRSRYYRQRQRSRRRRRRS

Linker: GGGGSGGGGS

Figure 2-1A. Construct map of LLO-PN fusion protein.

Oligonucleotides encoding cathepsin D-recognition sequence (CD) and human protamine fragment (PN) were inserted into 3'-end of LLO at restriction sites BglII and NotI. The DNA encoding the linker sequence was inserted before and after cathepsin D-recognition sequence by SLIM PCR.

Forward oligonucleotide: 5'-GATCTGCGTGGTTTCTTTCCGCGTTCTCAATCT CGATCAAGATATTACCGTCAACGCCAGCGATCTCGTCGGAGACGCCGTAGG TCAGC-3'

Reverse oligonucleotide: 5'-GGCCGCTGACCTACGGCGTCTCCGACGAGA TCGCTGGCGTTGACGGTAATATCTTGATCGAGATTGAGAACGCGGAAAGAA ACCACGCA-3'

Figure 2-1B. DNA sequences of cathepsin D recognition sequence and the first protamine fragment with two ligated joints of BglII and NotI.

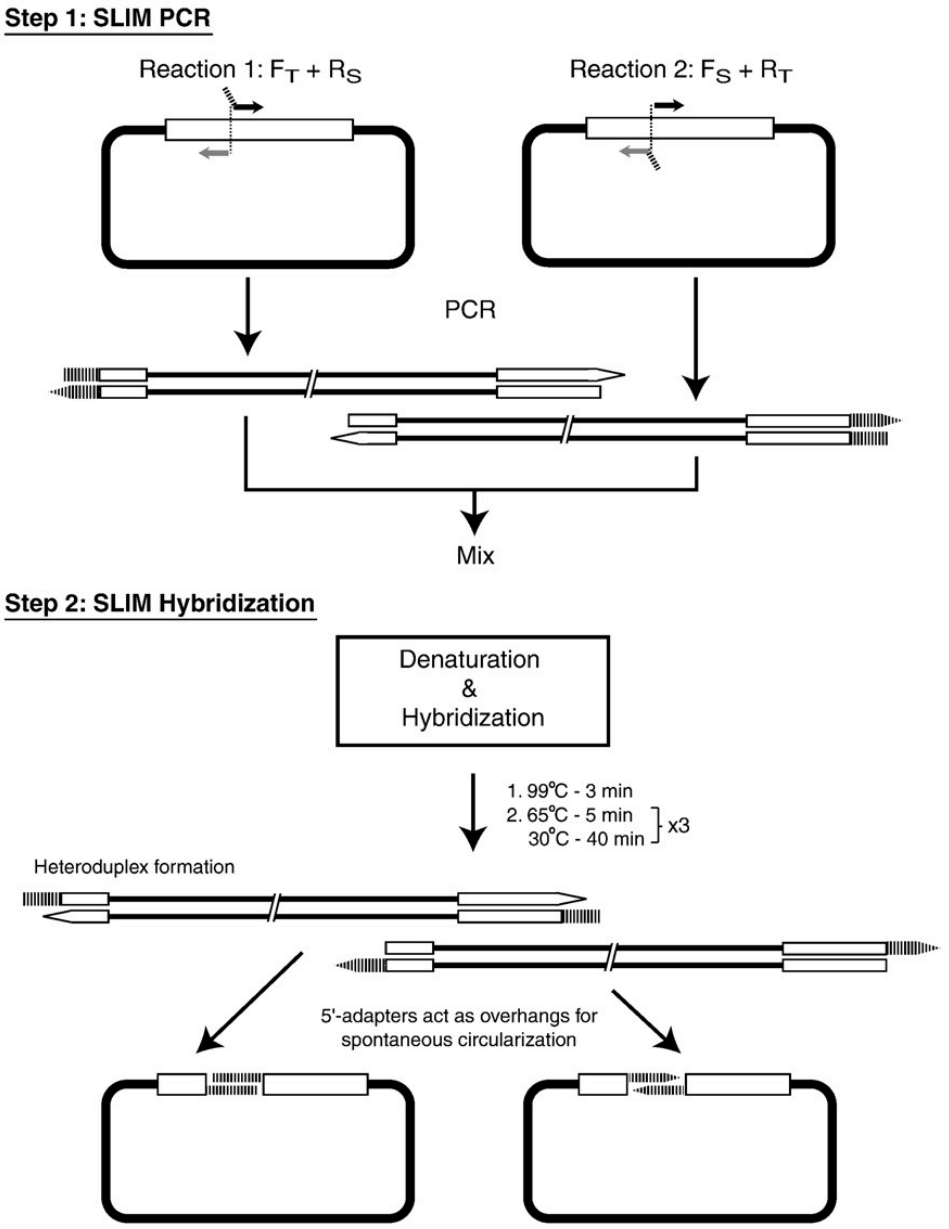


Figure 2-2A. Schematic of two-reaction SLIM PCR; adapted from Chiu J. *et al*., J. Microbiol. Methods. 73 (2008) 195-198.

Forward and reverse primers are in gray and black, respectively. The hashed line is the 5'-adapter of the tailed primer, which encodes for the inserted linker sequences. The gene-specific portion of each primer is represented by an arrow. The open box denotes the gene targeted for insertion, LLOCDPN.

A.

F_T: 5'-**GGCGGAGGCGGTTCAGGAGGTGGCGGAAGT**CGTGGTTTCTTTCCGC

GTTCTC-3'

F_S: 5'-CGTGGTTTCTTTCCGCGTTCTC-3'

R_T: 5'-**ACTTCCGCCACCTCCTGAACCGCCTCCGCC**CAGATCTCCTTCGATTG

GATTATCTACTTT-3'

R_S: 5'-CAGATCTCCTTCGATTGGATTATCTACTTT-3'

B.

F_T: 5'-**GGTGGCGGAGGCAGCGGAGGCGGTGGCTCT**CGTTCTCAATCTCGAT

CAAGATATTACCGTC-3'

F_S: 5'-CGTTCTCAATCTCGATCAAGATATTACCGTC-3'

R_T: 5'-**AGAGCCACCGCCTCCGCTGCCTCCGCCACC**CGGAAAGAAACCACGA

CTTCCGCCA-3'

R_S: 5'-CGGAAAGAAACCACGACTTCCGCCA-3'

Figure 2-2B. DNA sequences of primers used for SLIM PCR.

F_T and R_T are tailed forward and reverse primers. F_S and R_S are short forward and reverse primers. Inserted linker sequences denoted in bold. A: Primers for linker insertion between LLO and cathepsin D recognition sequence. B: Primers for linker insertion between cathepsin D recognition sequence and protamine fragment.

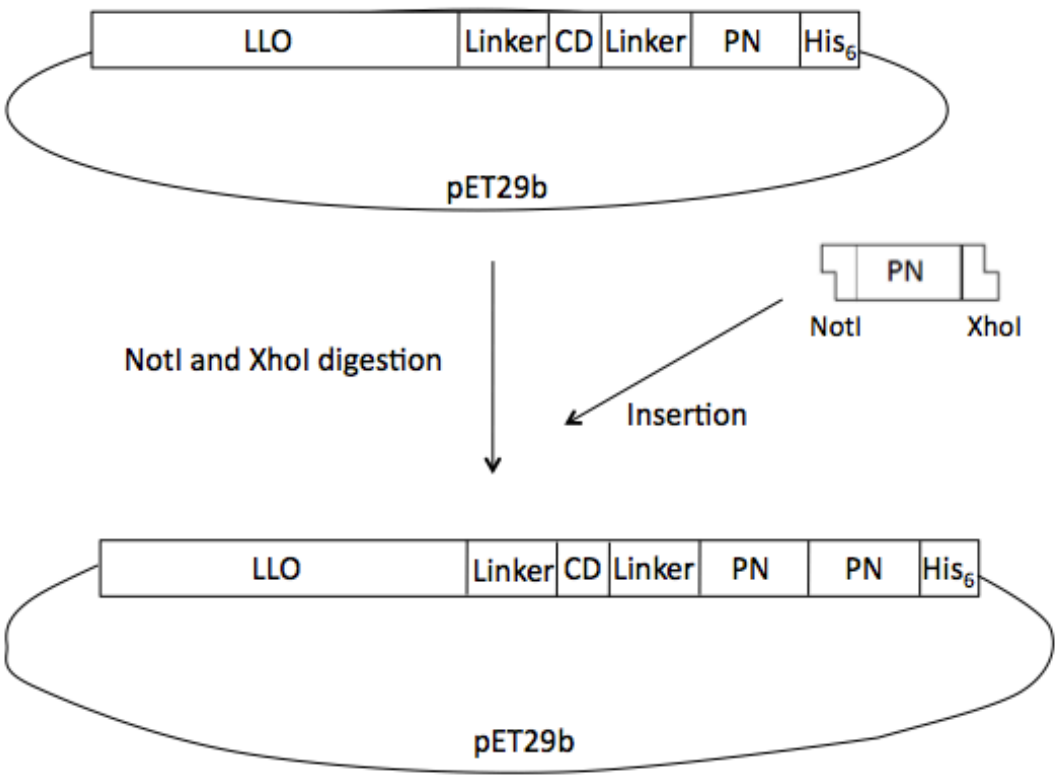


Figure 2-3A. Construct map of LLO-PNPN fusion protein.

Oligonucleotides encoding human protamine fragment were inserted into 3'-end of the first protamine fragment with restriction sites of NotI and XhoI.

Forward oligonucleotide: 5’-GGCCGCACGTTCTCAATCTCGATCAAGATATTAC

CGTCAACGCCAGCGATCTCGTCGGAGACGCCGTAGGTCAC-3’

Reverse oligonucleotide: 5’-TCGAGTGACCTACGGCGTCTCCGACGAGATCG

CTGGCGTTGACGGTAATATCTTGATCGAGATTGAGAACGTGC-3’

Figure 2-3B. DNA sequences of the second protamine fragment with two ligated joints of NotI and XhoI.

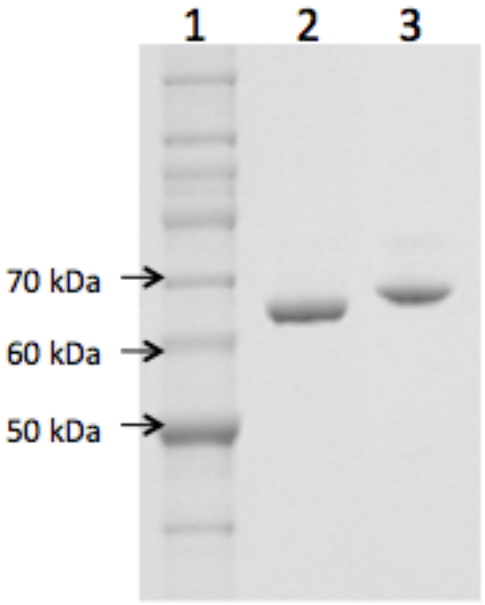


Figure 2-4. SDS-PAGE of expressed and purified LLO-PN and LLO-PNPN fusion proteins.

Lane 1: protein molecular weight standards; lane 2: LLO-PN (63 kDa); lane 3: LLO-PNPN (66 kDa)

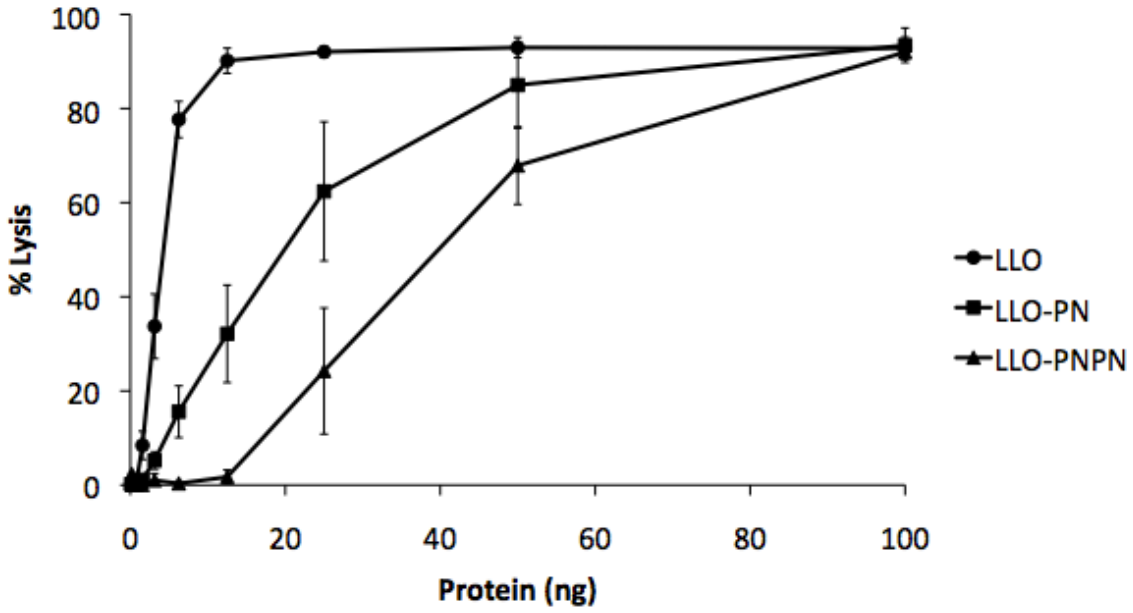


Figure 2-5. Hemolytic activities of LLO, LLO-PN and LLO-PNPN.

The fusion proteins were assayed for their ability to perforate membranes by monitoring lysis of RBCs. Various amounts of LLO or fusion proteins were incubated with RBCs at 37°C for 15 min, and the release of hemoglobin from lysed RBCs was monitored by the absorbance at 450 nm. Protamine alone, without LLO, at comparable amounts did not show any detectable hemolytic activity (not shown in the figure). (n=2, mean ± SD)

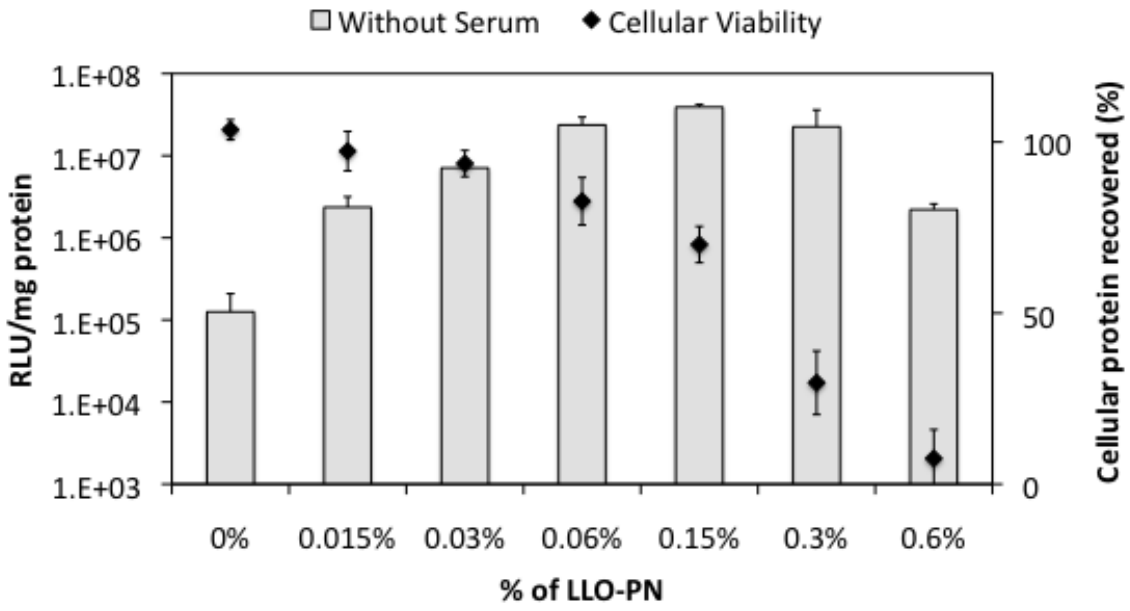


Figure 2-6. Effect of various amounts of LLO-PN on transfection efficiency and viability in P388D1 cells.

Increasing amounts of LLO-PN were mixed with protamine/DNA complexes while maintaining a 1.6/1 (+/-) charge ratio. P388D1 cells were incubated with the complexes (2 μg DNA/well) in the absence of serum. Luciferase activity of cell lysates was determined 24 h after transfection. Dots indicate the average ± SD total cellular protein recovered after transfection. (n=3, mean ± SD)

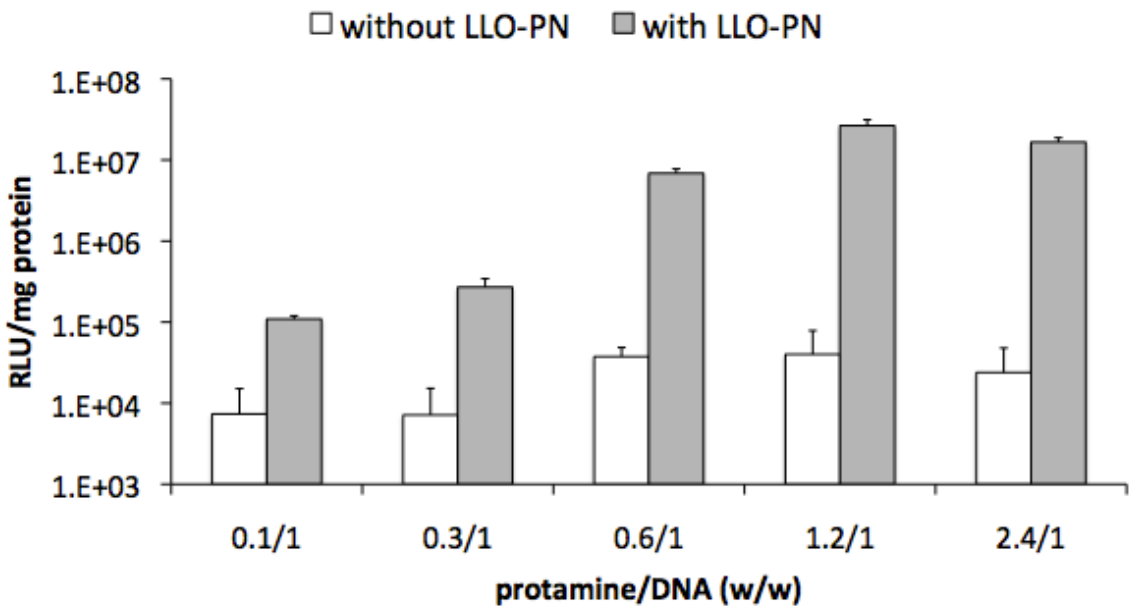


Figure 2-7. Effect of LLO-PN on transfection efficiency at various protamine/DNA weight ratios.

Grey bars: LLO-PN/protamine/DNA complexes with fixed amount of LLO-PN (0.15%) and increasing amount of protamine were incubated with P388D1 cells, and 24 h after transfection luciferase gene expression was measured. Open bars: Control luciferase gene expression of protamine/DNA complexes with increasing amount of protamine, without LLO-PN. (n=3, mean ± SD)

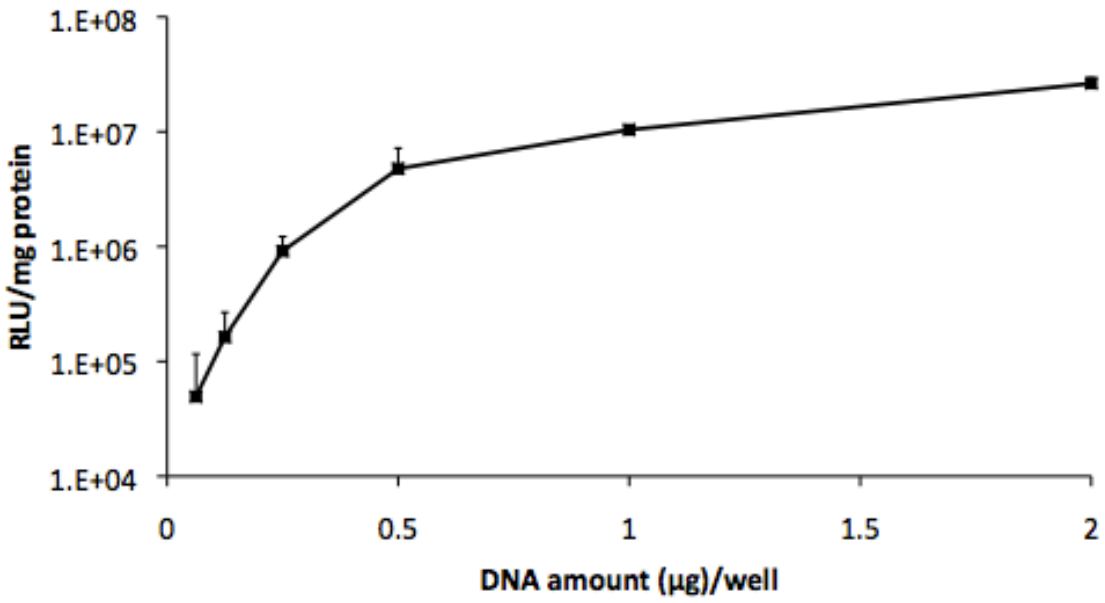


Figure 2-8. Effect of DNA dose on transfection efficiency.

Protamine/DNA complexes (1.2/1 (w/w)) with 0.15% of LLO-PN were serially diluted from 2 µg to 0.0625 µg of DNA/well. Luciferase activity was determined 24 h after transfection. (n=3, mean ± SD)

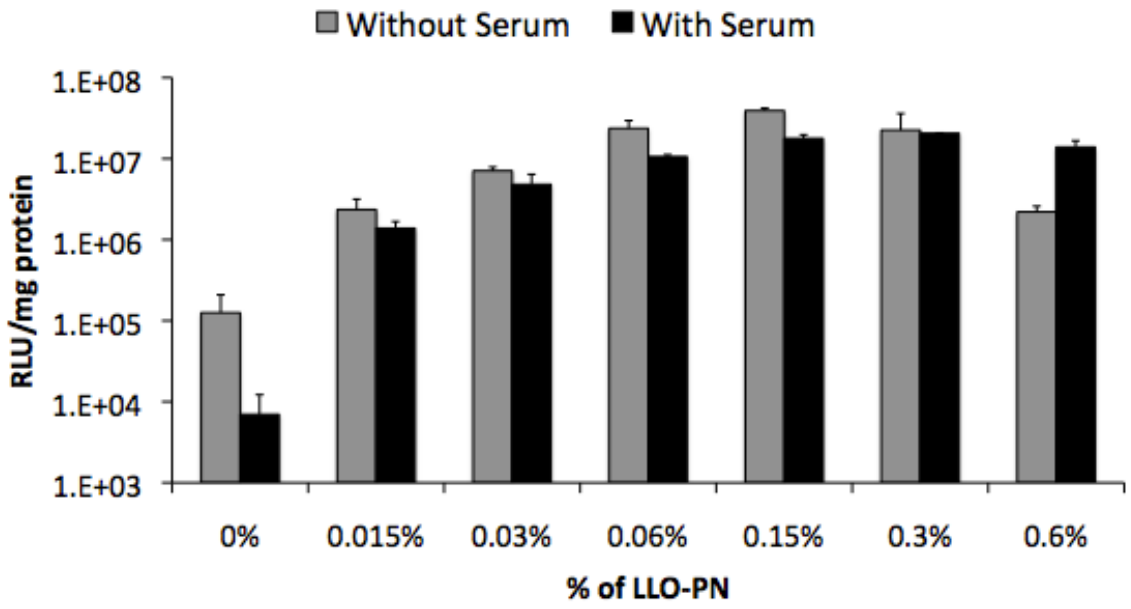


Figure 2-9. Effect of serum on transfection efficiency of LLO-PN/protamine/DNA.

LLO-PN/protamine/DNA complexes with varying amounts of LLO-PN were incubated with P388D1 cells in the absence or presence of 10% FBS. Luciferase activity was analyzed 24 h after transfection. (n=3, mean ± SD)

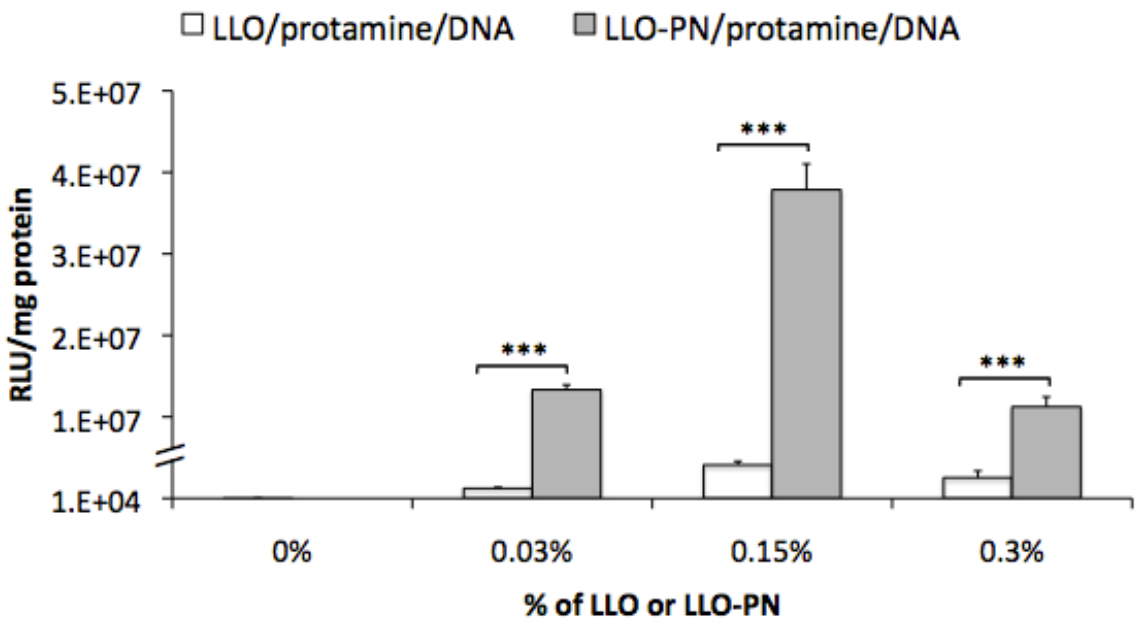


Figure 2-10A. Effect of protamine fragment on transfection efficiency: LLO vs. LLO-PN fusion protein.

Wild type LLO or fusion protein LLO-PN was mixed with protamine/DNA complexes and transfected into P388D1 cells in serum-free medium, and luciferase gene expression was determined one day after transfection. (***$p < 0.001$) (n=3, mean ± SD)

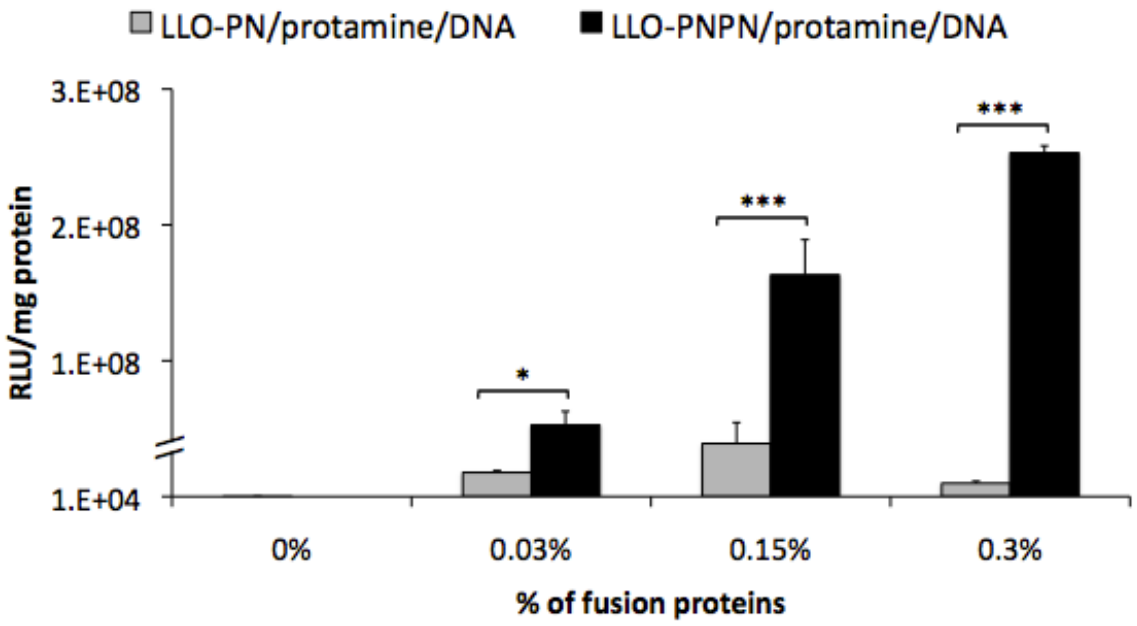


Figure 2-10B. Effect of protamine fragment on transfection efficiency: LLO-PN vs. LLO-PNPN fusion protein.

Protamine/complexes were mixed with various amounts of LLO-PN or LLO-PNPN fusion proteins and incubated with P388D1 cells in serum-free medium. Luciferase gene expression was determined one day after transfection. (*$p < 0.05$, ***$p < 0.001$) (n=3, mean ± SD)

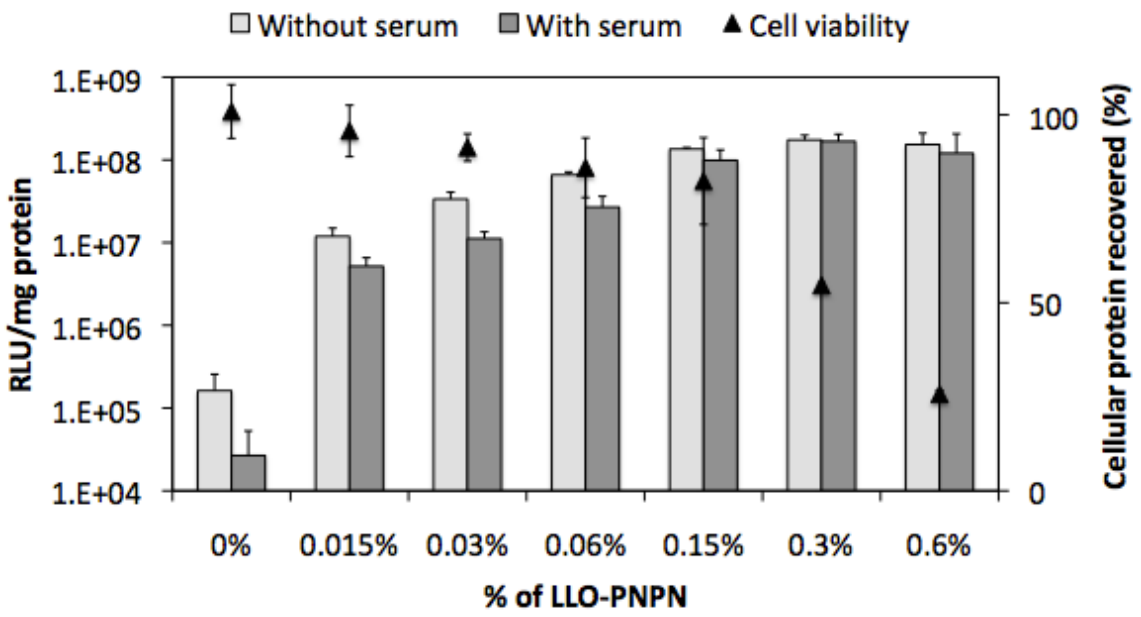


Figure 2-11. Effect of various amounts of LLO-PNPN on transfection efficiency and cell viability in P388D1 cells.

Increasing amounts (0-0.6%) of LLO-PNPN were mixed with protamine/DNA complexes while maintaining a 1.6/1 (+/-) charge ratio. P388D1 cells were incubated with the complexes (2 μg DNA/well) in the absence or presence of serum. Luciferase activity of cell lysates was determined 24 h after transfection. Dots indicate the average ± SD total cellular protein recovered after transfection. (n=3, mean ± SD)

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CHAPTER III

IN VIVO LUCIFERASE GENE EXPRESSION AND APPLICATION OF LLO-PROTAMINE FUSION PROTEIN-INCORPORATED NON-VIRAL VECTOR TO DNA VACCINE

SUMMARY

We recently showed the enhanced gene transfection efficiency of protamine/DNA complexes incorporating recombinant fusion protein consisting of listeriolysin O (LLO), the endosomolytic pore-forming protein from *Listeria monocytogenes*, and a fragment of the DNA binding protein protamine in a murine macrophage-like cell line. In this chapter, we investigated the delivery capability of this gene delivery system *in vivo* and evaluated its potential applicability as a DNA-based vaccine in mice. By the association of positively charged complex LLO-PNPN/protamine/DNA with anionic liposomes composed of PE and CHEMS, the net surface charge of the vector was changed to negative, which can reduce non-specific interactions with negatively charged plasma proteins. The transfection efficiency of Liposome(LLO-PNPN/protamine/DNA) was improved in mice, showing the highest luciferase gene expression in spleen, as well as in cultured cells with serum compared to LLO-PNPN/protamine/DNA. The immune responses after subcutaneous injection into mice were examined by measuring cytotoxic T lymphocyte induction levels and antibody response, and Liposome(LLO-

PNPN/protamine/DNA) showed only weak enhancement of OVA-specific antibody response, suggesting the need for further modification and characterization of this vector for utility as a DNA vaccine. However, the improved *in vivo* gene delivery with our system suggests its potential promise as a candidate gene delivery system for clinical application.

INTRODUCTION

Efficient gene delivery in *in vivo* systems is a requirement for the clinical application of non-viral gene delivery. Cationic polymers or liposomes have been widely studied as relatively efficient non-viral vectors *in vitro* and *in vivo* [1, 2]. However, the *in vivo* instability and toxicity of cationic complexes limit their *in vivo* applicability [3, 4]. While cationic polyplexes or lipoplexes can efficiently condense and protect DNA from degradation, their cationic surfaces non-specifically interact with negatively charged plasma proteins and extracellular matrix components, resulting in size and surface charge changes, dissociation of the complex, or formation of large aggregates [5, 6]. The aggregation induces rapid clearance of the vector by RES, uptake into the lung and adverse inflammatory reactions [7, 8]. Therefore, in order to obtain effective gene transfer under *in vivo* conditions, there have been studies involving shielding the surface positive charges while maintaining the advantages of cationic polymers or liposomes. For example, after PEGylation to shield the surface positive charges of lipoplexes (DOTAP-DOPE/DNA complex), the *in vivo* antigen expression was improved through the reduction of the electrostatic interactions with negatively charged extracellular matrix components such as proteoglycans and hyaluronic acid after intradermal administration [9].

Anionic liposomes have also been used as an effective alternative to reduce non-specific interactions with negatively charged biological components [10]. For the preparation of anionic liposome formulation, DNA is mostly pre-condensed with polycations for efficient condensation, and then associated with anionic liposomes. LPDII (anionic liposome-polycation-DNA complexes) composed of polycation (such as

polylysine or protamine)-condensed DNA complexed with anionic liposomes such as DOPE/CHEMS, DOPE/PS/CHOL or diolein/CHEMS, showed higher transfection efficiency and less toxicity than cationic liposome formulations, and DNA was better protected from DNase I degradation [11-13]. Also, it has been reported that the transfection efficiency of LPDII using diolein/CHEMS was retained in 50% serum containing media [12]. A combination of coating cationic polymer, poly(2-(dimethylethylamine)ethyl methacrylate); p(DMAEMA)) and DNA polyplex with anionic lipids (EPC/EPG/CHOL) was protected from destabilization [14]. Furthermore, the transfection efficiency was not affected by a polyanion such as hyaluronic acid, which is a component in tumor ascitic fluid that has been shown to induce inactivation of cationic polyplexes [14].

Previously, we designed a non-viral gene delivery system consisting of protamine/DNA complexes incorporating LLO-protamine fusion proteins, and demonstrated the enhancement of luciferase gene transfer efficiency in cultured cells. Here, we associated cationic LLO-PNPN/protamine/DNA complexes with pH-sensitive anionic liposomes composed of phosphatidylethanolamine (PE) and cholesteryl hemisuccinate (CHEMS) in order to reduce potential non-specific interactions with negatively charged plasma proteins. We tested the hypothesis that changing the net surface charge to negative gives better transfection efficiency in cultured cells containing serum and in animal models.

In addition to *in vivo* luciferase gene delivery efficiency, we further investigated the potential of our gene delivery system for clinical application. DNA-based vaccination is one of the more promising applications against infectious disease or cancer

among the DNA-based therapies. DNA-based vaccines have advantages such as induction of both cellular and humoral (antibody) immune responses, safety, stability, cost-effectiveness, and the possibility to delivery multiple epitopes or antigens by recombinant DNA design [15-17]. Since Ulmer *et al.* demonstrated that the plasmid DNA encoding influenza nucleoprotein A induced cellular and humoral immune responses following intramuscular injection in mice, DNA-based vaccines have been intensively studied with significant progress in therapeutic and preventive immunity against infections and cancers [16, 18]. However, the success in small animals was not transferred to human clinical trials because of less potency. In order to improve the immunogenicity of DNA-based vaccines, various approaches have been researched including modification of plasmid DNA design, use of adjuvants, and development of delivery system [19, 20].

Although intramuscular injection, gene gun-mediated delivery or electroporation of naked DNA have been shown to induce immune responses, in order to enhance DNA delivery efficiency and stability *in vivo* and improve immune responses, DNA-based vaccines using delivery vectors such as liposomes and polymers have been studied [16, 20]. We previously reported the enhanced antigen-specific CD8+ T cell response by LPDII containing LLO using a DNA-prime and protein-boost protocol [21]. Here, we tested whether the gene delivery system incorporating recombinant fusion protein, LLO-PNPN/protamine/DNA complexes associated with liposomes, can be clinically applied for DNA-based vaccination, not necessarily for the best DNA-based vaccine delivery system.

In this study, we demonstrate the enhanced *in vitro* and *in vivo* luciferase gene expression of LLO-PNPN/protamine/DNA complexes associated with anionic liposomes and immune response in mice following its application as a DNA-based vaccine.

EXPERIMENTAL PROCEDURES

Preparation of plasmid DNA

The plasmid DNA pNGVL3 encoding firefly luciferase and green fluorescent protein (GFP) under the control of the cytomegalovirus promoter was a gift from Dr. Gary Nabel (Vaccine Research Center, National Institutes of Health, MD, USA). The ovalbumin (OVA) DNA was kindly provided by Dr. Kenneth Rock at the University of Massachusetts Medical School. The plasmid DNA pNGVL3 encoding OVA was previously constructed by replacing the luciferase gene at the restriction sites EcoRI and XbaI in the Lee lab [21]. Both plasmids were isolated and purified from *E.coli* using Qiagen Giga Endofree Plasmid Purification kits (Qiagen). Concentrations of plasmid DNA were spectrophotometrically determined using absorbance at 260 nm, and the ratio of absorbance at 260 nm to 280 nm was consistently over 1.8.

Preparation of Liposome(LLO-PNPN/protamine/DNA)

To prepare LLO-PNPN/protamine/DNA complexes, 0.15% of LLO-PNPN were mixed with DNA in HBG and incubated for 20 min at room temperature, and then an equal volume of protamine (Salmine, Sigma-Aldrich) in HBG was added at a weight ratio of 1.2 (which corresponds to positive/negative charge (+/-) ratio of 1.6), and the complexes were further incubated for 20 min. The final concentration of DNA in the complexes was 150 μg/mL. In order to prepare complexes associated with negatively charged liposomes, the thin lipid film composed of phosphatidylethanolamine (PE) (Avanti Polar Lipids) and cholesteryl hemisuccinate (CHEMS) (Sigma-Aldrich) was prepared. PE dissolved in chloroform and CHEMS dissolved in chloroform/methanol

(1/1) were mixed at 2:1 molar ratio and dried to a thin film using a Büchi Rotavapor R-200 rotary evaporator at 25°C under vacuum. The lipid film was hydrated with LLO-PNPN/protamine/DNA complexes by vortexing and sonicating for 30 seconds twice in a bath-type sonicator (Laboratory Supplies Co., Inc.). For each 1 μg of DNA, 2.25 nmol – 11.25 nmol of lipid were used. For the heat-inactivated negative controls, half of the samples were heated at 75°C for 10 min after complex formation or liposome association.

***In vitro* transfection study**

The murine macrophage-like cell line P388D1 (ATCC) were plated in 24-well plates at 1.5×10^5 cells per well and incubated in RPMI-1640 containing 10% FBS, antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) and 1 mM sodium pyruvate for 24 h before transfection. Cells were grown at 37°C in a 5% CO_2 humidified atmosphere and were typically ~70% confluent at the time of transfection. For transfection studies, 300 μL of the transfection sample containing 2 μg of DNA in serum-free or 10% serum-containing RPMI 1640 was added dropwise into each well. Lipofectamine™ 2000 (Invitrogen) was used as a positive control. All experiments were performed using triplicate samples. After a 4 h incubation with cells at 37°C, transfection samples were replaced with fresh complete medium and cells were further incubated for 24 h. Thereafter, the medium was removed and the cells were washed once with phosphate-buffered saline (PBS). The luciferase gene expression in cells was measured using a luciferase assay kit according to the manufacturer's protocol (Promega). Briefly, cells in each well were lysed with 100 μL of Cell Culture Lysis Buffer (Promega), and lysed cells were transferred to a microcentrifuge tube, vortexed for 10 seconds, and centrifuged at 12,000 × g for 1 min. Then, 20 μL of supernatant was

assayed for its luciferase activity with 100 μL of luciferase substrate (Promega) using a BioTek Synergy HT plate reader at 25 °C in luminescence mode. The luciferase activity was expressed as relative luminescence units (RLU) normalized by total cellular protein as determined by BCA assay. Cell viability was reported as the percentage of cellular protein recovery, determined by BCA assay, after transfection compared to untransfected control cells without DNA.

***In vivo* transfection study**

Female C57BL/6 mice, 6-7 weeks old, were obtained from Harlan Laboratories (Indianapolis, IN, USA). The mice were kept in filter-topped cages with freely available standard food and water and a 12 h light/dark cycle. The experiment protocols were reviewed and approved by the University Committee on Use and Care of Animals at University of Michigan (UCUCA).

Mice in groups of six were intraveneously injected via tail vein with 50 μg of DNA per mouse formulated as LLO-PNPN/protamine/DNA, Liposome(LLO-PNPN/protamine/DNA), heat-inactivated LLO-PNPN/protamine/DNA, heat-inactivated Liposome(LLO-PNPN/protamine/DNA) or HBG buffer only. Mice were sacrificed after 24 h and spleens, lungs and livers were harvested, washed with PBS twice at 4°C, and homogenized with Cell Culture Lysis Buffer (Promega). The homogenates were centrifuged at 12,000 × g for 10 min at 4°C, and 20 μL of supernatant was assayed for luciferase activity as described above. The total protein concentrations of tissue lysates were determined by BCA assay. The results of the luciferase expression in mice were reported as RLU (from which the RLU of buffer-injected control group was subtracted) per mg total tissue protein.

Preparation of liposomes containing LLO and OVA proteins

Liposomes containing LLO and OVA proteins were prepared as previously described [22]. Recombinant wild type LLO in the bacterial expression vector pET29b was expressed in *E.coli* strain BL21(DE3) with a C-terminal six-histidine tag, and purified using Ni^{2+}-NTA agarose. The purity of LLO was determined by SDS-PAGE, concentrations were measured by BCA assay, and the pore forming activity of LLO was monitored by hemolysis assay [23]. The purified LLO and OVA (Sigma-Aldrich) were encapsulated into PE/CHEMS liposomes (2/1 molar ratio) at 20 mg/mL and 0.25 mg/mL, respectively. Unencapsulated LLO and OVA were removed by Sepharose CL-4B gel filtration (GE Healthcare), and liposome fractions containing LLO and OVA were collected. The amount of encapsulated LLO and OVA proteins was determined by quantitative SDS-PAGE with Krypton fluorescence protein stain (Thermo Scientific) and quantified using ImageQuant software after visualization with a Typhoon 9200 fluorescence scanner (GE Hearthcare).

Immunization of mice

For DNA-prime and protein-boost immunization study, groups of six female C57BL/6 mice (7-8 weeks old) were subcutaneously injected with HBG buffer, Buffer; heat-inactivated Liposome(LLO-PNPN/protamine/DNA), HI-Liposome; Liposome(LLO-PNPN/protamine/DNA), Liposome; or protein formulation Liposome(LLO, OVA), Protein on day 0. On day 12, all four groups were boosted with protein formulation, Liposome(LLO, OVA) subcutaneously. The amount of OVA-DNA or OVA protein injected per mouse was 50 μg as previously described [21]. On day 24, mice were

sacrificed for the *in vivo* cytotoxic T lymphocyte (CTL) assay and interferon-γ secretion assay, and blood was collected through cardiac puncture for antibody response assay.

For DNA-prime and -boost study, mice (5-6 mice/group) were immunized with HBG buffer, HI-Liposome, Liposome or naked DNA on day 0 and day 7 (50 μg DNA/mouse). On day 14, mice were sacrificed for enzyme-linked immunosorbent spot (ELISPOT) assay, and blood was collected through cardiac puncture for antibody response assay.

In vivo CTL assay

Antigen-specific CD8+ T cells cytotoxic activity was determined by *in vivo* CTL assay as previously described by some modification [21, 24]. Splenocytes from naïve C57BL/6 mice were harvested and ground through a 70 μm mesh screen in a Petri dish containing mouse media (RPMI 1740, 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 55 μM 2-mercaptoethanol) to form a single cell suspension. RBCs were lysed using ACK lysing buffer (Invitrogen) 1mL/spleen. Then, the splenocytes (2×10^7 cells/mL) were pulsed with either 10 μg/mL of OVA peptide (SINFEKL, amino acids 257-264) (AnaSpec) as a specific peptide or NP (influenza nuclear protein) peptide (TYQRTRALV, amino acids 147-155) as a control at 37°C for 1 h. Each cell population was washed with mouse media and adjusted to 1×10^8 cells/mL in PBS containing 0.1% FBS. The OVA peptide-pulsed and NP peptide-pulsed populations were labeled with 4 μM or 0.4 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen), respectively for 10 min at RT. CFSE was diluted from 50 μM stock to 5 μM in PBS, then added to the cells while vortexing to achieve the desired final concentration. CFSE labeling was stopped by the addition of an equal volume of cold

FBS and incubation for 5 min on ice. Cells were washed three times with PBS containing 5% FBS, counted and diluted to 1×10^7 cells/mL in PBS. Equal numbers of OVA peptide-pulsed target cells ($CFSE^{high}$) and NP peptide-pulsed cells ($CFSE^{low}$) were mixed together, and 200 μL (1 ×106 cells/population) were injected intravenously via tail vein into naïve or immunized mice. At 18h after injection, mice were euthanized and spleens were harvested and ground through a 70 μm mesh screen in a Petri dish containing mouse media to obtain a single cell suspension. After washing with mouse media, splenocytes were resuspended in 5 mL of Leibovitz's L-15 media and placed in a 15 mL conical tube. Ficoll-Paque Premium, 5mL (GE Healthcare) was carefully added to the bottom of the tube, and centrifuged at 800 × g for 20 min with brake off to form a gradient. RBCs were pelleted and the lymphocyte layer was collected for analysis using FACSCalibur (BD Sciences). Ten thousand viable CFSE-labeled cells were analyzed for each mouse and the two populations $CFSE^{high}$ and $CFSE^{low}$ were distinguished by the differences in CFSE intensity. The percentage of specific lysis for each mouse was calculated as follows: 100×[1-(ratio of $CFSE^{high}$/$CFSE^{low}$ cells recovered from immunized mice/ratio of $CFSE^{high}$/$CFSE^{low}$ cells recovered from naïve mice)].

OVA-specific IFN-γ secretion assay

Splenocytes (5×10^5 cells/well) from the immunized mice were incubated in 96-well tissue culture plates in triplicate containing mouse media alone or mouse media containing NP peptide (5 μM, final), OVA peptide (5 μM, final), OVA protein (50 μg/mL, final) or the T cell mitogen concanavalin A (ConA, 5 μg/mL) for 72 h at 37°C. The supernatants of splenocytes from a given mouse and treatment were combined and analyzed for IFN-γ secretion by enzyme-linked immunosorbent assay (ELISA)

performed in duplicate. ELISA plates were coated with purified rat anti-mouse IFN-γ capture antibody (BD Biosciences), and supernatants were incubated with the coated plates. The IFN-γ from the supernatant was detected with biotinylated rat anti-mouse IFN-γ (BD Biosciences), followed by streptavidin-horseradish peroxidase (HRP), and then developed with SureBlue TMB 1-Component Microwell Peroxidase Substrate (KPL). The reaction was stopped using 2 N sulfuric acid, and the absorbance at 450 nm was measured using a plate reader (Molecular Devices Emax). IFN-γ concentrations were calculated based on the recombinant mouse IFN-γ standards.

Anti-OVA antibody response assay

Anti-OVA antibody response was measured by enzyme-linked immunosorbent assay (ELISA) in duplicate. ELISA plates were coated with 10 μg/mL OVA proteins in 0.1 M sodium phosphate pH 9.0 for overnight at 4°C. Plates were washed with PBS containing 0.05% Tween 20 (PBST), and blocked with PBS containing 1% BSA for overnight at 4°C. The plates were washed, and sera was added to the plates and serially diluted down the columns in PBST containing 1% BSA. OVA-specific antibody was detected with goat anti-mouse IgG $(Fab')_2$-biotin (Sigma-Aldrich), followed by streptavidin-HRP. The absorbance values were measured at 450 nm after developing with SureBlue TMB 1-Component Microwell Peroxidase.

ELISPOT assay

The frequency of OVA-specific IFN-γ secreting cells was determined using the IFN-γ ELISPOT Mouse Set (BD Biosciences) following the manufacturer's instructions. Briefly, ELISPOT plates were coated with 5 μg/mL of anti-mouse IFN-γ capture

antibody in PBS for overnight at 4°C, then washed and blocked with mouse media for 2 h at room temperature. Splenocytes from immunized mice (5×10^5 cells/well) were added to the plates in mouse media containing either media alone, NP peptide (2.5 μg/mL), OVA peptide (2.5 μg/mL), OVA protein (50 μg/mL) or the ConA (1.25 μg/mL) for 18 h at 37°C. The plates were extensively washed with MilliQ water to lyse cells, then with PBST using the ELX405 plate washer (BioTek). The plates were incubated with 2 μg/mL biotinylated anti-mouse IFN-γ detection antibody for 2 h at room temperature. After washing with PBST using the plate washer, the plates were incubated with 5 μg/mL streptavidin-HRP for 1 h at room temperature, followed by washing. The final substrate solution (3-amino-9-ethyl-carbazole (AEC) substrate set, BD Biosciences) was added. Color development was monitored by eye and stopped by washing the wells with distilled water. After drying the plates overnight at room temperature, the number of spot-forming units (SFU) in each well was determined using a computerized CTL ImmunoSpot Image Analyzer (Cellular Technology Limited).

Statistical analysis

Grubb's test was used to analyze for outliers in *in vivo* studies. Data were compared by ANOVA and Turkey's post-test for data with homogenous variance. In the case of non-homogeneous variance, the data were compared using the Kruskal-Wallis test followed by the Dunn's multiple comparison tests. A p-value less than 0.05 was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

RESULTS

Transfection efficiency of Liposome(LLO-PNPN/protamine/DNA) in P388D1 cells

In order to investigate whether the transfection efficiency could be affected by the addition of anionic liposomes to complexes, various amounts of PE/CHEMS liposomes were associated with the LLO-PNPN/protamine/DNA complexes (Figure 3-1). In this experiment, a base condensate formulation consisting of 0.15% LLO-PNPN and 1.2/1 (w/w) protamine/DNA was used, since this composition struck an acceptable balance between expression level and toxicity (Figure 2-11). The lowest amount of liposomes used was 2.25 nmol lipids/μg DNA, corresponding to a theoretical net charge ratio (+/-) of 1.31, and increasing amounts of liposomes were associated to give theoretical net charge ratios of 1.04 (5.25 nmol lipids/μg DNA), 0.88 (7.5 nmol lipids/μg DNA), and 0.73 (11.25 nmol lipids/μg DNA). With 5.25 or 7.5 nmol lipids, the luciferase gene expression was increased compared to that without liposomes in the presence of serum ($p<0.001$), while the association of small amount of liposomes (2.25 nmol lipids/μg DNA, net positive charge 1.31 (+/-)) did not affect the luciferase gene expression. With excess anionic liposomes (11.25 nmol lipids/μg DNA), the transfection efficiency was decreased showing similar luciferase gene expression to that without liposome, suggesting a possibility that the excess liposomes which do not associate with LLO-PNPN/protamine/DNA exist and compete with the liposomes associated with the complexes for cellular binding and uptake. In the following experiments, LLO-PNPN/protamine/DNA complex associated with liposomes composed of 7.5 nmol lipids/μg DNA was used, which have a net negative charge and showed high luciferase gene expression. The LLO-PNPN/protamine/DNA gene delivery systems were

compared with negative and positive controls in the presence and absence of serum (Figure 3-2). As a negative control, heat-inactivated formulations were used in order to demonstrate LLO's enhancement of the transfection efficiency, as our heat-inactivation conditions (75°C for 10 min) abolish LLO's hemolytic activity without negatively impacting the rest of the complex (data not shown). Lipofectamine was also used as a positive control in order to examine the relative efficiency of the gene delivery system compared to a commonly used DNA delivery vector. The luciferase gene expression with heat-inactivated LLO-PNPN/protamine/DNA or Liposome(LLO-PNPN/protamine/DNA) in P388D1 was significantly decreased compared to that without heat-inactivation ($p<0.0001$), while heat-inactivation did not affect the transfection efficiency of Liposome(protamine/DNA) without LLO-PNPN. Lipofectamine showed higher transfection efficiency than our gene delivery system in the absence of serum ($p<0.001$). However, in the presence of 10% serum the transfection efficiency of Lipofectamine was significantly decreased ($p<0.0001$) resulting in comparable luciferase gene expression to our gene delivery system containing LLO-PNPN: luciferase activity with LLO-PNPN/protamine/DNA was similar to that with Lipofectamine ($p>0.05$); luciferase activity with Liposome(LLO-PNPN/protamine/DNA) was higher than that with Lipofectamine ($p<0.01$).

In vivo luciferase gene expression

In order to further investigate the feasibility of the gene delivery vector for *in vivo* application, LLO-PNPN/protamine/DNA with or without liposomes was administered to C57BL/6J mice intravenously and luciferase activity was measured in spleen, liver and lung. Since blood components such as plasma proteins or enzymes are one of the major

factors that affect the *in vivo* delivery efficiency of non-viral vector by causing destabilization or degradation of the vector, the *in vivo* activity of our gene delivery system was tested after systemic administration via tail vein injection [5]. Heat-inactivated formulations served as a negative control in these experiments as well. The luciferase gene expression of Liposome(LLO-PNPN/protamine/DNA) was detected in spleen and lung, with higher luciferase activity in spleen, while luciferase activity of LLO-PNPN/protamine/DNA was only detected in lung (Figure 3-3). Consistent with their *in vitro* transfection efficiencies, heat-inactivated controls *in vivo* showed much lower or non-detectable luciferase gene expression in spleen or lung.

Immunization study: DNA-prime and protein-boost

CTL response

Because the enhancement of luciferase gene expression with our gene delivery system in mice was observed, we further investigated the possibility of using this gene delivery system as a DNA-based vaccine. For immunization studies, plasmid DNA expressing ovalbumin as a model antigen (OVA DNA) was used, and mice were primed with one of the following: (1) Buffer; (2) heat-inactivated (HI) Liposome(LLO-PNPN/protamine/DNA) as a negative control, HI-Liposome; (3) Liposome(LLO-PNPN/protamine/DNA), Liposomes; or (4) OVA protein encapsulated in LLO-containing liposomes as a positive control, Protein. All four groups were boosted with the same formulation: OVA protein encapsulated in LLO-containing liposomes on day 12. OVA-specific CTLs were then determined by *in vivo* CTL assay using CFSE. A mixture containing equal populations of target cells, $CFSE^{high}$ (pulsed with OVA peptide), and control cells, $CFSE^{low}$ (pulsed with non-specific NP peptide), was injected intravenously.

The carboxyfluorescein diacetate succinimidyl ester passively diffuses into cells and generates high fluorescent carboxyfluorescein succinimidyl ester after cleavage of the acetate groups by intracellular esterases [25]. The succinimidyl ester group reacts covalently with amines of intracellular macromolecules, resulting in retention of the CFSE dye inside cells until the cells are lysed by the OVA-specific CTL response, allowing the diffusion of CFSE out of cells. The mice were sacrificed 18 h after injection, and the specific lysis of OVA peptide-pulsed splenocytes was monitored by flow cytometry. As shown in Figure 3-4, the lysis of target cells (OVA peptide-pulsed splenocytes) in only the protein formulation-primed group was significantly higher than that of other groups ($p<0.001$). The Liposome(LLO-PNPN/protamine/DNA)-primed group did not enhance OVA-specific lysis compared to buffer-primed or heat-inactivated Liposome(LLO-PNPN/protamine/DNA)-primed groups.

OVA-specific IFN-γ response

In order to further examine the cellular immune response, the antigen-specific IFN-γ secretion was determined by ELISA by stimulating splenocytes from immunized mice with OVA CD8 peptide or OVA protein (Figure 3-5). Splenocytes from protein formulation-primed mice stimulated with OVA protein exhibited higher IFN-γ generation compared to buffer-primed mice ($p<0.01$). The IFN-γ response from Liposome(LLO-PNPN/protamine/DNA)-primed group was similar to buffer-primed or heat-inactivated formulation. Splenocytes from all four groups exhibited negligible IFN-γ response when treated with media only or non-specific NP peptide, and strong IFN-γ response with the T cell mitogen ConA (data not shown).

OVA-specific antibody response

OVA-specific IgG responses were monitored from the immunized mouse serum samples by ELISA (Figure 3-6). The DNA-primed group, irrespective of heat-inactivation of the liposomal formulation, showed an increased IgG response similar to the protein formulation-primed group, as compared to the buffer-primed group.

Immunization study: DNA-prime and -boost

In the previous immunization study with DNA-prime and protein-boost, both Liposome(LLO-PNPN/protamine/DNA) and heat-inactivated Liposome(LLO-PNPN/protamine/DNA) showed enhanced OVA-specific IgG response, suggesting the effect of OVA DNA delivery on immune response. Therefore, in order to further demonstrate the effect of our gene delivery system on OVA DNA-mediated immune response, we used the DNA-based formulation for both prime and boost instead of boosting with the protein formulation. Mice were immunized with buffer, heat-inactivated Liposome(LLO-PNPN/protamine/DNA), Liposome(LLO-PNPN/protamine/DNA) or naked OVA DNA as a control, as naked DNA without a vector has been used for DNA-based vaccination [16, 26]. The induction of IFN-γ secretion was evaluated by ELISPOT assay, which is considered more sensitive for the detection of cytokine release as compared to the measurement of bulk IFN-γ secretion by ELISA. The number of SFUs as measured by ELISPOT from splenocytes stimulated with OVA CD8 peptide or OVA protein was not statistically different in all four groups (Figure 3-7). The number of SFUs from splenocytes stimulated with media alone was subtracted from that with stimulants. Splenocytes stimulated with non-specific NP peptide exhibited the same level of SFUs as with media alone, while splenocytes with the

T cell mitogen ConA exhibited a very strong IFN-γ response generating many SFUs. OVA-specific IgG response was also determined by ELISA using serum samples from immunized mice (Figure 3-8). The IgG response was increased in mice immunized with Liposome(LLO-PNPN/protamine/DNA) compared to buffer group.

DISCUSSION

We demonstrated the improvement of DNA delivery in protamine/DNA complexes by the addition of LLO-PNPN in mice (Figure 3-3) as well as in P388D1 cells in the presence and absence of serum (Figure 2-11). Taken together, the results showing that heat-inactivated controls had transfection efficiencies similar to that of protamine/DNA complexes without LLO-PNPN, along with the improved luciferase gene expression by various amounts of LLO-PN or LLO-PNPN, suggest that the fusion proteins play a key role in the enhanced gene delivery. However, it is worth noting that in mice the relative luciferase expression was highest in lung, which we attribute to the net positive charge of the LLO-PNPN/protamine/DNA complexes. The positive charge of protamine is necessary for the neutralization and condensation of DNA as well as protection of DNA from degradation. The interaction of cationic surfaces of complexes with the negatively charged plasma membrane can also induce cellular uptake, resulting in relatively high transfection efficiency, but the rapid and non-specific interactions between positively charged vector and negatively charged RBCs form agglutinates that can result in the highest gene expression in lung after intravenous injection [8, 27].

In order to reduce these unwanted interactions with plasma proteins, we associated the complexes with anionic liposomes to change the theoretical net charge from positive to negative. The *in vitro* transfection efficiency with anionic Liposome(LLO-PNPN/protamine/DNA) was higher than that with cationic LLO-PNPN/protamine/DNA or the cationic lipid formulations with Lipofectamine, in the presence of serum (Figure 3-2). In addition, serum did not affect the transfection efficiency of protamine/DNA complexes associated with anionic liposomes, while

protamine/DNA complexes without anionic liposomes showed decreased transfection efficiency with 10% serum. In the preliminary study, the transfection efficiency of Liposome(LLO-PNPN/protamine/DNA) decreased about two to five-fold with increasing serum concentrations up to 50%, but was still higher than that of cationic complexes (data not shown). These results, consistent with other studies using anionic liposome formulations, suggest its potential compatibility and ability to function in physiological environments [12, 13, 28]. After intravenous injection of Liposome(LLO-PNPN/protamine/DNA) into mice, the highest gene expression was observed in spleen, not lung, consistent with a reduction in non-specific interactions between the vector and serum proteins resulting from a net anionic surface charge (Figure 3-3). Following intravenous injection, foreign particles are generally recognized and phagocytosed by antigen presenting cells (APCs) of the reticuloendothelial system [29]. The fenestrated endothelia that line the capillaries of the spleen and liver allow particles to diffuse into the tissues. Once in the liver or spleen, the particles would encounter and be taken up by Kupffer cells or resident macrophages, which are likely the primary cells transfected by the vector. This result (highest luciferase expression in spleen) is consistent with the transfection results using LLO-LPDII consisting of protamine/DNA complexed with anionic liposomes containing encapsulated LLO [21]. LLO-LPDII showed luciferase gene expression in liver as well, while the gene expression in liver with Liposome(LLO-PNPN/protamine/DNA) was not detectable; one plausible explanation for this observation is that the rate of clearance or biodistribution of liposomes is dependent on liposome composition and size [30, 31].

The enhancement of transfection efficiency with Liposome(LLO-PNPN/protamine/DNA) was observed in the murine macrophage-like cell line, P388D1. This cell line was chosen because it is relatively difficult to transfect macrophage-like cells with non-viral vectors in the previous studies as well as macrophage-like cells represent one of the antigen-presenting cells inducing immune response [44, 45]. Therefore, with the enhancement of transfection efficiency in the murine macrophage-like cell line as well as in mice, we further investigated the potential of this gene delivery system for clinical use as a DNA-based vaccine. The samples were subcutaneously administered, which is one of the most commonly investigated routes for vaccination using liposomes in the mouse model, thereby delivering the injected samples to the lymphatic system including the tissue-draining lymph nodes where phagocytosis by APCs actively occurs [32, 33]. First, we tried a DNA-prime and protein-boost immunization protocol which has been shown to produce stronger immune responses than DNA-prime and DNA-boost protocols in several studies [34-37], and compared the immune responses among mice groups primed with different formulations, keeping the boost formulation constant. The protein formulation used was pH-sensitive liposomes containing LLO and OVA protein, which demonstrated strong OVA-specific CTL activity and antibody response in mice following subcutaneous injection [22]. In this study, the Liposome(LLO-PNPN/protamine/DNA)-primed group did not generate a strong antigen-specific CTL response or IFN-γ secretion compared to the buffer-primed or heat-inactivated Liposome(LLO-PNPN/protamine/DNA)-primed group (Figure 3-4 and 3-5). In a humoral immune response test, three groups other than the buffer-primed

group showed an improved anti-OVA IgG response, suggesting the possibility of OVA DNA delivery and protein expression inducing an immune response (Figure 3-6).

In order to further investigate the effect of DNA delivery on the immune response using our gene delivery vector, we also tried a DNA-prime and DNA-boost immunization to exclude the effect of the protein boost. An increase in OVA-specific IFN-γ secretion by activated T cells from all four groups was not detected using ELISPOT assay (Figure 3-7). However, Liposome(LLO-PNPN/protamine/DNA)-immunized mice showed significantly ($p < 0.01$) higher antibody response compared to buffer-immunized mice, while the antibody response from naked DNA or heat-inactivated Liposome(LLO-PNPN/protamine/DNA)-immunized mice was not statistically significant compared to that from buffer-immunized mice (Figure 3-8). With these results, it may be suggested that Liposome(LLO-PNPN/protamine/DNA) can generate only weak immune responses. In addition to the immunization, we also examined the luciferase gene expression of Liposome(LLO-PNPN/protamine/DNA) after s.c. injection, which showed non-detectable luciferase activity in liver, lung, spleen and lymph nodes (data not shown). Following s.c. injection, liposomes are absorbed by lymphatic capillaries draining the injection sites and reach the general circulation, or captured in regional lymph nodes, or remain at the site of injection, depending on the liposome size [32]. While less than 30% of small liposomes (< 100 nm) remained at the site of injection, about 80 % of the injected dose remained in the case of 400 nm liposomes. Considering that the Liposome(LLO-PNPN/protamine/DNA) has a mean diameter of 242 ± 94 nm, only a small portion of the injected dose may be transported through the interstitium and reach

lymphatic capillaries, resulting in inefficient uptake by APCs and a relatively weak immune response.

The low cellular immune response compared to the antibody response generated by Liposome(LLO-PNPN/protamine/DNA) may also be partly explained by the OVA DNA used in this study containing a secretion signal sequence. The cellular localization of antigen can affect the type of immune response that is generated. Cytosolic localization of antigen increased intracellular accumulation and MHC class I presentation for cellular immune responses compared to secreted or membrane bound antigen [19]. It was also reported that the DNA encoding secreted OVA generated higher IgG responses compared to IgG generated by DNA encoding cytoplasmic or membrane-bound OVA [38]. However, since secreted OVA showed an increased CTL response as well, this may not be the main reason for the weak immune response. The immunization frequency may be another factor that can affect the immune response. Although various immunization protocols, with intervals of less than one week to four weeks with twice to several times injection [35, 39, 40] have been used, frequent immunization with relatively high doses over longer periods showed higher immune responses [41-43].

In conclusion, anionic liposome association with protamine/DNA complexes incorporating LLO-PNPN fusion protein enhanced the luciferase gene expression in cultured cells containing serum and in mice with different expression profiles from cationic complexes. Our gene delivery system showed limited success in generating immune responses, but it is of sufficient interest to investigate whether the *in vivo* immune response can be improved via some modifications such as reducing particle size with freezing/thawing and sonication or using different immunization protocols.

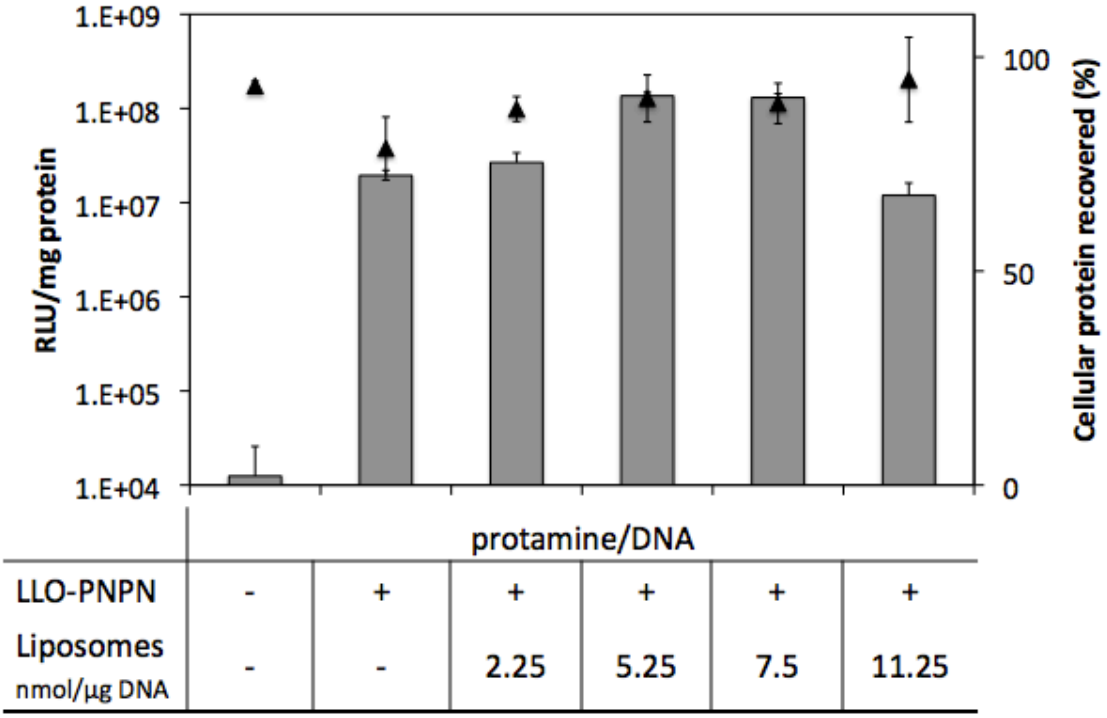


Figure 3-1. Effect of anionic liposomes on transfection efficiency of LLO-PNPN/protamine/DNA complexes in P388D1 cells.

LLO-PNPN/protamine/DNA complexes were prepared with 0.15 % LLO-PNPN and 1.2/1 (w/w) protamine. The LLO-PNPN/protamine/DNA complexes were used to hydrate lipid films composed of various amounts of PE and CHEMS (2.25 – 11.25 nmol lipids/µg DNA) by vortexing and sonication. Plasmid DNA in various formulations was incubated with P388D1 cells at 2 µg DNA per well with 10% serum, and luciferase activity of cell lysates was assayed 24 h after transfection. (Gray bars: luciferase activity, Black dots: cell viability) (n=3, mean ± SD)

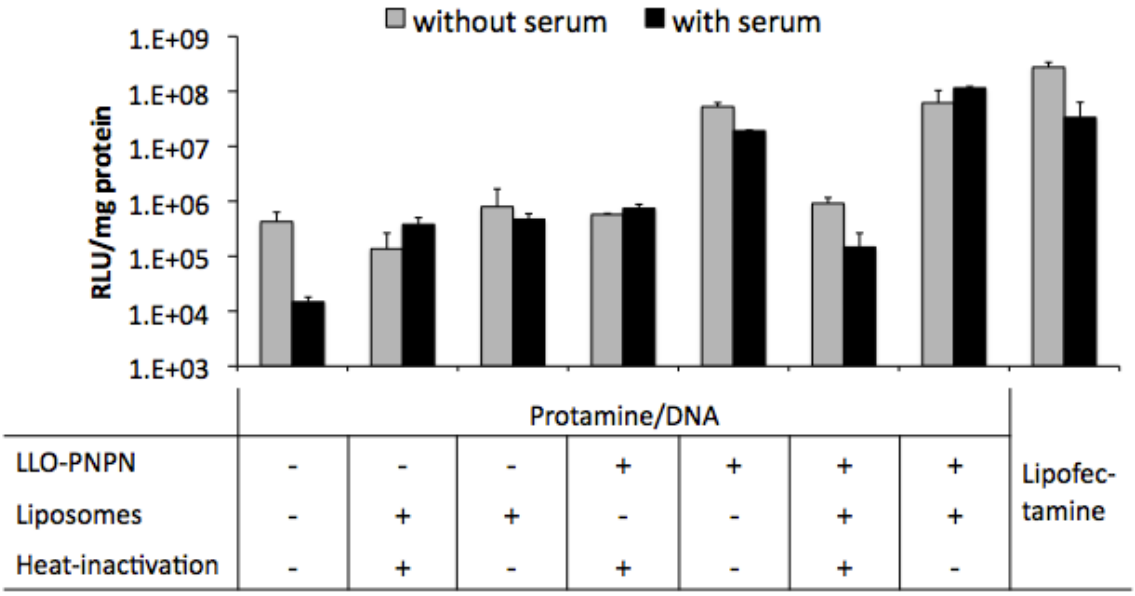


Figure 3-2. Comparison of transfection efficiency with heat-inactivated controls and Lipofectamine.

The complexes were prepared with 0.15 % LLO-PNPN and used to hydrate lipid films composed of PE and CHEMS (7.5 nmol lipids/μg DNA) by vortexing and sonication. Protamine/DNA complexes or Liposome(protamine/DNA) without LLO-PNPN was also prepared for comparison. As a negative control, samples were heat-inactivated at 75°C for 10 min in order to abolish LLO's hemolytic activity; Lipofectamine was used as a positive control. Plasmid DNA in various formulations was incubated with P388D1 cells at 2 μg DNA per well without or with 10% serum, and luciferase activity of cell lysates was assayed 24 h after transfection. (n=3, mean ± SD)

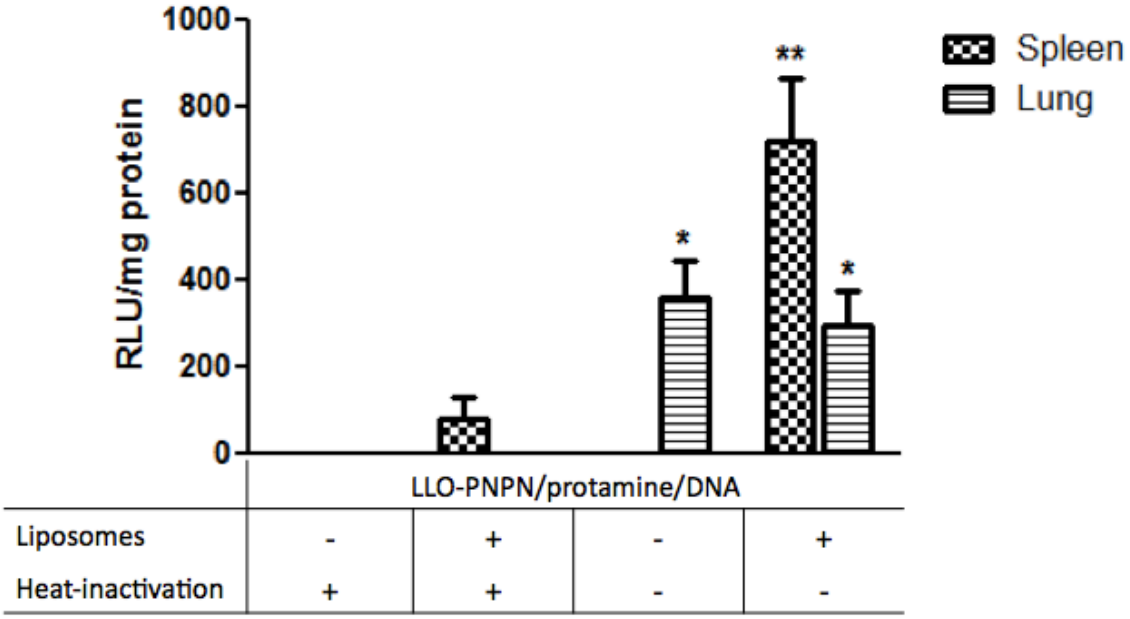


Figure 3-3. ***In vivo*** **luciferase gene expression.**

LLO-PNPN/protamine/DNA or Liposome(LLO-PNPN/protamine/DNA) was injected intravenously into mice (50 μg DNA/mouse), and mice were sacrificed 24 h following injection. The spleens, lungs and livers were harvested and homogenized in lysis buffer, and the supernatants were assayed for luciferase activity (n=6, mean ± SEM). The RLU of buffer-injected control group was subtracted from the RLU of each sample. * $p<0.05$, ** $p<0.01$ (compared to heat-inactivated Liposome(LLO-PNPN/protamine/DNA)

A

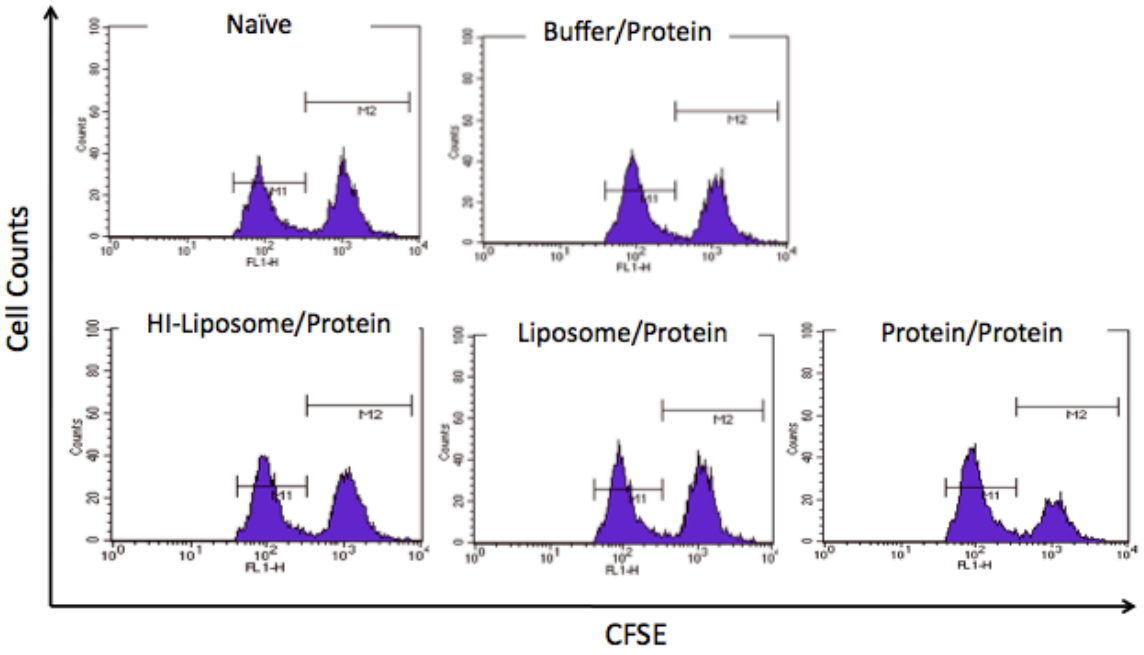


Figure 3-4. OVA-specific CTL response in immunized mice. (A) Representative results of flow cytometric analysis of each group.

Mice (n=6) were primed on day 0 with buffer; Buffer, 50 μg of OVA plasmid DNA in Liposome(LLO-PNPN/protamine/DNA); Liposome or HI-Liposome(LLO-PNPN/protamine/DNA); HI-Liposome, or 50 μg of OVA protein encapsulated in LLO-containing liposomes; Protein, respectively. On day 12, all of the mice were boosted with 50 μg of OVA protein encapsulated in LLO-containing liposomes, Protein. On day 24, mice were injected intravenously with $CFSE^{high}$ cells pulsed with OVA peptide and $CFSE^{low}$ cells pulsed with NP peptide. After 18 h, splenocytes were prepared from the immunized mice, and the proportions of the $CFSE^{high}$ cells and $CFSE^{low}$ cells were analyzed by flow cytometry. The histograms show counts of remaining OVA peptide-pulsed $CFSE^{high}$ (right peak) and NP peptide-pulsed $CFSE^{low}$(left peak)

B

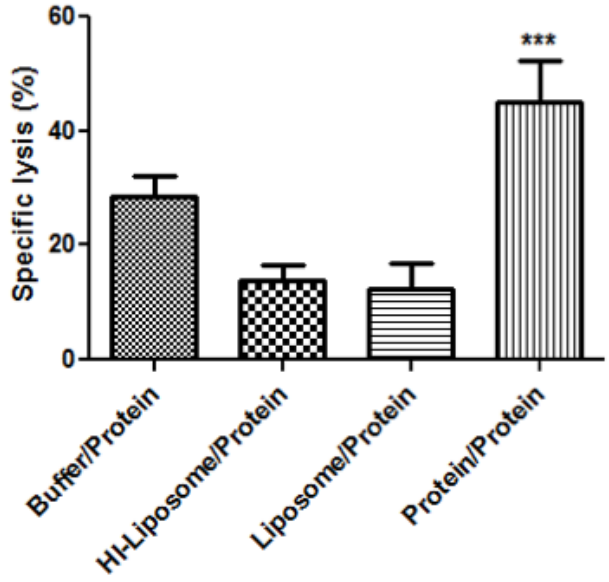
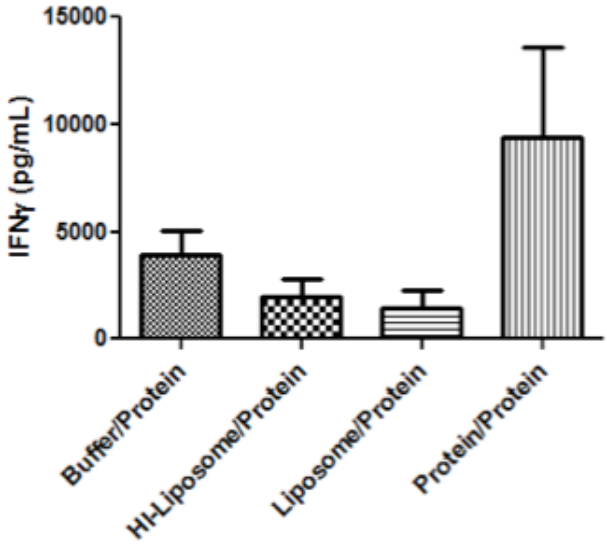


Figure 3-4. OVA-specific CTL response in immunized mice. (B) Percentage of OVA-specific cell lysis.

The percentage of specific lysis for each mouse was calculated as follows: 100×[1-(ratio of $CFSE^{high}/CFSE^{low}$ cells recovered from immunized mice/ratio of $CFSE^{high}/CFSE^{low}$ cells recovered from naïve mice)]. The mean ± SEM for each group is shown.
*** $p<0.001$

A



B

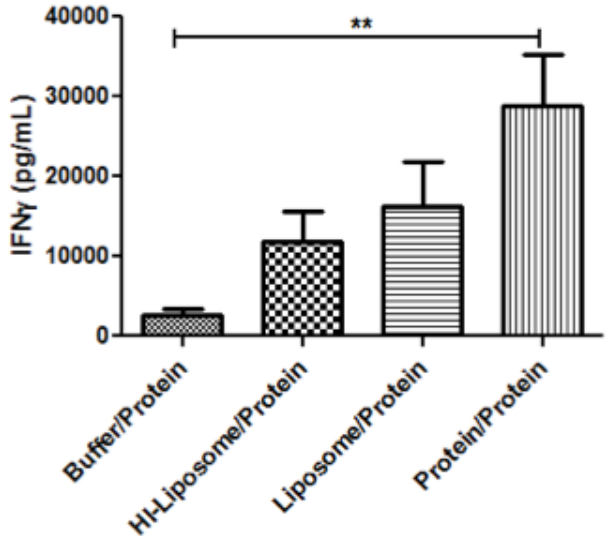


Figure 3-5. OVA-specific IFN-γ secretion generated by immunization.

Mice were immunized as described in Figure 3-4. Splenocytes from the immunized mice were stimulated with (A) OVA CD8 peptide (5 μM) or (B) OVA protein (50 μg/mL). The IFN-γ secreted in supernatant was determined by ELISA. The mean ± SEM for each group is shown. ** $p<0.01$

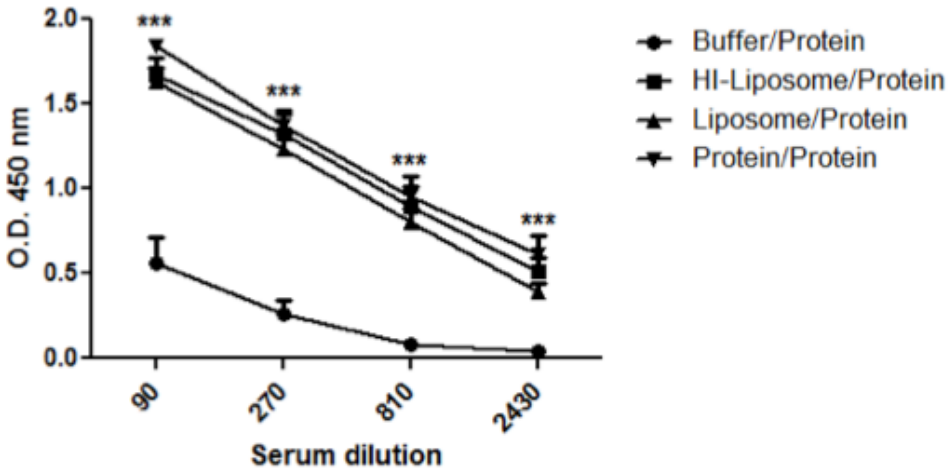
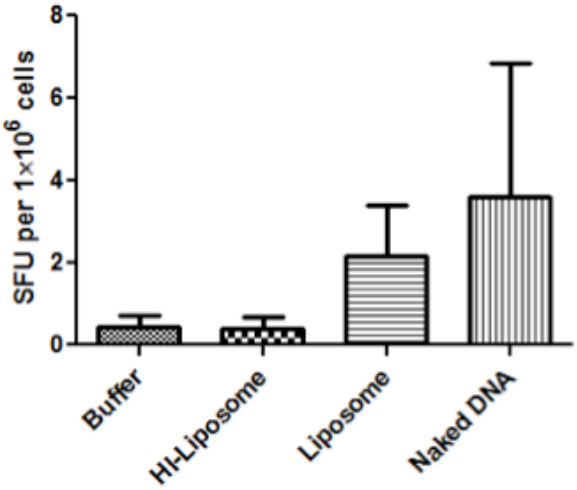


Figure 3-6. OVA-specific IgG response in immunized mice.

Mice were immunized as described in Figure 3-4, and anti-OVA IgG response in the serum samples collected from immunized mice was determined by ELISA. Data shown as mean ± SEM for each group. HI-Liposome-, Liposome-, Protein-primed groups were significantly higher than the control Buffer-primed group. *** $p<0.001$

A



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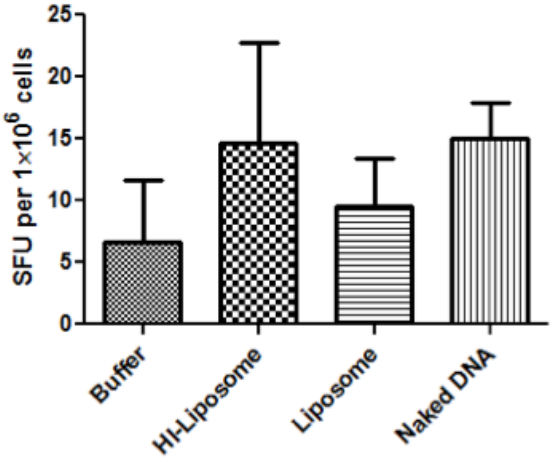

Figure 3-7. OVA-specific IFN-γ secreting T cell frequency generated by DNA-based immunization.

Mice (n=5-6) were immunized with Buffer, 50 μg of OVA DNA in HI-Liposome(LLO-PNPN/protamine/DNA); HI-Liposome or Liposome(LLO-PNPN/protamine/DNA); Liposome, 50 μg OVA DNA alone; Naked DNA on day 0 and day 7. On day 14, mice were euthanized and splenocytes were harvested and stimulated with (A) OVA CD8 peptide (2.5 μg/mL) or (B) OVA protein (50 μg/mL). IFN-γ spot forming units (SFU) were monitored by ELISPOT. The mean ± SEM of each group is shown.

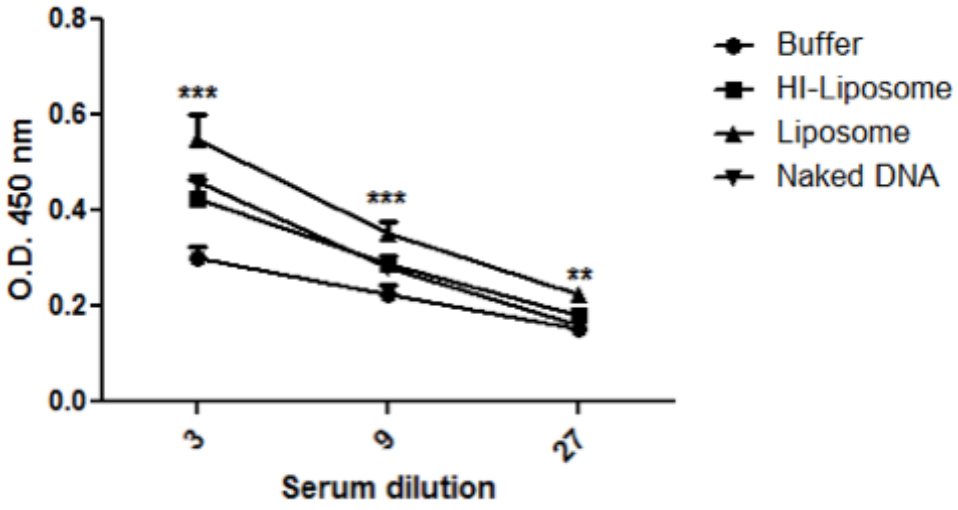

Figure 3-8. OVA-specific IgG response in mice immunized with DNA.

Mice were immunized as described in Figure 3-7. Sera were obtained from the immunized mice on day 14 and anti-OVA IgG was determined by ELISA. IgG response of mice immunized with Liposome(LLO-PNPN/protamine/DNA) was significantly higher than that with Buffer. ** $p<0.01$, *** $p<0.001$

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CHAPTER IV

CHARACTERIZATION OF LLO-PROTAMINE FUSION PROTEINS FOR THE IMPROVEMENT OF GENE DELIVERY

SUMMARY

The physical and biochemical characteristics of LLO-PN fusion protein and the complexes were further studied in this chapter. The cathepsin D recognition sequence, RGFFP between LLO and protamine of LLO-PN fusion protein, was characterized in terms of cathepsin D cleavability and effect on the transfection efficiency. The cleavage of cathepsin D recognition sequence of LLO-PN was not observed in SDS-PAGE after incubation of LLO-PN with cathepsin D, and the transfection efficiency of protamine/DNA complexes with LLO-PN containing cathepsin D cleavable linker was similar to that with LLO-control-PN containing cathepsin D non-cleavable linker, suggesting the cathepsin D-cleavable linker did not affect the DNA transfer in this gene delivery system. In addition to a linker effect, the recently developed gene delivery system incorporating LLO-PNPN/protamine/DNA complexes and Liposome(LLO-PNPN/protamine/DNA) were also characterized in terms of particle size, zeta potential, protection from DNase, and effect of DNA and protamine on hemolytic activity of LLO-PNPN. With additional characterization studies, this gene delivery system is expected to become further optimized as an efficient gene delivery system.

INTRODUCTION

We have recently developed a gene delivery system incorporating listeriolysin O (LLO) - protamine fragment (PN) recombinant fusion proteins. These protein chimeras bind to DNA via electrostatic interaction between the positively charged protamine fragment and negatively charged DNA, and enhance cytosolic delivery of DNA through the activity of the endosomolytic pore-forming protein LLO from *Listeria monocytogenes*. In order to further optimize this gene delivery carrier, in addition to the functional components LLO and PN we introduced a cathepsin D-cleavable linker into the fusion proteins between LLO and PN. Enzyme-cleavable linkers, which are designed to be stable in the circulation and degraded under specified conditions, have been used for targeted delivery or controlled release of active cytotoxic agents [1]. For example, introduction of cathepsin-degradable peptide linkers between radiometal chelate and targeting antibody increased the clearance of radioactivity from the liver and body and improved the therapeutic index [2, 3], and matrix metalloproteinase (MMP)-cleavable linkers were also used for targeting gene delivery systems to tumors [4, 5].

Cathepsin D is an aspartyl endopeptidase in endosomes and lysosomes of most of human cells and cleaves peptide bonds formed by hydrophobic amino acids, especially aromatic amino acids such as phenylalanine [6, 7]. LLO is a member of the family of pore-forming cholesterol-dependent cytolysins that bind to cholesterol-rich regions of lipid bilayers with subsequent oligomerization and pore formation [8, 9]. The C-terminal domain 4 of LLO has been implicated in the membrane binding step of LLO's pore formation, and some modifications of the tip of domain 4 have been shown to prevent the binding step [8]. Based on this characteristic, we hypothesized that if LLO's dissociation

from protamine/DNA complexes is promoted inside endosomes through the cleavage of cathepsin D-cleavable linker, thus hypothetically exposing the C-terminus of LLO, this would allow more efficient release and endosomal escape of DNA and result in improved exogenous gene expression. Therefore, our LLO and PN fusion proteins were designed and made with an intervening cathepsin D recognition sequence from the B-chain of insulin, RGFFP [10, 11]. As a negative control with non-cleavable linker, the two hydrophobic amino acids (FF) were mutated to positively and negatively charged amino acids (RE), thus keeping the same length and overall charge of the fusion protein.

Here, we examined the linker cleavability of LLO-PN fusion protein by cathepsin D and the effect of cathepsin D-cleavable linker on transfection efficiency in cultured cells. In addition, we examined particle size, zeta potential, DNase protection, and the effect of binding of the fusion protein to DNA on LLO's hemolytic activity.

EXPERIMENTAL PROCEDURES

Construction of LLO-PN, LLO-control-PN and PN-LLO

The DNA encoding LLO-PN fusion protein with spacers was constructed as described in the Experimental Procedures section of Chapter II. In order to construct DNA encoding LLO-PN with a cathepsin D non-cleavable linker, the nucleotides encoding Phe-Phe of RGFFP were mutated to Arg-Glu (RGREP) using the SLIM method as previously described, with some modification (Figure 2-2A) [12, 13]. Briefly, four primers were used for amplification: two gene-specific sequences that recognize the template, F_S and R_S; two tailed primers F_T and R_T carrying the mutations to be made on the target sequences and having gene-specific sequences identical to F_S and R_S (Figure 4-2A). Each set of primers (10 pmol each), F_T and R_S or F_S and R_T, was mixed with 100 ng of plasmid template (LLOPN/pET29b) in 25 μL solution containing 2.5 μL of 10× PfuUltra HF reaction buffer, 200 μM each dNTP, 1.25U PfuUltra Hotstart DNA polymerase (Stratagene). The PCR reactions were started by heating to 98°C for 1 min, and then subjected to 25 cycles of 95°C for 30 s, 50°C for 1 min and 72°C for 7 min (1 min per kb), with a final 10 min extension step at 72°C. Plasmid template was then digested by incubation with DpnI (New England Biolabs) at 37°C for 1 h. Two DpnI digested PCR products were mixed for denaturation and hybridization. The reaction contained 10 μL of each PCR product, 10 μL of 5 × H-Buffer (700 mM NaCl, 125 mM Tris pH 9.0 and 100 mM EDTA, pH 8.0) and water to a final volume of 50 μL. The denaturation was performed at 99°C for 3 min, followed by 3 cycles of 65°C for 5 min and 30°C for 30 min for hybridization. After hybridization, the double-stranded products

with complementary overhang spontaneously circularized, and the circular DNA molecules containing desired sequences were transformed into One Shot® Mach1™-T1 chemically competent *E.coli* (Invitrogen) and gave rise to colonies. The plasmid DNA containing LLO-PN with cathepsin D non-cleavable linker (LLO-control-PN) was isolated using a miniprep kit (Qiagen) and the sequence of resultant construct was verified by DNA sequencing at the University of Michigan Sequencing Core.

In order to construct DNA encoding PN-LLO (fusion proteins containing protamine fragment at N-terminus and LLO at C-terminus), the DNA encoding protamine fragment was inserted into the 5'-end of the LLO DNA in the bacterial expression vector pET29b by the SLIM method as described above. The DNA encoding the first half of protamine fragment was inserted using four primers (Figure 4-2B), then the second half of protamine fragment was inserted between the first half and LLO using the same SLIM procedures (Figure 4-2C). The sequence of human protamine fragment (RSQSRSRYYRQRQRSRRRRRRS) of PN-LLO was identical to that of LLO-PN.

Expression and purification of fusion proteins

The expression construct containing LLOPN, LLO-PN, LLO-control-PN, or PN-LLO was transformed into *E.coli* strain BL21(DE3) RIPL (Stratagene), expressed as His-tagged fusion proteins, and purified using Ni^{2+}-NTA agarose. Briefly, starter cultures from single colonies grown in 50 mL LB media at 37°C overnight with 30 μg/mL kanamycin and 25 μg/mL chloramphenicol were diluted 1:50 into 2 L LB media with 30 μg/mL kanamycin, and incubated at 37°C until the absorbance at 600 nm reached ~ 0.7. The culture was induced at 30°C for 6 h with 1 mM IPTG, and then centrifuged at 6,000 × g for 10 min at 4°C and the bacterial cell pellet was frozen at -80°C until purification.

The bacterial pellet was resuspended in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, 200 μM PMSF) and lysed using a French press (Thermo Spectronic). The lysate was centrifuged at 10,000 × g for 40 min and the supernatant was incubated with Ni^{2+}-NTA agarose (Qiagen) for 2 h. The Ni^{2+}-NTA agarose was washed with a total of 400 mL wash buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole) and eluted with wash buffer containing 250 mM imidazole. The fusion proteins were added to PD-10 desalting column (GE Healthcare Life Sciences) for buffer exchange (50 mM sodium phosphate, 300 mM NaCl), and stored in 40% glycerol at -80°C. The expression of the fusion protein was confirmed by SDS-PAGE with Simply Blue (Invitrogen) staining, and protein concentration was determined by a bicinchoninic acid (BCA) protein assay using bovine serum albumin as a standard (Pierce).

Recombinant wild type LLO in the bacterial expression vector pET29b was expressed in *E.coli* strain BL21(DE3) with a C-terminal six-histidine tag, and purified using Ni^{2+}-NTA agarose. The purity of LLO was determined by SDS-PAGE, concentrations were measured by BCA assay.

Hemolysis assay

The membrane pore-forming activity of LLO-PN, LLO-control-PN, PN-LLO fusion proteins alone or in complexes with DNA, protamine/DNA or liposomes was examined using an *in vitro* red blood cell (RBC) hemolysis assay as previously described [14]. Briefly, RBCs were washed three times with phosphate-buffered saline (PBS, pH 7.4) and resuspended at a concentration of 2×10^8 cells/mL in MBSE (10 mM MES pH 5.5 containing 140 mM NaCl and 1 mM EDTA) with 2 mM DTT. The 100 μL of RBCs was added to 0 – 100 ng of fusion protein with or without complexes in 100 μL of

HEPES-buffered glucose (HBG: 280 mM glucose, 10 mM HEPES, pH 8.4) and incubated for 15 min at 37°C. The released hemoglobin from lysed RBCs was measured by absorbance at 450 nm.

Cathepsin D cleavage assay of cathepsin D substrate

The cathepsin D substrate, Bz-RGFFP-4-methoxy-2-naphthylamine (Bz-RGFFP-4-MNA, Calbiochem®, EMD Biosciences), was used for the demonstration of the linker cleavability by cathepsin D. The cathepsin D cleavage assay of the substrate was followed as manufacturer's protocol. Briefly, the substrate (final concentration 350 μM) was incubated with various amounts of cathepsin D (Bovine, spleen, Calbiochem®, EMD Biosciences) in 500 μl of reaction buffer (20 mM glycine-HCl, pH 3.0) at 37°C for 1 h, 2 h or 4 h. Then, the reaction was stopped by adding 50 μl of 1% KOH. After adding 850 μL of reaction buffer 2 (100 mM Tris-HCl, pH 7.8), 1 mU dipeptidyl peptidase IV (DPP IV, Invitrogen) was added and incubated for 2 h at 37°C. The release of 4-MNA was monitored at 345 nm (excitation) and 425 nm (emission) using a Fluoromax 2 fluorometer (Horiba Scientific).

Cathepsin D cleavage assay of fusion proteins

The fusion proteins, LLOPN or LLO-PN or LLO-PN/protamine/DNA complexes were incubated with various amounts of cathepsin D enzyme (protein:cathepsin D, 1:0 to 1:10) in MBS (10 mM MES pH 5 containing 140 mM NaCl) for various periods of time (0 h to overnight) at 37°C. The reaction was stopped by the addition of pepstatin A (1:1 mole ratio of cathepsin D to pepstatin A). The linker cleavage was monitored by SDS-PAGE with Simply Blue (Invitrogen) or Krypton (Pierce) staining.

Transfection studies

To prepare LLO-PN/protamine/DNA, LLO-control-PN/protamine/DNA or PN-LLO/protamine/DNA complexes, 0.015 – 0.15% of LLO-PN, LLO-control-PN or PN-LLO was mixed with DNA in HBG and incubated for 20 min at room temperature, and then an equal volume of protamine (Salmine, Sigma-Aldrich) in HBG was added at a weight ratio of 1.2 (which corresponds to positive/negative charge (+/-) ratio of 1.6), and the complexes were further incubated for 20 min. The final concentration of DNA in the complexes was 150 μg/mL.

The murine macrophage-like cell line P388D1 (ATCC) were plated in 24-well plates at 1.5×10^5 cells per well and incubated in RPMI-1640 containing 10% FBS, antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) and 1 mM sodium pyruvate for 24 h before transfection. Cells were grown at 37°C in a 5% CO_2 humidified atmosphere and were typically ~70% confluent at the time of transfection. For transfection studies, 300 μL of the transfection sample containing 2 μg of DNA in serum-free RPMI 1640 was added dropwise into each well. All experiments were performed using triplicate samples. After a 4 h incubation with cells at 37°C, transfection samples were replaced with fresh complete medium and cells were further incubated for 24 h. Thereafter, the medium was removed and the cells were washed once with PBS. The luciferase gene expression in cells was measured using a luciferase assay kit according to the manufacturer's protocol (Promega). The luciferase activity was expressed as relative luminescence units (RLU) normalized by total cellular protein as determined by BCA assay.

Particle size and zeta potential measurement

The protamine/DNA, LLO-PNPN/protamine/DNA complexes or Liposome(LLO-PNPN/protamine/DNA) were prepared at a DNA concentration of 40 μg/mL in HBG with 1.6 (+/-) ratio of protamine. For LLO-PNPN/protamine/DNA, 0.15% of LLO-PNPN was used (keeping the 1.6 (+/-) ratio of protamine to DNA), and 7.5 nmol of lipid film composed of PE and CHEMS were hydrated with the complexes by vortexing and sonication. The samples were diluted to 5 μg/mL with HBG, and the particle size was determined by quasi-elastic light scattering using Nicomp 380 ZLS zeta potential/particle sizer equipped with an avalanche photodiode detector. Zeta potential was also measured with the Nicomp 380 ZLS.

DNase I protection assay

DNase I protection assay was performed as previously described [15]. Naked DNA, Protamine/DNA, LLO-PNPN/protamine/DNA complexes or Liposome(LLO-PNPN/protamine/DNA) were incubated in the presence or absence of DNase I (1U/μg DNA) at 37°C for 30 min. The samples were then mixed with 2 × stop solution (80 mM EDTA and 2% SDS) to dissociate DNA from complexes. Each sample was electrophoresed in an ethidium bromide-containing 1% agarose gel at 90 V for 1 h. The samples without SDS were also electrophoresed as a control.

Sucrose gradient centrifugation

Sucrose gradient centrifugation was performed as described previously [16]. LLO-PNPN fusion protein alone or LLO-PNPN/protamine/DNA complexes were

prepared in 200 μL of HBG. The samples were layered on 200 μL of 30% sucrose in an ultracentrifuge tube, and then centrifuged at 100,000 × g (Optima MAX Ultracentrifuge, Beckman coulter) for 30 min at 4°C. HBG and sucrose fractions were separated and monitored for the level of LLO hemolytic activities by hemolysis assay.

RESULTS AND DISCUSSION

Characterization of cathepsin D-cleavable linker

Expression, purification and hemolytic activity of fusion proteins

The DNA encoding cathepsin D recognition sequence, RGFFP and human protamine fragment PN (Arg-8 to Ser29) was inserted into the 3'-end of the LLO DNA and subcloned into the bacterial expression vector pET29b. We also inserted flexible linkers, GGGGSGGGGS, before and after the cathepsin D recognition sequence in order to give more flexibility and accessibility to cathepsin D enzyme (Figure 4-1B) [17]. The DNA encoding LLO-cathepsin D non-cleavable control sequence, RGREP-PN was constructed by mutation of nucleotides encoding hydrophobic aromatic amino acids Phe-Phe to charged amino acids Arg-Glu, since cathepsin D cleaves peptide bonds formed by carboxyl groups of hydrophobic amino acids (Figure 4-1C) [6]. We also constructed an N-terminus PN and LLO fusion protein (PN-LLO) which binds to plasmid DNA through electrostatic interaction between PN and DNA, but by design leaves the C-terminus position of LLO exposed as wild type LLO (Figure 4-1D). The DNA encoding PN-LLO fusion protein was constructed by inserting the PN DNA into the 5'-end of the LLO DNA using the SLIM method. The sequences of all DNA constructs were verified by DNA sequencing. His-tagged LLO-PN, LLO-control-PN and PN-LLO fusion proteins were expressed in *E.coli* strain BL21(DE3) RIPL, and purified using Ni^{2+}-NTA agarose with a typical yield of 5 mg/L culture for LLO-PN and LLO-control-PN, and 0.5 mg/L for PN-LLO. The relatively low yield of PN-LLO can be explained by the effect of the high density of positive charges from the N-terminal arginines causing the decreased

expression, delayed translocation or misfolding [18-20]. The successful expression and purification of each fusion protein was confirmed by SDS-PAGE (Figure 4-3).

The membrane pore-forming activity of each fusion protein was determined by an *in vitro* RBC hemolysis assay (Figure 4-4). As expected, the hemolytic activity of N-terminal PN-LLO fusion protein was retained, showing similar hemolytic activity to that of wild type LLO at a range of concentrations, while the C-terminal PN fusion proteins, LLO-PN or LLO-control-PN, showed reduced hemolytic activities at relatively lower ranges of concentrations and similar hemolytic activities at high concentrations. The position of the protamine fragment at the C-terminus of LLO may explain the reduced hemolytic activities, since the C-terminal domain 4 of LLO likely plays an important role in binding to cholesterol-containing target membranes.

Cathepsin D cleavage assay

In order to examine whether the cathepsin D recognition sequence RGFFP in the fusion protein LLO-PN can be cleaved by cathepsin D, we first tested the cleavability of the recognition sequence RGFFP itself by cathepsin D enzyme using a fluorometric assay. The cathepsin D substrate Bz-RGFFP-4-methoxy-2-naphthylamine (Bz-RGFFP-4-MNA) was incubated with various amounts of cathepsin D at 37°C. Upon cleavage of the Phe-Phe bond by cathepsin D, the dipeptide derivative Phe-Pro-4-MNA was formed and then the addition of dipeptidylpeptidase IV hydrolyzed the Phe-Pro-4-MNA to Phe-Pro-OH and 4MNA and the release of 4MNA was monitored at 345 nm (excitation) and 425 nm (emission) [10]. A corresponding increase in fluorescence intensity was observed when

incubating the substrate with various amounts of cathepsin D for one to four hours, which confirmed the cleavability of Phe-Phe bond of RGFFP sequence.

The fusion protein LLO-PN was incubated with cathepsin D (1:1.6 mole ratio) at 37°C in pH 5.0 since cathepsin D shows the highest activity in an acidic pH (pH 3.5-5.5) [6]. After incubation for various periods of time, the SDS-PAGE results suggested that cathepsin D did not specifically cleave the cathepsin D recognition site between LLO and PN. We expected to observe an LLO band after cleavage of the cathepsin D recognition sequence of LLO-PN fusion proteins, but there was no detected band other than LLO-PN and cathepsin D enzyme (Figure 4-6A). The bovine cathepsin D used in this study exists in two forms, a single polypeptide chain (47 kDa) and a two-polypeptide chain of H-chain (34 kDa) and L-chain (12 kDa) [21]. The cleavage of cathepsin D recognition sequence in LLO-PN fusion protein was also not detected after incubation of various amounts of cathepsin D with either LLO-PN fusion protein alone (data not shown) or LLO-PN/protamine/DNA complexes (Figure 4-6B).

Effect of cathepsin D-cleavable linker of fusion protein on transfection efficiency

Although the cleavage of cathepsin D recognition sequence of LLO-PN was not demonstrated by SDS-PAGE following incubation with cathepsin D, we decided to examine the effect of the cathepsin D cleavable linker on transfection efficiency in P388D1 cells (Figure 4-7), as the *in vitro* cathepsin D activity may not accurately reflect that in cells. Transfection complexes of protamine/DNA were prepared with either LLO-PN with cathepsin D cleavable linker (RGFFP) or control linker (RGREP), and incubated with P388D1 cells. The luciferase gene expression of LLO-PN/protamine/DNA was

similar to that of LLO-control-PN/protamine/DNA. We also compared the luciferase gene expression of protamine/DNA with PN-LLO whose C-terminus is not attached to PN and which showed higher relative hemolytic activity at lower concentrations (Figure 4-7). The luciferase gene expression of PN-LLO/protamine/DNA was also comparable to that with LLO-PN or LLO-control-PN. In this study, the effect of cathepsin D cleavable linker or the position of PN fragment was not demonstrated *in vitro*. Without cleavage of the cathepsin D recognition sequence and separation of LLO from C-terminal PN, the LLO-PN fusion protein itself was hemolytic, suggesting that linker cleavage might not be necessary for the pore-forming ability of LLO-PN for endosomal escape of DNA. The mechanism of action of LLO-PN fusion protein inside endosomes should be further elucidated with regard to how the LLO-PN fusion protein of protamine/DNA complexes binds to endosomal membranes and makes pores, or whether LLO-PN fusion protein indeed dissociates from protamine/DNA complexes prior to membrane binding.

Characterization of LLO-PNPN/protamine/DNA complexes

In addition to characterizing the cathepsin D cleavable linker, we also examined the characteristics of our gene delivery system containing 0.15% of LLO-PNPN, which showed improved gene expression in both cultured cells and animal models. As shown in Table 4-1, the average diameter of LLO-PNPN/protamine/DNA complexes formed with 0.15% of LLO-PNPN and 1.6/1 (+/-) of protamine was 166 ± 47 nm, which was similar to that of protamine/DNA complexes without LLO-PNPN (162 ± 24 nm). When associated with anionic PE:CHEMS liposomes (7.5 nmol lipids/μg DNA), the average diameter of Liposome(LLO-PNPN/protamine/DNA), not surprisingly, increased to 242 ± 94 nm, consistent with previous work [22]. The zeta potential of LLO-

PNPN/protamine/DNA complexes was 20.1 ± 0.9 mV, and the change in the theoretical net charge from positive (1.6 (+/-)) to negative (0.88 (+/-)) by the addition of anionic PE:CHEMS liposomes was confirmed by measuring the zeta potential of the liposome-containing complexes: -27.9 ± 2.0 mV.

The effect of DNA binding on the pore-forming activity of LLO-PNPN was also examined. As shown in Figure 4-8, the hemolytic activity of LLO-PNPN complexed with plasmid DNA was reduced compared to that without DNA, while the hemolytic activity of wild type LLO or N-terminal PN-LLO was not decreased after mixing with DNA (data not shown). This observation suggests that DNA binds to the protamine fragment of LLO-PNPN fusion protein and the binding at the C-terminus of LLO affects its pore-forming activity, consistent with the C-terminal domain of LLO being involved in the membrane binding step of pore-formation. When complexed with protamine/DNA, the hemolytic activity of LLO-PNPN was increased at relatively high concentrations, while protamine/DNA complexes without LLO-PNPN showed less than 5% lysis of RBCs at the highest concentration. The reason for increased hemolytic activity of LLO-PNPN/protamine/DNA complexes is not clear, but one possibility is some degree of dissociation of LLO-PNPN from DNA by competitive binding with protamine to DNA. The improved *in vitro* and *in vivo* transfection efficiency of protamine/DNA complexes with LLO-PNPN fusion proteins compared to that with LLO-PN or with wild-type LLO, as shown in Chapters II and III, suggests the ability of fusion protein to remain associated with protamine/DNA complexes and to be internalized together into cells. However, in order to examine whether free, unbound LLO-PNPN exists in LLO-PNPN/protamine/DNA complexes in solution, we tried to separate the unbound LLO-

PNPN fusion proteins from LLO-PNPN/protamine/DNA complexes using sucrose density gradient centrifugation. When LLO-PNPN alone was run in a single-step sucrose ultracentrifugation gradient (30% sucrose), 68% of the total hemolytic activity was detected in the upper layer, while 57% of the total hemolytic activity was detected in the lower layer (sucrose layer) after centrifugation of LLO-PNPN/protamine/DNA complexes (total hemolytic activity: hemolytic activity of upper layer and lower layer). This result suggests that LLO-PNPN stayed with complexes and migrated into the sucrose layer since protamine/DNA complexes migrated to sucrose layer, but there is still the possibility that unbound free LLO-PNPN exist in our sample. Further characterization studies such as quantification of the amount of LLO-PNPN remaining with protamine/DNA complexes or separation of unbound LLO-PNPN fusion protein from complexes could help understand the structure and characteristics of this gene delivery vector and prepare homogenous system for potential clinical application.

Since DNA can be easily degraded by nucleases in serum or cytosol, the gene delivery carrier should be able to protect DNA from degradation [23]. In order to demonstrate the protection of DNA in our gene delivery system from degradation by DNase I, the LLO-PNPN/protamine/DNA complexes were incubated with DNase I. Because the movement of DNA in agarose gel electrophoresis was completely retarded by the condensation with protamine (Figure 4-9, lanes without DNase I and SDS), 1% SDS, anionic surfactant, was added to dissociate DNA from protamine in order to monitor the degree of degradation of DNA by DNase I incubation. As shown by the results from agarose gel electrophoresis (Figure 4-9), naked DNA was degraded after 30 min incubation with DNase I. However, DNA complexed with protamine with or

without LLO-PNPN as well as associated with liposomes was not degraded by DNase I incubation, suggesting this system can protect DNA from DNase I degradation.

The reduced hemolytic activity of Liposome(LLO-PNPN/protamine/DNA) even at the highest tested concentration of LLO-PNPN suggests the complexes were associated with or encapsulated within liposomes (Figure 4-8). Kogure *et al*. demonstrated that polylysine/DNA complexes could be packaged within DOPE/CHEMS liposomes prepared by hydration and sonication using freeze-fracture electron microscopy and sucrose density gradient centrifugation [22]. Using rhodamine-labeled DOPE and FITC-labeled DNA, they demonstrated co-localization of DNA and lipid peaks and FRET between rhodamine-labeled DOPE and FITC-labeled DNA resolved in the gradients. Taken together with these published data, our results concerning hemolytic activity, particle size and zeta potential, as well as the *in vitro* and *in vivo* transfections using Liposome(LLO-PNPN/protamine/DNA), suggests that the LLO-PNPN/protamine/DNA complexes were associated with liposomes. However, additional studies of liposome coating or encapsulation efficiency will help to understand the characteristics of this formulation better and optimize for further improvement.

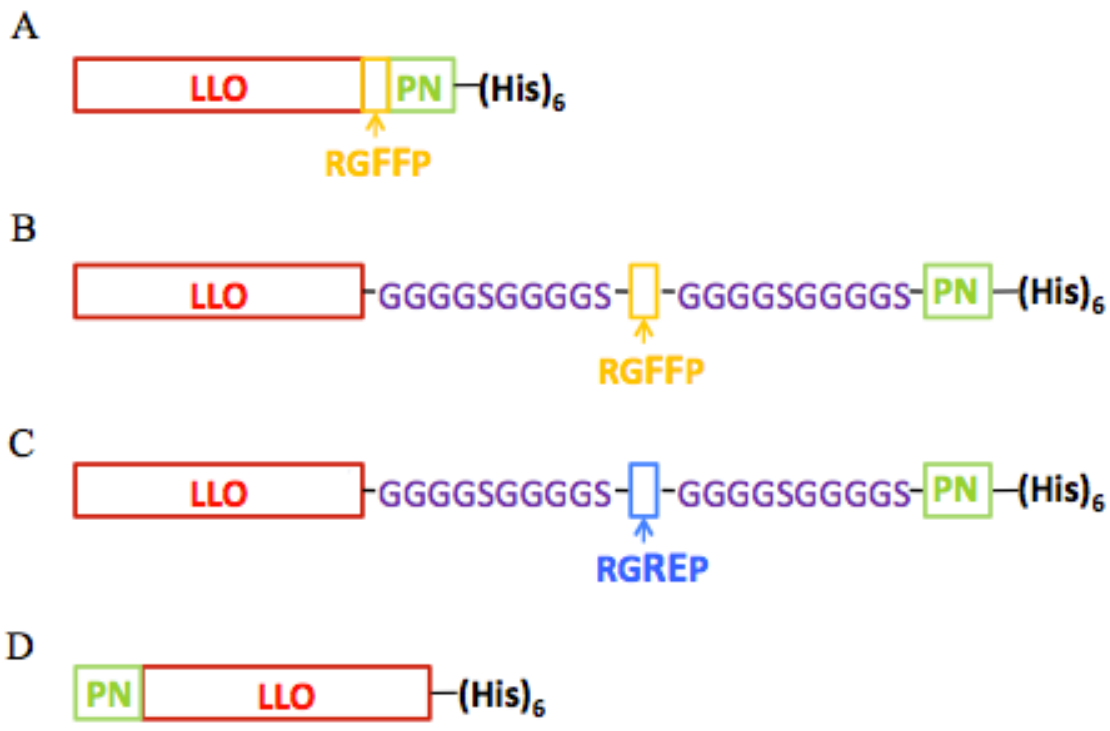


Figure 4-1. Schematic structures of LLO and protamine fusion proteins.

(A) LLOPN: fusion protein with cathepsin D-cleavable linker, RGFFP. (B) LLO-PN: fusion protein with spacers before and after cathepsin D-cleavable linker. (C) LLO-control-PN: fusion protein with cathepsin D-non-cleavable (control) linker, RGREP. (D) PN-LLO: fusion protein with protamine fragment at N-terminus and LLO at C-terminus.

A

F_T: 5'-CGTGGTCGCGAGCCGGGTGGCGGAGGCAGCGGAGGCG-3'

F_S: 5'-GGCGGAGGCAGCGGAGGCG-3'

R_T: 5'-ACCCGGCTCGCGACCACGACTTCCGCCACCTCCTGAACCGCC-3'

R_S: 5'-ACTTCCGCCACCTCCTGAACCGCC-3'

B

F_T: 5'-CGTTCTCAATCTCGATCAAGATATTACCGTCAAAAGGATGCATCTGCA
TTCAATAAAGAA-3'

F_S: 5'-AAGGATGCATCTGCATTCAATAAAGAA-3'

R_T: 5'-TTGACGGTAATATCTTGATCGAGATTGAGAACGCATATGTATATCTCCT
TCTTAAAGTTAAAC-3'

R_S: 5'-CATATGTATATCTCCTTCTTAAAGTTAAAC-3'

C

F_T: 5'-CGCCAGCGATCTCGTCGGAGACGCCGTAGGTCAAAGGATGCATCTGCA
TTCAATAAAGAAAATTCA-3'

F_S: 5'-AAGGATGCATCTGCATTCAATAAAGAAAATTCA-3'

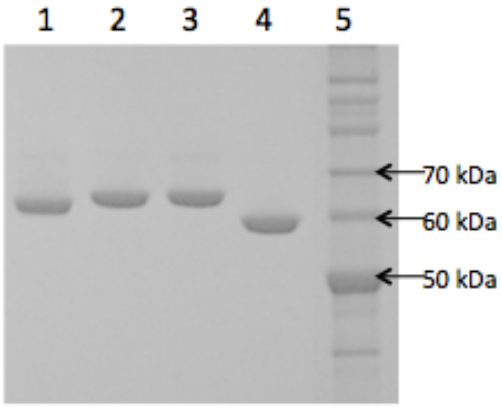
R_T: 5'-TGACCTACGGCGTCTCCGACGAGATCGCTGGCGTTGACGGTAATATCTT
GATCGAGATTGAGA-3'

R_S: 5'-TTGACGGTAATATCTTGATCGAGATTGAGA-3'

Figure 4-2. DNA sequences of primers used for SLIM PCR.

A. Primers for mutation to cathepsin D non-cleavable linker (RGREP). B. Primers to insert protamine fragment to N-terminus of LLO (1st half of protamine fragment). C. Primers to insert 2nd half of protamine fragment to N-terminus of LLO. Mutated or inserted sequences denoted as underlined.

A



B

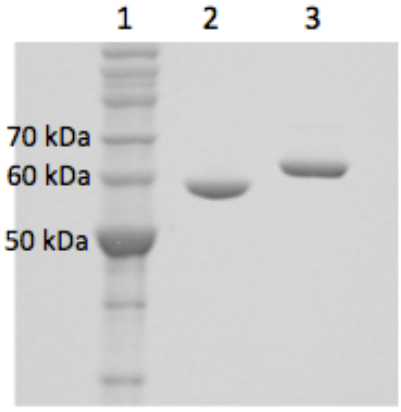


Figure 4-3. SDS-PAGE of expressed and purified fusion proteins.

A. Lane 1: LLOPN, lane 2: LLO-PN, lane 3: LLO-control-PN, lane 4: LLO, lane 5: protein molecular weight standards. B. Lane 1: protein molecular weight standards, lane 2: LLO, lane 3: PN-LLO.

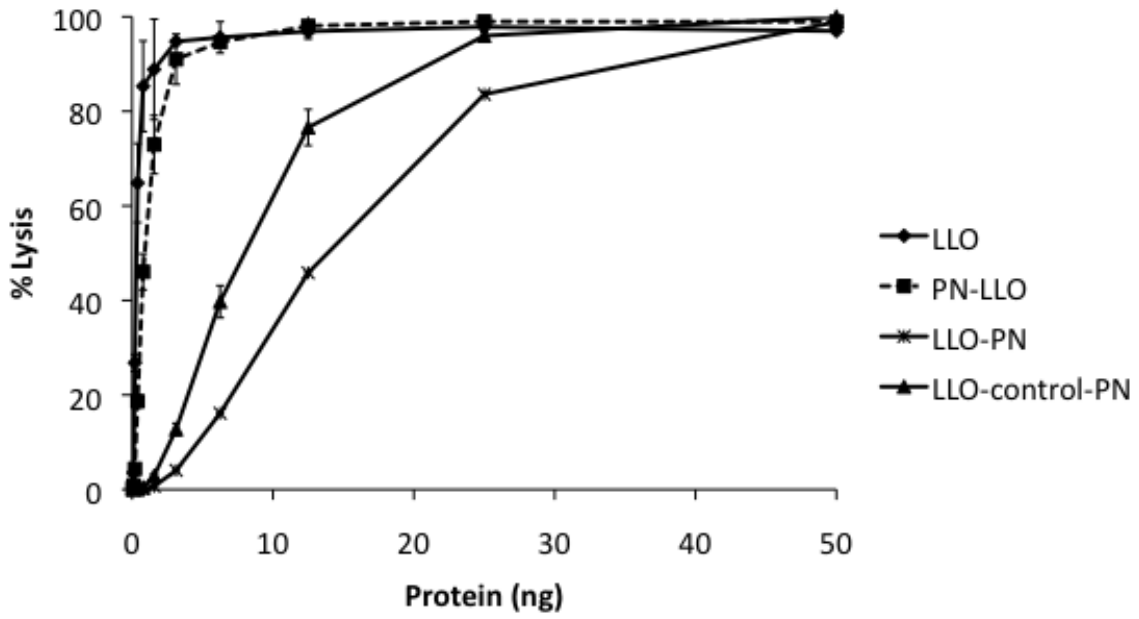


Figure 4-4. Hemolytic activities of fusion proteins.

The fusion proteins, LLO-PN, LLO-control-PN and PN-LLO and wild type LLO were assayed for their ability to perforate membranes by monitoring lysis of RBCs. Various amounts of LLO or fusion proteins were incubated with RBCs at 37°C for 15 min, and the release of hemoglobin from lysed RBCs was monitored by the absorbance at 450 nm. Protamine alone, without LLO, at compatible amounts did not show any detectable hemolytic activity (not shown in the figure). (n=2, mean ± SD)

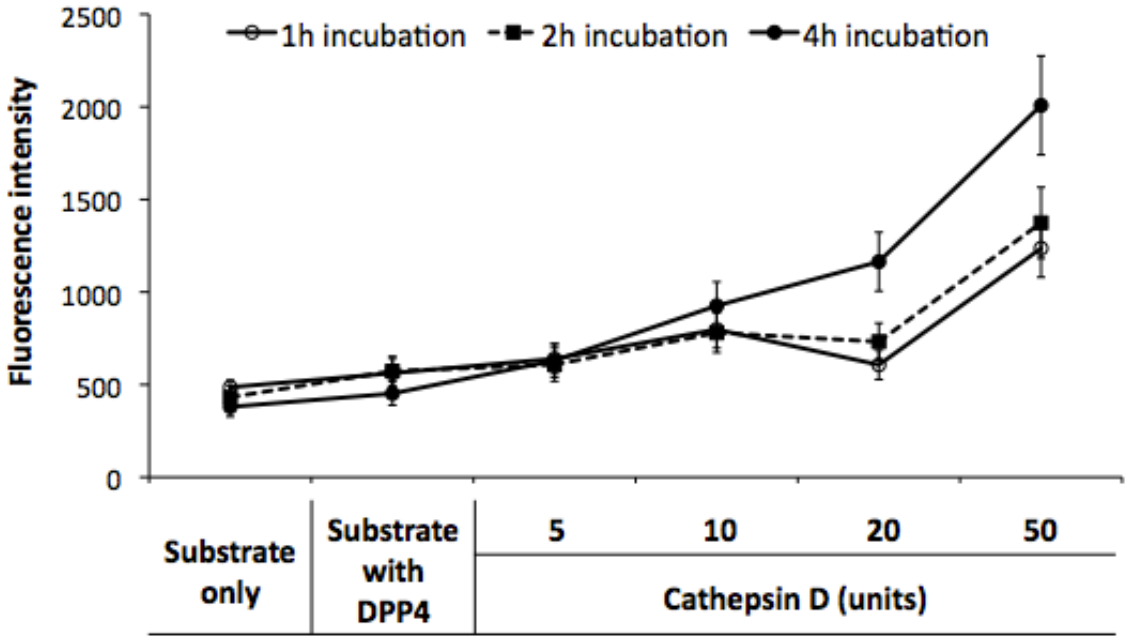
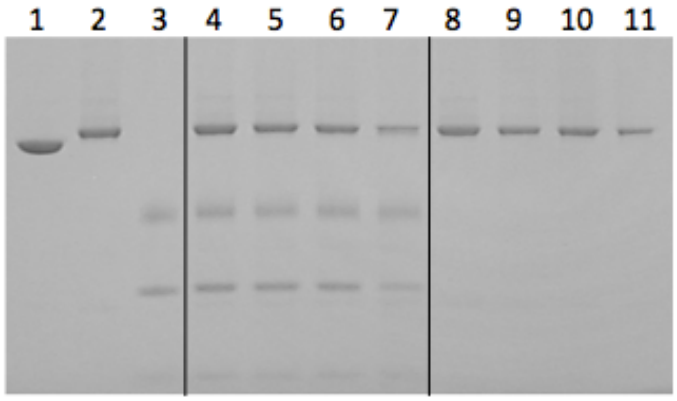


Figure 4-5. Cathepsin D cleavage of cathepsin D substrate.

The cathepsin D substrate, Bz-RGFFP-4-methoxy-2-naphthylamine (Bz-RGFFP-4-MNA) was incubated with various amounts of cathepsin D at 37°C for 1 h, 2 h or 4 h. Dipeptidyl peptidase IV was added to hydrolyze the Phe-Pro-4-MNA to Phe-Pro-OH and 4MNA and the release of 4MNA is monitored at 345 nm (excitation) and 425 nm (emission).

A



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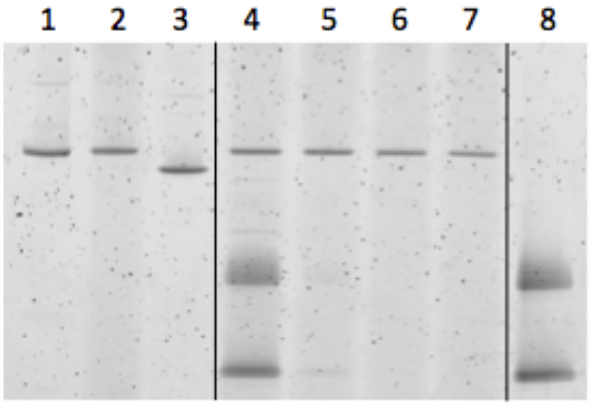

Figure 4-6. Cathepsin D cleavage assay of LLO-PN fusion protein.

A. LLO-PN fusion protein was incubated with cathepsin D (lanes 4-7) or without cathepsin D (lanes 8-11) at 37°C and the cleavage was monitored by SDS-PAGE. Lane 1: LLO, lane 2: LLO-PN, lane 3: cathepsin D, lanes 4-7: LLO-PN incubated with cathepsin D for 0 h (4), 1 h (5), 2.5 h (6) or overnight (7), lanes 8-11: LLO-PN incubated without cathepsin D for 0 h (8), 1 h (9), 2.5 h (10) or overnight (11). B. LLO-PN/protamine/DNA complex was incubated with various amounts of cathepsin D at 37°C for 1 h and monitored by SDS-PAGE. Lane 1: LLO-PN, lane 2: LLO-PN/protamine/DNA, lane 3: LLO, lanes 4-7: LLO-PN/protamine/DNA incubated with cathepsin D at 1:10 (4), 1:1 (5), 1:0.1 (6), 1:0 (7) ratios of LLO-PN to cathepsin D, lane 8: cathepsin D (same amount with lane 4).

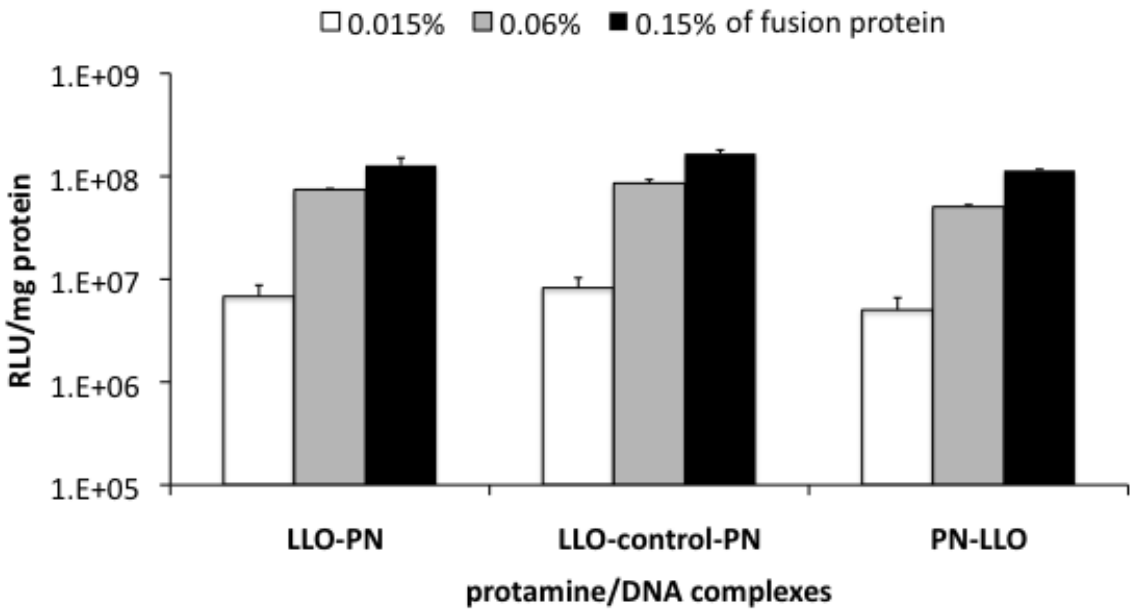

Figure 4-7. Effect of cathepsin D-cleavable linker on transfection efficiency.

LLO-PN, LLO-control-PN or PN-LLO fusion proteins (0.015 – 0.15%) were mixed with protamine/DNA complexes while maintaining a 1.6/1 (+/-) charge ratio, and the complexes (2 μg/well) were incubated with P388D1 cells. Luciferase activity of cell lysates was determined 24 h after transfection. (n=3, mean ± SD)

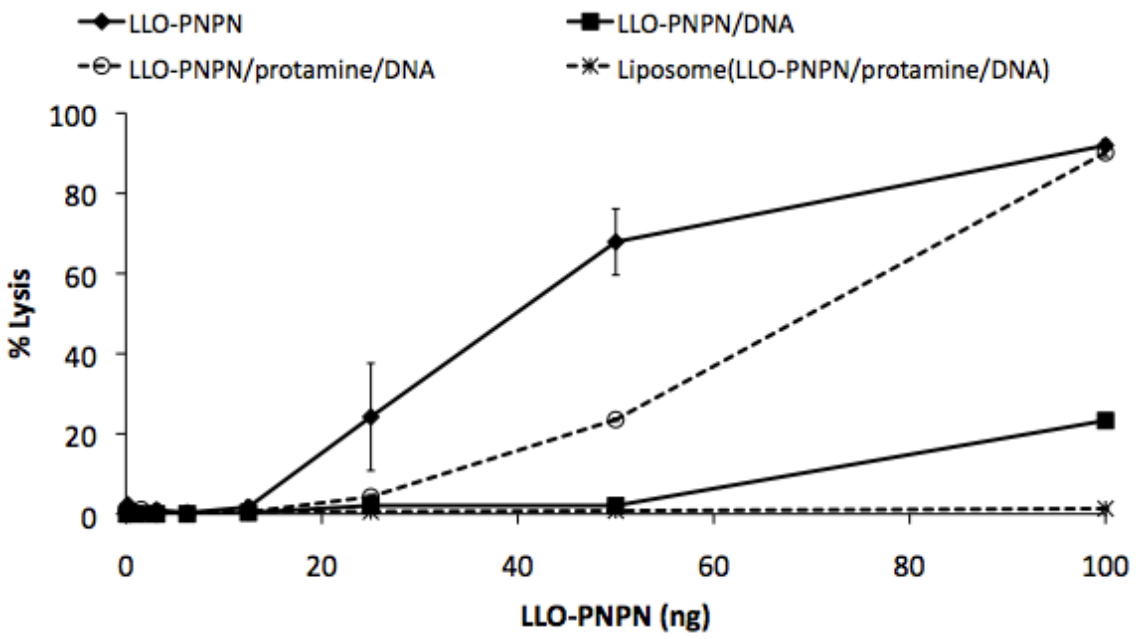

Figure 4-8. Effect of complex formation on hemolytic activities of fusion proteins.

Hemolytic activities of LLO-PNPN, LLO-PNPN with DNA, LLO-PNPN/protamine/DNA complexes, or Liposome(LLO-PNPN/protamine/DNA) were incubated with RBCs and the pore-forming ability of LLO-PNPN was monitored by measuring the absorbance of hemoglobin released from lysed RBCs at 450 nm. The protamine/DNA complexes without LLO-PNPN showed less than 5% lysis of RBCs at the highest concentration (not shown in the figure). (n=2, mean ± SD)

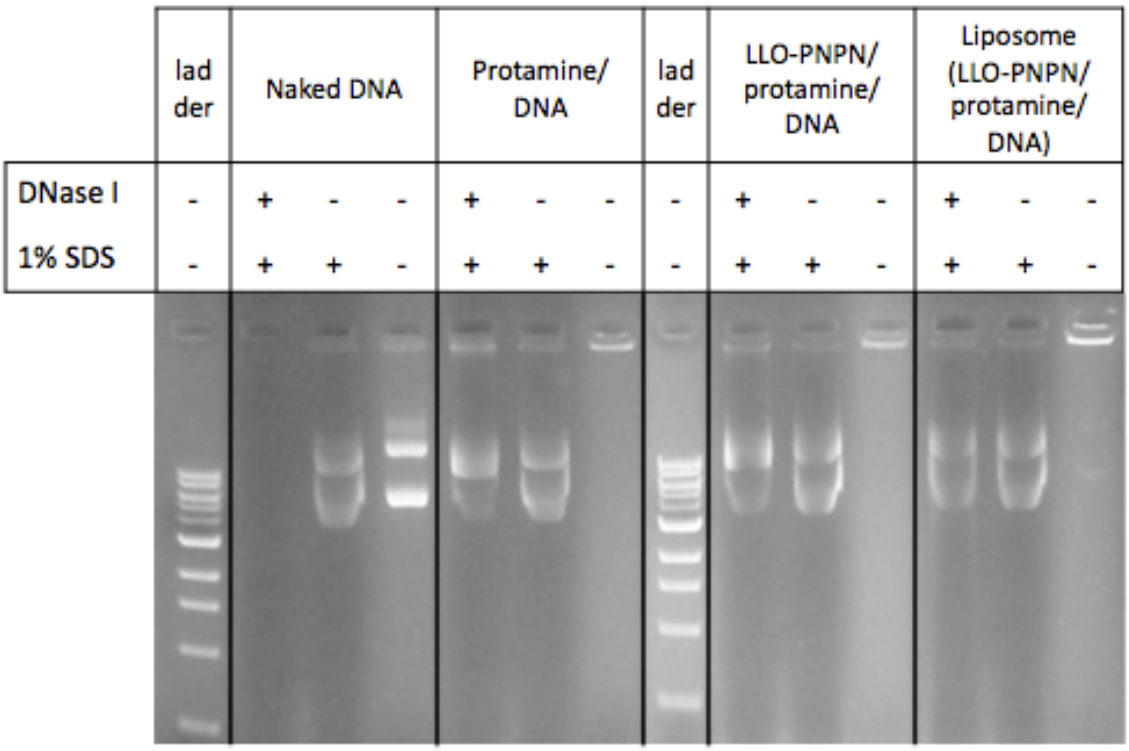

Figure 4-9. DNase I protection assay by LLO-PNPN/protamine/DNA complexes.

Naked DNA, protamine/DNA, LLO-PNPN/protamine/DNA or Liposome(LLO-PNPN/protamine/DNA) was incubated with or without DNase I (1 U/μg DNA) at 37°C for 30 min, and analyzed by 1% agarose gel electrophoresis with 1% SDS for dissociation of DNA from protamine. Each sample without either DNase I or 1% SDS was also analyzed by agarose gel as a control.

Table 4-1. Particle size and zeta potential of complexes.

The Protamine/DNA, LLO-PNPN/protamine/DNA complexes were prepared in HBG with 1.6 (+/-) ratio of protamine and with or without 0.15% of LLO-PNPN. For Liposome(LLO-PNPN/protamine/DNA), lipid film composed of PE and CHEMS (7.5 nmol of lipids/μg DNA) was associated with LLO-PNPN/protamine/DNA complexes by hydration and sonication. The particle size and zeta potential were measured by Nicomp 380 ZLS zeta potential/particle sizer (n=3, mean ± SD).

	Diameter (nm)	Zeta potential (mV)
Protamine/DNA	162 ± 24	27.2 ± 8.4
LLO-PNPN/protamine/DNA	166 ± 47	20.1 ± 0.9
Liposome(LLO-PNPN/protamine/DNA)	242 ± 94	-27.9 ± 2.0

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CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

This is the first study to incorporate genetically engineered LLO-protamine fusion protein into a non-viral gene delivery system and demonstrate the enhanced *in vitro* and *in vivo* DNA delivery efficiency. This particular construct is the potential candidate of recombinant fusion proteins with multiple functional domains to be utilized as DNA carriers or for augmenting the efficiency of existing platforms for DNA delivery.

Endosomal escape is a necessary step to achieve efficient cytosolic delivery of non-viral vectors. Our laboratory has previously demonstrated improved transfection efficiency using the endosomolytic pore-forming protein, LLO, conjugated with polycation or encapsulated in liposomes [1-4]. Compared to the previous strategies, the use of genetically engineered LLO-protamine fusion protein is advantageous as it is possible to produce large amounts of homogeneous fusion protein or various fusion proteins with different functional groups using relatively simple methods, and to incorporate consistent amounts of fusion proteins in gene delivery systems. In this study, we designed bifunctional fusion proteins that incorporate LLO and a 22 amino acid fragment of the DNA-condensing polypeptide protamine (PN), single (LLO-PN) or in tandem (LLO-PNPN) as a non-viral vector.

The recombinant fusion proteins LLO-PN and LLO-PNPN were successfully expressed and purified, and determined to be as active as wild type LLO at relatively high concentrations. We demonstrated that the luciferase gene expression of protamine/DNA complexes with a small amount of LLO-PN fusion protein incorporated was dramatically enhanced in a murine macrophage-like cell line. LLO-PNPN fusion protein exhibited more efficient augmentation of luciferase gene expression, in comparison with LLO-PN, both in these cultured cells and in mice. In addition, the association of anionic liposomes with the cationic LLO-PNPN/protamine/DNA complexes, yielding a net negative surface charge, resulted in better *in vitro* transfection efficiency in the presence of serum and improved *in vivo* luciferase gene expression in spleen and lung. The potential of this gene delivery system for application to DNA-based vaccines was also tested during the thesis work and demonstrated, though apparently limited because a weak immune response was generated in mice.

There are several outstanding questions that can shed lights on the mechanism of the enhancement of gene delivery by the studied LLO-protamine recombinant proteins; these questions were not fully addressed due to time limitations. Although the amount of fusion protein (0.15%), full-length protamine (1.2 w/w), or liposomes (7.5 nmol lipids/μg DNA) incorporated in this gene delivery system was optimized by monitoring transfection efficiency and cell viability with various amounts of each component, there is the possibility that our samples contain a small percent of unbound free fusion protein and/or free uncomplexed protamine, or complexes that were not associated with liposomes. Boeckle *et al*. purified PEI/DNA complexes from free PEI using size-exclusion chromatography [5]. Although the purified PEI/DNA complex showed low

cellular and systemic toxicity, its transfection efficiency at low DNA concentrations was lower than that with free PEI. Mechanistic studies suggest that free PEI contributes to the endosomal escape step by a proton sponge effect that is essential for intracellular delivery of DNA [5]. The LPDII consisting of DNA/polylysine complex and DOPE/CHEMS was also fractionated using discontinuous sucrose gradient centrifugation [6]. The transfection efficiency of the purified LPDII fraction separated from free DNA and lipid was similar to that of unpurified LPDII particles, suggesting that the presence of relatively small amounts of free DNA and lipid did not affect the overall gene transfer by LPDII, and purification may not be necessary for the improvement of gene delivery. Based on these previous studies, the purification of our gene delivery system by separation of free LLO-PNPN or uncoated complex may not affect the transfection efficiency, but it is worthwhile to understand the structure and characteristics of this gene delivery system and to prepare homogeneous systems for potential clinical application.

When we designed this fusion protein, a cathepsin D recognition sequence was introduced between LLO and C-terminal PN in keeping with the hypothesis that endosomal pore formation by the fusion protein is facilitated with the cleavage of the cathepsin D recognition sequence in endosomes and release of LLO from the complex, since the C-terminus of LLO is likely involved in membrane binding and pore formation. However, cleavage of the cathepsin D-recognition sequence incubated with cathepsin D was not observed in SDS-PAGE, and the transfection efficiency with LLO-PN was similar to that with LLO-control-PN or PN-LLO. Since intact LLO-PN fusion protein itself had hemolytic activity, the release of LLO by the cathepsin D-mediated cleavage may not be necessary for pore-formation in endosomes. An interesting outstanding

mechanistic question regarding LLO's *in vivo* activity is whether release from PN is required for perforation of endosomal membranes or if the LLO-PN fusion proteins are sufficiently active to see LLO-dependent enhancement of gene expresssion. One possible method to address that is, for example, to study intracellular trafficking of fluorescence labeled fusion protein such as YFP-LLO-PN-CFP, or YFP-LLO-PN and rhodamine labeled-DNA complex. In addition, because LLO-control-PN with cathepsin D non-cleavable linker also showed similar transfection efficiency, the intracellular trafficking of each component of LLO-PN/protamine/DNA complex will be helpful to understand the mechanism of pore formation of LLO-PN in protamine/DNA complex; whether LLO-PN dissociates from protamine/DNA complexes or whole LLO-PN/protamine/DNA complex bind to endosomal membrane.

The investigation of biodistribution of DNA *in vivo*, including in various tissues and cell types, following subcutaneous injection of Liposome(LLO-PNPN/protamine/DNA) into mice using fluorescence- or radio-labeled DNA remains to be addressed, since luciferase gene expression was not detected in major organs and lymph nodes and only weak antibody response against OVA antigen was observed after s.c. injection into mice. The potential for application as a DNA-based vaccine can be further investigated by immunization for longer periods such as three injections of 50-200 μg DNA each with two-week intervals, a protocol that has been reported to show higher immune responses [7-9], together with the careful characterization of the size of liposome formulation by freezing/thawing and extrusion. Dileo *et al*. reported that following i.v. injection, LPD particles accumulated in the marginal zone of spleen and taken up mainly by dendritic cells and macrophages [10]. The Liposome(LLO-PNPN/protamine/DNA)

formulation also showed the highest luciferase gene expression in spleen, suggesting that predominantly dendritic cells and macrophages were transfected, but further investigation of cell types transfected following i.v. injection remains to be addressed.

This initial study suggests that LLO-protamine fusion proteins have great potential as efficient non-viral gene delivery vectors. It is conceivable that with further modification and characterization the LLO-protamine chimeras could be tailored to achieve specific aims such as increasing/decreasing the PN fragment's affinity for DNA, mutagenesis of LLO to further limit potential damage to non-endosomal membranes, higher and controlled efficiency of endosomal escape, and less immunogenicity toward the vector components. Since other functional domains such as targeting ligands or nuclear-localization signals can also be added or replaced, and expressed as a single recombinant protein, this recombinant fusion protein as a non-viral vector has a possibility to be improved by incorporating additional multiple functional components and can be applied for various gene delivery needs.

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