國 立 交 通 大 學

生 物 科 技 系 暨 研 究 所

碩士論文

建立一種新型微脂體與聚合物之複合體來做為核酸疫苗與次單位疫苗的共同載體

Development of a novel liposome-polymer transfection complex as co-delivery system for DNA and subunit vaccine

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摘　　要

近年來多盛行以非病毒性載體來當作基因治療及疫苗開發等載體系統。 因此在本篇研究當中，主要是建立一種新型微脂體與聚合物之複合體，進而使其當作核酸或是蛋白質的載體系統。新型的複合體則是由兩種聚合物聚乙烯亞胺(PEI)及聚乙二醇(PEG)與大豆沙拉油經過快速的超音波震盪製作而成。 進一步分析其基本物理性質包括利用穿透式電子顯微鏡觀察複合體之外觀以及使用動態粒徑散射儀分析其顆粒大小分布約在212.2奈米至312.1奈米範圍之中，並且偵測此複合體之表面電位來確立其具有正電性質。此外，透過洋菜膠電泳方式證明此複合體具有緊密吸附核酸的能力並且可以保護核酸免於核酸酶的破壞。同時，經由細胞轉染實驗發現此複合體可以有效的在纖維母細胞中提升核酸的轉染效率並且增強基因表現。 再者，經由實驗證明此複合體確實可有效的提升巨噬細胞對抗原的吞噬能力與誘發老鼠免疫細胞較高量的腫瘤壞死因子, 這都說明了此複合體具有擔任疫苗佐劑功能的潛力。 然而在動物實驗上, 我們使用胃幽門螺旋桿菌相關的熱休克蛋白與尿素酶蛋白當作抗原, 進一步觀察核酸抗原及蛋白質抗原與複合體共同進行免疫老鼠的實驗, 則證實可誘發較高量的免疫反應產生。 因此，在本篇研究中已建立了一個核酸與蛋白質傳遞系統, 此

系統則是以微脂體與聚合物之複合體來當作載體，並且評估此載體在未來疫苗開發的潛力上，可由於製作的方便性及較低的材料價格，使其可當作動物農場所使用的核酸疫苗與次單位疫苗之共同傳輸系統。

Development of a novel liposome-polymer transfection complex as co-delivery system for DNA and subunit vaccine

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ABSTRACT

In this study, a novel liposome-polymer transfection complex (LPTC) was developed with inexpensive cost and used as DNA or protein delivery system. LPTC was fabricated via the sonication and composed of soybean oil, polyethylenimine (PEI) and polyethylene glycol (PEG). After preparation of LPTC, the morphology of particles and their physical properties were conducted with TEM, DLS and Zeta-Sizer. The particles of LPTC showed round shape and fuzzy edge around the surface of particle captured by TEM. In addition, the particle sizes of LPTC were in the major range of 212.2 nm to 312.1 nm and the zeta-potential (surface charge) was strongly positive (+38.7 mV). Moreover, we examined the ability of DNA condensation and the protection from DNase I digestion in agarose gel electrophoresis. In the *in vitro* tests, to clarify the gene delivery efficacy, we evaluated the transfection efficiency of LPTC/DNA complexes in Balb/3T3 cells and the transfection efficiency increased as the charge ratios of LPTC to DNA increasing. LPTC enhanced the cellular uptake of antigen in mouse macrophage cells and also stimulated TNF-alpha release in naïve mice splenocyte that both showed the potential to be adjuvant in vaccine development. *In vivo* studies, using *H. pyrori* relative Hsp60 and Urease B as antigen model, we observed that vaccination of BALB/c mice with LPTC complexed DNA and protein enhanced humoral immune response. Therefore,

these results showed that we have developed a DNA and protein delivery system by liposome-polymer transfection complex with inexpensive cost and successfully applied in the development of DNA and subunit vaccine. The success of this design may provide an economical vaccination alternative for farm animal use.

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Abbreviations

AAV	Adeno-associated virus
PEI	Polyethylenimine
PEG	Polyetheylene glycol
LPTC	Liposome polymer transfection complex
kDa	Kilo dalton
APCs	Antigen presenting cells
Hp	Helicobacter pylori
rHsp60	Recombinant Heat-shock protein 60
rUreB	Recombinant Urease B

1. Introduction

1.1 Gene delivery system

Gene therapy is the treatment or prevention of diseases by gene transfer, which has been rapidly studied and the central concept of gene therapy is the ability to deliver exogenous nucleic acids to cells of various tissues. Over the past ten years, the research of gene therapy has been moving quickly from the laboratory to the clinic. However, a successful gene therapy is depended on the efficiency of gene transfer. Although some success has been reported in the uptake of "naked" DNA, efficient delivery and persistent expression have been limited to only a few tissues such as muscle [1]. Thus, many approaches have been developed to enhance the efficacy of gene delivery, involving the injection of vectors.

There are generally two types of vector systems used for gene therapeutic applications – viral and non-viral. Viral vectors are able to achieve high-efficiency gene transfer. There are four types of virus currently in clinical trials-retroviruses, adenoviruses (AdV), herpes-simplex viruses and adeno-associated viruses (AAV) [2, 3]. AdV and AAV viral vectors have been found to be the most efficient for transducing muscle fibers. AAV viral vectors appear to be the least immunogenic and can sustain longer periods of transgene expression than other viral vectors. But several aspects need to be improved to achieve

therapeutic benefit in virus vectors. These include better efficiency and reliability of procedures for the preparation of virus, freedom from helper virus contamination, avoidance of immunogenicity of the viral particle itself, and ways of overcoming the limitation of transgene size. Hence, non-viral vectors are developed and offer many advantages over viral delivery systems shown in Table 1. In generally, non-viral vectors were divided into two types, one is cationic lipid and the other one is cationic polymer. Both of them are used to improve the efficiency of naked plasmid DNA for gene delivery and protect DNA from degradation.

1.2 Non-viral vectors for gene delivery

Cationic lipids or cationic liposomes are the most widely used and have been shown to raise the efficiency of in vitro gene delivery in many cell types. Lipofectin®, LipofectAMINE™ and CeLLFECTIN® are frequently used as lipid transfection reagents [4]. The mechanisms of lipids and liposomes improve gene transfer is not clearly understood. However, some of the improving transgene delivery reasons are listed in the following mechanisms. Positive charged lipid can bind and condense negative charged DNA to form a complex called "lipoplex", in which the DNA is protected against extracellular degradation [5]. And furthermore, the positively charged lipoplex binds to the negatively charged cell surface molecules facilitating endocytosis. Once lipoplex in the endosome, some lipid may destabilize the endosome membrane and encourage the release

of DNA into the cytosol, thus avoiding the lysosomal degradation pathway [6, 7].

The cationic polymer based transfection enhancing reagents called "polyplexes" are particular attractive for the delivery of nuclei acids in recent year [8]. Among the polycations presently used for gene delivery, polyethyleneimine (PEI), takes an important role [9]. Due to every third atom of PEI is a nitrogen atom capable of protonation, thus it shows high cationic charge density. But higher molecular weights lead to increased cytotoxicity, presumably due to aggregation of huge clusters of the cationic polymer on the outer cell membrane, which thereby induces necrosis [10]. It is commonly believed that the molecular weight of PEI most suitable for gene transfer ranges between 5 and 25 kDa. Thus, PEI has three common transfection systems that differing in the molecular mass and degree of branching including linear PEI (22kDa) (ExGen^{TM}500), linear PEI (50kDa) and branched PEI (25kDa).

In the biological environment (pH= 7.4), the amine groups of PEI are protonated to form quaternary amines that cationic charges are generated. Thus leading to a correlation between environmental pH and cationic charge density. All of linear PEI as well as branched PEI show the high buffering capacity above pH 7 that was attributed to the secondary amines. In addition to higher amounts of primary and secondary amines can be correlated with higher pka values, due to their higher protonation and, therefore, a higher number and density of positive charges. PEI is also capable of condensing plasmid DNA

into stable complexes via electrostatic interactions that offers some advantages for gene delivery. Such as the complexation of DNA with PEI form small particles that is a necessary prerequisite for the efficient delivery of the DNA into cells. The complexation and condensation behavior is dependent on several polymer characteristics, such as molecular weight, number and the density of charges, in addition to the composition of the complexes, e.g. the ratio of polymer to DNA. In fact, a lower charge density, as well as a lower molecular weight, can impair the condensation capability. Besides, the complexation of DNA with PEI could protect the cleavage form nuclease. PEI/DNA polyplexes are internalized by large variety of cells, delivering polymer/DNA complexes to endosomal compartment. The intrinsic endosomolytic activity of PEI is related to its capacity to buffer the endosomal environment, prompting the osmotic swelling of the vesicle and finally its rupture, which leads to the liberation of the polyplexes into the cytoplasm, that so called “proton sponge” hypothesis [11].

1.3 Vaccine development

Animal vaccine is a good strategy to prevent infectious disease of animal [12]. Especially the intensive feeding easily induces a great quantity of infection between animals. Throughout history, most vaccines have been developed using live attenuated organisms, killed whole organsims or inactivated toxins. Due to live vaccines may induce a serious risk of reverting back to their virulent form and intrinsic instability, making them difficult to deliver. In addition to

killed or inactivated whole organism vaccines generate a weaker immune response and typically require multiple doses. Thus, recent efforts have focused on utilizing technologies such as recombinant DNA methods to develop DNA and subunit vaccines [13, 14]. Theses vaccines are attractive because of their increased safety since they cannot revert to a virulent form and their lack of contaminants remaining form the original pathogenic organism. Additionally, the ability of consistently produce large, well defined quantities of antigen form recombinant methods is highly desirable.

1.4 DNA vaccine and subunit vaccine

DNA vaccine is an antigen-encoding plasmid that upon introduction into the body is capable of directing in vivo expression of that protein. This may induce the antigen-specific immune responses. DNA vaccines are able to induce cellular immune (cell-mediated immunity) in addition to antibody response (humoral immunity). Generally, cellular immunity is better able to fight viruses and parasites. DNA plasmid vector carry the genetic information encoding an antigen, allowing the antigen to be produced inside of host cell. The DNA vaccine-derived protein antigen is then degraded by proteosomes into intracellular peptides, following to bind MHC class I molecules on cell surface. The peptide antigen/MHC complexes bind to cytotoxic $CD8^+$ lymphocytes and induce a cell-mediated immune response. DNA-vaccines may offer new possibilities for prevention and control of diseases. They can be designed to

express the associated protein from infectious bacteria or viruses, which is recognized by the immune system. There are many advantages of DNA vaccines than traditional vaccines. DNA vaccines are able to induce the expression of antigens that resemble the natural epitopes more closely than standard vaccines. DNA vaccination could trigger both of cellular and humoral immune response after the internal production and presentation of the protein. Rapid and large-scale productions are available at costs considerably lower than traditional vaccines. They are also very temperature stable for making storage and transportation much easier.

Subunit vaccines use only the parts of an organism to stimulate a strong immune response. To create a subunit vaccine, the gene which code for appropriate subunits from the genome of the infectious agent are placed into bacteria or yeast host cells to produce large quantities of subunit molecules. These foreign molecules can be isolated, purified from host cells and used as a vaccine. Hepatits B vaccine is an example of this type of vaccine. With the development of these new types of vaccines, there exists a critical need for additional delivery vehicles as well as new adjuvants.

1.5 Liposome-mediated vaccine delivery

Liposomes are spherical entities composed of a phospholipid bilayer shell with an aqueous core and the potency of liposomes depends on the number of lipid layers, electric charge, composition and method of preparation. For vaccine

delivery, an antigen (or adjuvant) maybe either encapsulated in the core of the liposome, buried within the lipid bilayer or adsorbed on the surface for presentation to antigen presenting cells. Thus, for vaccine purposes, these particles are considered most useful for delivering antigens and adjuvants [15]. Cationic liposomes have been used extensively in both drug delivery and vaccine research. This is based on the assumption that cationic liposomes are able to deliver the antigen to the antigen-presenting cells (APCs) and thus enhance antigen-specific immune responses. Moreover, Nakanishi et al. has demonstrated that cationic liposomes were much more potent than anionic or neutral liposomes for generating a cell-mediated immune response [16]. Hence, the adsorption of antigen onto the cationic liposome increase the efficiency of antigen presentation, it has been suggested by the primary adjuvant mechanism of cationic liposomes, which targeting the cell membrane of antigen-presenting cells, then subsequently leads to enhanced uptake and presentation of antigen [17]. An interesting preclinical study in mice conducted by Guan et al. evaluated the effect of the liposome formulation on the type of immune response generated for a MUC1 therapeutic cancer vaccine. This study revealed that liposome-associated (either encapsulated or surface-exposed MUC1 peptide (BP25) produced a strong specific CTL response [18]. In addition to cationic lipids such as DC-Chol and DDA have been effectively used as adjuvants. It has been claimed that DC-Chol is able to overcome the observed non-responsivness to hepatitis B vaccine. Brunel et al. have outlined its use in a liposomal adjuvant

where, if used in conjunction with genetically engineered Hepatitis B, the levels of specific antibodies (IgG1 and IgG2a) and cell factors can be increase [19]. Although liposomes constituted one of most studied antigen delivery systems, they are still the subject of novel results on enhancing strategies. The synergistic effect of liposomally co-entrapped DNA and protein has been shown to exceed the well-known adjuvant effects of plasmid DNA and liposomes. This new approach to vaccination has been termed ‘codelivery’ and it may derive from the simultaneous presentation of antigen via MHC class-I (DNA) and MHC class-II (protein) pathways to CD8+ and CD4+ cells at the same antigen presenting cell mode of presentation that would commonly occur with live viral pathogens, opening new uses for this technology.

Here, we evaluated the novel liposome-polymer transfection complex as a delivery system and applied in the development of DNA and subunit protein antigen vaccine. The delivery system had the advantages of ease manufacture and low cost in comparison to general cationic liposome of antigen delivery system. Thus, it showed the potential to apply in the development of animal vaccine for farm animals that need large quantity of vaccine product with low cost to prevent the infectious disease, particularly for species where a large number of animals with a relatively low commercial value are utilized such as chickens.

2. Materials & Methods

2.1 Chemicals

PEI (branched form, average molecular weight of 25 kDa) was purchased from Aldrich Chemical Co. (St. Louis, MO, USA), PEG (average molecular weight of 8,000) and Propium ioide (PI) were purchased from Sigma Co. (St. Louis, MO, USA). Soybean oil was purchased from Uni-President® Co. (Taiwan, ROC). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, antibiotics (penicillin 100U/ml; streptomycin 100 μg/ml), SYBER green and Trypan blue stain were purchased from GIBCO (Grand Island, NY, USA)

2.2 Cells and Culture, Plasmids

The mouse embryo fibroblast cell line Balb/3T3 was cultured in DMEM with 10% FBS and 1% PSA. The mouse lymphoblast cell line P338/D1 was cultured in RPMI with 10% FBS and 1% PSA. Cells were cultured in a 37°c incubator with 5% CO_2. The plasmid of pAAV-MCS-hrGFP was from Dr. Liao's Lab (the department of biological science and technology, NCTU).

2.3 Mice and Immunogens

Female BALB/cByJNarI mice 3-5 week of age were purchased from National Laboratory Animal Center (NLAC). All mice were maintained under standard pathogen-free conditions. Female mice were used at 9-10 weeks of age. For DNA vaccine, both of the plasmids of pCJ3- HpHsp60 and pCJ3-Urease B were gifts from Dr. Wu's Lab (the department of food science, NTOU). Plasmids DNA was purified by anion exchange chromatography (Qiagen-tip 2500 Mega Prep Kit, Qiagen. Germany). For subunit protein vaccine, recombinant *H pylori* heat-shock protein 60 (rHpHsp60) and recombinant Urease B protein (rUreB) were encoded by pET 30a-HpHsp60 and pET 30a-Urease B DNA which from Dr. Liao's Lab (the department of biological science and technology, NCTU).

2.4 Preparation of Liposome-Polymer Transfection Complex

Liposome-polymer transfection complex (LPTC) was formed by two hydrophilic polymers (PEI, PEG) and soybean oil, two phases were mixed by sonication. Briefly, the first step was to make separately oil and aqueous phases. In aqueous phase, polyethyleneimine (PEI) and polyethylene glycol (PEG) at 1:1 molar ratio dissolved in ddH_2O until the mixture dissolved well. After aqueous phase was prepared, soybean oil added to the aqueous phase with 10% of total volume of mixture. Then, the mixture was vortexed for 10min and then, sonicated at 25W for 30min at room temperature until it formed milky white appearance and stored at 4°C.

2.5 Particle Size and Zeta-Potential Measurement

The particle size and zeta-potential of LPTC was measured by the laser light scattering measurement using a Malvern Zetasizer 3000HS (Malvern Instruments, UK). Here, we used N/P ratios to describe the PEI/DNA complexes (PEI contained in LPTC) that were the ratios of moles of the amine groups of PEI to those of the phosphate groups of DNA. The calculation of N/P ratios was described in the following section. The LPTC/DNA binary complexes were prepared at N/P ratios ranging from 1 to 30 containing 50μg DNA. Then the prepared complexes were incubated for 30 minutes and diluted by distilled water to 1ml volume for the size and zeta potential measure respectively. The distribution of particle size was measured by dynamic light scattering (90 plus, Brookhaven Instruments Corp., USA)

The calculation of the N/P ratio is based on the assumption that one repeating unit of PEI featuring one nitrogen corresponds to 43.1 g/mol, and one repeating unit of DNA featuring one phosphate corresponds to 330 g/mol. To calculate the N/P ratio for a 1 mg/mL solution of 25kDa PEI, the equation is as follows:

$$\text{N/P} = \frac{(\mu\text{L PEI stock solution}) \times 23.2\ \text{mM nitrogen residues}}{(\mu\text{g plasmid DNA}) \times 3\ \text{nmol phosphate}}$$

In this example, for 100 μg plasmid DNA and 200 μg PEI (= 200 μL), the N/P ratio is 15.5.

2.6 Transmission Electron Microscopy (TEM)

The mixture was placed onto a 400 mesh copper grid coated with carbon. About 2 min after deposition, the grid was tapped with a filter paper to remove surface water and placed into dry box for two days.

2.7 Gel Electrophoresis Assay

The LPTC/DNA binary complexes at varying N/P ratios ranging from 1 to 30 were prepared by adding appropriate volumes of LPTC to 300 ng plasmid DNA pre-stained by 1μl SYBR Green in ddH_2O. The binary complexes were incubated at 37°C for 30min, and then heparin (Sigma, St. Louis, MO) was added in different dosage from 150 μg to 1 μg. After 10min, the complexes were in a total volume of 10 μl and then were loaded on the 0.8% (w/v) agarose gel with Tris-acetate (TAE) running buffer at 100V for 30min. DNA was visualized with a UV lamp using Uni-photo gel image system (EZ lab, Taiwan, ROC).

2.8 DNase I Protection Assay

LPTC was complexed with 300 ng DNA at N/P = 10, then the complexes were treated either (1) DNase I alone (1 units), (2) DNase I and heparin at the same time, (3) DNase I then heparin or (4) heparin alone. For the treatment of DNAase I, 300ng DNA was incubated with 1 units of DNase I in a 20 μl reaction mixture at 37°C for 30 min, and then 0.5 M EDTA was used to stop the

reaction followed by heparin or buffer. The DNA complexes were analyzed with 0.8% agarose gel eletrophoresis.

2.9 Plasmid DNA Extraction- Midi Preparation Method

The midipreparation was performed by NucleoBond PC 100 kit (Macherey-Nagel, Duran, Germany) following the manufacturer's protocol. At first, a single colony of *E. coli* was inoculated in 100 ml of LB broth contained antibiotics and grew overnight at 37°C with agitation. The cells were recovered by centrifugation at 8,000 rpm for 15 minutes at 4°C. The pellet was collected, and 4 ml buffer S1 (with RNase) (Macherey-Nagel, Duran, Germany) was added to dispense the pellet. Then 4 ml buffer S2 (Macherey-Nagel, Duran, Germany) was added to the suspension**.** The lysate was mixed gently and incubated at room temperature for 3 minutes (no more than 5 minutes). The pre-cooled 4 ml buffer S3 (Macherey-Nagel, Duran, Germany) was then added to the solution and mixed gently until a homogeneous suspension containing an off-white flocculate was formed. The mixture was incubated on ice for 5 minutes and then spun at 13,000 rpm for 25 minutes at 4°C. The supernatant was loaded onto the NucleoBond AX 100 Midi column which was equilibrated with 2.5 ml buffer N2 (Macherey-Nagel, Duran, Germany). The flow-through was emptied by gravity flow and discarded. 10 ml buffer N3 (Macherey-Nagel, Duran, Germany) was added to wash the column twice. The DNA was eluted by 5 ml buffer N5 (Macherey-Nagel, Duran, Germany) Then 3.5 ml isopropanol

was added to precipitate the DNA. The mixture was incubated on ice for 10 minutes and recovered by centrifugation at 13,000 rpm for 30 minutes at 4°C. 6 ml 70% ethanol was added to the pellet and the solution was spun at 13,000 rpm for 5 minutes. Finally, the pellet was dissolved in appropriate amount of ddH2O and stored at -20°C.

2.10 Plasmid DNA Extraction- Mega Preparation Method

A single colony was picked from a freshly streaked selective plate and inoculated a starter culture of 5ml LB medium containing the appropriate selective antibiotic. After incubation for 8 hr at 37°C with vigorous shaking (225rpm), 2.5ml of starter culture was inoculated to 2.5 liters LB broth contained selective antibiotic for 12–16 h with vigorous shaking (225rpm). . The cells were recovered by centrifugation at 8,000 rpm for 15 minutes at 4°C. The cell pellet was resuspended in 50 ml buffer S1. Then 50 ml buffer S2 was added and mixed thoroughly by vigorously inverting 4–6 times, and incubated at room temperature for 5 min. The 50ml pre-cooled buffer S3 was added immediately and thoroughly by vigorously inverting 4–6 times, and incubated on ice for 30 min. After centrifugation at 12,000rpm for 30 min at 4°C, the supernatant was loaded on to a QIAGEN-tip 2500 column which was equilibrated by applying 35 ml buffer N2, and allowed the column to empty by gravity flow. 100 ml buffer N3 was added to QIAGEN-tip 2500 column and wash twice. DNA was eluted with 35 ml buffer N5, then adding 24.5ml (0.7 volumes) room-

temperature isopropanol to precipitate the eluted DNA. Mix and centrifuge immediately at 12,000 rpm for 30 min at 4°C. Carefully decant the supernatant. Wash DNA pellet with 7 ml in room-temperature 70% ethanol, and centrifuge at 12,000 rpm for 10 min. Carefully decant the supernatant without disturbing the pellet. Air-dry the pellet for 10–20 min, and re-dissolve the DNA in a suitable volume of DDW

2.11 *In Vitro* Cytotoxicity Assay

The cytotoxicity assay was performed with Balb/3T3 cells by MTT assay. Briefly, the cells were seeded in a 96-well plate at a density of 2×10^4 cells/well, and then cells were incubated in 100 μl DMEM containing 10% FBS for 3hr prior to adding the LPTC. After LPTC were added for 3 days, the medium was replaced with 100μl of fresh medium and 10 μl MTT (5mg/ml) solutions was added to each well. After 4 hr of incubation, the medium was removed and 100μl DMSO was added. The absorbance was measured at 595 nm using a microplate reader (Tecan). The relative cell viability was calculated as:

$$\textbf{Cell Viability (\%)} = (OD_{595(sample)} / OD_{595(control)}) \times 100$$

$OD_{595(control)}$ was obtained in the absence of polymers and $OD_{595(sample)}$ was obtained in the presence of LPTC.

2.12 *In Vitro* Transfection Assay

Cells were seeded in the 6-well plate at a density of 2.5×10^5 cells/well and cultured with 2ml growth medium for 24 hours respectively. Cells were transfected plasmid DNA encoding GFP gene by LPTC with various N/P ratios. Briefly, 3 μg plasmid DNA and 5 μl of LPTC in different concentrations were each diluted into 100 μl of 150mM NaCl and vortexed. The LPTC solution was added into DNA solution for 5 minutes (Notice: not the reverse order), and then vortexed. After 20 minutes, the cells were rinsed and supplemented with 200 μl Opti-MEM I medium (Gibco, Grand Island, NY). The LPTC/DNA mixture was gently and equally added to each well. Finally, Opti-MEM I medium (600 μl) (Gibco, Grand Island, NY) were added to each well. After 12 hours incubation, 2ml fresh growth medium were added into each well. After 48 hours, the gene expressions were measured by FACScan flow cytometry (Becton Dickinson, Moutain View, CA).

2.13 Measurement of Transfection Efficiency by Flow Cytometry

After 48 hours of transfection, cells were harvested to measure the gene expression. Briefly, the medium was discarded and each well was rinsed with 1ml PBS. 1ml trypsin was then added, and the cells were incubated at 37°C for 5 minutes. 1ml PBS was added into each well and the cells were recovered by centrifugation at 1,500rpm for 5 minutes at 4°C. The supernatant was discarded and the pellet was re-suspended by 1ml PBS in FACS tube. The reporter gene

expression was measured by FACScan flow cytometry (Becton Dickinson, Moutain View, CA). Fluorescence intensities were analyzed with CELLQUEST software (Becton Dickinson).

2.14 *In Vitro* Cellular Uptake Assay

BSA-FITC (14 μg/ml) mixed with LPTC (4.8 μg) for 30min in room temperature and kept in dark. P338/D1 cells (2×10^6 cells/ml) were pulsed with BSA-FITC/LTPC complexes and BSA-FITC alone in a humidified atmosphere at 5% CO_2 and 37°C or at 4°C. After incubation for 2hr, cell were centrifuged in 4,000 rpm for 5 min and washed with 1ml cold PBS for two times. After the final steps of washing procedure were finished, the supernatant was discarded and the pellet was re-suspended in 1ml cold PBS in FACS tube. To remove the surface-associated BSA-FITC on the cell membrane, 20 μl trypan blue were added to quench and kept in dark on ice for the following measurement of FACScan flow cytometry (Becton Dickinson, Moutain View, CA).

2.15 Recombinant HpHsp60 Protein Expression

pET-HpHsp60 were transformed into *E. Coli* BL21, then grown on LB plates containing kanamycin (30 mg/ml) at 37°C. After 16 hr incubation, five colonies were inoculated into 100ml LB medium containing kanamycin (30 mg/ml) at 37°C for 16 hr. The culture broth were refreshed in 900 ml LB

medium with shaking at 37°C, 225 rpm until the value of OD 600 reaches 0.6 (about 40 min). IPTG diluting from stock concentration of 800 mM to a final concentration of 1 mM were added to culture broth for 4 hr incubation. Cells were harvested by centrifugation at 8,000 rpm for 15 min at 4°C. Supernatant were discarded and the pellet were resuspended into 30 ml binding buffer. Then, total cell lysates were sonicated for 15 min and centrifuged at 12,000 rpm for 30 min at 4°C. The purification of protein was performed on HisTrap^{TM} HP column. All of the buffer used in the experiment should be filtered with 0.45 μm syringe filter. Column were firstly washed by 5 column volumes of DDW and then equilibrated by 5 column volume of binding buffer at the flow rate about 1ml/min. Pretreated sample were loaded into column and were washed by wash buffer about 60 column volume. Then, column eluted with elution buffer and each fraction was collected for protein detection by coomasie reagent. The positive fractions were collected and loaded into G25 column to remove unnecessary salt from the solution with PBS. Each fraction was collected and was detected by coomasie reagent for protein content. Then the recombinant protein was checked by SDS-PAGE (Fig.14).

- **Elution buffer** (20 mM Na_2HPO_4, 0.5 M NaCl, 200 mM imidazole, pH 7.4)
- **Binding buffer** (20 mM Na_2HPO_4, 0.5 M NaCl, 40 mM imidazole, pH 7.4)
- **Wash buffer** (20 mM Na_2HPO_4, 0.5 M NaCl, 60 mM imidazole, pH 7.4)

2.16 Recombinant Urease B Protein Expression

pET-30-Urease B plasmid were transformed into *E. Coli* BL21, then grown on LB plates containing kanamycin (30 mg/ml) at 37°C. After 16 hr incubation, five colonies were inoculated into 100ml LB medium containing kanamycin (30 mg/ml) at 37°C for 16 hr. The culture broth were refreshed in 900 ml LB medium with shaking at 37°C, 225 rpm until the value of OD 600 reaches 0.6 (about 40 min). IPTG diluting from stock concentration of 800 mM to a final concentration of 1 mM were added to culture broth for 4 hr incubation. Cells were harvested by centrifugation at 8,000 rpm for 15 min at 4°C. Supernatant were discarded and the pellet were resuspended into 30 ml lysis buffer. Then, total cell lysates were sonicated for 15 min and centrifuged at 12,000 rpm for 30 min at 4°C. The pellet was resuspended by 10 ml wash buffer I and centrifugated at 12,000 rpm for 20 min. Repeat the same step of above mentioned process. Then, the pellet was resuspended by 10 ml wash buffer II and the mixture was shake in 4 °C for 20 min (55 rpm). After the mixture was well mixed, the mixture was centrifugated at 12,000 rpm for 20 min. Repeat the same step of above mentioned process, again. The collected pellet was resuspened by 10 ml lysis buffer and centrifugated at 12,000 rpm for 20 min. The pellet was resuspened by 10 ml binding buffer (6N urea) and the mixture was shake in 4 °C for 12~16 hr (55 rpm). The mixture was collected and centrifugated at 12,000 rpm for 30 min. Then the supernant should be filtered with 0.45 μm syringe filter. The purification of protein was performed on

HisTrap™ HP column. All of the buffer used in the experiment should be filtered with 0.45 μm syringe filter. Column were firstly washed by 5 column volumes of DDW and charged by 5 column volume of charge buffer, then 5 column volumes of binding buffer at the flow rate about 1ml/min. Pretreated sample were loaded into column at 1ml/min and were washed by wash buffer (6N urea) about 10 column volumes at 1ml/min. Then, column eluted with elution buffer and each fraction was collected for protein detection by coomasie reagent. The positive fractions were collected and loaded into dialysis membrane (Spectrum Laboratories. Inc., USA) to exchange the urea contented solution Each fraction was collected and was detected by coomasie reagent for protein content. Then the recombinant protein was checked by SDS-PAGE (Fig. 14).

- **Wash buffer I (**50mM Tris-HCl , 1mM EDTA , 100mM NaCl , 1% Triton X-100 pH 8.0)
- **Wash buffer II (**50mM Tris-HCl , 1mM EDTA , 100mM NaCl , 2% Sodium deoxycholate pH8.0)
- **Lysis buffer (**50mM Tris-HCl , 1mM EDTA , 100mM NaCl , pH8.0)
- **Binding buffer with 6N urea (**50mM Tris-HCl , 1mM EDTA , 100mM NaCl , 6N urea pH8.0)
- **Wash buffer (**20mM Tris-HCl , 0.5M NaCl , 6N urea , 20mM Imidazole pH8.0)
- **Elution Buffer (**20mM Tris-HCl , 0.5M NaCl, 6N urea , 200mM Imidazole pH8.0)

2.17 *Ex Vivo* Splenocyte Stimulation of LPTC

Spleens were isolated aseptically in a laminar flow hood. Organs were cut in several pieces and clumps were further dispersed by drawing and expelling the suspension for several times through a sterile syringe with a 19-G needle. Suspensions were filtered through sterile gauzes. After 5min centrifugation with 1500rpm, pellets were resuspended with 10ml ACK lysis buffer (1X) in DDW and incubated for 10min. Suspensions were centrifugated with 1500rpm for 10min, then the pellets were washed by 5ml PBS and centrifugated (1500rpm, 5min) to remove the supernatant. Cells were resuspended with RPMI-1640 medium and immediately used. Splenocytes were seeded in 24 well at a density of 4×10^6 cells/ well, then 80μg LPTC were added into cells and further cultured with 1ml RPMI-1640 medium for 48 hr.

2.18 Cytokine Release From Splenocytes

TNF-alpha in cell-culture supernatants was collected by centrifugation and assayed by ELISA using a mouse TNF-alpha ELISA development kit (R&D). The concentration of TNF-alpha was determined using a standard curve. ELISA was performed as according to the manufacturer's instruction.

2.19 *In Vivo* immunization regimen

For heterologous immunization protocol, naïve mice were prime immunized with DNA (ie, pCJ3-Hphsp60 and pCJ3-Urease B) and were boosted

with protein (ie, rHpHsp60 and rUreB) (Fig.11). Control group received sterile normal saline solution. DNA was administered as 12.5 μg/dose with or without formulated with 10μl LPTC (3.28 mg/mL) in 5% glucose PBS and then protein was administered as 100 μg/dose with or without formulated with 10μl LPTC (100 mg/mL) in 5% glucose PBS by subcutaneous inoculation. One additional group received 25 μg DNA with formulated with 20μl LPTC (3.28 mg/mL) in 5% glucose PBS and boosted by 100 μg protein formulated with 10μl LPTC (100 mg/mL) in 5% glucose PBS. Each mouse was inoculated with 100μl formulation.

For co-delivery of DNA and protein antigen immunization protocol, we injected 100 μg protein antigen (ie, rHpHsp60 and rUreB) and 50 μg DNA (ie, pCJ3-Hphsp60 and pCJ3-Urease B) in 5% glucose PBS, or formulated with 10μl LPTC (25 mg/mL) in 5% glucose PBS buffer via subcutaneous route (Fig.15). Control group received sterile normal saline solution. Each mouse was inoculated with 100μl formulation. Mice were immunized at 0 and 2 weeks. Blood were collected before each immunization and 2 weeks after the last dose.

2.20 Determination of Serum Antibody Levels

Blood sample were collected from the retro-orbital plexus of mice. Serum anti-HpHsp60-specific antibodies and anti-Urease B-specific antibodies were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, micro-

ELISA plates (Nunc-Maxisorp, Nunc, Wiesbaden, Germany) were coated with 100 ng rHpHsp60 or rUreB protein per well in 100 μl PBS. Serial dilutions of the sera in PBS were added to the antigen-coated wells. And then incubation for 1 h at room temperature followed by three washes with PBS supplemented with 0.05% Tween 20. Bound serum antibodies were detected using horseradish peroxidase conjugated anti-mouse total Ig antibodies at a dilution of 1:5000 followed by incubation with TMB. The reaction was stopped by 1 N HCl and the extinction was determined at 450 nm. End-point titers were defined as the highest serum dilution that resulted in an absorbance value three times greater than that of negative control sera (derived form non-immunized mice).

2.21 Statistical analysis

Results were expressed as mean ± SE. Statistical significance of differences between mean values was estimated using the Student's *t*-test (Microsoft Excel). $p < 0.05$ was considered significant.

3. Results

3.1 Physical characterization of Liposome-polymer transfection complex

The liposome-polymer transfection complex (LTPC) was constructed from two hydrophilic polymers (PEG, PEI) and soybean oil. After sonication, LPTC could be rapidly produced and appeared as white emulsion (Fig. 2). Sequentially, the sizes of LPTC were measured by dynamic light scattering (DLS) and the results showed that the major particle sizes were distributed between 212.2 nm and 320.1nm. And the second range was from 1099.5 nm to 1658.9 nm (Fig. 3). The surface charge of the particles showed cationic property and the strength was +38.6 mv measured by zeta-sizer. Besides, the complex's pH was measured and the value was 10.98 (data not shown).

The structure of LPTC was also observed by transmission electron microscopy (TEM), which showed hollow structure and round shape (Fig. 4a). Furthermore, the outer surface of the particles were high dense as hair-like structure (Fig. 4b). The hair-like structure was considered as made by polymers.

3.2 The effect of DNA bound to LPTC on particle size and zeta-potential

The cationic LPTC may bind to anionic DNA via electrostatic interaction. Thus, different amounts of LPTC were reacted with 50 mg DNA to observe the changes on their particle size and zeta-potential. As the amounts of LPTC were

increased, the sizes of LPTC/DNA complexes were firstly increased from 1 to 5 of N/P ratio, in contrast to the higher N/P ratio (10 and 30) that would decrease their sizes (Fig. 5a). In addition to the changes of zeta-potentials (surface charge), the surface charges were firstly decreased from 1 to 5 of N/P ratio and then increased at 10 to 30 of N/P ratio (Fig. 5b). We also captured the appearance of LPTC/DNA complex by TEM (Fig. 6).

3.3 Gel retardation of LPTC/DNA complex

The binding capability of DNA is essential for gene transferring. Hence, the DNA binding ability of LPTC was examined by agarose gel electrophoresis in different N/P ratios (Fig. 7a). The result showed that at N/P ratios higher than 5, there was no band on the gel. It referred that plasmid DNA was adsorbed by LPTC. Moreover, heparin was also used to compete with the electrostatic interaction of DNA and LPTC (Fig. 7b). However, when the complexes were incubated with higher dosage of heparin, the migration of DNA was observed obviously at N/P ratios ranging from 1 to 30. Therefore, the results indicated that DNA did bind to LPTC.

3.4 Protective effect of LPTC/DNA complex on DNase I digestion

To investigate whether DNA complexed to LPTC could be protected from DNase I digestion was further proceeding. Hence, LPTC/DNA complex was treated with DNase I and examined the integrity of DNA bound on LPTC (Fig.

8). Gel electrophoresis revealed that DNA alone without LPTC adsorption was completely degraded by DNase I but not affected by heparin treatment (lanes 7 and 8). In the counter part, LPTC/DNA complexes treated with DNase I (lane 2) showed the same intensity band as untreated LPTC/DNA complex (lane 1). In addition, heparin was added to release bound DNA from LPTC with or without DNase I treatment (lane 3 and 5), then these results showed that DNA bound on LPTC was not affected by DNase I. By contrast to the bound DNA released from LPTC by heparin was digested by DNase I (lane 4). Therefore, we proved that LPTC showed the ability to protect bound DNA from nuclease's digestion.

3.5 *In vitro* transfection efficiency of LPTC/DNA complex

In the *in vitro* transfection tests, LPTCs were complexed with plasmid pAAV-MCS-hrGFP DNA encoded green fluorescent protein as reporter gene and then transfected into Balb/3T3 cells in the absence of serum conditions. By different N/P ratios, the reporter gene expressions were low efficiency (2% or 13% at 1 or 5 N/P ratio), in contrast to high trasnfection efficiency 32% or 63% at 10 or 30 N/P ratio (Fig. 10). However, the gene expressions of green fluorescent protein were observed by fluorescent microscopy and the results showed at N/P ratio of 5 and 10 had high expression (Fig. 11). The results indicated that N/P ratio of LPTC could affect the transfection efficiency and the expression of transfectants.

Furthermore, the cytotoxicity of LPTC in Balb/3T3 cells was also measured by MTT assay and the results showed that the dosage of LPTC at N/P ratio of 30 and 10, cell viability was 70.4% and 85%, respectively (Fig. 9).

3.6 Cellular uptake of LPTC/BSA-FITC complex

Here, whether LPTC could enhance cellular uptake was investigated. BSA conjugated FITC was complexed with different dosage of LPTC to interact with macrophage cells (P338/D1). The intracellular fluorescence represented the degree of internalization. At 37°C, the cellular uptake efficiency of LPTC/BSA-FITC complex was increased 38.7% compared with BSA-FITC alone (Fig. 12a). However, cellular uptake efficiency of LPTC/BSA-FITC complex was not different with BSA-FITC alone at 4°C (Fig. 12b).

3.7 LPTC induces TNF-alpha secretion in splenocytes of naïve mice

Naïve mice splenocyte were taken out and treated with LPTC for 48 h, then TNF-alpha secretion were detected in supernatant by cytokine ELISA. The result showed that LPTC could stimulate TNF-alpha secretion in splenocytes and show higher concentration (105 pg/ml) of TNF-alpha secretion than control group (26 pg/ml) (Fig. 13).

3.8 The *in vivo* adjuvant effects of LPTC on heterologous immunization

In vitro studies showed that LPTC could enhance the gene transferring efficacy, the cellular uptake of antigen by macrophage cells and also stimulate the pro-inflammatory cytokine (TNF-alpha) secretion. Thus, we further investigated whether LPTC could be an effective vector to deliver DNA or antigen *in vivo*. Firstly, a heterologous immunization protocol was developed by priming with DNA and boosting with protein antigen to investigate the effect of LPTC on the induction of humoral response (Fig. 15). Specific anti-Hphsp60 responses and anti-Urease B responses were measured, respectively. The results showed that the treatment of LPTC complexed with DNA or antigen could enhance about 1-fold of anti-Hphsp60 Ig responses compared to immunization without LPTC facilitation (Fig.16a). Furthermore, LPTC complexed with 25 μg of DNA could increase anti-Hphsp60 Ig responses and show to enhance about 2-fold of Ig responses compared to 12.5 μg of DNA at weeks 4 (Fig.16b). By contrast, the anti-Urease B Ig responses were both induced at low levels at 2nd and 4th weeks (Fig. 17a, 17b). Thus, we continued to booste of 100 μg DNA with or without LPTC formulation (Fig. 18). Both of LPTC formulated groups enhance about 1 –fold of anti-Urease B Ig response.

3.9 The *in vivo* adjuvant effect of LPTC on co-delivery immunization

However, what a vaccine strategy could induce antigen specific immune response in the short time was an important issue in clinical trials. Here, co-delivery immunization protocol was developed by prime or boost with DNA and

protein antigen at the same time to investigate the effect of LPTC on the induction of humoral response (Fig. 19). After 2 weeks of last boost, the results showed that the treatment of LPTC complexed with DNA and protein antigen could enhance about 2-fold of anti-Hphsp60 Ig responses (Fig. 20) and about 3-fold of anti-Urease B Ig responses compared to immunization without LPTC facilitation (Fig. 21).

4. Discussion

In recent years, the development of vaccine was mostly focused on liposome that can encapsulate the antigens within the lipid bilayer. The encapsulated antigens would slowly release from the vesicle to continuously stimulate host immunity, which can trigger more efficient immune responses than non-capsulated antigens. In addition, Korsholm KS and colleagues also demonstrated that cationic liposome-capsulated antigen will facilitate the antigen uptake and presentation [17]. Furthermore, cationic liposomes have more potency than anionic or neutral liposomes in inducing cell-mediated immune response to soluble proteins [16]. Besides, the various phospholipids had differential adjuvant activities to induce protective immunity. Together these results, they suggested that the structures or components of liposomes may be determined the strengths of the immune responses or adjuvant effects.

Liposome-based vaccines have been investigated in human trials including vaccines against malaria, HIV, hepatitis A and influenza and these vaccines were safe and highly immunogenic [20]. However, liposome-based vaccines for animal are few and far between in vaccine development. In the intensive farm of livestock breeding, the prevention of infectious disease would become very important to avoid economic loss. Thus, how to develop an inexpensive and an efficient liposome-based animal vaccine would be an important issue to apply in intensive farm of livestock [12]. Here, we developed a novel liposome-polymer

transfection complex (LPTC) which have the components including high molecular weight branched polyethylenimine (PEI, 25 kDa) (Fig. 1), polyethyleneglycol (PEG) and soybean oil. The cationic polymer, PEI was an effective transfection reagent and had some particular advantages such as, (1) the high cationic charge-density potential is able to compact DNA efficiently for enhancement of DNA delivery [10], and (2) its "proton-sponge" effect could release DNA from the endosome to protect transgenes from degradation [11]. The other hydrophilic molecules polymer, polyethylenglycol (PEG) has been used to coat liposome and the PEG coating liposome could significantly diminish the uptake by macrophage of liver and spleen leading to an increase blood circulation time [21]. Generally, the third component is soybean oil that composed of three unsaturated fatty acid (linolenic acid, linoleic acid and oleic acid) and two saturated fatty acid (stearic acid and palmitic acid). Here, soybean oil was used to form liposome structure via sonication and may also provide an modification that is suitable for extra addition of immunostimulatory components such as the saponin adjuvant Quil A, which is derived from the bark of the South American *Quillaia saponaria* Molina tree and has been used as component of immunostimulating complex (ISCOM) [22]. The immunostimulatory agents typically showed the hydrophobic nature and immunogenic property, hence easily mixing with soybean oil through apolar interactions to improve the adjuvant effect of vaccine. In addition, all of these components in LPTC are very inexpensive, which can largely reduce the cost of

animal vaccine and suit to apply in massive inoculation of animal vaccine for prevention of infectious disease between animals.

However, for gene delivery vectors included cationic liposome, cationic polymer and polycationic liposome, they should be examined in the following criteria: (1) DNA adsorptive ability, (2) the efficiency of gene delivery, (3) low cytotoxic, and (4) large-scale commercial manufacture. These criteria were important for efficient gene expression and possible practical application. Here, we constructed the novel LPTC with the satisfactions for above criteria and offered an efficient and rapid preparation procedural to produce the gene delivery vectors. We used LPTC that showed high positive charge to absorb DNA and formed smaller complexes. In addition, the complexes showed the higher efficiency of gene transferring in N/P ratio of 10 and 30. Besides, we found LPTC showed the adjuvant effect and efficiently enhanced antigen specific antibody responses in both strategies of immunization (heterlogous and codelivery).

The structure and dimensions of LPTC were round, nanometer scale for size (Fig. 4) and showed the positive charge. We speculated the positive charge that was arisen from the cationic polymer (PEI) and it may exist on the surface of particles as the high density of hair-like filaments (Fig. 4b). The assumption of the interaction between PEI polymers and liposome was due to the hydrophobic force between large branched chains of PEI and lipid layer. Furthermore, the dimensional changes and surface charge changes occurring by varying the N/P

ratios of LPTC/DNA complex formation was examined (Fig. 5). Here, the result showed higher N/P ratio that could form smaller complexes and we presumed that DNA rigidly bound on LPTC at the existence of large amounts of LPTC. And also another view pointed that high positive charge may reduce the aggregation by electronic repulsion, which helpt the smaller LPTC/DNA complexes formation. Furthermore, for positive charged complex in surface charge detection, which can facilitate adherence to cellular membranes, inducing and increasing intracellular uptake.

A successful gene therapy or DNA vaccine relies on an efficient DNA liberation from the endocytotic vesicles and the following DNA nuclear localization for final gene expression. However, D Lechardeur1 et al. proposeed that cytosol nucleases are responsible for the rapid degradation of plasmid DNA, which limits the ability of DNA nuclear localization [23]. Our data shown that LPTC was not only complexed with DNA efficiently in high N/P ratio but also protected DNA from the DNase I digestion (Fig. 7 and Fig. 8). Godbey et al have been implied that the protection of DNA by PEI resulted from a physical or electrostatic barrier to enzymatic degradation with DNAse I [24]. Thus, we speculated that PEI exhibited an important role in LPTC for DNA complexation and the protection of DNase I degradation.

Furthermore, *in vitro* transfection results showed that as the N/P ratios increasing could increase the transfection efficiency (Fig. 9). The gene expression could be easily visualized at N/P ratio of 5 and 10. (Fig. 10c,d).

However, the higher transfection efficiency was at N/P=30 but exhibited low gene expression in fluorescence microcopy observation (Fig. 10e). The possible explanation was that less DNA bound on LPTC in large amounts of LPTC existence, so less DNA entered into each cell. Nevertheless, large amounts of LPTC enhanced the probability of LPTC/DNA complexes entering into cells. We considered N/P ratio of 10 that showed the highest gene expression in this result, it was the most suitable charge ratio of LPTC/DNA complex for following DNA vaccine treatment *in vivo* studies.

Cationic lipid and cationic liposome had been used as vaccine adjuvants included DNA vaccines and protein-based vaccines [25, 26]. Thus, antigen adsorbed on cationic LPTC was investigated whether could effectively enhance the cellular uptake of antigen-presenting cells (APCs). We showed that LPTC could enhance the efficiency of cellular uptake (Fig. 11a). We presumed that the cationic property of LPTC was good for targeting to the cell membrane of antigen-presenting cells, which subsequently leads to enhanced uptake. The explanation was also examined and confirmed the primary adjuvant effect of cationic liposome by other studies [17].

A variety of DNA vaccine prime and recombinant viral boost immunization strategies have been developed to enhance immune responses in humans. This prime and boost vaccination strategy has been used to overcome the ineffective induction of immune responses displayed by DNA immunization alone in nonhuman primates and humans [21]. And furthermore, the advantages of this

approach include a synergistic effect on the induction of immune responses and the generation of a robust T cell-mediated immune response [27]. Here, we used LPTC as a dual vector to carry DNA for priming immunization and carry protein for boosting immunization, and indeed enhanced higher antigen specific immune response. However, the animal vaccine strategy should be considered that how to induce higher immune response in short time for wide range of animals. Hence, we explored the possibility of efficiently co-delivering DNA vaccine and protein-based vaccine by carrying with LPTC. And the result showed that this vaccine strategy was successful to stimulate immune response after once immunization and induced higher immune response of twice immunization (Fig. 18). Both of the immune response after once and twice immunization in co-delivery vaccine strategy showed higher antibody response than heterologous immunization strategy. Furthermore, the co-delivery vaccine strategy was also confirmed by other studies and was examined to prime an enhanced and balanced specific immunity of Th1 and Th2-biased responses [28].

Thus, theses results confirm the usefulness of LPTC in *in vitro* and *in vivo* gene delivery, and provide the adjuvant effect. Furthermore, LPTC showed the potential to apply in the development of animal vaccine for farm animals that need large quantity of vaccine product with low cost to prevent the infectious disease, particularly for species where a large number of animals with a relatively low commercial value are utilized such as chickens.

Figures

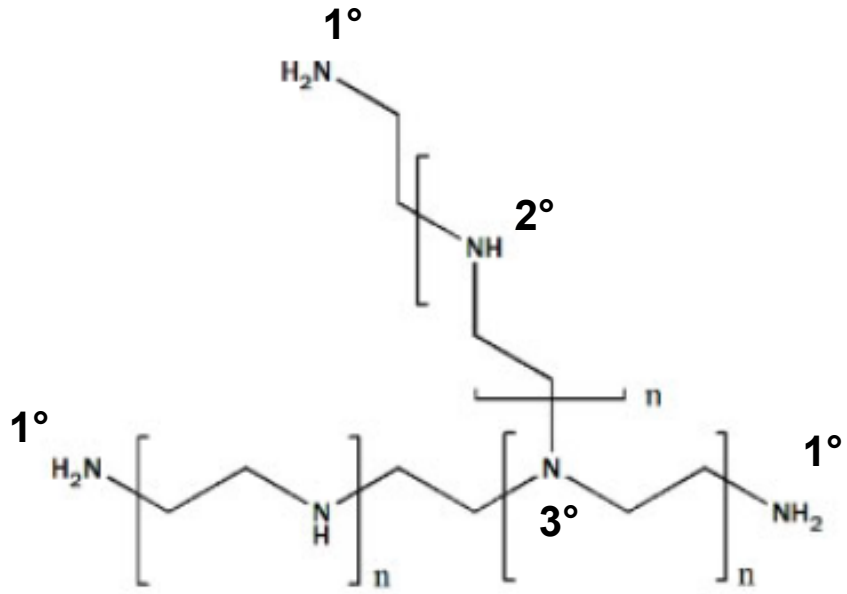


Figure 1. Structure of branched polyethylenimine (bPEI)

The branched form of PEI shows a theoretical ratio of primary (1°) to secondary (2°) to tertiary (3°) nitrogen atoms of 1:2:1. Every third atom of PEI is a nitrogen atom capable of protonation. [10]

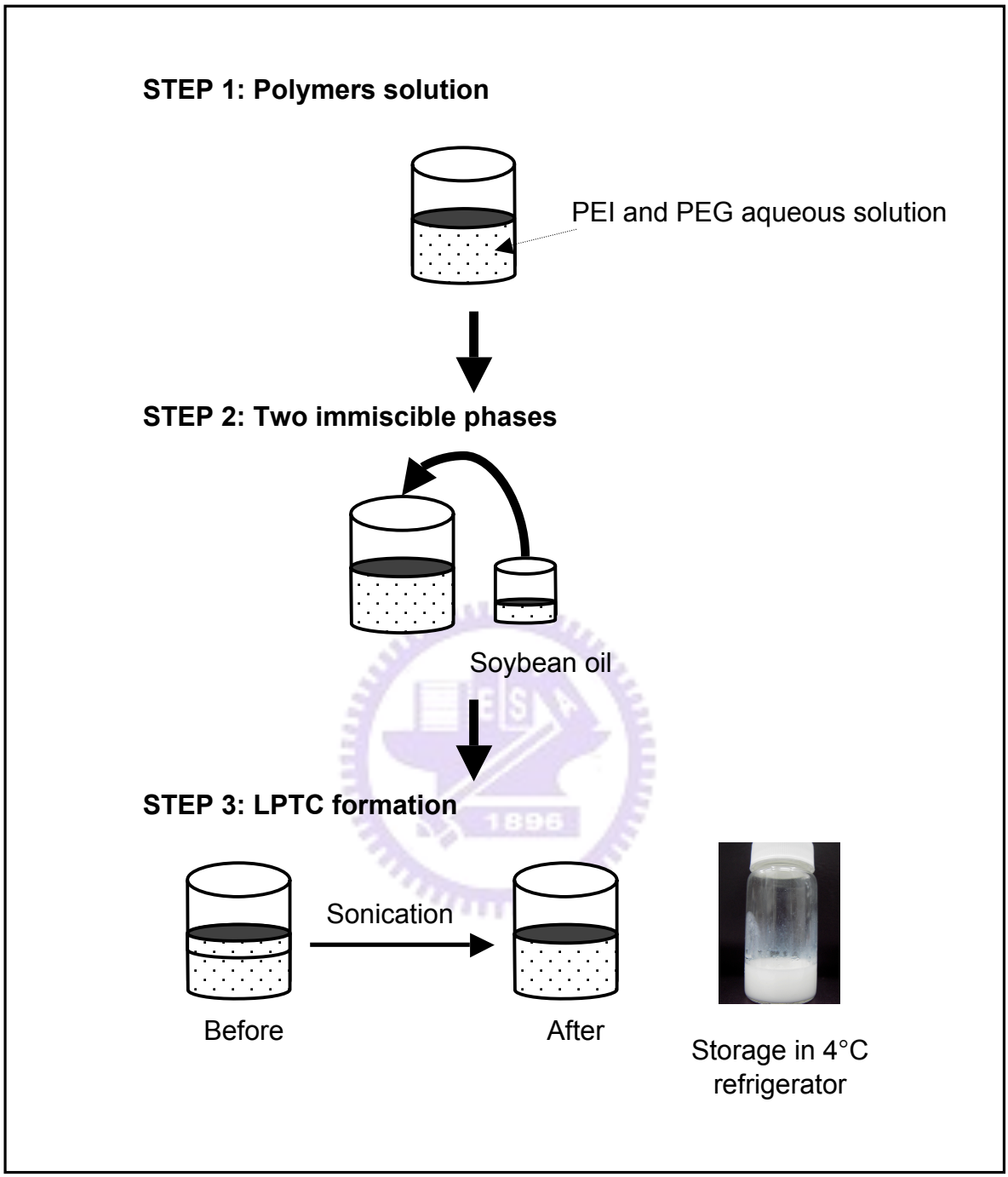

STEP 1: Polymers solution
PEI and PEG aqueous solution
STEP 2: Two immiscible phases
Soybean oil
STEP 3: LPTC formation
Sonication
Before
After
Storage in 4°C refrigerator

Figure 2. The liposome-polymer transfection complex (LPTC) preparation

STEP 1: Polymers solution, both of PEI and PEG were well dissolved in ddH_2O to form aqueous phase. **STEP 2: Two immiscible phases,** soybean oil was added to the polymer solution that formed two separate phases. **STEP 3: LPTC formation**, the solution was sonicated for 30 min that formed a uniform phase and showed the milky white appearance. PEI: polyethylenimin; PEG: polyethylene glycol.

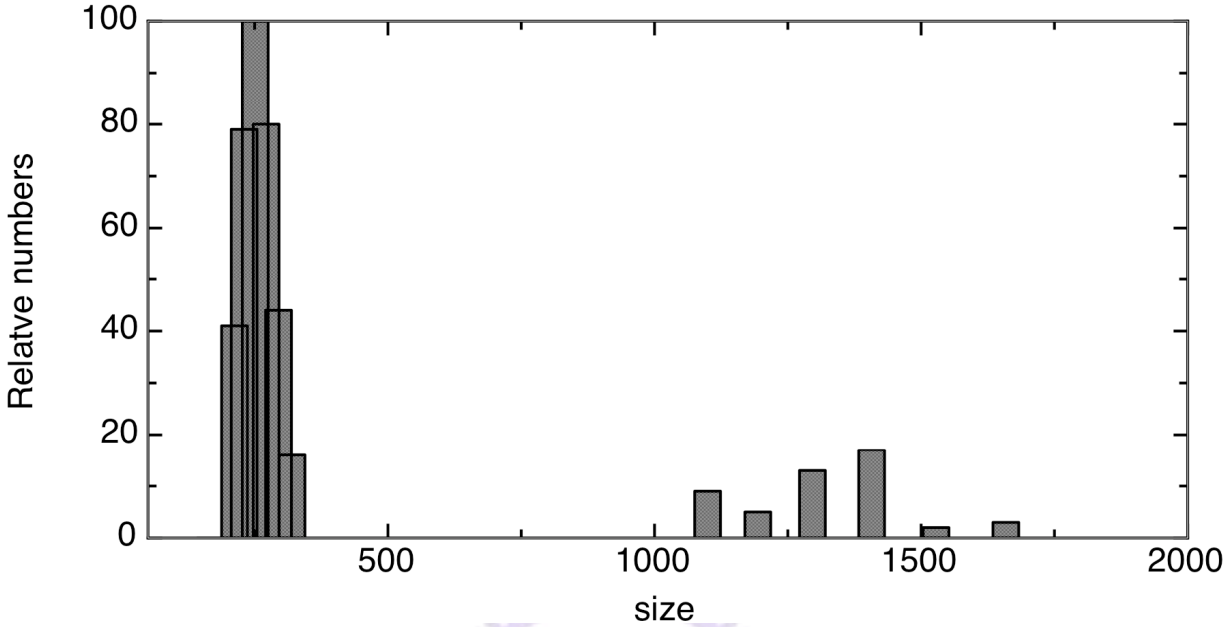


Figure 3. The particle distribution of LPTC

The particles distribution was measured by dynamic light scattering (DLS). Y axis was represented the relative numbers of particles and X axis was represented diameter (nm) of particles. The particle size was distributed into two populations, the major population was from 212.2 nm to 320.1nm and the minor range was from 1099.5 nm to 1658.9 nm.

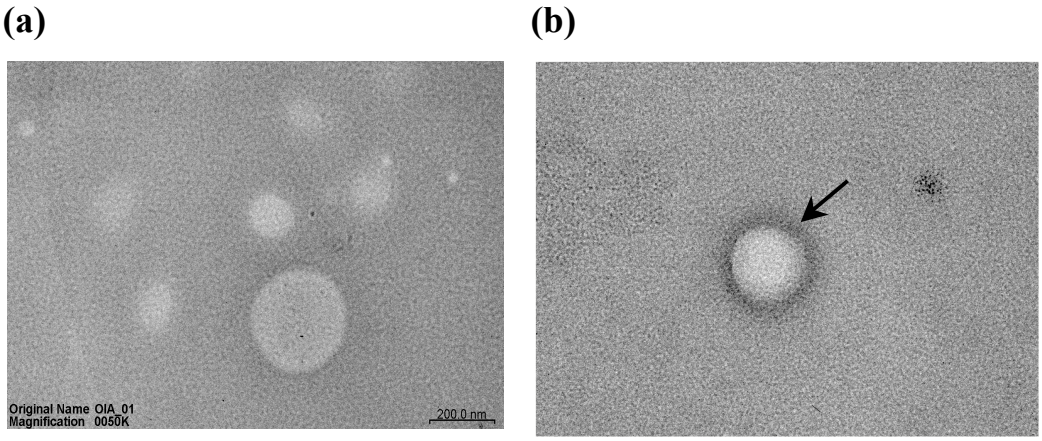


Figure 4. Transmission electron microcopy of LPTC particles

In Fig. 4(a), LPTC particles showed a round shape and particle size were ranging from 100 nm to 300 nm. In high magnification (Fig. 4b), the arrow pointed the hair-like filaments that existed on the outer surface of LPTC.

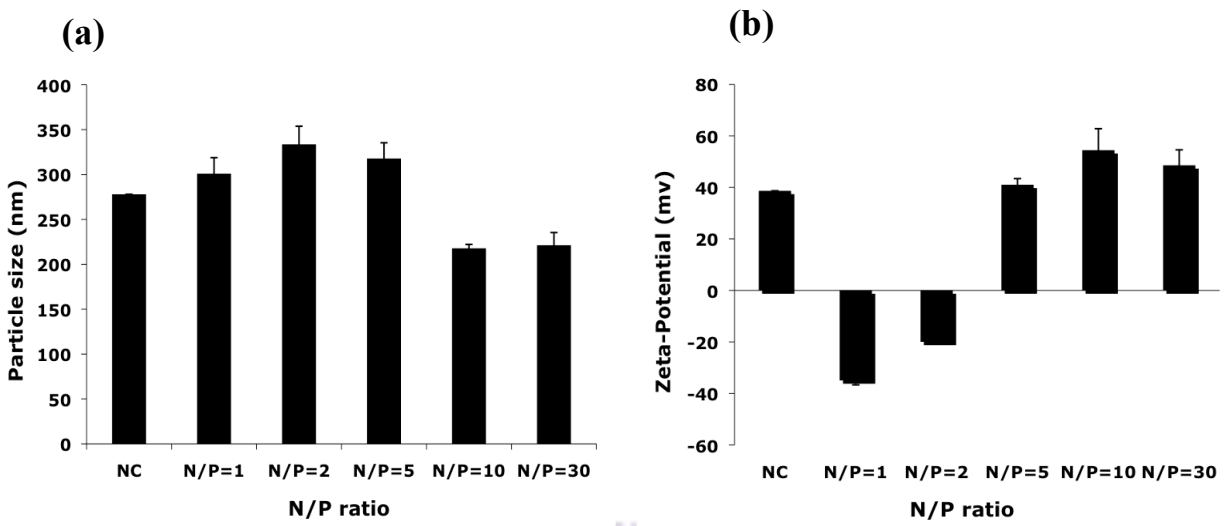


Figure 5. Particle size and zeta-potential of LPTC/DNA complexes

In Fig. 5a and 5b, different dosages of LPTC were mixed with 50 μg DNA and then formed different N/P ratios in order of 1 to 30. After DNA incubated with LPTC for 30 min, the particle size (Fig. 5a) and zeta-potential (Fig. 5b) was measured by Zeta-sizer. NC represented the LPTC alone.

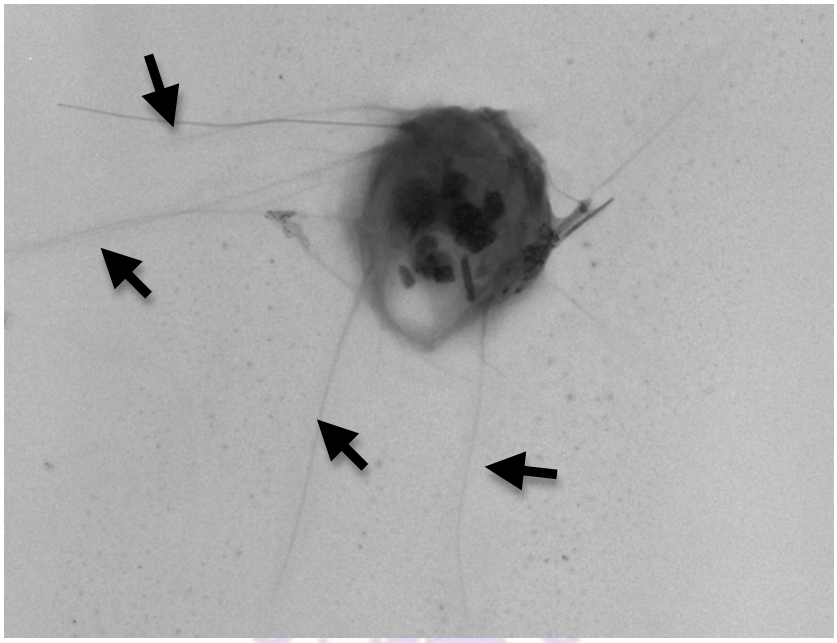


Figure 6. Transmission electron microcopy of LPTC/DNA complex

LPTC/DNA complex photograph was took by TEM and the black arrow pointed the DNA fragment bound on LPTC. The size of complex was about 1.5μm.

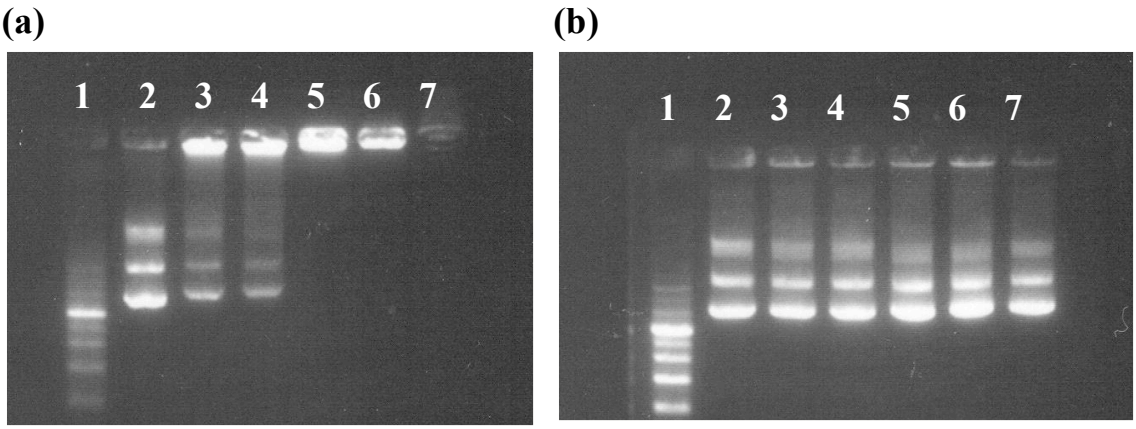


Figure 7. Gel retardation assay of LPTC/DNA complexes

In Fig. 7a, LPTC at different concentrations were incubated with a fixed amount of DNA for 30 min at room temperature, and the complexes were ran on an agarose gel. Lane 1 was DNA marker and lane 2 was naked DNA, lane 3 to lane 7 were represented at N/P ratios from 1, 2, 5, 10 and 30, respectively. The displacement of DNA from complexes by heparin competition was shown on Fig. 7b. DNA complexes after treatment were analyzed with 0.8% agarose gel electrophoresis.

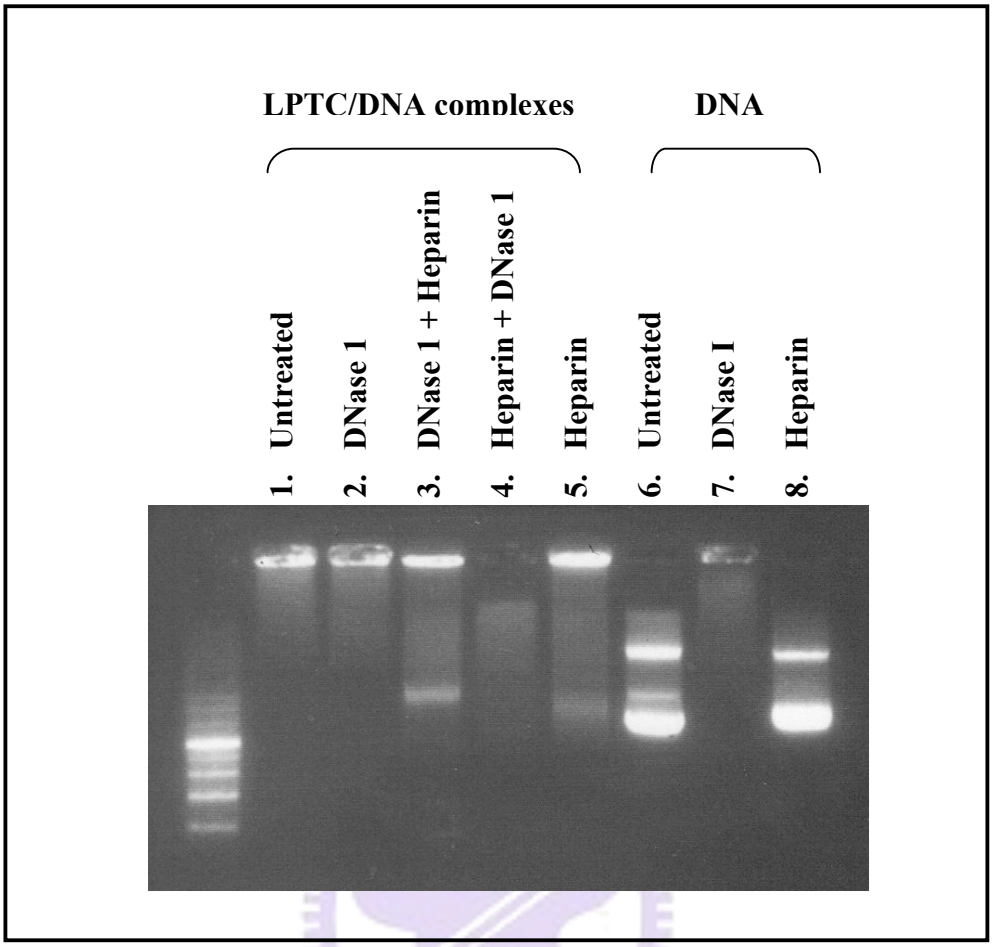


Figure 8. LPTC protects DNA from DNase I digestion.

LPTC protected DNA from DNase I digestion was assessed by treatment with DNase I, DNase I and heparin, DNase I then heparin and heparin alone. LPTC/DNA complexes were formed at N/P=10 with 300ng of DNA. DNA complexes after treatment were analyzed with 0.8% agarose gel electrophoresis.

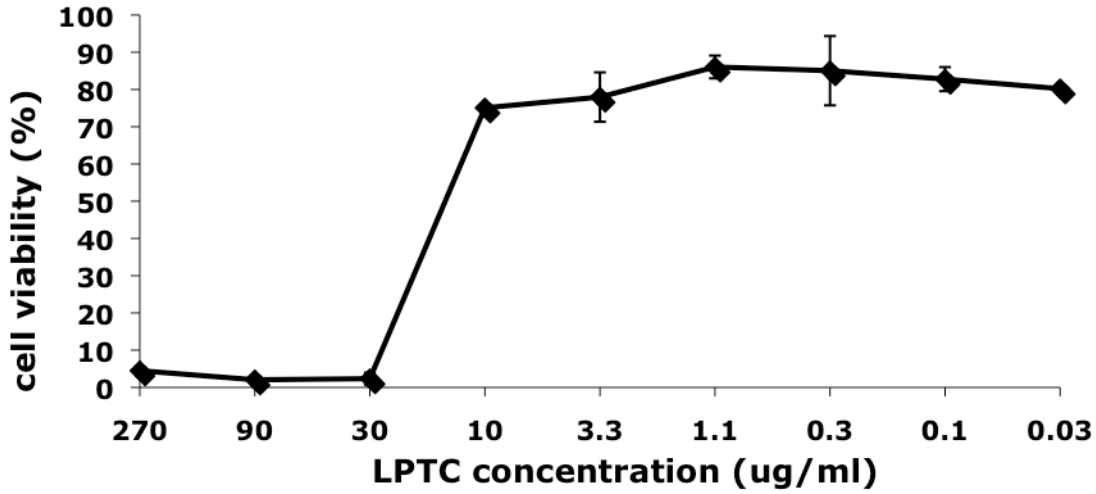


Figure 9. Cytotoxicity tests of LPTC on Balb/3T3 cells

Cell viability of cells treated with different dosages of LPTC were measured by MTT assay. Cells were incubated with various dosages of LPTC and cell viability was measured at 72 hr after treatment. Data represent the percentage to untreated cell.

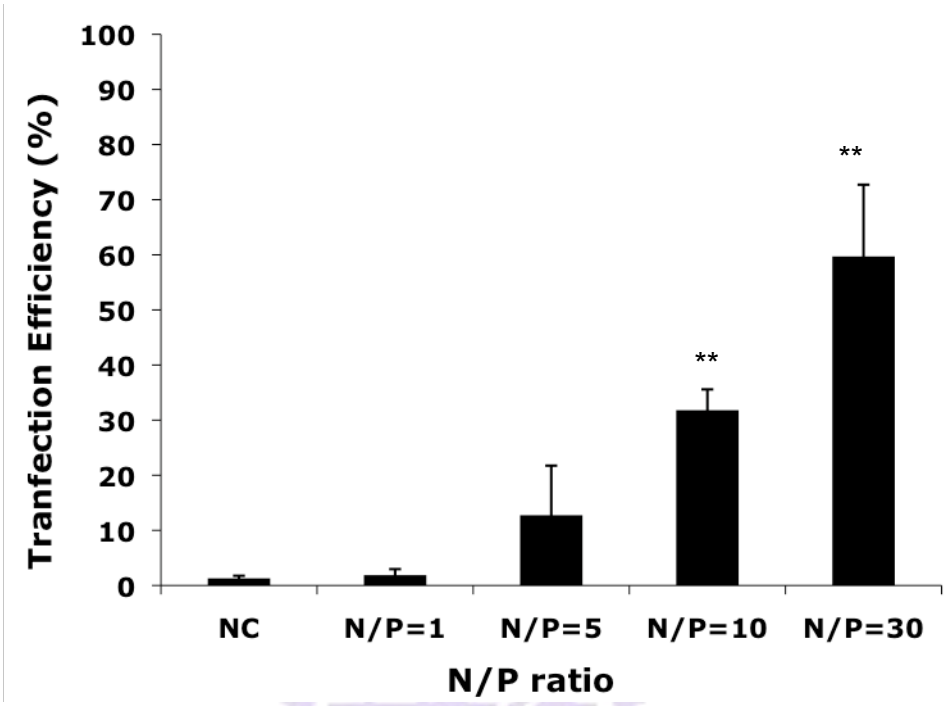


Figure 10. ***In vitro*** **Transfection efficiency of LPTC/DNA complexes**

Using 3 μg DNA and different dosages of LPTC formed each N/P ratio in order from 1 to 30. LPTC/DNA complexes transfected into Balb/3T3 cells, after 48 hr the transfection efficiency was measured by flow cytometry. NC: cell alone, Data represents the mean ± S.E. of six experiments. **P<0.01 v.s. Negative Control

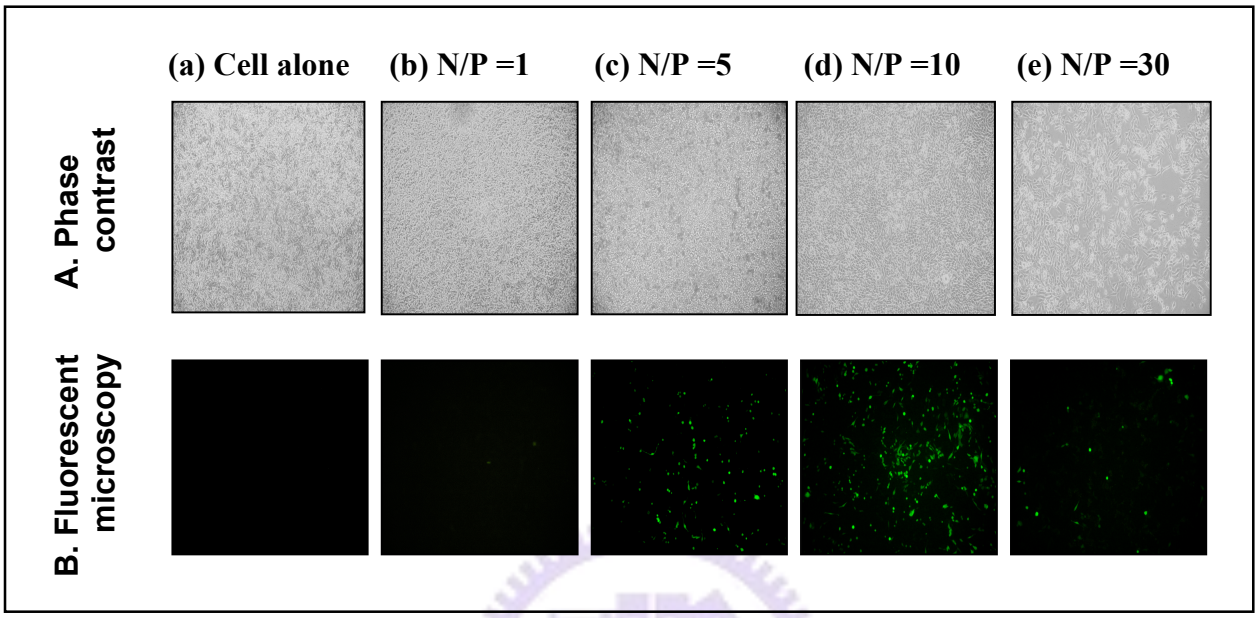


Figure 11. Fluorescent microscopy of gene expression after *in vitro* transfection

Cell were transfected with each LPTC/DNA complex in order of 1 to 30. Forty-eight hours after transfection, phase contrast image (A), fluorescent microscopy images (B) of transfectants were monitored (200x magnification).

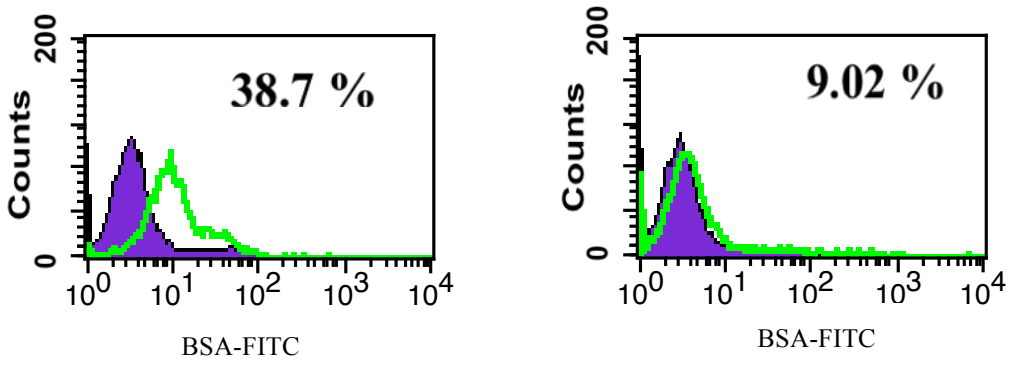


Figure 12. Cell uptake assay of LPTC/BSA-FITC complexes in P338/D1 cells

P338/D1 cells were cultured for 1 hr at 37°C (Fig. 12 a) or 4°C (Fig. 12 b) with 14 μg/ml BSA-FITC alone (dark side) or complexed with LPTC (grey line). Trypan blue were added to quench surface-associated BSA-FITC, then the fluorescent intensity was measured by flow cytometry. BSA-FITC: bovine serum albumin conjugated fluorescein isothiocyanate (FITC).

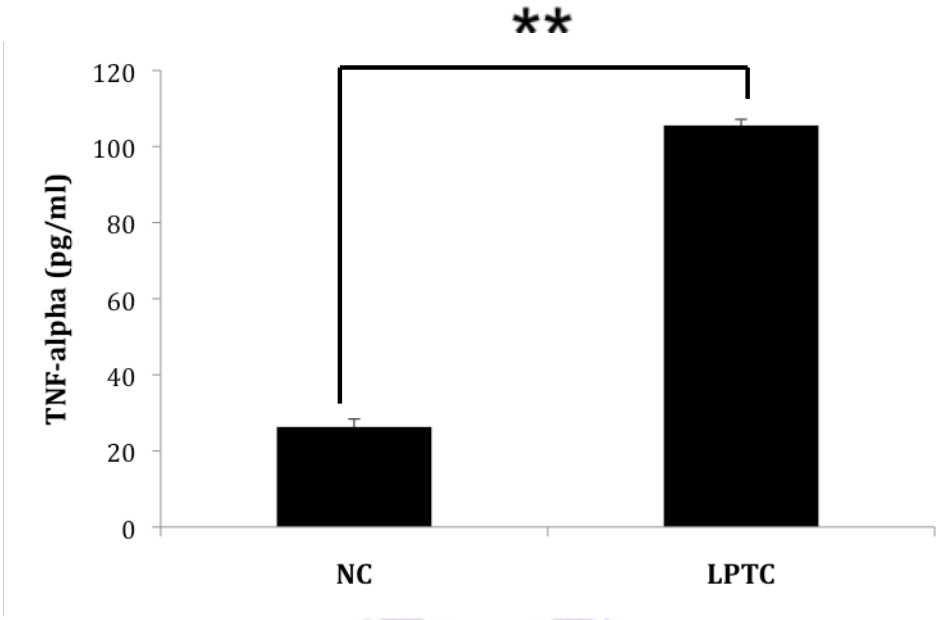


Figure 13. Induction of TNF-alpha from naïve mice splenocytes stimulated with LPTC.

The splenocytes of naïve mice (4×10^6 cells/well) were incubated with LPTC (80μg) or without in 24 well. After stimulation for 48 hr, the culture supernatant was collected and the production of TNF-alpha was measured by ELISA. NC: cell alone, Data are expressed as means ± SEM of three wells. **P<0.01 v.s. Negative Control

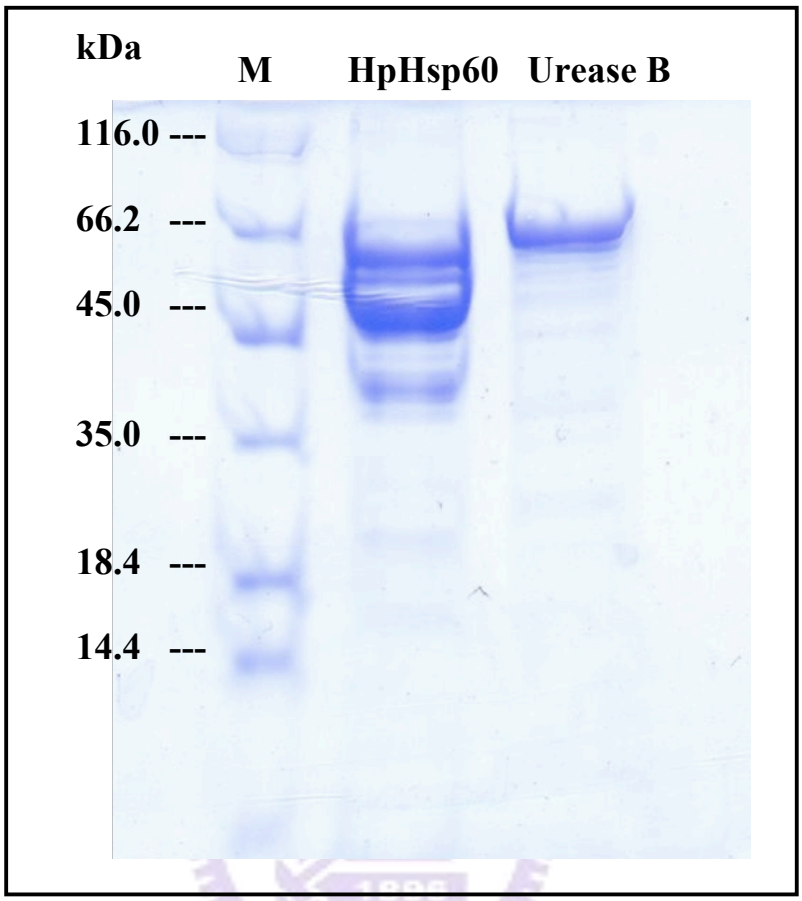


Figure 14. Protein purification of HpHsp60 and urease B protein

After purification and quantification of proteins, ten micrograms of HpHsp60 and Urease B were loaded into 10% SDS-PAGE and run out in 100V for 90min. The molecular mass of HpHsp60 is 60kDa and urease B is 62kDa. M: protein unstained marker.

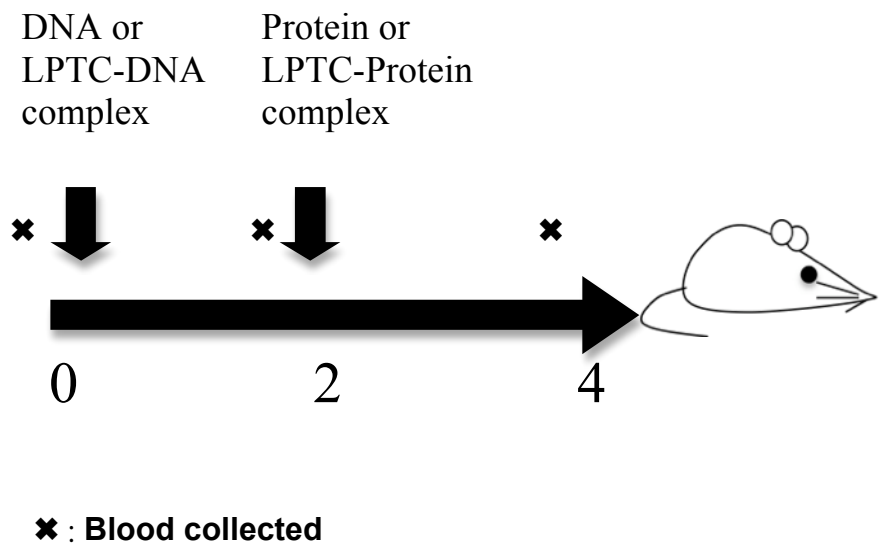
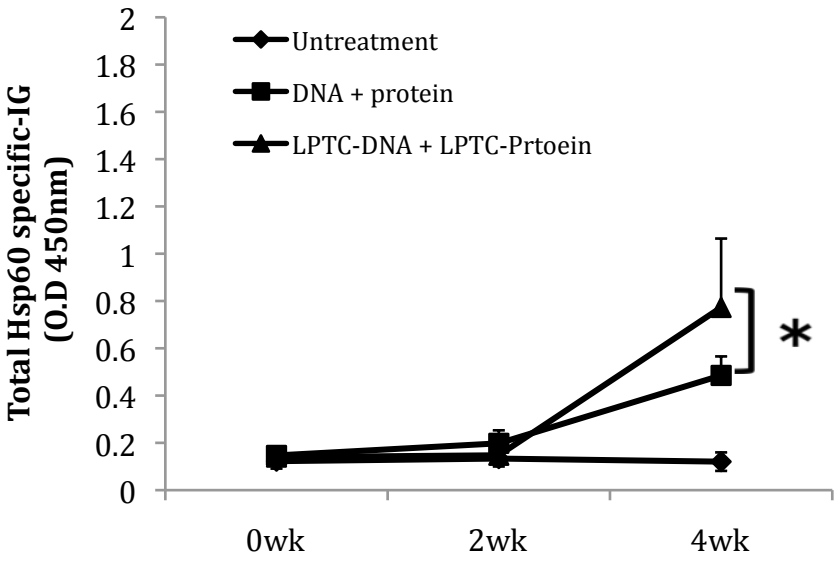


Figure 15. The heterologous vaccine protocol of prime boost schedule

Naïve mice were immunized with one dose of DNA alone or LPTC-DNA complex (12.5 μg-25 μg/dose) followed by one dose of protein alone or LPTC-protein complex (100 μg/dose) at week 0 and week 2, respectively via subcutaneous route. Ag-specific immune responses were measured at 0, 2 and 4 week, respectively. DNA consisted of pCJ3-HpHsp60 and pCJ3-Urease B. Proteins consisted of rHpHsp60 and rUreB proteins.

(a)


Total Hsp60 specific-IG
(O.D 450nm)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Untreatment
DNA + protein
LPTC-DNA + LPTC-Prtoein
*
0wk
2wk
4wk

(b)

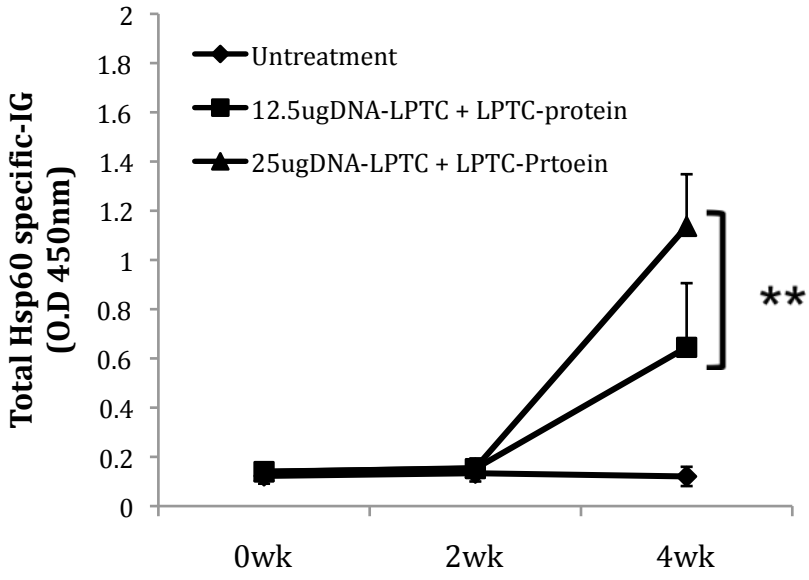
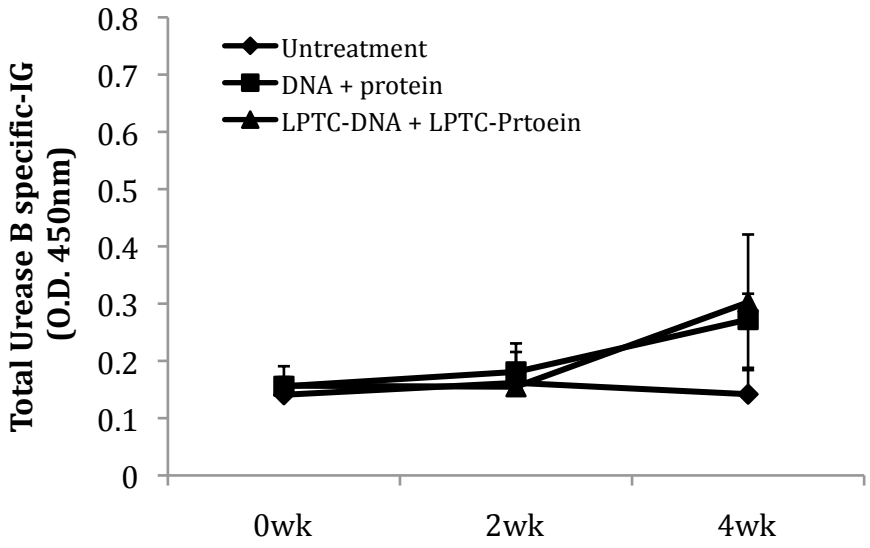

Total Hsp60 specific-IG
(O.D 450nm)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Untreatment
12.5ugDNA-LPTC + LPTC-protein
25ugDNA-LPTC + LPTC-Prtoein
**
0wk
2wk
4wk

Figure 16. LPTC/DNA and LPTC/protein complex induce hsp60-specific IG response by heterologous immunization regimen

Three groups of female BALB/c mice were immunized on weeks 0 with DNA alone (12.5 μg/100 μl), LPTC-DNA complex (12.5 μg/100 μl) (Fig. 16a) and LPTC-DNA complex (25 μg/100 μl) (Fig. 16b) respectively and then boosted on weeks 2 with protein alone (100 μg/100 μl) and LPTC- protein complex (100 μg/100 μl) via subcutaneous route. Serum was collected on weeks 0, 2, 4 and Hsp60-specific total Ig (IgG, IgM and IgA) responses were measured by Ag-specific ELISA. Negative control: untreated mice; DNA: pCJ3-HpHsp60/ pCJ3-Urease B (7.5 μg/7.5 μg or 12.5 μg/12.5μg); protein: rHpHsp60 and rUreB proteins (50μg/50μg). *P<0.05 V.S. DNA/Protein group, **P<0.01 V.S. 12.5μg DNA-LPTC group.

(a)


Total Urease B specific-IG (O.D. 450nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Untreatment
DNA + protein
LPTC-DNA + LPTC-Prtoein
0wk
2wk
4wk

(b)

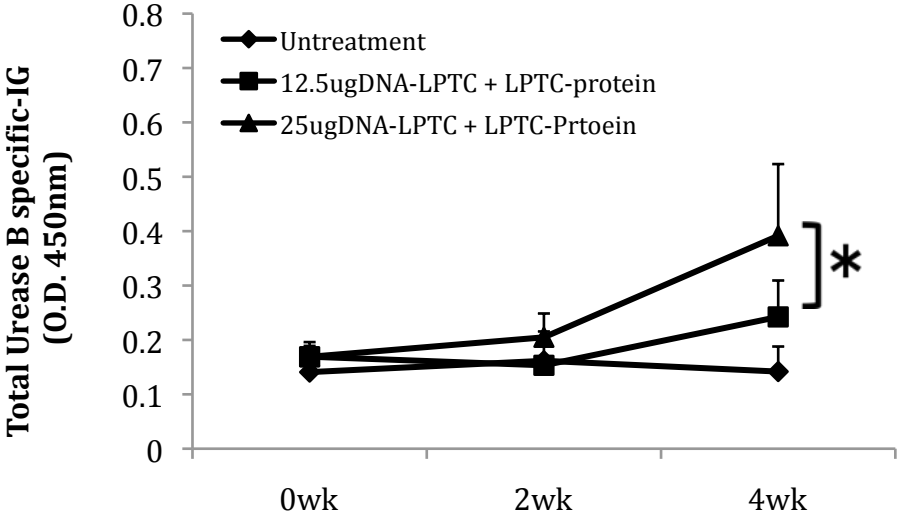

Total Urease B specific-IG (O.D. 450nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Untreatment
12.5ugDNA-LPTC + LPTC-protein
25ugDNA-LPTC + LPTC-Prtoein
*
0wk
2wk
4wk

Figure 17. LPTC/DNA and LPTC/protein complex induce Urease B specific IG response by heterologous immunization regimen

Three groups of female BALB/c mice were immunized on weeks 0 with DNA alone (12.5 μg/100 μl) (Fig. 17a), LPTC-DNA complex (12.5 μg/100 μl) and LPTC-DNA complex (25 μg/100 μl) (Fig. 17b) respectively and then boosted on weeks 2 with protein alone (100 μg/100 μl) and LPTC- protein complex (100 μg/100 μl) via subcutaneous route. Serum was collected on weeks 0, 2, 4 and urease B-specific total Ig (IgG, IgM and IgA) responses were measured by ELISA. Negative control: untreated mice; DNA: pCJ3-HpHsp60/ pCJ3-Urease B (7.5 μg/7.5 μg or 12.5 μg/12.5 μg); protein: rHpHsp60 and rUreB proteins (50 μg/50 μg).

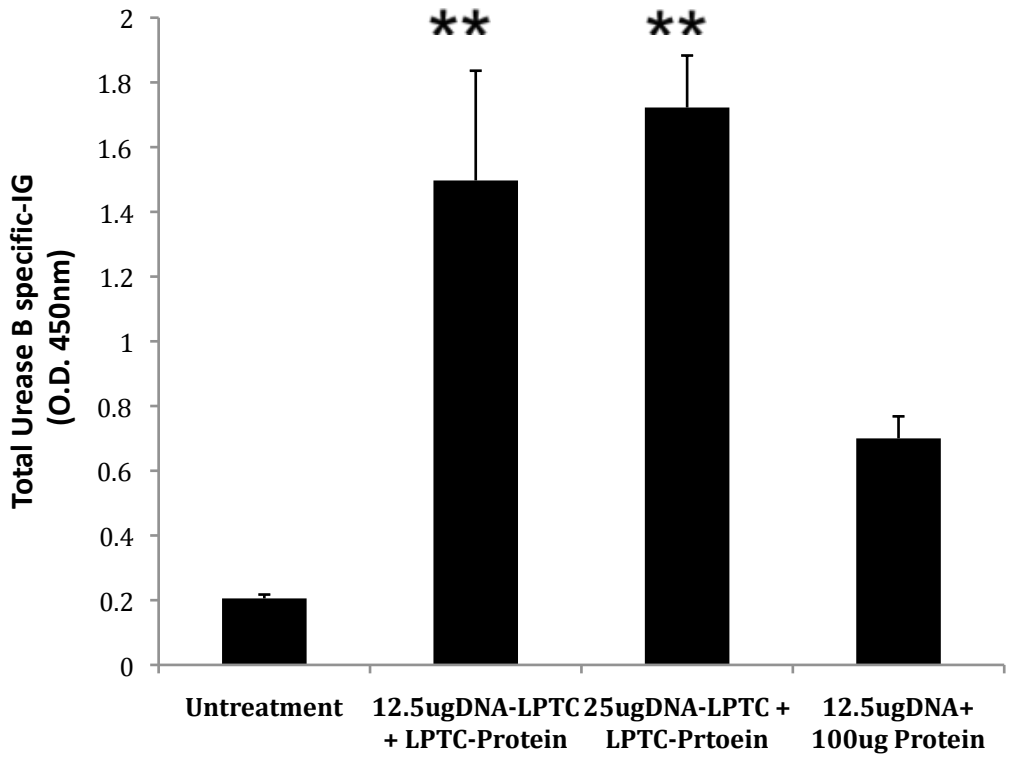


Figure 18. LPTC/DNA and LPTC/protein complex induce Urease Bspecific IG response by heterologous immunization regimen after 4th week boosted

Three groups of female BALB/c mice were boosted on weeks 4 with protein alone (100 μg/100 μl) and LPTC- protein complex (100 μg/100 μl) via subcutaneous route. Serum was collected on weeks 6 and Urease B-specific total Ig (IgG, IgM and IgA) responses were measured by ELISA. Negative control: untreated mice; DNA: pCJ3-HpHsp60/ pCJ3-Urease B (7.5 μg/7.5 μg or 12.5 μg/12.5 μg); protein: rHpHsp60 and rUreB proteins (50 μg/50 μg). **P<0.01 v.s. 12.5 μg DNA+100 μg protein group

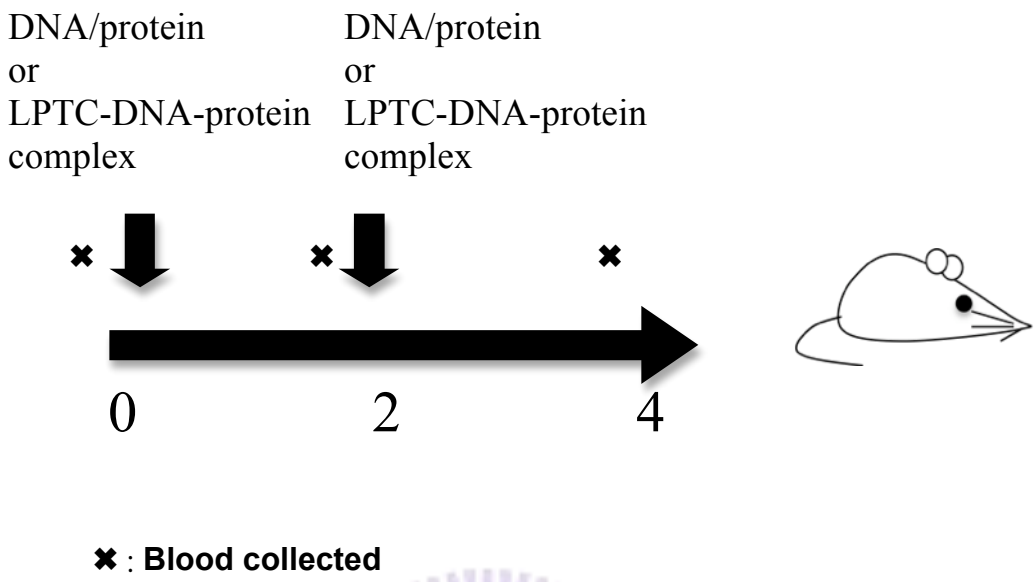


Figure 19. The co-delivery vaccine protocol of prime boost schedule

Naïve mice were immunized with two doses of DNA (50 μg)/ protein (100 μg) or the co-delivery of LPTC-DNA (50 μg)-protein (100 μg) complex at weeks 0 and weeks 2 via subcutaneous route. Ag-specific immune responses were measured at 0, 2 and 4 week, respectively. DNA consisted of pCJ3-HpHsp60 and pCJ3-Urease B. Proteins consisted of rHpHsp60 and rUreB proteins.

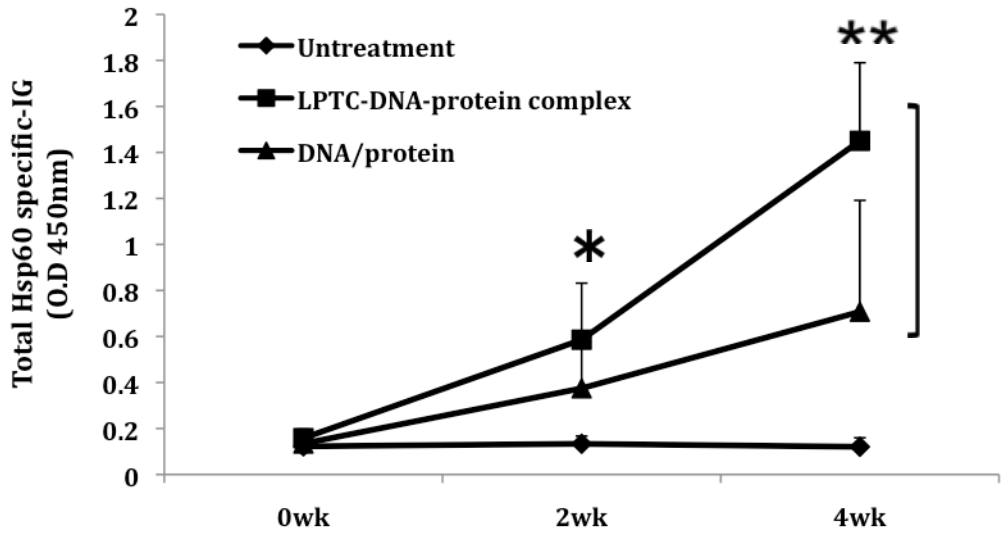


Figure 20. LPTC/DNA and LPTC/protein complex induce hsp60-specific IG response by co-delivery immunization regimen

Two groups of female BALB/c mice were immunized on weeks 0 and week 2 with DNA (50μg)/ protein (100μg) and the co-delivery of LPTC-DNA (50μg)-protein (100μg) complex via subcutaneous route. Serum was collected at 0, 2, 4 week and then total Hsp60-specific Ig responses were measured by Ag-specific ELISA. Negative control: untreated mice; DNA: pCJ3-hphsp60/ pCJ3-urease B (25 μg/25 μg); protein: rHpHsp60 and rUreB proteins (50 μg/50 μg). **P<0.01 V.S DNA/Protein group

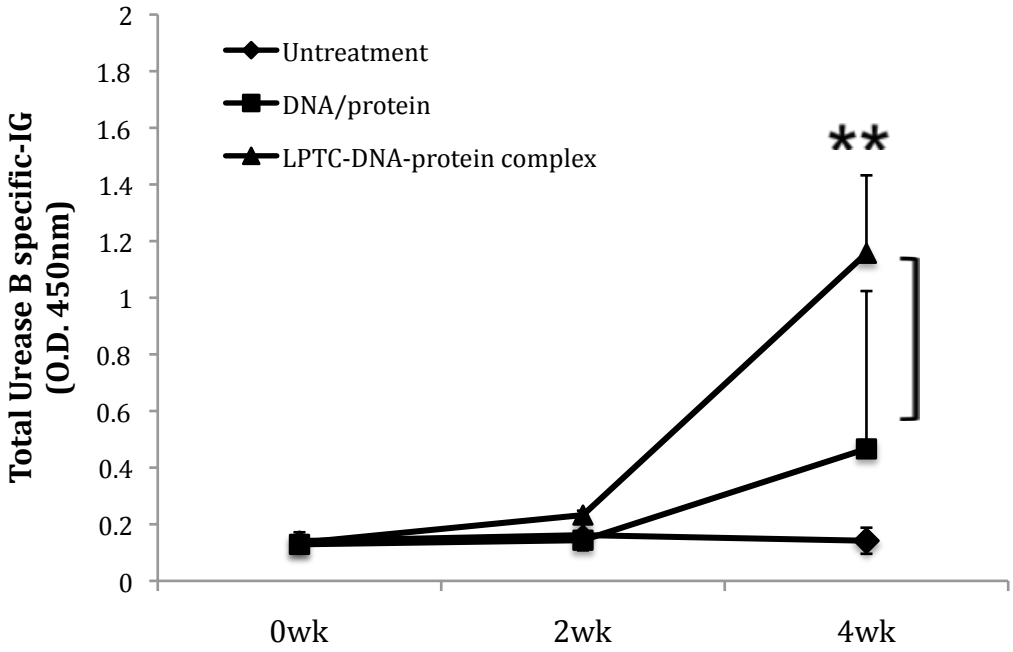


Figure 21. LPTC/DNA and LPTC/protein complex induce Urease B-specific IG response by co-delivery immunization regimen

Three groups of female BALB/c mice (N=3 /group) were immunized on weeks 0 and week 2 with DNA (50μg) /protein (100μg) and the co-delivery of LPTC-DNA (50μg)-protein (100μg) complex via subcutaneous route Serum was collected at 0, 2, 4 week and then total Hsp60-specific Ig responses were measured by Ag-specific ELISA. Negative control: untreated mice; DNA: pCJ3-hphsp60/ pCJ3-urease B (25 μg/25 μg); protein: rHpHsp60 and rUreB proteins (50 μg/50 μg). **P<0.01 V.S DNA/Protein group

Table

Table 1. The advantages and disadvantages of viral vector and non-viral vector

	Viral	Non-viral
Advantage	◆ High transfection efficiency ◆ Natural transportation of DNA into nucleus ◆ Endosomal escape mechanism ◆ Ability to infect different cells	◆ Low immunogenicity ◆ Easy to synthesize: massive production ◆Potential targetable ◆ No limit on plasmid size
Disadvantage	◆ Complicated sythesis process ◆ Chromosomal insertion and proto-oncogene activation ◆ Toxicity, contamination of live virus ◆ Limitation on gene size	◆ Low transfection efficiency ◆ Most vehicles are toxic at high doses ◆ No natural tropism, endosomal escape or nuclear transport mechanisms

Table 2. The price comparison of commercial cationic liposome and LPTC

Transfection Reagent	Company	Component	Price (per reaction, rx)
Lipofectamine™ 2000	Invitrogen	Cationic lipid: DOSPA	NTD $145.2 /rx
Cellfectin®	Invitrogen	Cationic lipid: TM-TPS; DOPE	NTD $ 61.6 /rx
LyoVec™	InvivoGen	Cationic lipid: DTCPTA; neutral lipid: DiPPE.	NTD $ 23.4 /rx
TransFast™	Promaga	Cationic lipid: (+)-N, N [bis (2-hydroxyethyl)]-N-methyl-N- [2,3 -di(tetradecanoyloxy)propyl] ammonium iodide ; neutral lipid: DOPE.	NTD $ 49.8 /rx
Tfx™ Reagents	Promaga	Cationic lipid: [N,N,N´,N´-tetramethyl-N,N´-bis(2-hydroxyethyl)-2,3-di(oleoyloxy)-1,4-butanediammonium iodide] ; neutral lipid: DOPE	NTD $ 172.5 /rx

Commercial transfection reagent	**Range: NTD $ 172.5 ~ NTD $23.4 /rx**
LPTC	**NTD $ 1 /rx**

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Appendices

A1. The map of pAAV-MCS-hrGFP