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Research Article

Development and Evaluation of Chitosan-Coated Liposomes for Oral DNA Vaccine: The Improvement of Peyer's Patch Targeting Using a Polyplex-Loaded Liposomes

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Abstract. The aim of this study was to develop chitosan-coated and polyplex-loaded liposomes (PLLs) containing DNA vaccine for Peyer's patch targeting. Plain liposomes carrying plasmid pRc/CMV-HBs were prepared by the reverse-phase evaporation method. Chitosan coating was carried out by incubation of the liposomal suspensions with chitosan solution. Main lipid components of liposomes were phosphatidylcholine/cholesterol. Sodium deoxycholate and dicetyl phosphate were used as negative charge inducers. The zeta potentials of plain liposomes were strongly affected by the pH of the medium. Coating with chitosan variably increased the surface charges of the liposomes. To increase the zeta potential and stability of the liposome, chitosan was also used as a DNA condensing agent to form a polyplex. The PLLs were coated with chitosan solution. *In vivo* study of PLLs was carried out in comparison with chitosan-coated liposomes using plasmid encoding green fluorescence protein as a reporter. A single dose of plasmid equal to 100 μg was intragastrically inoculated into BALB/c mice. The expression of green fluorescence protein (GFP) was detected after 24 h using a confocal laser scanning microscope. The signal of GFP was obtained from positively charged chitosan-coated liposomes but found only at the upper part of duodenum. With chitosan-coated PLL540, the signal of GFP was found throughout the intestine. Chitosan-coated PLL demonstrated a higher potential to deliver the DNA to the distal intestine than the chitosan-coated liposomes due to the increase in permanent positive surface charges and the decreased enzymatic degradation.

KEY WORDS: chitosan; DNA vaccine; liposome; oral immunization; polyplex-loaded liposome.

INTRODUCTION

DNA vaccines have recently been investigated as a high potential new generation of vaccine for humans. DNA vaccines are generally regarded as being potentially safe, relatively cheap, and easy to manufacture (1,2). Manipulating DNA into a particulate delivery system potentially renders DNA useful for mucosal immunization, by which it is possible to stimulate both humoral and cell-mediated responses. Mucosal immunization also induces mucosal and systemic immunity simultaneously. It provides the first line of protective immune through secretory IgA that prevents the attachment of bacteria and viruses to the mucosa (3–5). The oral route is attractive for vaccination because the GI tract of a human has over 300 m^2 of mucosal surface containing immune inductive tissue, such as the intraepithelial lymphocytes and lymphoid follicles at the laminar propria and Peyer's patches, known as the gut associated lymphoid tissues (GALT) (6). However, most protein, peptide, and plasmid DNA delivery via the oral route remains relatively unsuccessful due to their instability and low permeability in the intestinal mucosa. Large doses are always required in order to stimulate adequate immune responses. The oral delivery system of a DNA vaccine must overcome the harsh environment and barriers in the gastrointestinal (GI) tract. Recently, many studies have shown that oral delivery systems in the form of nanoparticulates have the potential to increase the stability and absorption of protein and plasmid DNA via the GI tract (7–11).

Liposomes have been extensively considered as a useful delivery system of antigens and genes via many routes (8,12). Due to their unique properties, such as biocompatibility and biodegradability, together with a high variability of surface charge and membrane fluidity, liposomes have become attractive as oral vaccination adjuvants. Conventional liposomes and cationic lipid-based systems are not very popular for oral gene delivery (13) due to their instability in gastric acidity, intestinal protease, and enzymes including bile salts solution. Despite the fact that cationic liposome–DNA (lipoplex) always has shown good transfection efficiency *in vitro*, various cationic lipids have been studied and reported for their toxicity (14,15). Recently, low toxicity cationic lipid-

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based particles or noncationic liposomes for gene delivery such as niosome (7) and solid lipid particles (16,17) have become more interesting. The most viable mechanism of liposome is adsorptive endocytosis and the retentive property of the particles at the absorption site. Many studies in the past decade have suggested that liposomes fused with the plasma membrane and mediated the entry of DNA into the cell compartment. The liposome–cell membrane fusion is a major mechanism to facilitate internalization into the cytoplasm (18).

In this study, we increased the stability of plasmid DNA throughout the GI tract by encapsulating in liposome and coating with chitosan. Since chitosan is a positive charge biopolymer, it can interact with the negative charge of the liposome by electrostatic interaction and protect the DNA from extracellular endonuclease. Chitosan has been studied as nonviral gene delivery vector by forming a chitosan–DNA complex (polyplex) which was found to be efficient in transfection *in vitro* (19). As a coating material, chitosan has been used for surface modification and as a mucoadhesive enhancer in the form of biodegradable microspheres carrying peptides and proteins for mucosal and oral delivery (20,21). The bioadhesive property of chitosan increased the residence time and prolonged the adsorption at mucosal site (22–24). The aims of this study were to formulate and evaluate chitosan-coated liposomal preparations including polyplex-loaded liposomes for DNA delivery via oral by investigating the characteristics and the stability *in vitro* using pRc/CMV-HBs. The efficacy of the chitosan-coated liposomes was studied *in vivo* in BALB/c mice using plasmid encoding green fluorescence protein (pEGFP-C2) as a reporter DNA. The localization of the delivery system was revealed by the protein expression of the encoded gene.

MATERIALS AND METHODS

Materials

Phosphatidylcholine (PC; Phospholipon® 80), was a gift from Lipoid, GmbH, Germany. Cholesterol (CHO), dicetyl phosphate (DCP), and sodium deoxycholate (DC) were purchased from Sigma (USA). Chitosan (medium MW, ca 300 kDa) with a deacetylation grade of about 87% was purchased from Fluka (Germany). Plasmid pRc/CMV-HBs (5.6 kb) expressing the hepatitis B surface protein (HBsAg subtype *ayw*) cloned by Whalen *et al.* (25) was a gift from Aldevron (USA). Plasmid pEGFP-C2 (5.4 kb) encoding green fluorescence protein was purchased from Bioscience (USA). All chemicals, bacterial culture media, and components of buffer solutions were analytical or biochemical grade products. *Escherichia coli* (DH5α) was purchased from Invitrogen (USA).

Animals

Male inbred BALB/c mice, 10–12 weeks of age were purchased from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. The animal study was in accordance with ethical guidelines for the care and use of laboratory animals.

Plasmid Propagation and Isolation

Plasmid pRc/CMV-HBs encoding the small protein of hepatitis B virus (HBsAg, subtype *ayw*) was used as a DNA vaccine for the development study. pEGFP-C2 plasmid was used as the reporter gene for the *in vivo* study which was separately performed during the experiment with different groups of mice. Each of the plasmids was separately transformed into *E. coli* (DH5α) for propagation. The plasmid isolation was carried out using the alkaline lysis method according to the manufacturer's instructions using an EndoFree Mega Kit (Qiagen GmbH, Germany) followed by ethanol precipitation. The quantity and quality of the purified plasmids were assessed using UV spectrophotometer (GeneQuant Pro, Amersham/Bioscience, USA) at 260 and 280 nm and by agarose gel electrophoresis after an incubation with restriction enzymes. The DNA ladder was used as size reference. Only the pRc/CMV-HBs plasmid was confirmed by sequencing. In the routine study, both of the transformed and isolated plasmids were confirmed by cutting with restriction enzymes and running on agarose gel electrophoresis concurrently with DNA ladders.

Preparation of Chitosan-Coated Liposomes

Liposomes were composed of PC and CHO in a molar ratio of 5:4, and the liposome was named LP540. DCP was supplemented as negative charge inducer with the molar ratio of PC/CHO/DCP equal to 5:4:1, and the liposome was named LP541. When necessary, DC was added, and the liposomes were named LP540DC or LP541DC. The lipid stock solution was prepared by dissolving all lipid components in chloroform. The liposome containing plasmid DNA with or without DC was prepared by the reverse-phase evaporation method. In a typical procedure, 10 ml of lipid solution (150 μmol of total lipid) was taken from the stock solution and mixed with 5 ml of diethyl ether. Five milliliters of DNA solution (containing 1 mg of plasmid pRc/CMV-HBs) with or without 50 mg of DC was added. The emulsion size reduction was performed by using an ultrasonicator (Bransonic®, AS200 Digit, Retch, Germany) for 15 min in an ice bath. The emulsion mixture was evaporated to form a gel at room temperature under low pressure using a Rotary Evaporator (EYELA® Aspirator A-3S, Japan). The gel then was subjected to vigorous mechanical agitation on a vortex mixer until the gel collapsed and transformed into a fluid. The liposome suspension was additionally evaporated to remove the trace organic solvent at room temperature for 30 min. The chitosan-coated liposomes were prepared by mixing the liposome suspensions with an equal volume of 0.1% *w/v* chitosan solution and then stored at 4°C until characterized.

Preparation of Polyplex-Loaded Liposome

Polyplex was prepared using the complex coacervation method (26) with some modification. In the optimization study, various amounts of chitosan were prepared by diluting the 0.5% *w/v* stock solution with a 25-mM sodium acetate buffer pH 5.4 to the final volume of 5 ml. Chitosan stock solution was prepared by dissolving 0.5 g of chitosan in 100 ml of 1% *v/v* acetic acid. The polyplex was formed by mixing plasmid pRc/CMV-HBs (1 mg/5 ml) in 5 ml of chitosan

solution under gentle stirring for 30 min. The phase separation was enhanced by adding 200 μl of 10% *w/v* Na_2SO_4 into the mixture. The particle was concentrated by centrifuging at 8,000×*g* for 10 min. The packed particle was separated by sonication at low intensity until a clearly opalescent suspension was obtained. The polyplex was adjusted to a desired concentration using distilled water. Loading efficiency was calculated by measuring the difference between the total amount of DNA added in the reaction medium and the amount of nonentrapped DNA remaining in the supernatant after centrifugation. The supernatant was analyzed using a spectrophotometer at 260 nm for DNA concentration. The obtained nanoparticles were characterized by sizes and size distributions, zeta potentials, and morphology to investigate the optimal condition for chitosan–DNA condensation.

Polyplex-loaded liposomes (PLLs) were prepared using thin film method. The lipid components were the same as the chitosan-coated liposomes of LP540 and LP541. Ten milliliters of lipid stock solution (150 μmol of total lipids) was placed in a 250-ml round-bottomed flask. The flask was connected to a rotary evaporator and rotated in the water bath at 40°C to remove the solvent until a dry lipid film was obtained on the inner wall of the flask. Evaporation was additionally performed at ambient temperature for 30 min to remove the residual solvent. A 3 ml of polyplex suspension was added into the flask. The lipid film was rehydrated by hand shaking until a milky white suspension without visible particles was obtained. PLL540 and PLL541 were the chitosan–DNA complex entrapped in lipid vesicles composed of PC/CHO in molar ratio of 5:4 and PC/CHO/DCP in molar ratio of 5:4:1, respectively. The resulting PLLs were kept at 4°C until characterized. Prior to use, PLL540 and PLL541 were coated by gently mixing with an equal volume of 0.1% *w/v* chitosan solution. After coating, the preparation codes were transformed to CS-PLL540 and CS-PLL541, respectively.

Particle Characterization

Particle sizes and size distributions were measured by a submicron particle size analyzer (Coulter® Model N4D, Germany). Zeta potentials of the preparations were measured at 25°C in double-distilled water, pH 7.0, using a Zetasizer (Nano-ZS, Malvern Instruments, UK). Particle morphology and shape were investigated by a transmission electron microscope (TECNAI 20, FEI Co., The Netherlands) after negative staining using 2% *w/v* uranyl acetate for 2 min.

Effect of pH and Chitosan Concentrations on Surface Charge of Plain Liposomes

The surface charges of the plain and coated liposomes were measured in distilled water. To study the effect of pH on the zeta potential, the medium was adjusted with 0.1 N HCl or 0.1 N NaOH to a different pH ranging from 8 to 2.

The effect of chitosan concentration on the zeta potential of liposome preparations was studied by incubating the liposomal suspension with an equal volume of chitosan solution under continuous stirring for 10 min at 25°C. The concentrations of chitosan solution were 0.05%, 0.1%, and 0.2% *w/v*. The final concentrations of both liposome suspension and chitosan solution were half of the original concentration. The coated liposomes were stored at 4°C until used.

Protection Test and Stability Study

To assess the efficiency of encapsulation and stability against nuclease enzyme, plain liposomes, chitosan-coated liposomes, and polyplex-loaded liposomes containing pRc/CMV-HBs (equivalent to 5 mcg of DNA) were separately incubated with 5 unit of DNaseI at 37°C, for 30 min. After incubation, 20 μl of the sample was mixed with 2 μl of ×10 loading dye including 0.5 mcg/ml of ethidium bromide and applied on a 0.8% *w/v* agarose gel. The naked plasmid DNA was used as a reference. The gel was run at 50 mV for 30 min using an electrophoretic chamber (3000/300, Bio-Rad Lab, USA). The degraded or released products of DNA from the particles were visualized with ultraviolet light and photographed using a Gel Doc 2000 (Bio-Rad Lab, USA).

The stability of liposomal preparations for oral vaccine delivery system was investigated in simulated gastric fluid (SGF; pH 1.2) and simulated intestinal fluid (SIF; pH 6.8). Each of the plain and chitosan-coated liposomal suspensions was incubated with equal volume of SGF and SIF at 37°C for 1 and 2 h, respectively. The integrity of the particles was studied using agarose gel electrophoresis as described above.

In Vivo Study of Chitosan-Coated PLLs Using pEGFP-C2

In vivo study was performed only with pEGFP-C2 to evaluate the ability of the DNA delivery system to the targeting site of Peyer's patches. The chitosan-coated liposomes and chitosan-coated PLLs containing pEGFP-C2 were separately prepared using the same procedures as the previous study of plasmid pRc/CMV-HBs and coated with 0.1% *w/v* chitosan solution. The resulting liposomes were named in the same codes as in the preparation and characterization studies of plasmid pRc/CMV-HBs as shown in Table I. Five types of the pEGFP-C2 preparations (CS-LP540, CS-LP540DC, CS-LP541, CS-LP541DC, and CS-PLL540) were selected. Each preparation was intragastrically inoculated into two BALB/c mice per group. Mice were deprived of food 8 h before and 1 h after inoculation. The volume used was 0.5 ml per dose. The mice were sacrificed after 24 h. The intestines were removed and washed two times with cooled phosphate buffer saline (PBS) pH 7.4. Then the intestines were fixed in cooled 4% *v/v* paraformaldehyde in PBS pH 7.4 for 1 h and then immerged in cooled PBS until used. The protein expression was examined using a confocal laser

Table I. Sizes and Size Distributions of 0.1% Chitosan-Coated Liposomal Preparations Carrying pEGFP-C2

Preparations	Size (nm ± SD)	PI
CS-LP540	1,090±300	0.76
CS-LP540DC	320±180	0.72
CS-LP541	809±260	0.62
CS-LP541DC	329±101	0.78
CS-PLL540	5,165±805	1.00[a]
CS-PLL540DC	314±170	0.75
CS-PLL541	1,490±200	1.00
CS-PLL541DC	295±59	0.63

[a] The PI value was not valid due to a high agglomeration of the coated particles

scanning microscope (FV1000, Olympus, Japan). The experiment was performed in duplicate. The animals were treated in full compliance with the regulations of the "The Ethical Principles of the Use of Animals for Scientific Purpose" of the National Research Council of Thailand under protocol approved by the Animal Ethics Committee (Faculty of Pharmacy, Mahidol University).

RESULTS AND DISCUSSION

Chitosan-Coated Liposome Preparation and Morphology

Liposomes containing pRc/CMV-HBs were prepared using the reverse-phase evaporation technique. This method provides high reproducibility in size range and more homogeneous in size distribution without additional size reduction. The major lipid components of all formulas were PC and CHO. CHO was used for almost half of the total lipids to provide more stable lipid bilayers to decrease the membrane fluidity as reported elsewhere (27,28). DCP and sodium DC were used as helper lipids to induce a negative surface charge on the liposomes. The hydrophobic steroidal backbone of DC has been used to stabilize the membrane from bile salt solution in GI tract (4,8) and has also been used as a solubilizing agent (29). In this study, DC was used as a negative charge inducer; nevertheless, it predominantly performed as a solubilizing agent to yield nanoscale liposomes.

Figure 1 shows the transmission electron micrographs of chitosan-coated liposomes containing pRc/CMV-HBs. The shape of both plain liposomes and chitosan-coated liposomes was observed to be similarly spherical particles. At the concentration of 0.1% *w/v* chitosan solution, interaction between the polymer bridge among the spheres was not observed. When DC was incorporated, the higher concentration of chitosan solution caused a higher degree of aggregation, which could be immediately observed after adding the coating solution due to the interaction between the excessive chitosan and the DC in the mixture.

The differences observed in liposomes produced by adding DCP as a negative charge modifier (LP541) clearly appeared in the form of multilamellar vesicles as shown in Fig. 1b. The orientation of the plasmid was supposed to be in-between lipid bilayers. In general, liposomes produced from anionic lipid provided wider spaces between the lipid bilayers due to the repulsion of the net negative charges of the phospholipid polar head group.

Size and Zeta Potential of Plain Liposomes

The average sizes of all liposomes containing plasmid pRc/CMV-HBs were about 66–497 nm (Table II). Liposomes made from DC resulted in nanosizes of between 66 and 69 nm. The polydispersity indices (PI) of all preparations were between 0.43 and 0.68. Liposome preparations containing DC and DCP resulted in more homogenous sizes than those without these lipids. Zeta potentials of plain liposomes varied dependent on the incorporation of modifier lipids (DCP and/or DC). The zeta potentials of liposomes without DCP and DC (LP540) were −13.5 mV by which resulted from the impurity of the PC, which might be fatty acids or the degradation products of PC by aging and oxidation. In previous study, the existence of fatty acids has resulted in the negative charge of the nonionic liposome (29). When the anionic phospholipid DCP was incorporated into the lipid membrane, named as LP541, the zeta potentials substantially decreased from −13.5 to −52.0 mV. Similarly, DC is a negative charge bile salt that significantly lowered the zeta potential of the typical vesicles. When DC was incorporated into the lipid membrane it named as LP541DC. The zeta potential decreased from −13.5 to −62.0 mV. The resulting negative charge of liposomes might be useful for enhancing ionic interaction between the vesicle surfaces and the positive charged chitosan.

In the present study, the effect of pH on the zeta potential of the plain liposomes was studied to mimic the surface charge of the vesicles after oral administration since the environment in the GI tract is extremely variable. The instability of the vesicles, such as the zeta potential, might alter the internalization of the liposomes.

Figure 2 demonstrates the difference in behavior of the increase of zeta potentials following the decrease of pH from 8 to 2 according to the lipid components. The sizes of the plain liposomes were found to be increased with the decrease of pH due to the aggregation of the particles in the formulation containing DC (data not shown). After titration with 0.1 N NaOH to pH 8, the zeta potential of LP540 decreased to about −40 mV, and after adding 0.1 N HCl to adjust to the lower range of pH, the zeta potentials gradually increased due to the acid neutralization of phosphate polar

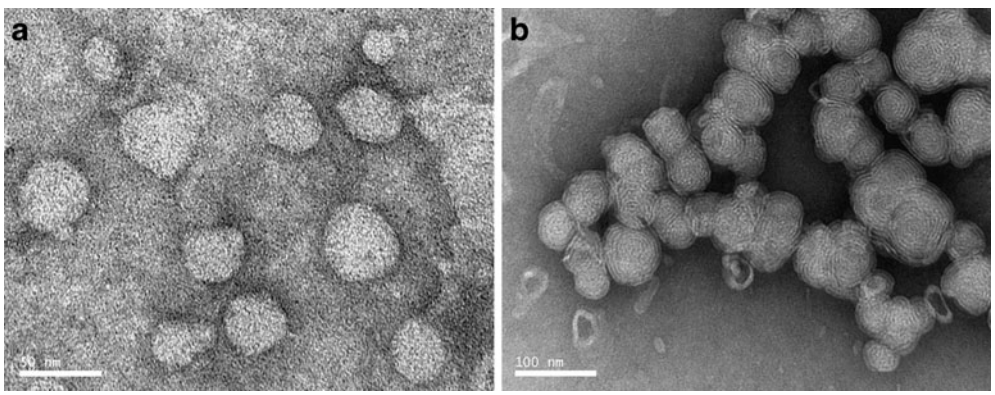

Fig. 1. Transmission electron micrographs of chitosan-coated liposomes. **a** LP540DC; **b** LP541DC

Table II. Lipid Compositions and Characteristics of the Liposomes Containing pRc/CMV-HBs

Composition in 150 μmol total lipids	Liposome preparation	Plasmid DNA (mg)	Zeta potential (mV ± SD)	Size and size distribution	
				Size (nm ± SD)	PI
PC/CHO (5:4)	LP540	1	−13.5±0.3	497±9	0.68
PC/CHO (5:4)+DC	LP540DC	1	−62.0±1	69±0.3	0.46
PC/CHO/DCP (5:4:1)	LP541	1	−52.0±2.5	441±41	0.47
PC/CHO/DCP (5:4:1)+DC	LP541DC	1	−71.2±0.2	66±14	0.43

head group of the phosphatidylcholine. Adsorption of H^+ ions at the surface occurred and induced higher positive charge at low pH. DC has pK_a about 6.58, it less ionized at low pH. From the titration curve, both LP540 and LP540DC exhibited the same isoelectric point at pH ~3.

The zeta potentials of liposomes containing the negative charge lipid (LP541 and LP541DC) exhibited large negative charges at pH 7.0 resulted from the ionization of DCP at higher pH values. As shown in the titration curve, the zeta potentials of LP541 and LP541DC were gradually increased with the decrease of pH from 8 to 4. At a pH lower than 4, the zeta potentials strikingly increased. In this case, the increase of the surface charges were due to the neutralization and ionization of the phosphate groups of both DCP and PC in the present of H^+ ions. The isoelectric point might be obtained at the pH lower than those made from PC. The results imply that in the gastric environment, neutral liposomes (LP540 and LP540DC) exhibited a little more positive charge than the negative charge liposomes (LP541 and LP541DC). When they passed through small intestine where the pH gradually increased up to 7.0, the surface charge of each formulation changed to more negative in different degrees rely on the composition of the lipid vesicles. The negative charges always restrained the interaction between the particles and the mucous membrane, in consequence barricaded the internalization of the DNA particles.

PLLs and the Characteristics

Chitosan was first reported as a cationic polymeric condensing carrier by Mumper (30). The chitosan–DNA complex (polyplex) has been studied by many researchers and is deemed to possess a high potential for *in vitro* and *in vivo* transfection (31,32). The polyplex provides the benefits of the protection of the DNA from nuclease degradation and a mucoadhesive property that permits a sustained interaction between the encapsulated materials and the membrane epithelia, which promotes more efficient uptake (33). The loading of PLL and the coating with chitosan were expected to improve the stability in harsh environment throughout the intestine. Optimization study of polyplexes were formulated using various amount of chitosan (5–10 mg) and fixed amounts of DNA (1 mg). The loading efficiency of all polyplex preparations was higher than 84%. The sizes of the particles increased with the increase of chitosan amounts ranging from 346 to 554 nm (Table III). Higher concentration of chitosan resulted in more elongated shapes and clusters of the particles.

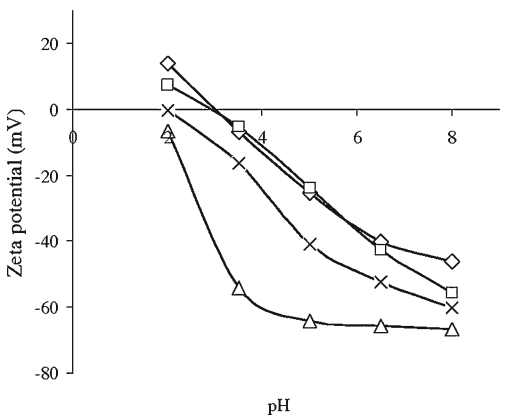

Fig. 2. The effect of pH on surface charges of plain liposomes. *Diamonds*, LP540; *squares*, LP540DC; *error marks*, LP541; *triangles*, LP541DC

To study the efficiency of PLLs for targeting the DNA to Peyer's patch, 5 mg of chitosan and 1 mg DNA was selected for preparing polyplex. PLL was prepared by encapsulating the polyplex in the same lipid components as the previous study using thin film method. Figure 3 shows a transmission electron micrograph of polyplex entrapped in lipid vesicles prepared from LP541.

In order to increase the zeta potential and mucoadhesive property of the particles, the resulting PLLs were coated with chitosan solution to form chitosan-coated PLLs. Figure 4 shows the effect of chitosan concentrations on surface charge of the liposomal preparations. The adsorption of chitosan on the liposome surface resulted in the increase of the zeta potential proportionally to the increase of chitosan concentration. The net positive charge of coated liposome was obtained from the neutral liposome (LP540). Chitosan concentration up to 0.2% *w/v* could not completely conduct the positive charge of the liposome particles containing DCP and DC as membrane modifiers. Thus, coating with a 0.1% *w/v* concentration was selected for the subsequent study. As shown in Fig. 4, the dashed lines illustrate the effect of chitosan concentrations on the zeta potential of coated PLLs. The zeta potential of coated LP540, PLL-540, and PLL-541 using 0.1% *w/v* chitosan

Table III. Characteristics of Chitosan–DNA (polyplex) Containing pRc/CMV-HBs

Amount of CS (mg) per DNA 1 mg	Loading efficiency (%)	Size and size distribution	
		Size (nm ± SD)	PI
5.0	86	346±4	0.19
7.5	84	429±10	0.29
10.0	87	554±10	0.37

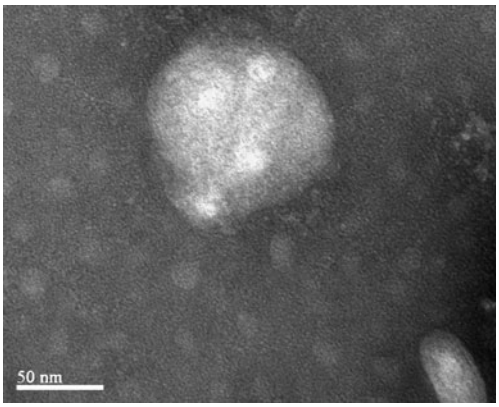

Fig. 3. Transmission electron micrograph of PLL-541. The *bar* represents 50 nm

solution were about 22 mV. The existing positive charge of chitosan-coated PLLs was expected to be more stable than chitosan-coated liposome along the distance to the target sites in the GI tract.

Integrity and Stability Study

The stability of the delivery system carrying pRc/CMV-HBs gene was previously studied and the efficacy of this DNA vaccine was evaluated elsewhere (data not shown). Regarding the protection ability of the liposomes against the endonuclease enzyme, it was found that both the plain and chitosan-coated liposomes protected the DNA from the endonuclease enzyme. Figure 5 shows the integrity of the plain (LP541 and LP541DC) and chitosan-coated liposomes after incubation with DNaseI. It clearly shows that both the plain and coated liposomes could protect DNA from endonuclease digestion.

Figure 6 shows the stability of DNA after incubation in simulated gastric fluid pH 1.2 (a) and simulated intestinal fluid pH 6.8 (b). DNA in plain LP540 and LP541 was more stable in gastric fluid. Some leakage of DNA appeared from the liposomes containing DC (lanes 3 and 5 in a). In the case of intestinal fluid containing lipase, protease and pancreas enzymes, all plain liposome preparations were less protective than those in gastric fluid, as shown in Fig. 6b. The smear of the band indicates the leakage and/or degradation of the

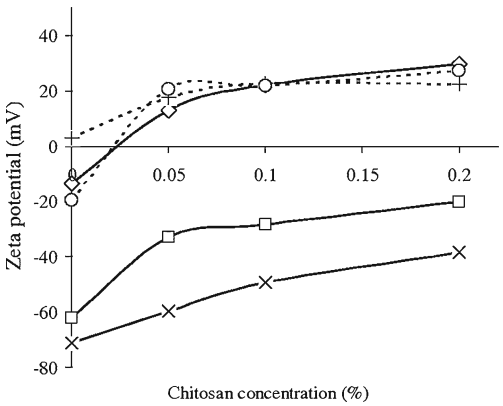

Fig. 4. The effect of chitosan concentration on surface charge of liposomal formulations. *Diamonds*, LP540; *squares*, LP540DC; *error marks*, LP541; *plus sign*, PLL-540; *circles*, PLL-541

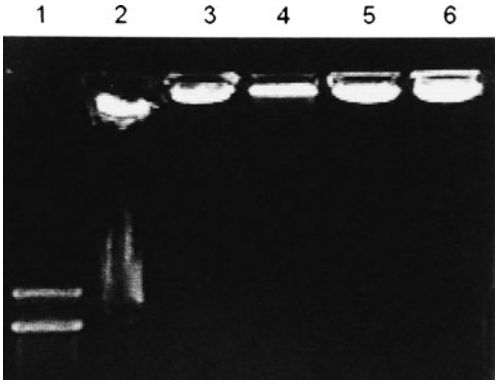

Fig. 5. Integrity of plain and coated liposomes carrying pRc/CMV-HBs after incubation with DNaseI (1 U/μg DNA) at 37°C, for 30 min. *Lane 1*, naked plasmid as reference; *lane 2*, plain LP541; *lane 3*, plain LP541DC; *lanes 4–6*, LP541DC coated with 0.05% *w/v*, 0.1% *w/v*, and 0.2% *w/v* chitosan solutions, respectively

DNA. DNA ladder was not included in the gel electrophoresis running because the well characterized plasmid pRc/CMV-HBs was used as reference itself. The two bands indicated the circular and supercoiled forms of the plasmid after purification. The plasmid pRc/CMV-HBs was prior characterized by DNA sequencing before the transformation into the host and by gel electrophoresis after cutting with restriction enzymes after purification in the propagation step.

The liposomes containing DC in this study did not give any benefit against intestinal enzymes protection. However, all the formulas of chitosan-coated liposomes with 0.1% *w/v* chitosan solution appeared to be protective in both gastric and intestinal fluid as shown in Fig. 6 (lanes 6–9). After formulating DNA into PLLs and coating with 0.1% *w/v* chitosan, the DNA was found highly stable in both SGF and SIF.

In Vivo Study of Chitosan-Coated Liposomes Carrying GFP

To evaluate the efficiency of the chitosan-coated liposomes and PLLs as oral gene delivery systems, each formulation of liposome carrying pEGFP-C2 was investigated in BALB/c mice.

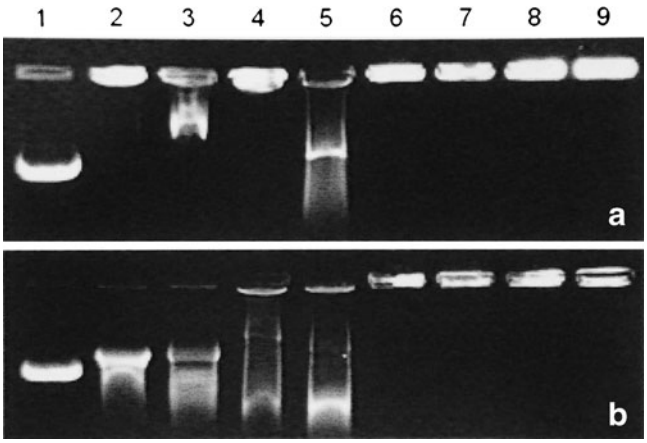

Fig. 6. Integrity of plain liposomes (*lanes 2–5*) and 0.1% *w/v* chitosan-coated liposomes (*lanes 6–9*) in SGF (**a**) and SIF (**b**) after incubation at 37°C for 1 and 2 h, respectively. *Lane 1*, naked pRc/CMV-HBs; *lanes 2* and *6*, LP540 and CS-LP540; *lanes 3* and *7*, LP540DC and CS-LP540DC; *lanes 4* and *8*, LP541 and CS-LP541; *lanes 5* and *9*, LP541DC and CS-LP541DC

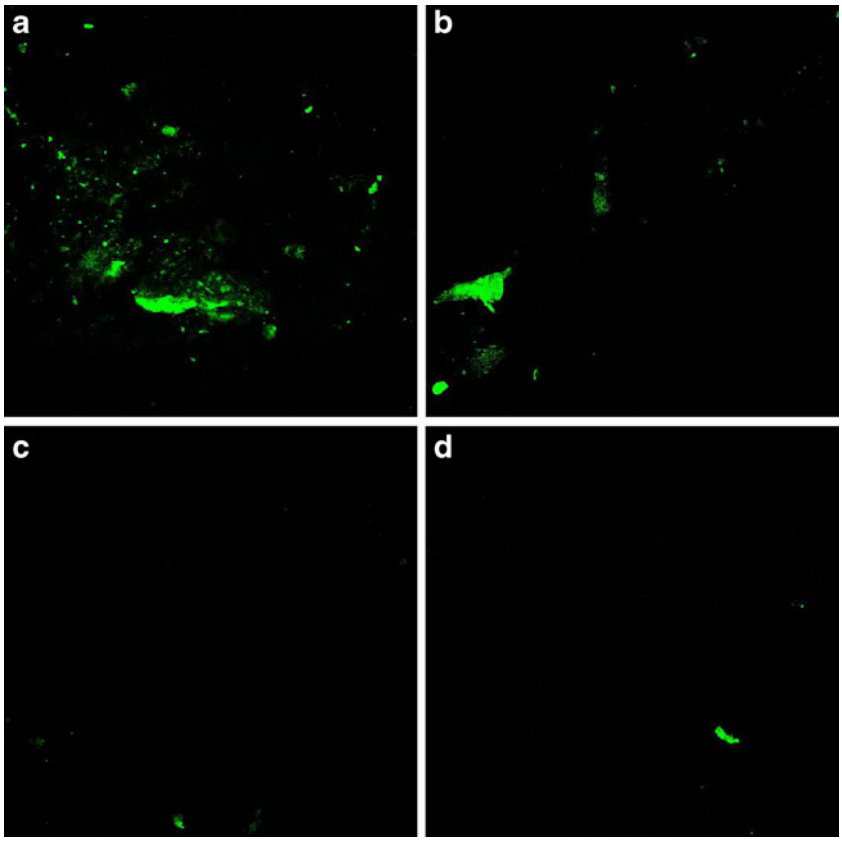

Fig. 7. Confocal laser scanning microscopy photographs of BALB/c mice intestinal tract (duodenum) 24 h after an oral inoculation with chitosan-coated liposomes containing 100 μg of pEGFP-C2 plasmid DNA. **a** CS-LP540; **b** CS-LP540DC; **c** CS-LP541; **d** CS-LP541DC

Table I shows the sizes and size distributions of 0.1% *w/v* chitosan-coated liposomal preparations carrying pEGFP-C2. After coating with chitosan, the sizes and size distributions (PI) were larger due to the layers of coating polymer.

Figure 7 shows the confocal laser scanning microscope images of intracellular GFP expression after inoculation with chitosan-coated liposomes. The protein expression level reflected the ability of the particulate carrier in terms of DNA protection and internalization of the DNA into the cell compartment. The protein signal was detected only from the upper part of the duodenum. The highest signal was observed from chitosan-coated LP540 (CS-LP540). The result was in agreement with the former study, in that the zeta potential of the LP540 was positive in the acid environment, which might promote liposome-epithelial cell fusion better than the negative charge liposomes. Although it was demonstrated in a previous study that chitosan-coated liposomes were resistant to simulated GI fluids, it could be hypothesized that the

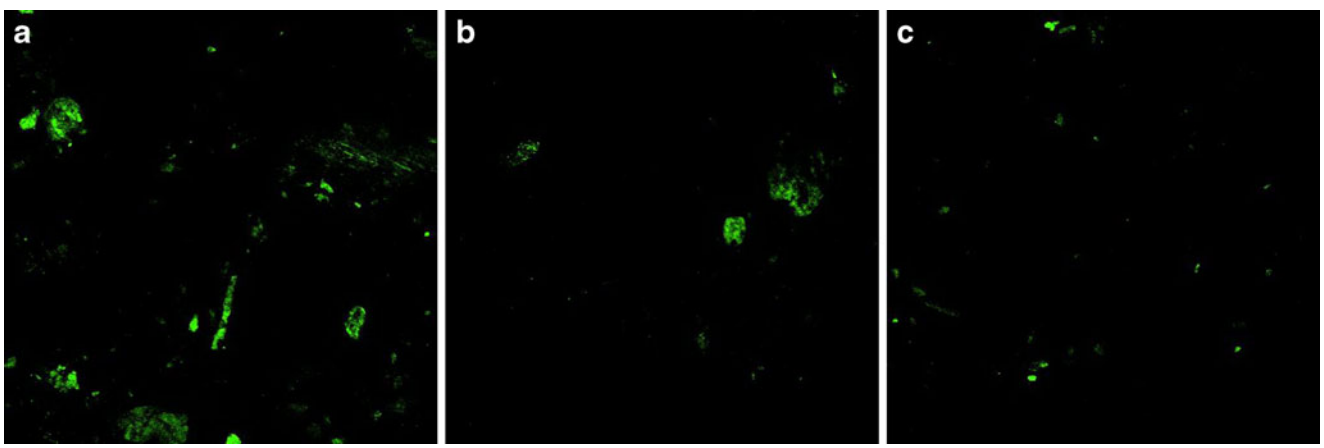

Fig. 8. Confocal laser scanning microscopy photographs of BALB/c mice intestinal tract 24 h after an oral inoculation with 100 μg of pEGFP-C2 in chitosan-coated PLL540. The *green color* indicates the luminescent signals of the expressed protein that were found from different areas: **a** duodenum; **b** jejunum; **c** ileum

zeta potential was too low to promote the internalization of the DNA. Thus, PLL was fabricated to counteract the weak point of the former particulate systems.

Figure 8 shows the improved results of the delivery systems for oral DNA delivery. CS-PLL540 and CS-PLL540DC exhibited higher efficiency of DNA delivery throughout the small intestine. The expressed protein signals were found to be similar in the duodenum, jejunum, and ileum. Meanwhile, the GFP expression from naked DNA, plain liposome, and CS-PLL541 and CS-PLL541DC was poor (data not shown).

To succeed in gene delivery to the target site of Peyer's patches, many strategies such as larger doses of DNA with higher volume of the DNA preparation to cover the area of adsorption as well as the emptiness of the GI tract of the animals should be taken in the study. The uses of chitosan as cationic complexing agent and as coating polymer have been extensively studied for oral drug delivery systems (9,17,34). As coating agent, the chitosan polymer decreased the chance of lipid vesicles to be attacked by enzymes. The mucoadhesive property of chitosan has been referred to the success of delivery in GI tract because of the longer residence time of the delivery system on the mucosal tissues resulted in higher concentration of the targeting particles at the site of adhesion–adsorption which consequently created a delivery force for paracellular and transcellular internalization (35).

CONCLUSIONS

Oral vaccination is not straightforwardly successful due to many factors not only from the delivery system but also to the sufficient uptake of the antigen particles at M cells as well as the stability of the targeting antigen until it reaches to the target site of Peyer's patches. In this study, we developed a simple and biodegradable delivery system by combining the benefits of chitosan and liposome together for DNA delivery through the Peyer's patches, which are mostly located in the ileum. Polyplex was first prepared by complex coacervation of the DNA with chitosan to provide a more permanent positive charge to the particles. For better protection in gastric environment, polyplex was loaded into lipid vesicles and finally coated with 0.1% *w/v* chitosan solution since the liposome is normally less resistant in the intestinal environment. Coating with chitosan improved DNA internalization efficiency and reduced the rate of DNA deterioration. In addition, the coated chitosan helped to counteract the negative charge which resulted from the ionization of lipid at elevated pH in ileum, since highly negative charge played less beneficial due to the electrostatic repulsion of the particles and the mucus layers. From the GFP protein expression results, the chitosan-coated PLL demonstrated the highest potential of the DNA delivery to the distal intestine in consequence of an upsurge in robustness, and the extended stability of surface charge of the particles which were crucially important for oral DNA vaccine delivery.

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