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Lecithin-based cationic nanoparticles as a potential DNA delivery system

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Abstract

Previously, we have reported a novel nanoparticle-based DNA vaccine delivery system, which elicited strong immune responses against antigens of interest encoded by the DNA. The nanoparticles were engineered by cooling pre-formed warm microemulsions comprised of emulsifying wax as the oil phase and hexadecyltrimethyl ammonium bromide (CTAB) as the surfactant. However, the poor aqueous stability of the nanoparticles and the emulsifying wax in the nanoparticles may severely limit the applications of the nanoparticles. In the present study, we used lecithin, a more biocompatible material, instead of emulsifying wax, to prepared lecithin-based cationic nanoparticles. The 50% growth inhibition concentration (IC_{50}) of the lecithin-based nanoparticles was found to be more than 1000-fold higher than that of the emulsifying wax-based nanoparticles. Moreover, the stability of the lecithin nanoparticles was also significantly increased. The size of the nanoparticles did not significantly change during a 6-month storage period at room temperature. Finally, when plasmid DNA was adsorbed on their surface, the lecithin nanoparticles successfully transfected cells in culture. These lecithin-based nanoparticles may hold great potentials as a DNA (vaccine) delivery system.

Keywords: Cytotoxicity; Stability; Transfection

1. Introduction

Nanoparticles have attracted much attention recently due to its different, and in most cases, more desirable properties. For example, nanoparticles prepared using various methods have been successfully applied in many recent studies as a delivery system for plasmid DNA for gene therapy and DNA vaccination purposes (Bisht et al., 2005; Cui and Mumper, 2002a; Hattori and Maitani, 2005; Kaul and Amiji, 2005a,b; Kim et al., 2005; Locher et al., 2003; Rudolph et al., 2004). Nanoparticles as a carrier for plasmid DNA are advantageous in that the particles can generally enhance the chemical stability of the DNA, regardless of whether the DNA is incorporated inside the nanoparticles or simply adsorbed on the surface of the nanoparticles (Singh et al., 2000). Moreover, nanoparticles can provide an opportunity for targeting the DNA of interest into specific tissues or cells by attaching specific ligands or antibodies on the surface of the nanoparticles. In the case of DNA vaccination using plasmid DNA carried by nanoparticles, it is generally understood that immune cells, such as the immature dendritic cells (DCs) that play a pivotal role in priming immune responses, take up particles more efficiently than molecules, such as naked plasmid DNA (Banchereau and Steinman, 1998). Also, it was previously shown that plasmid DNA adsorbed onto smaller poly(lactic-*co*-glycolic) acid (PLGA) nanoparticles (300 nm) induced significantly stronger immune responses than the same DNA adsorbed onto larger PLGA microspheres (1 and 30 μm) (Singh et al., 2000).

Previously, we have reported a novel method to prepare cationic nanoparticles by simple cooling microemulsions preformed using non-ionic emulsifying wax as the oil phase and CTAB as the cationic surfactant at increased temperature (50–55 °C) (Cui and Mumper, 2002a). This nanoparticle engineering method has many advantages, in part: (i) well defined and uniform solid nanoparticles can be readily and reproducibly engineered without the use of expensive and sometimes damaging high torque mixing procedures, (ii) the engineering process is potentially scalable, and (iii) toxic and organic solvents are not required. The resulted cationic nanoparticles were around 100 nm in diameter. When plasmid DNA encoding an antigen of interest was adsorbed on the surface of the nanoparticles and used to immunize mice via many different routes including

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subcutaneous, intradermal, intranasal, or topical, the DNA-adsorbed nanoparticles elicited significantly enhanced humoral and cellular immune responses against the encoded antigen, when compared to immunization with the plasmid DNA alone (Cui et al., 2003; Cui and Mumper, 2002a,b,c), pointing out the potential of these cationic nanoparticles in DNA vaccine delivery (Cui and Mumper, 2003).

Unfortunately, there are factors that may severely limit the applications of these nanoparticles, namely its lack of stability in aqueous suspension and its potential toxicity. For example, when the nanoparticles were stored at room temperature in an aqueous suspension, their size increased by as much as 70% in a short 6-day storage period (Cui and Mumper, 2002a), presumably due to the aggregation of particles. Moreover, although the CTAB component in the nanoparticles (i) has a defined toxicity profile, (ii) had been previously used in pharmaceutical products such as eye drops (Bron et al., 1998), and (iii) may be replaced with more biocompatible cationic lipids or proportionally decreased by adding other non-ionic co-surfactants (Cui and Mumper, 2002b), the applications of the emulsifying wax in pharmaceutical products had been strictly limited to topical products, such as creams and ointments (Wade and Weller, 1994), but we expect to administer the nanoparticles as injectables. Thus, there exists a clear need to search for alternative, more biocompatible or biodegradable materials to replace the emulsifying wax. The United States Pharmacopeia and National Formulary (USP/NF) described lecithin as a combination of acetone-insoluble phosphatides, which consist mainly of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI), combined with various amounts of other substances such as triglycerides and fatty acids. For example, soy lecithin contains 21% PC, 22% PE, and 19% PI, along with other components (Wade and Weller, 1994). Lecithins are components of cell membranes and are thus consumed as a normal part of diet. It had been used in a wide variety of pharmaceutical applications as dispersing, emulsifying, and stabilizing agents and was included in intramuscular and intravenous injectables, parenteral nutrition formulations, as well as topical products (De Muynck et al., 1994; Drejer et al., 1992; Growdon et al., 1978; Williams et al., 1984).

In this present study, we proposed to prepare cationic nanoparticles by replacing the emulsifying wax in our previously reported nanoparticles with soy lecithin. These lecithin-based cationic nanoparticles were over 1000-fold less toxic than the emulsifying wax-based nanoparticles and were more stable when stored in aqueous suspension at room temperature.

2. Materials and methods

2.1. Materials

Plasmid, pNGVL-luc, containing the cDNA of firefly luciferase driven by the cytomegalovirus (CMV) promoter was from the National Gene Vector Laboratories (Indianapolis, IN) (Lemoine et al., 2005). It was transfected into *E. coli* DH5*a*, amplified, and purified using a Qiagen Endo Free plasmid purification kit (Qiagen, Valencia, CA). Emulsifying wax (melting point, ∼55 °C) and lecithin (NF, m.p., ∼200 °C) were purchased from the Spectrum Chemicals & Laboratory Products (New Brunswick, NJ). CTAB (m.p. > 250 °C), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) kit, and Sephadex G-75 were from Sigma-Aldrich (St. Louis, MO).

2.2. Methods

2.2.1. Engineering of lecithin-based nanoparticles

To prepare the lecithin-based nanoparticles, exactly 4 mg of lecithin was weighed and placed into a 7-mL glass scintillation vial. A predetermined volume of de-ionized, filtrated (0.22 μm) warm water (50–55 °C) was added into the vial and stirred at 50–55 °C on a hot plate until a milky suspension was formed. A required volume of CTAB (50 mM in water) was then added into the lecithin-in-water suspension while stirring to reach a final CTAB concentration of 0, 2.5, 5, 7.5, or 10 mM, while maintaining the final volume of the nanoparticle suspension at 1 mL. Stirring was maintained until a transparent solution-like system was formed (usually within about 5 min), which was then allowed to cool down to room temperature.

Emulsifying wax-based nanoparticles were prepared as previously described except that the amount of the emulsifying wax was increased to 4 mg/mL (Cui and Mumper, 2002a) for easy comparison with the lecithin-based nanoparticles.

2.2.2. Physical characterization of the size and surface charge of the nanoparticles

The size of the nanoparticles was measured by photon correlation spectroscopy (PCS) using a Coulter N4 Plus Submicron Particle Sizer (Beckman Coulter Inc., Fullerton, CA) by scattering light at 90° angle at 25 °C for 120 s. The zeta potentials of the nanoparticles were measured using a Zeta Sizer from Malvern Instruments (Southborough, MA).

2.2.3. Transmission electron microscopy (TEM)

The size and morphology of the nanoparticles were observed using a transmission electron microscope (PHILIPS CM12 TEM/STEM) in the Oregon State University Electron Microscope Facility. A carbon-coated 200-mesh copper specimen grid (Ted Pella Inc., Redding, CA) was glow discharged for 1.5 min. One drop of nanoparticles suspension (lecithin, 4 mg/mL; CTAB, 7.5 mM) was deposited on the grid and left to stand for 1.5 min, and all excess fluid was removed with filter paper. The grid was then stained with one drop of 1% uranyl acetate solution (0.2 mm-filtrated) for 30 s, and all excess uranyl acetate was again removed with filter paper. The grid was allowed to dry at room temperature for an additional 30–60 min before being examined.

2.2.4. Differential scanning calorimetry

The thermal analysis was performed in a differential scanning calorimeter (TA Instruments DSC 2920, New Castle, DE). Approximately 5–10 mg of sample was weighed into the DSC aluminum pans. An empty pan was used as reference. Heating curves were recorded with a scan rate of 10 °C/min from 10 to

300 °C. Each sample was cooled to 10 °C before proceeding to change pans and run further samples.

2.2.5. Stability of the nanoparticles in aqueous suspension

The lecithin-based nanoparticle suspensions prepared as described above were sealed into glass vials and stored at room temperature in the dark for 6 months. The size of the particles was measured on days 0, 15, as well as after 6 months. Immediately prior to particle sizing, the nanoparticle suspension was diluted with water to adjust the total light scattering intensity (in counts per second, cps) so that it was within the specified range of the particle sizer (5×10^4 to 1×10^6 cps). The emulsifying wax-based nanoparticles were monitored for only 5 days because their size was already significantly increased after those 5 days of storage.

2.2.6. Gel permeation chromatography

To separate the nanoparticles from free CTAB, a gel permeation chromatography (GPC) using Sephadex G-75 was performed. Sephadex G-75 was pre-soaked with de-ionized and filtrated (0.22 μm) water overnight and packed into a 15 mm × 100 mm plastic column. The column was equilibrated with water. Two hundred and fifty microliters (250 μL) of the nanoparticle suspension was applied to the column and eluted with water. The elution was collected in 1-mL fractions in plastic vials. The collected fractions were analyzed by laser light scattering to identify the fraction(s) that contained nanoparticles. In all cases, the fourth fraction contained the greatest number of nanoparticles, as evidenced by the highest particle counts per sec in it. The concentration of the CTAB in every elution fraction was also determined.

2.2.7. Determination of CTAB concentration

The concentration of CTAB in each fraction was quantified using the methyl orange spectrophotometric method as previously described (Wang and Langley, 1977). Briefly, a standard curve was constructed by making a series of CTAB solutions (5 mL) with concentrations ranging from 0 to 2 mM in glass tubes. Then, 0.1 mL of methyl orange (Sigma, 0.1% w/v), 0.5 mL buffer solution (0.5 M citric acid and 0.2 M disodium hydrogen phosphate mixed in equal volume), and 2.5 mL chloroform were added into each tube. The tubes were vigorously vortexed for 1 min and allowed to stand undisturbed for 20 min for the chloroform and aqueous layers to separate. After the upper aqueous layer was removed, the lower chloroform layer that contained the CTAB-bound methyl orange was read at 415 nm in a DU® 640 UV–vis spectrophotometer (Beckman Coulter). The GPC elution samples were treated similarly, and the CTAB concentration in each fraction was derived from the standard curve.

2.2.8. Cytotoxicity assay

TC-1 cell line from Dr. Wu at the Johns Hopkins University (Baltimore, MD) was engineered from C57BL/6 mouse lung endothelial cells. Cells were grown in Roswell Park Memorial Institute (RPMI) medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/mL of penicillin (Invitrogen), and 100 μg/mL of streptomycin (Invitrogen) and cultured at 37 °C in a humidified incubator containing 5% CO_2. The cells (4×10^5) were co-incubated in 96-well plates with 200 μL of RPMI medium supplemented with phosphate buffered saline (PBS, 10 mM, pH 7.4), emulsifying wax nanoparticles, lecithin nanoparticles, or CTAB (the final concentrations of them in the culture medium were adjusted to 1, 0.1, 0.01, 0.001, and 0.0001 mg/mL) for 16 h. To quantify the number of viable cells, the cells were incubated for two more hours with an MTT solution (0.01 mL, 5 mg/mL in PBS) at 37 °C. At the end of the incubation, 0.1 mL of Solution C (isopropyl alcohol with 0.04N HCl) was added into each well. The o.d. values at 570 and 630 nm were measured using a SpectraMax plate reader (Molecular Devices Inc., Sunnyvale, CA). The percentage of viable cells was calculated as: $(\text{o.d.}_{570} - \text{o.d.}_{630})_{\text{Sample}}/(\text{o.d.}_{570} - \text{o.d.}_{630})_{\text{PBS}} \times 100$. The cytotoxicity was expressed as the concentration of particles or CTAB that led to 50% cell growth inhibition (IC_{50}) compared with the growth of cells co-incubated with PBS.

Both lecithin-based nanoparticles and the emulsifying wax-based nanoparticles were prepared with 4 mg/mL of lecithin or emulsifying wax and a final CTAB concentration of 7.5 mM. The particles were GPC-purified, lyophilized, weighed, and reconstituted in PBS (10 mM, pH 7.4).

2.2.9. Surface adsorption of plasmid DNA on the nanoparticles

Lecithin-based nanoparticles prepared with a final CTAB concentration of 7.5 mM were GPC-purified. To the purified nanoparticle suspension (fraction 4), an equal volume of pNGVL-luc solution containing a required amount of pNGVL-luc was added to obtain final DNA concentrations ranging from 0 to 200 μg/mL. The mixture was gently pipetted, briefly vortexed, and allowed to stay at room temperature for at least 30 min for DNA binding. The particle sizes and zeta potentials of the DNA-adsorbed nanoparticles were then measured as described earlier.

2.2.10. Stability of the DNA-adsorbed nanoparticles in simulated biological media

In order to evaluate the stability of the nanoparticles in simulated biological media, pNGVL-luc-adsorbed lecithin nanoparticle suspensions were diluted 10-fold with either 10% lactose (w/v), PBS (10 mM, pH 7.4), or 10% (v/v) of FBS in normal saline and incubated at 37 °C for 30 min. The particle sizes at 0 and 30 min were measured.

2.2.11. In vitro cell transfection

The human 293 cell line (CRL-1573™) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in minimum essential medium (Eagle) (EMEM, Invitrogen) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, and FBS (10%). Transfections were performed with confluent cells. Cells were plated in 48-well plates at a density of 5×10^5 cells/well and incubated at 37 °C, 5% CO_2 overnight. The cells were

then co-incubated with EMEM supplemented with pNGVL-luc alone, pNGVL-luc adsorbed on lecithin nanoparticles (25 μg of pNGVL-luc per mL), or pNGVL-luc complexed with Lipofectin® (Invitrogen) as a control. In all cases, the final pNGVL-luc dose was 2.5 μg/well. Four hours later, the medium was refreshed. The culture medium was removed 48 more hours later; cells were washed with cold PBS buffer twice, and lysed by incubating with 200 mL of 1 × Lysis Buffer (Promega, Madison, WI) for 2 h, followed by five cycles of freeze-and-thawing. Luciferase activity was assayed as previously described (Cui and Huang, 2005; Cui and Mumper, 2002a).

2.2.12. Statistical analyses

Statistical analyses were completed using one-way analysis of variance (ANOVA) followed by the Fischer's protected least significant difference (PLSD) procedure. A p-value of ≤ 0.05 (two-tailed) was considered to be statistically significant.

3. Results and discussions

Previously, we have reported the engineering of cationic nanoparticles from microemulsion precursors using emulsifying wax as the matrix material and CTAB as the surfactant (Cui and Mumper, 2002a). These cationic emulsifying wax-based nanoparticles, when adsorbed with plasmid DNA on their surface, have been demonstrated to be a potential delivery system for DNA vaccine. Unfortunately, as mentioned earlier, the nanoparticles, when stored in aqueous suspension, were found to be very unstable (Cui and Mumper, 2002a). Moreover, the matrix material of the nanoparticles, emulsifying wax, was approved for use only in cosmetics and topical pharmaceutical formulations, such as creams and ointments. Thus, there is a need to search for alternative materials to generate more stable nanoparticles with improved biocompatibility. Lecithin is a component of cell membranes and is used in a wide variety of pharmaceutical products including intramuscular and intravenous injectables as emulsifying or solubilizing agent (Wade and Weller, 1994). In this present study, we sought to replace the emulsifying wax in our previous nanoparticles with lecithin and to evaluate the resulting lecithin-based nanoparticles.

As shown in Fig. 1, particles with diameters in the range of 100–200 nm were readily engineered using soy lecithin as the matrix material and CTAB as the surfactant. As expected, the zeta potential of the particles was positive, and reached a maximal value of about 60 mV at the CTAB concentration of 7.5 mM (Fig. 1). TEM showed the particles were spherical (Fig. 2), although the size derived from the TEM micrograph tended to be smaller than that when measured using the particle sizer. This is understandable because the photon correlation spectroscopic particle sizer determines the size of the particles by measuring the movement of the particles due to Brownian motion. Therefore, the particle size determined using the particles sizer was in fact the size of the particles with their surrounding aqueous boundary layer, which moved together with the particles. In contrast, the particle size derived from the TEM micrograph was the size of the particles alone.

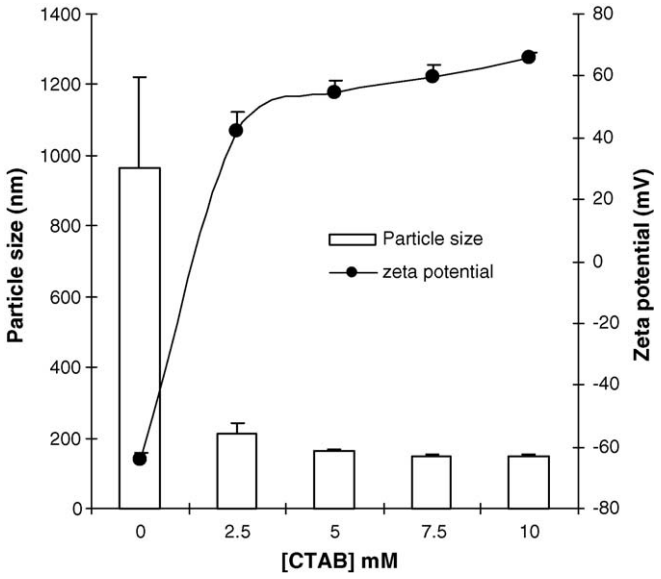

Fig. 1. Effect of the concentration of CTAB on the size and zeta potential of the lecithin nanoparticles. Cationic nanoparticles were prepared with lecithin (4 mg/mL) and various concentration of CTAB. The size and zeta potential of the particles were measured and reported as mean ± S.D. ($n = 3$). For both particle size and zeta potential, the values at the CTAB concentration of 7.5 mM were not different from that at the 10 mM CTAB concentration ($p = 0.89$ and 0.06, respectively).

It needs to be pointed out that, although the procedure for preparing the lecithin-based nanoparticles was similar to that for the preparation of the emulsifying wax-based nanoparticles, it is unclear whether these lecithin nanoparticles were formed mechanistically similar to the emulsifying wax-based nanoparticles as

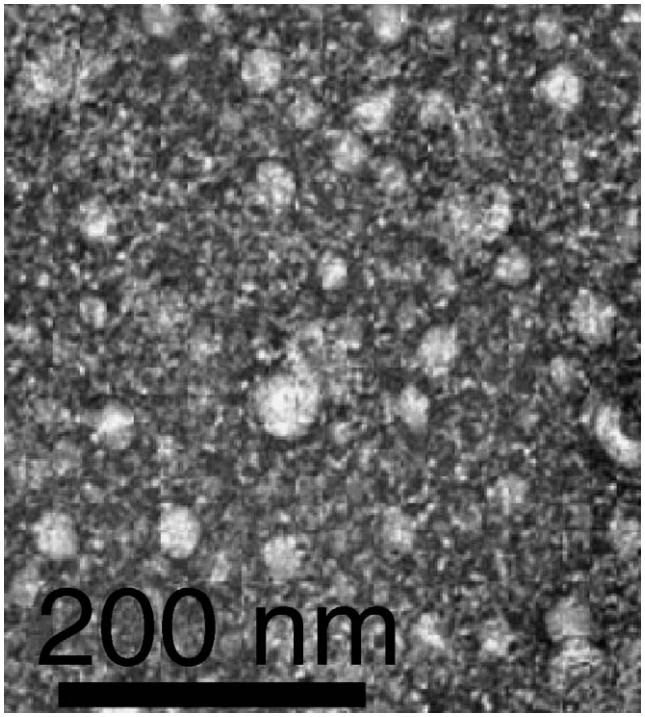

Fig. 2. Transmission electron micrograph of the lecithin-based cationic nanoparticles.

we previously reported (Cui and Mumper, 2002a). Differential scanning calorimetry (DSC) analyses revealed that the melting point of emulsifying wax was around 50–55 °C, while the melting point of the soy lecithin (NF) we used was around 200 °C. When heated to 50–55 °C, the emulsifying wax was melted. It was believed that the melted emulsifying wax, with CTAB as a surfactant, formed microemulsions, which were then solidified into nanoparticles when being cooled down to room temperature. However, in the case of the lecithin-based nanoparticles, the lecithin could not have melted at 50–55 °C. In fact, we did not observe the melting of the lecithin when it was placed in 50–55 °C. The warm temperature might have only helped the dispersion of the lecithin in the warm water prior to the addition of the CTAB as a surfactant. Moreover, it is also unreasonable to believe that a liquid to solid phase transition was involved when the lecithin-based preparation was cooled to room temperature from 50 to 55 °C because the melting point of the lecithin nanoparticles was determined to be more than 100 °C using DSC. Thus, we do not believe that the final lecithin-CTAB product was simply some microemulsion droplets. Finally, the particles formed were very unlikely to be simple CTAB micelles because the diameter of CTAB micelles was previously shown to be around 6–8 nm using TEM (Singh et al., 2004). Future studies will be directed towards defining the mechanisms behind the particle formation.

Interestingly and to our surprise, the lecithin nanoparticles were found to be very stable in aqueous suspension in a 6-month storage period (Fig. 3A). In fact, even after 1 year at room temperature, the lecithin nanoparticles prepared with a final CTAB concentration of 7.5 mM remained to be 136 ± 63 nm (polydispersity index, 0.28), as compared to its original size of around 146 ± 60 nm (PI, 0.26). This was in sharp contrast to the nanoparticles prepared with emulsifying wax as the matrix material, whose particle size increased by 28–64%, depending on the final concentration of CTAB, within a short 5-day storage period in identical conditions (Fig. 3B). Although more experiments have to be completed to fully elucidate the mechanisms behind the improved stability of the lecithin nanoparticles, we speculate that the higher melting point of the lecithin might have partially contributed to it.

Fig. 4 demonstrated that the lecithin nanoparticles can be separated from the unincorporated CTAB molecules using the GPC method. Although free CTAB may have formed micelles at the concentration used, the hydrodynamic radius (R_h) of CTAB micelles at a CTAB concentration of 10 mM was estimated to be only 3.5 nm (Zhang et al., 2000), which may explain why CTAB micelles were successfully separated from the lecithin nanoparticles using the GPC column (Fig. 4). Using the trapezoid rule, it was estimated that around 25% of the added CTAB was associated with the lecithin nanoparticles. Also, in elution fraction number 4, which was used to adsorb plasmid DNA in studies followed, the CTAB concentration was around 0.2 ± 0.03 mM.

To evaluate the toxicity of the lecithin nanoparticles, the growth inhibition of a tumor cell line (TC-1) was monitored after the cells were exposed to the nanoparticles for 16 h. As shown in Fig. 5, the 50% growth inhibition concentration (IC_{50}) of the

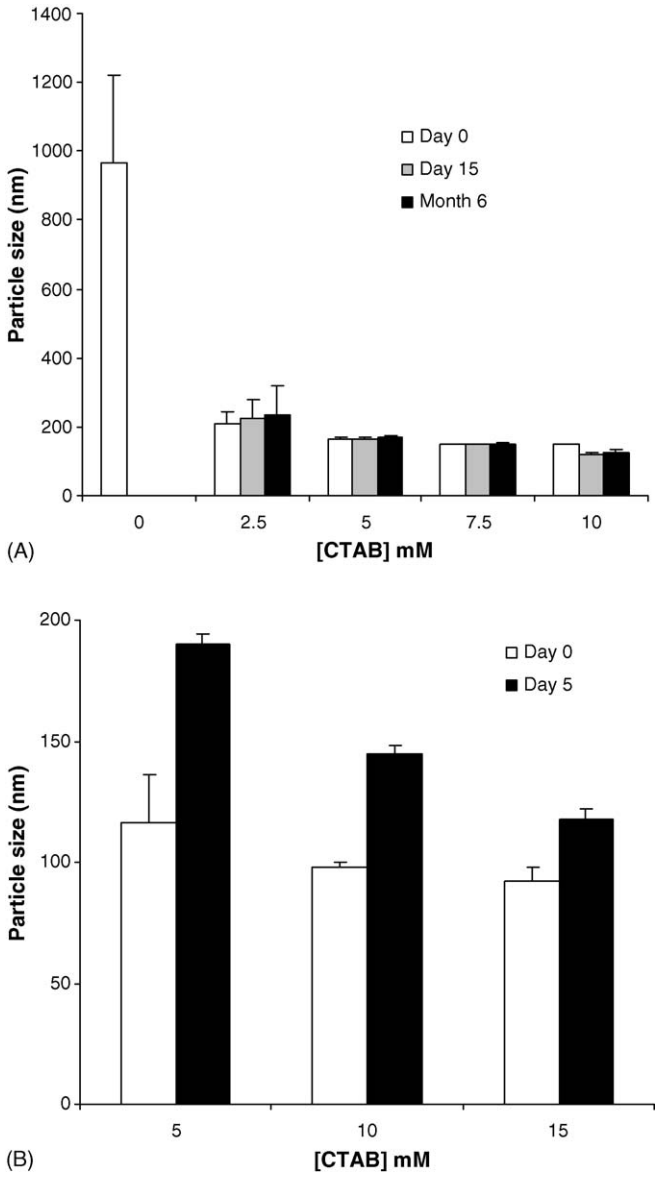

Fig. 3. The stability of the nanoparticles in aqueous suspension. (A) The lecithin nanoparticles prepared with various final concentrations of CTAB were stored at room temperature in the dark for 6 months. The sizes of the nanoparticles were determined 15 days and 6 months after the preparation. The size of the particles prepared with CTAB did not significantly change after the storage. The size of the preparation without CTAB was not followed. (B) For comparison, the sizes of the nanoparticles prepared with emulsifying wax were measured 0 and 5 days after the preparation. Their sizes were all significantly increased after 5 days of storage in conditions identical to that for the lecithin-based nanoparticles. Data shown are mean ± S.D. ($n = 3$).

lecithin nanoparticles was estimated to be 2.6×10^{-3} mg/mL, which was over 1000-fold higher than the IC_{50} of the emulsifying wax-based nanoparticles (2.4×10^{-6} mg/mL). CTAB had an IC_{50} value of 4.8×10^{-10} mg/mL. Thus, the lecithin nanoparticles appeared to be much less toxic than the emulsifying wax-based nanoparticles, although an alternative positively charged surfactants or lipids, such as DC-chol, DOTAP, and ethyl-PC, that are less toxic are still needed to replace the CTAB or to decrease the amount of CTAB to further improve the safety of the lecithin nanoparticles. Nevertheless, CTAB has been used

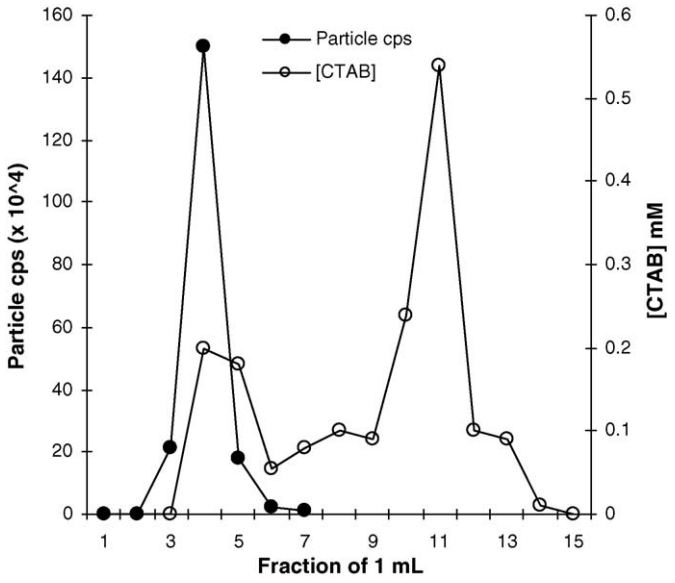

Fig. 4. The gel permeation chromatograph of the lecithin nanoparticles. The lecithin nanoparticle suspension (250 μL) prepared with 7.5 mM of CTAB was applied to a Sephadex G75 GPC column and eluted with water. The elution fractions (1 mL/fraction) were collected. The particle counts per sec (cps) and the concentration of CTAB in each fraction were determined. Data reported were the mean values from three independent determinations. The cps in fraction 7 and thereafter were below the accurate detection limit of the particle sizer.

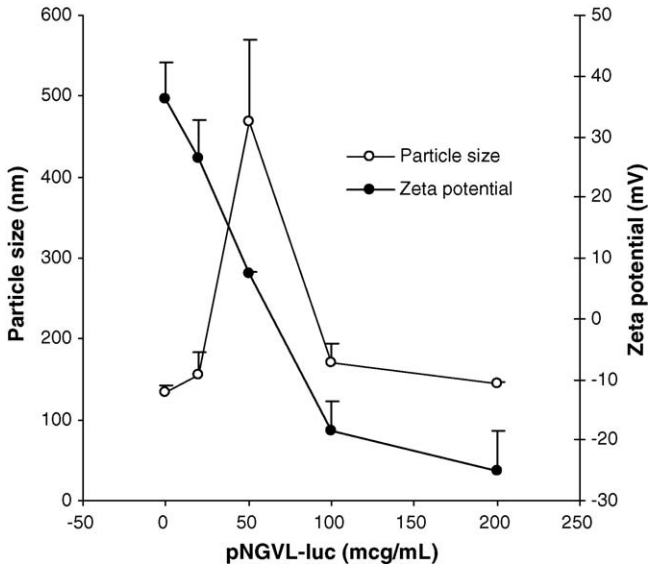

Fig. 6. The adsorption of pNGVL-luc on the lecithin nanoparticles. Lecithin nanoparticles were prepared with lecithin (4 mg/mL) and a final CTAB concentration of 7.5 mM. The GPC-purified particle suspension (fraction 4) was gently mixed with an equal volume of pNGVL-luc solution to reach a final pNGVL-luc concentration of 0, 25, 50, 100, and 200 μg/mL. The mixture was allowed to stay at room temperature for at least 30 min for DNA adsorption. The size and zeta potential of the mixture were then measured and reported (mean ± S.D., $n = 3$).

for a range of biomedical applications, including as an antibacterial agent in eye drops (Bron et al., 1998). Its toxicity is well defined. Moreover, we have previously reported that, using a co-surfactant such as Brij 78, a final CTAB concentration of 0.8 mM was enough to prepare the nanoparticles (Cui and Mumper, 2002b). Thus, the amount of CTAB in the nanoparticles may be further limited accordingly.

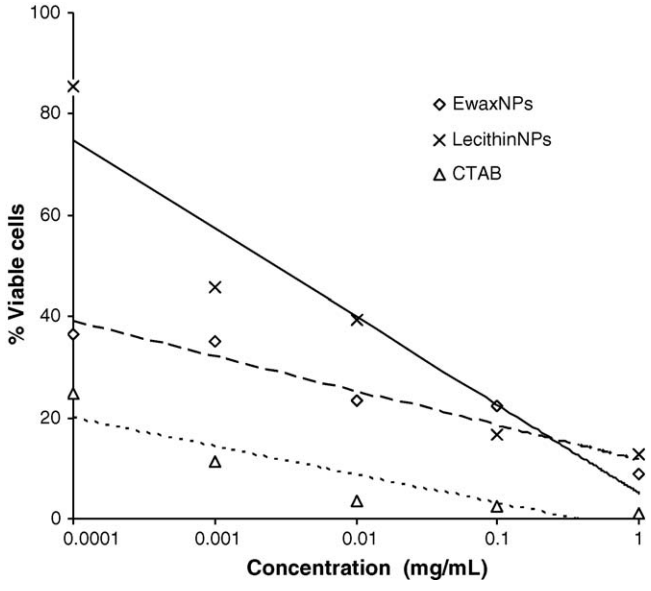

Fig. 5. Cytotoxicity of the lecithin nanoparticles. TC-1 cells (4×10^5) were incubated with different concentrations of CTAB, lecithin-based nanoparticles (lecithinNPs), or emulsifying wax-based nanoparticles (EwaxNPs) for 16 h at 37 °C with 5% of CO_2. The percentage of viable cells was estimated using an MTT assay. LecithinNPs and EwaxNPs were prepared with 4 mg of lecithin or emulsifying wax with a final CTAB concentration of 7.5 mM. The particles were GPC-purified, lyophilized, and reconstituted in sterile PBS prior to addition to the TC-1 cell culture. Data showed were the mean from 6 to 10 replicates.

Because the cationic lecithin nanoparticles were engineered as a potential DNA delivery system, the adsorption of a plasmid DNA on the surface of the nanoparticles was evaluated. As expected, when the GPC-purified lecithin nanoparticles were mixed with an equal volume of a plasmid DNA solution that contained increasing amount of DNA, the zeta potential of the resulted DNA-adsorbed nanoparticles decreased and changed from positive to negative, while the size of the resulting particles increased to a maximum when the zeta potential was about zero, and then decreased again (Fig. 6). The lecithin nanoparticles were positively charged; while the plasmid DNA was negatively charged. When an electro-neutral point was reached, their mixture may precipitate out. When the DNA concentration was 50 μg/mL, the zeta potential of the resulting DNA-adsorbed nanoparticles approached zero, and the size of the particles dramatically increased (Fig. 6). In fact, precipitation occurred at this DNA concentration. Thus, we can conclude that the electro-neutral point was reached when the DNA concentration was around 50–60 μg/mL. This agreed well with our calculated value. As shown in Fig. 4, the CTAB concentration in fraction number 4 was about 0.20 ± 0.03 mM. Thus, the concentration of the positively charged groups in this fraction should be around 0.2 mM (i.e., one positive charge per CTAB molecule). Also, it is known that the average molecular weight per base pair of DNA is 660, and that a base pair of DNA has two negative charges. Thus, a DNA concentration of 50–60 μg/mL corresponded to 0.15–0.18 mM of negative charges.

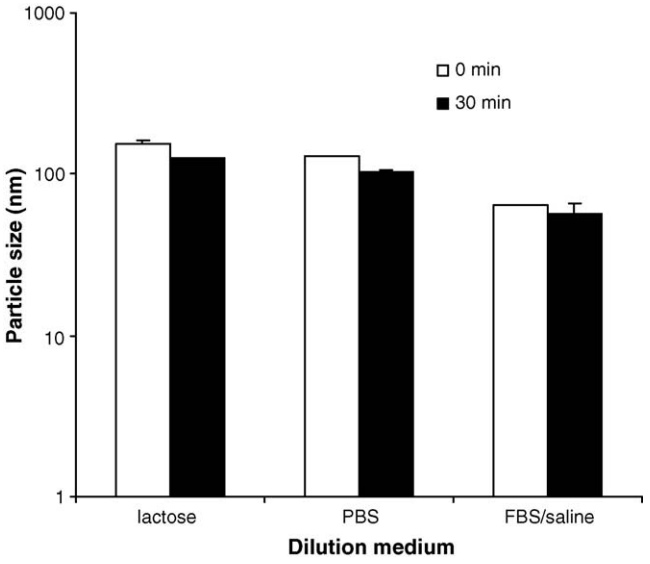

Fig. 7. The stability of the pNGVL-luc-adsorbed lecithin nanoparticles in lactose (10% w/v), PBS (10 mM, pH 7.4), or FBS (10%) in normal saline. The pNGVL-luc-adsorbed lecithin nanoparticles that contained 25 μg/mL of DNA were diluted (10-fold) into lactose solution, PBS, or FBS in saline. The particle sizes were measured immediately after the dilution and after 30 min of incubation at 37 °C. Data reported were mean ± S.D. ($n = 3$).

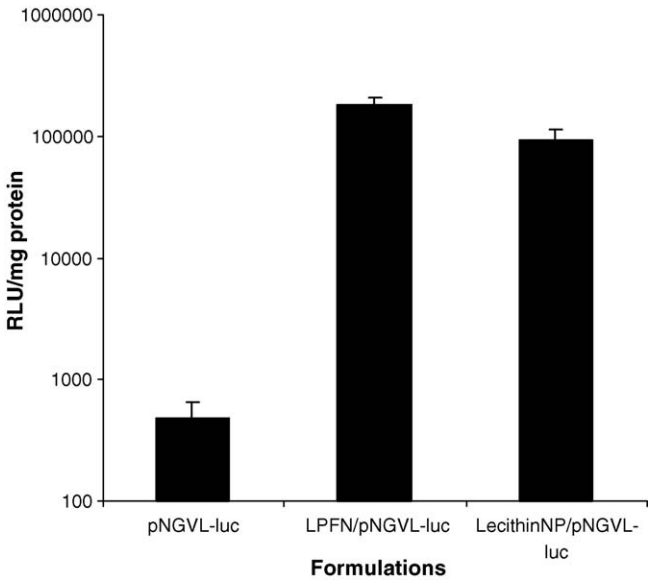

Fig. 8. In vitro transfection of 293 cells by the pNGVL-luc-adsorbed lecithin nanoparticles. Human 293 cells (5×10^5/well) were co-incubated with pNGVL-luc alone, pNGVL-luc-adsorbed lecithin nanoparticles, or pNGVL-luc complexed with Lipofectin® (LPFN) for 4 h. The amount of pNGVL-luc was maintained at 2.5 μg/well. The incubation medium was refreshed, and luciferase expression was measured 48 h later. The RLU values were normalized to the content of total proteins. Data reported were mean ± S.D. ($n = 3$). The values from LPFN–pNGVL-luc and LecithinNPs–pNGVL-luc were significantly different from each other ($p = 0.002$, t-test).

Fig. 7 demonstrated that the pNGVL-luc-adsorbed lecithin nanoparticles did not significantly aggregate when dispersed in media, such as 10% (w/v) lactose, PBS (10 mM, pH 7.4), or FBS (10% v/v) in normal saline. This is important because it is expected that we can disperse the nanoparticles into these solutions to maintain isotonicity. Also, the fact that the DNA-adsorbed nanoparticles did not extensively aggregate in the FBS/normal saline solution indicated that they will not aggregate when injected into hosts. Fig. 8 showed that the lecithin nanoparticles successfully helped to transfer the pNGVL-luc plasmid into the 293 cells for luciferase gene expression. Although the transfection efficiency of the lecithin nanoparticles was not as high as that of the commercial Lipofectin®, it was much higher than that of the pNGVL-luc plasmid alone (Fig. 8), presumably due to the enhanced uptake of the plasmid DNA via the endocytosis of the nanoparticles. Moreover, that fact that the pNGVL-luc-adsorbed lecithin nanoparticles were net positive charged (Fig. 6) may also help to explain their enhanced transfection efficiency because cell surface is generally negatively charged. Finally, the observation that the pNGVL-luc-adsorbed lecithin nanoparticles did not perform as well as the commercially available Lipofectin® does not mean that the lecithin nanoparticles will not be a good DNA delivery system in vivo. More experiments will have to be carried out in the future to evaluate the in vivo performance of the lecithin-based nanoparticles as a DNA delivery system.

In conclusion, we have significantly improved the stability and safety of our previously reported nanoparticle-based DNA delivery system by replacing the emulsifying wax component of the nanoparticles with lecithin. When plasmid DNA was adsorbed onto the surface of the lecithin nanoparticles, the nanoparticles successfully transfected cells in culture. These lecithin-based nanoparticles should have an improved potential for DNA vaccine delivery.

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