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Development of nanosomes using high-pressure homogenization for gene therapy

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Abstract

Objectives The aim of this project was to develop a novel lipid-based formulation suitable for gene therapy.

Methods Novel nanosize liposome (nanosome) formulations containing pDNA (plasmid DNA) were developed using high-pressure homogenization (HPH). The effect of lipid concentration was studied at two levels: 3 mM and 20 mM. The preformed nanosomes were incubated for 18–20 h with pDNA or pDNA/protamine sulfate (PS) complex. The physical properties of the pDNA nanosomes were compared by particle size distribution and zeta-potential measurements. Their biological properties were also compared by pDNA efficiency of encapsulation/complexation, integrity, nuclease digestion, transfection efficiency and cell cytotoxicity.

Key findings pDNA nanosomes prepared with 20 mM lipid (nanosomes : pDNA : PS at a ratio of 8.6 : 1 : 2) had particle sizes of 170–422 nm (90% confidence). The zeta-potential of the formulation was 49.2 ± 1.5 mV, and the pDNA encapsulation/complexation efficiency was ~98%. pDNA nanosomes prepared with 3 mM lipid (nanosomes : pDNA : PS at a ratio of 2.09 : 1 : 2) had particle sizes of 140–263 nm (90% confidence). The zeta-potential of this formulation was 36.4 ± 1.2 mV, and the pDNA encapsulation/complexation efficiency was ~100%. However, a comparison of the efficiency of transfection indicated that pDNA nanosomes prepared with low-concentration lipids (3 mM) showed significantly higher transfection efficiency compared with the pDNA nanosomes prepared with high-concentration lipids (20 mM), as well as those prepared with Fugene-6 (a commercially available transfection reagent). This particular formulation (pDNA nanosomes, 3 mM lipids) also showed significantly less cytotoxicity compared with the other pDNA nanosome formulations.

Conclusions To conclude, these results indicate that condensing pDNA with PS followed by subsequent complexation with low-concentration nanosomes generated from HPH can produce a pDNA nanosome formulation that will boost transfection efficiency, while minimizing cytotoxicity. This new technology appears to be an efficient tool for future commercial or large-scale manufacture of DNA delivery systems for gene therapy.

Keywords high pressure homogenizer; nanoparticles; nanosomes; pDNA delivery; transfection

Introduction

Gene therapy has led to significant advances in the treatment of infectious disease, viral disease and cancer.[1,2] Delivery of genes into the target cells or organs by escaping the endogenous nuclease digestion is important as it maintains the functional integrity. A number of techniques for DNA delivery have been attempted, such as electroporation,[3] viral genomes,[4,5] ballistic gold particles,[6] liposomal and polymeric nanoparticles and even direct injection of naked DNA.[7] Viral vectors have been observed to be highly efficient, but they are also associated with high toxicity[8] and immunogenicity.[9] These limitations of using viral vectors for effective DNA delivery led to the development of nonviral vectors, such as liposomal (lipid vesicles)[10,11] and polymeric delivery vehicles.[12,13] Liposomal delivery vehicles were preferred for decades because of their safety, non-immunogenicity, comparatively easy assembly and commercial large-scale production capability.[14,15]

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Extrusion and sonication are two techniques that have been widely used for liposome preparation.[16–19] While extrusion produces fairly reproducible vesicle size, as well as size distribution from batch to batch, it is not a practical approach for commercial large-scale production. Moreover, generating particle sizes less than 100 nm by using the extrusion technique is time consuming, laborious and inconvenient for commercial production. On the other hand, sonication allows generation of small particles less than 100 nm, but the generated liposomes vary in the mean diameter and size distribution from batch to batch. Therefore, development of new technology that will generate lipid-based nanosomal formulations, which have been defined as 'nanosomes' because of their nanometer size range, and which are fairly reproducible in mean diameter and size distribution, has a potential for commercial large-scale application. High-pressure homogenization (HPH) is one such technique that produces smaller nanosomes (20–50 nm) with a narrow size distribution.[20,21] Most of the nanosomes produced by HPH are small unilamellar vesicles (SUVs) that are less rapidly removed from the bloodstream and thus are advantageous for sustained delivery of drugs or genes. Because of this unique characteristic of producing small, as well as uniform, nanosome particles,[22,23] HPH will have an enormous effect in formulating non-viral delivery vehicles for future gene therapy application.

For efficient nanosomal gene delivery, physiochemical properties such as high +/– charge ratio between a cationic formulation and the entrapped DNA has proven to be important for intravenous delivery.[24] Compositional aspects, such as inclusion of cholesterol in the formulation, have been shown to improve the transfection efficiency, whereas the inclusion of some other lipids, such as dioleoylphosphatidylethanolamine (DOPE), significantly decreases its activity.[25,26] Therefore, in addition to the size and uniformity of a nanosomal formulation, the composition of the nanosome itself, as well as its overall charge density after entrapping DNA, also plays a significant role in the overall transfection efficiency. In this context, inclusion of cationic peptides, such as protamine sulfate (PS),[27] in the formulation has been reported to help reduce particle size, increase positive zeta-potential of the particles and enhance overall gene transfection capacity.[28] Since cationic lipids and cationic peptides both confer a positive charge to the particles, it is important to determine the optimum combination of peptides and lipids for effective cell transfection. Cationic agents also have been reported to be toxic to cells,[29] thus a thorough study is required to identify the optimum combination of peptides, lipids and DNA that will promote efficient gene delivery while minimizing cell toxicity.

The goal of this project is to produce novel pDNA encapsulated nanosomal formulations by HPH that will overcome the current limitations of extrusion and sonication methods. We hypothesize that the pDNA nanosomes (i.e. pDNA encapsulated nanosomes) will maintain their smaller size as well as their effective gene transfection capabilities, without generating toxicity to the cells. For our experiment, we chose two different concentrations of nanosomes (blank liposomal particles). In two of the four formulations, the pDNA was precondensed with PS before encapsulating into nanosomes. Different pDNA nanosomes were prepared by changing their composition, following which a rigorous study was carried out with respect to both physiochemical characterization and biological potency of those formulations. Our experimental results demonstrate that condensing pDNA with PS followed by subsequent complexation with low-concentration nanosomes prepared by HPH can produce a novel nanosome formulation that boosts transfection efficiency while minimizing cytotoxicity.

Materials and Methods

Materials

1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) and cholesterol were purchased from Avanti Polar-lipids Inc. (Birmingham, USA). Protamine sulfate salt Grade X, trehalose dihydrate and HPLC-grade chloroform were obtained from Sigma Chemical Co. (St Louis, USA). The Picogreen assay kit was supplied by Molecular Probes (Eugene, USA).

The luciferase assay kit and reporter lysis buffer were purchased from Promega (Madison, USA). The pDNA (pEGFP-Luc, 6.4 kb) encoding both enhanced green fluorescent protein (GFP) and luciferase marker gene was obtained from Clontech Laboratories, Inc. (Mountain View, USA). Fugene-6, fetal bovine serum albumin (BSA), Dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin antibiotics were purchased from Gibco, Invitrogen Corp. (Carlsbad, USA). All other reagents were of analytical grade and supplied by Sigma Chemical Co. (St Louis, USA).

Preparation of nanosomes

The nanosomes were prepared using an EmulsiFlex-B3 high-pressure homogenizer (Avestin Inc., Ottawa, Canada).[20,30] Briefly, the nanosomes were prepared from a mixture of two lipids, cholesterol and DOTAP, at a molar ratio of 1 : 1. The effect of the lipid amount was studied at two levels: 2.09 and 8.6 μg, respectively (Table 1). The effect of PS was also studied at each of these two lipid levels. The lipids (containing 10.47 mg DOTAP and 5.81 mg cholesterol for B1 and B2; and 50 mg DOTAP and 26.7 mg cholesterol for B3 and B4) were dissolved in 10 ml and 20 ml HPLC-grade chloroform, respectively, in a round bottom flask and then dried under nitrogen gas and vacuum overnight. The resulting films of the lipids were hydrated in de-ionized water to give a final concentration of 3 mM and 20 mM, respectively. The lipid dispersions were warmed and mixed by rotation at 50°C for 45 min, and warmed again at 35°C for another 10 min. The resultant dispersion was stored at room temperature for 3 h before it was transferred into a scintillation vial and warmed again at 50°C for 10 min. The final lipid dispersion was homogenized by using HPH at 20 000 PSI for five cycles. Each time, 2 ml of lipid dispersion was subjected to homogenization and the resultant nanosomes (i.e. nanometer-size lipid vesicles) were collected in another scintillation vial. The nanosomes were kept at room temperature for 1 h before overnight storage at 4°C.

Preparation of pDNA-loaded nanosomes

The pDNA-loaded nanosomes were prepared by using two different concentrations of nanosomes (3 mM and 20 mM), PS

Table 1 Composition, particle size and zeta-potential of different pDNA-entrapped nanosome formulations

Nanosomes	DOTAP : cholesterol (1 : 1 molar ratio)	PS	Particle size (nm)			Zeta-potential (mV)
			50%	70%	90%	
B1	2.09 μg		209 ± 13	285 ± 28	451 ± 67	–29.4 ± 4.0
B2	2.09 μg	2 μg	140 ± 17	181 ± 13	263 ± 14	36.4 ± 1.2
B3	8.60 μg		191 ± 11	291 ± 15	520 ± 62	42.0 ± 2.0
B4	8.60 μg	2 μg	171 ± 35	250 ± 59	422 ± 129	49.2 ± 1.5

PS, protamine sulfate. All pDNA nanosomes (B1–B4) contained pDNA (pEGFP-Luc) 1 μg and trehalose 10 μg. The particle size was reported as the mean ± SD, $n = 4$. The zeta-potential data represents mean ± SD of five experiments.

and pDNA. The composition of the different formulations is listed in Table 1. Briefly, freshly prepared PS solution in de-ionized water was added drop-wise to 52 μl aqueous solution of pDNA (70 μg) while vortexing the solution at a moderate speed. To study the effect of condensation of pDNA with PS, two batches of pDNA nanosomes (B2 and B4) were prepared using PS. The condensation of pDNA with PS was performed by incubation of the mixture for 40 min at room temperature. Following the pDNA–protamine condensation, the pre-warmed nanosomes were added to the mixture. The final preparations were mixed rapidly by pipetting up and down 30 times. Two batches of pDNA nanosomes (B1 and B3) were also prepared without PS, in which the pre-warmed nanosomes were added to the pDNA solution and mixed in a similar fashion. The resultant pDNA nanosomes were then stored at 4°C overnight. Finally, freshly prepared trehalose solution was added to the pDNA nanosomes followed by vortexing twice to ensure thorough mixing of the formulations with trehalose. The resultant mixtures were stored at 4°C. Before experimental use, the pDNA nanosomes were always sonicated in an ice-cold water bath for 5 min.

Measurement of particle size and zeta-potential

The mean particle size of pDNA nanosomes was determined by the dynamic laser light scattering method at room temperature using a Delsa Nano C Particle Analyzer (Beckman Coulter Inc., Fullerton, USA). For each measurement, 100 μl of pDNA nansomes was diluted up to 1 ml using de-ionized water. The resultant mixture was sonicated for 5 min in a bath sonicator filled with ice-cold water. The surface charge of the particles was measured by examining the zeta-potential with a Malvern Zetasizer 2000 (Malvern Instruments, Malvern, UK). The system was initially calibrated with standards and all samples were measured five times.

Measurement of pDNA encapsulation efficiency

The amount of pDNA, either complexed or encapsulated, was determined for each nanosome concentration studied. The efficiency of encapsulation was calculated by comparing the total amount of pDNA measured in the sample with the actual amount of pDNA added to the sample. Briefly, the pDNA nanosomes were centrifuged at 10 000 rev/min (Allegra Centrifuge; Beckman Coulter Inc., Fullerton, USA) for 15 min at 4°C. Supernatants containing the free pDNA were separated from the pellets. Five-hundred microlitres of 1% sodium dodecyl sulfate (SDS) was added to the pellets and to the supernatants. Samples were then incubated at 37°C for 18 h with gentle agitation (50 rev/min). The amount of pDNA from both supernatants and pellets was measured by using a Pico green assay following the manufacturer's protocol. Blank particles for every batch were also prepared without pDNA, and their fluorescence reading was subtracted from that of the sample batch to get the actual amount of pDNA present in both the free and encapsulated states. The results were reported as the mean ± standard deviation ($n = 3$).

DNase I treatment of nanosomes

The extent of integration of pDNA into nanosomes was determined by DNase I digestion assay (1 U of DNase I/μg of DNA). Briefly, different batches of pDNA nanosomes (150 μl) were freshly prepared by using 35 μg of pDNA and stored overnight at 4°C. The encapsulated pDNA particles were then separated from free unencapsulated pDNA by centrifugation at 10 000 rev/min for 15 min at 4°C. Following careful removal of the supernatants from the pellets, 150 μl of de-ionized water was added to the pellets and the pellet suspensions were incubated with 15 μl DNase buffer (10×). This was followed by addition of 35 μl DNase I (Invitrogen Corp., Carlsbad, USA). The samples were incubated at 37°C for 30 min, following which the reaction was terminated by using EDTA (0.5 M). Finally, pDNA was extracted from PS in these formulations by using 1% SDS in a manner similar to the procedure outlined in the previous section.

Agarose-gel electrophoresis

The structural integrity of pDNA that was complexed/encapsulated into pDNA nanosomes was assessed by agarose gel electrophoresis. In brief, after extracting pDNA from different batches using 500 μl of 1% SDS, the solution was mixed with an equal volume of chloroform and then vortexed for 1 min using the Vortex Genie Mixer (Scientific Industries Inc., Bohemia, USA) and stored at room temperature for an additional 30 min. The resultant mixture was centrifuged at 12 000 rev/min for 30 min and the aqueous layer was collected. The pDNA present in the aqueous layer was precipitated with a mixture of absolute ethanol (200 Proof) and 3 M sodium acetate. The precipitated pDNA was collected by centrifugation at 12 000 rev/min for 30 min at room temperature. Finally, the precipitated pDNA was purified by washing with 70% ethanol. An equal amount of pDNA (0.75 μg) was run on a 0.8% agarose gel for 50 min at 114 V and the pDNA band picture was captured using a digital camera.

In-vitro pDNA release study

For each formulation, 240 μl samples containing 70 μg pDNA were taken into 1.5-ml Eppendorf tubes and diluted to make 1 ml with phosphate buffer (pH 6.8). The tubes were then placed in a shaking water bath (50 rev/min) at 37°C. After each selected time period, the samples were centrifuged at 10 000 rev/min for 15 min. The supernatants were collected and replaced with 1 ml fresh phosphate buffer. The collected supernatants were mixed with 1% SDS to decomplex pDNA. The pDNA present in each sample was determined using the Pico green assay method.

Routine cell culture

The highly metastatic murine breast cancer 4T1 cells were obtained from Dr Fred Miller (Karmanos Cancer Institute, Detroit, USA). The cells were routinely cultured and expanded in high-glucose DMEM supplemented with 5% fetal bovine serum (FBS), non-essential amino acids, sodium pyruvate, 100 U/ml penicillin and 0.1 mg/ml streptomycin.

Cytotoxicity assay

The toxicity of the pDNA nanosomes to 4T1 cells was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay following the manufacturer's protocol (Sigma Chemical Co., St Louis, USA). Briefly, 4T1 cells (1×10^4) were cultured on 24-well tissue culture plates (TCP) in 5% FBS containing DMEM media for 24 h. The DMEM media was then replaced with fresh 2% FBS media. Cells were transfected with either 4 μg pDNA-loaded nanosomes or the same amount of pDNA loaded Fugene-6 for another 48 h. Cell viability was assessed by measuring the absorbance at 570 nm, and expressed as the ratio of the Abs_{570} of cells treated with pDNA entrapped nanosomes or Fugene-6 over the control (i.e. untreated cells).

In-vitro transfection study

In-vitro transfection efficiency of different formulations to 4T1 cells was performed by measuring both luciferase activity and GFP expression. For the luciferase activity assay, 1 and 4 μg pDNA containing nanosomes and Fugene-6 were used to transfect cells. Briefly, 4T1 cells were grown in 24-well plates in 5% FBS containing DMEM medium for 24 h. The DMEM medium was replaced with 2% serum medium before transfection. For Fugene-6 treatment, 3 μl of Fugene-6 per μg of pDNA was used, and the experiments were carried out according to the manufacturer's instructions. Cell culture was continued for another 48 h before harvesting them with 100 μl reporter lysis buffer. Ten microlitres of each sample was analysed for luciferase expression using the Promega luciferase assay system on a luminometer (Luman LB9507; EG&G Berthold, Berlin, Germany). For normalization purposes, the amount of protein present in each sample was measured by Bio-Rad protein assay kit (Hercules, USA) using equal amount of cell lysates.

To determine GFP expression, 4T1 cells were similarly cultured and transfected with 1 and 4 μg pDNA-loaded nanosomes and Fugene-6 for 48 h. GFP expression was monitored by using a fluorescence microscope (Olympus America, Inc., New York, USA) and photographs were taken at 10× magnification.

Statistical analysis

All statistical analyses were performed using Prism software package (GraphPad Software Inc., La Jolla, USA). Results were expressed as mean ± standard deviation. Statistical significance between groups with respect to luciferase activities in transfection studies was compared using one-way analysis of variance followed by Tukey's multiple comparison test. $P < 0.05$ was considered as evidence of a significant difference.

Results and Discussion

Physiochemical characterization of nanosomes prepared by high-pressure homogenization

In this study, the mean vesicle size of the low-concentration nanosomes (3 mM) and high-concentration nanosomes (20 mM) was found to be 117 ± 13 nm (90%) and 170 ± 20 nm (90%), respectively. The zeta-potential was also measured and it was noted that high-concentration nanosomes (20 mM) showed a higher charge density (68.0 ± 3 mV) than the low-concentration nanosomes (49.0 ± 2 mV). This difference in zeta-potential was due to the presence of a comparatively higher amount of DOTAP in the 20 mM nanosomes. Transmission electron microscope observation showed that the vesicles in both nanosomes (i.e. 3 mM and 20 mM) are discrete and spherical (data not shown).

Physiochemical characterization of different pDNA nanosome formulations

Particle size and zeta-potential: effect of lipid concentration and protamine sulfate

Four different pDNA nanosomes were prepared from these two sets of nanosomes using pDNA that was condensed with or without PS. The effects of lipid content and the presence or absence of PS on the particle size and zeta-potential of these formulations have been measured (Table 1). At low lipid content (3 mM), the size of B1 (devoid of PS) was 451 ± 67 nm (90%), whereas addition of PS (B2) decreased the particle size to 263 ± 14 nm (90%). Similarly, when a high concentration of lipid (20 mM) was used, the particle size of B3 (without PS) was 520 ± 62 nm (90%), whereas with PS (B4) it was 422 ± 129 nm (90%). In the overall comparison, it was shown that condensing pDNA with PS decreased the particle size in both B2 and B4 compared with their counterparts (without PS, i.e. B1 and B3). Since a relatively higher lipid amount was used in B4 compared with B2, some lipid in B4 may remain unoccupied by the pDNA. This excess lipid was assumed to fuse together with a tendency towards making larger particles. The difference in lipid contents between B2 and B4 also resulted in a significant difference ($P < 0.05$) in overall zeta-potential. B1 and B2 showed an average zeta-potential of –29.4 ± 4 and 36.4 ± 1.2 mV, respectively, whereas B3 and B4 showed overall zeta-potentials of 42.0 ± 2 and 49.2 ± 1.5 mV, respectively (Table 1). Due to the presence of a low amount of cationic lipid DOTAP, B1 rendered an overall negative zeta-potential (–29.4 ± 4 mV) whereas

the increased amount of DOTAP in B3 led to a positive zeta-potential (42.0 ± 2) as the total amount of anionic pDNA was same in both formulations. Similarly, addition of cationic peptide (PS) in B2 rendered a positive zeta-potential (36.4 ± 1.2 mV), though their lipid contents (B1 vs B2) were the same. On the other hand, addition of PS to the high-concentration pDNA nanosome (B3) increased the zeta-potential from 42.0 ± 2 mV (B3) to 49.2 ± 1.5 mV (B4). Therefore, the high lipid contents (i.e. cationic lipid) and the simultaneous inclusion of cationic peptide (PS) increased the overall zeta-potential of these formulations. The average zeta-potential of cationic formulations in the range of +20 to +25 mV has been reported to increase gene transfection efficiency and overall in-vivo gene delivery.[28,31] These results indicate that among the experimental formulations studied, B2 has been demonstrated to produce smaller particles and also maintain a zeta-potential close to the range that will be effective for better gene delivery applications.

Efficiency of encapsulation/complexation of pDNA into pDNA nanosomes

The pDNA encapsulation efficiency was measured by separating free pDNA from encapsulated particles (Figure 1). The experimental formulation B1 was observed to entrap modest levels of pDNA (54.6 ± 1.5%), while leaving a large quantity of free unbound pDNA (47.6 ± 1.7%) in the solution. B2 showed 104 ± 2.6% encapsulation efficiency. A similar level of pDNA encapsulation efficiency was also observed in B4 (98.1 ± 1.1%). In B3, the pDNA encapsulation efficiency was 57.0 ± 3.5% and the free unencapsulated pDNA was 45.2 ± 1.2%. Increasing the amount of lipid itself does not cause any significant changes in the pDNA encapsulation efficiency, as can be inferred by comparing B3 to B1. It is possible that the amount of pDNA that was used in B1 and B3 probably attained a saturation state with the lipid contents at some stage and the addition of more lipids barely influenced their encapsulation efficiency. However, when pDNA was precondensed with PS in B4, the pDNA encapsulation efficiency increased to 98.1 ± 1.1%. These observations showed that condensing pDNA with PS increased the efficiency of encapsulation whereas an increase in the amount of lipid was unlikely to improve the efficiency of encapsulation.

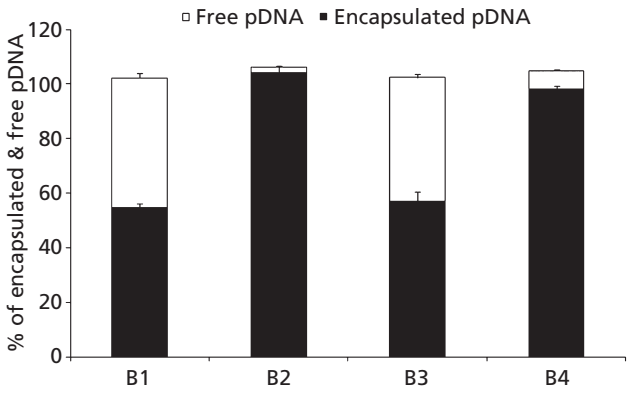

Figure 1 Efficiency of encapsulation/complexation of pDNA in nanosome formulations. Both free and encapsulated/complexed pDNA were exposed to 1% SDS and the decomplexed pDNA was then measured by Pico green assay. The results represent mean ± SD, $n = 3$.

Integrity of pDNA entrapped into pDNA nanosomes

During the encapsulation of pDNA into nanosomes, the pDNA is exposed to shear forces that may adversely affect its integrity. The effect of the inclusion of PS and varying the lipid content on pDNA integrity was examined. Generally, pDNA maintains three separate isoforms: super coiled isoform, linear isoform and the hinge region of pDNA, which can be differentiated via agarose gel electrophoresis. It is believed that due to shear stress, the super coiled isoform, which is primarily involved in biological activity (i.e. transfection), is broken and rearranged to form more linearized plasmids that show less transfection capability.[20] Therefore in this experiment we studied the relative abundance of the super coiled isoforms with respect to other isoforms in different formulations. The results obtained from agarose gel electrophoresis (Figure 2) indicate that all of the pDNA was released from B1 and B3. Compared with the naked pDNA, it was noted that both formulations retained a majority of the pDNA in the super coiled isoforms (Figure 2; left lanes). The amount of the super coiled isoform is higher in B3 than in B1 (visual assessment). However, both formulations also produced a neck DNA band immediately following the super coiled DNA band. This neck DNA formation is an undesirable outcome, as it potentially lowers the gene transfection capability. Further optimization of these formulations and the encapsulation method are therefore required.

In the case of B2 and B4, only a minute amount of pDNA was released from those formulations. Most of the condensed pDNA remained entrapped within the nanosomes. The low release of pDNA is explained as follows. It is postulated that SDS, through its anionic nature, can disrupt any nanosomal surface-bound pDNA as well as any loosely condensed pDNA, but it is unable to disrupt the actual nanosome membrane. As a result, the encapsulated pDNA within the nanosomes was not exposed to SDS and thus not decomplexed. Consequently, only the released pDNA would migrate and separate during agarose gel electrophoresis, but the encapsulated pDNA within the nanosomes that was not exposed to SDS would remain at the site of application.[32] Nevertheless, the results clearly showed that both B2 and B4 maintained super coiled DNA isoforms during the preparation. Most importantly these two formulations did not produce any neck DNA as was observed in the B1 and B3 formulations. Hence, the results from this experiment clearly indicate that precondensing pDNA with PS successfully maintained the integrity of pDNA. This experiment also indicates that most of the pDNA in B2 and B4 remained encapsulated within the nanosomes, whereas in B1 and B3, which are devoid of PS, the pDNA probably bound loosely to the nanosome surface.

Protection of pDNA in the formulations from nuclease degradation

The ability of pDNA nanosomes to work efficiently *in vivo* depends on their ability to protect pDNA from endonuclease

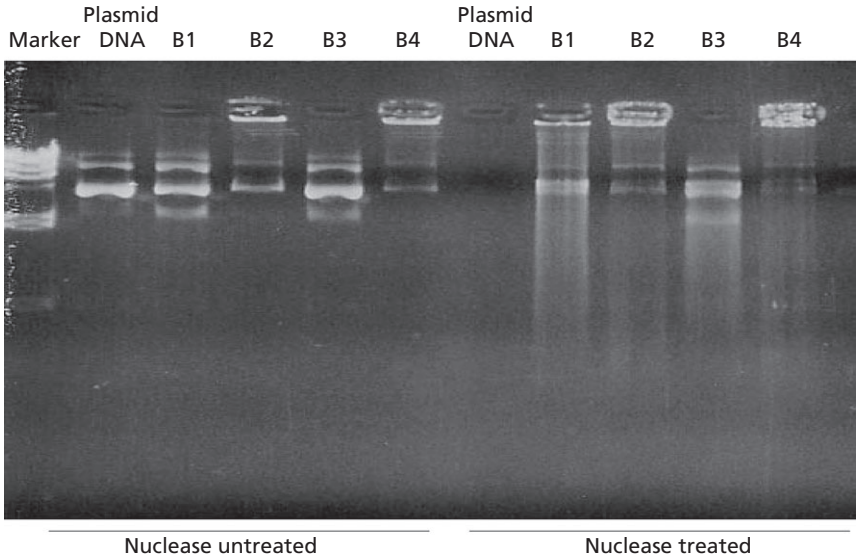

Figure 2 Measurement of pDNA integrity during pDNA nanosome formulation and post-nuclease digestion. Integrity of pDNA in the formulation without nuclease treatment (left lane), and post-nuclease treatment (right lane). The samples were incubated with DNase I (1 U/μg pDNA) at 37°C for 30 min, followed by extraction of pDNA with 1% SDS. pDNA (0.75 μg) was loaded into each well and separated by gel electrophoresis (0.8% gel).

digestion. The nuclease stability of pDNA in different formulations was studied (Figure 2; right lanes). As shown by the experimental results, the naked pDNA that was not complexed with nanosomes is completely digested and disappeared from the gel. B1 and B3, which were devoid of PS, guaranteed only a partial protection of the pDNA against nuclease digestion and a smear of DNA bands was observed in both formulations in the agarose gel. In the case of B2 and B4, most of the pDNA was not separated and remained on the spot point. Even the small amount of pDNA that was separated from B2 and B4 remained intact and was not broken by nuclease digestion. Therefore, these two formulations were successfully able to protect the encapsulated pDNA from nuclease digestion and maintain their structural integrity. Thus, protection of pDNA from endonuclease digestion by condensing with PS and encapsulating into nanosomes might help to improve the stability of pDNA.

In-vitro release of pDNA from encapsulated nanosomes

Figure 3 shows the dissolution profiles of pDNA from four different pDNA nanosomes. The results show that B1 released the maximum amount of pDNA, with 50% released in seven days (168 h). There was a significant ($P < 0.05$) decrease in pDNA release from B3 due to an increase in lipid content. Only 18% pDNA was released in seven days from B3. These results clearly indicate that an increase in lipid content in the formulation reduced the release of pDNA. The release of pDNA from the remaining two formulations, B2 and B4, was also examined. In both formulations, the release of pDNA within seven days was very low (~10%). The decrease in the release of pDNA from these two formulations was due to the high electrostatic interactions between the negatively charged pDNA and positively charged PS.

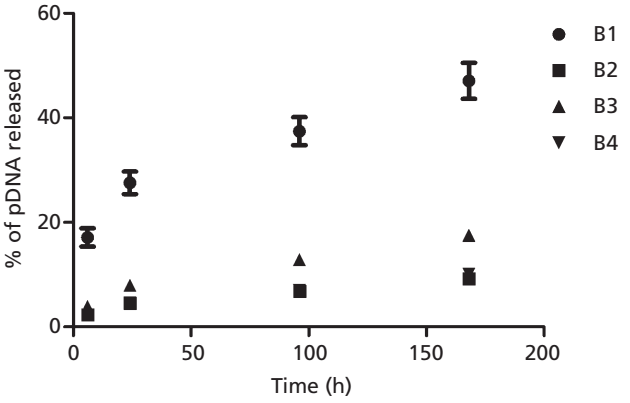

Figure 3 In-vitro release of pDNA at pH 6.8 from different nanosome formulations. The results are expressed as percentage of pDNA released from pDNA nanosomes over time ($n = 3$).

Cell viability of 4T1 cells transfected with different formulations

Since cationic nanoparticles carry a net positive charge, they can bind to the negatively charged cell membrane. Depending on the intensity of the net positive charge on the nanoparticles, their interactions with the cell membrane and proteins in the serum can vary. Fischer *et al*. have reported that cationic particles can induce significant cellular toxicity by interacting with the negatively charged cell membrane.[29] In this context, since the different pDNA nanosomes in our study have different surface charges, their influence on cell viability needed to be evaluated. The MTT assay indicated that the cell viability for B1 and B2 transfection was between 80% and 90% (Figure 4). However, in the case of Fugene-6 transfected cells,

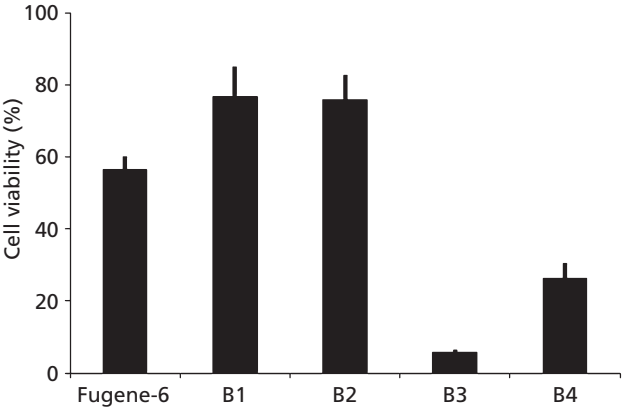

Figure 4 Cell viability of 4T1 cells transfected with 4 μg pDNA-loaded nanosomes, and with Fugene-6. The percentage of viable cells was compared with the untreated cells during 48 h post-transfection. The results represent mean ± SD, $n = 3$.

the cell viability was reduced to 50–60%. In case of B3, cell viability was observed to be less than 15%, whereas cell viability for B4 transfection was around 30–40%. The high amount of lipid present in B3 and B4 probably played a role in inducing toxicity to those cells. The excess amount of free lipid that is available following encapsulation with pDNA probably binds and penetrates into the cells, thereby generating toxicity. In general, the high lipid content in B3 and B4 made them significantly more toxic than the low-lipid-containing B1 and B2 formulations.

In-vitro transfection studies

4T1 cells were used to evaluate the transfection capability of the experimental formulations. The transfection efficiency was monitored by fluorescence imaging of GFP expression (Figure 5) as well as by luciferase activity assay (Figure 6). The transfection efficiency was also compared by transfecting cells with two different amounts of pDNA loading (i.e. 1 and 4 μg). Figure 5 shows that B2 produced the highest level of GFP expression when the cells were transfected with 4 μg pDNA. The level of GFP expression by B2 transfection was reduced when 1 μg pDNA was used. When the cells were transfected with B4 containing 1 μg pDNA, the GFP expression was higher (visual observation) than with B2 at the same pDNA loading. However, when 4 μg pDNA was used, the GFP expression of B4 was significantly reduced. The pDNA nanosomes in B1 or B3 formulations barely showed any GFP expression regardless of the doses (i.e. 1 or 4 μg pDNA). The transfection results were also compared with Fugene-6, a commercial DNA transfection reagent. When the cells were transfected with 1 μg pDNA-loaded Fugene-6, the level of GFP expression was low, but when the cells were transfected with 4 μg pDNA-loaded Fugene-6, the level of GFP expression increased. However, the expression of GFP was significantly lower than that of B2 at the same pDNA loading (i.e. 4 μg pDNA).

To confirm these observations, we also evaluated the luciferase activity of 4T1 cells transfected with different pDNA nanosomes, as well as with Fugene-6. The results shown in Figure 6 indicate that the luciferase activity for B2 transfection was significantly higher than that of Fugene-6 for both pDNA amounts (i.e. 1 and 4 μg pDNA) ($P < 0.0001$). Similarly, when the cells were transfected with B4 containing 1 μg pDNA, the luciferase activity was also significantly higher than that of Fugene-6 at the same pDNA loading ($P < 0.0001$). However, when 4 μg pDNA was used, the transfection efficiency of B4 was drastically reduced. Since the lipid content in B4 proportionately increased from 1 μg to 4 μg pDNA loading, these excess lipids resulted in localized toxicity to the cells. However, when the cells were transfected with B1, the luciferase activity was barely noticeable. For B3 transfection, higher luciferase activity compared with Fugene-6 was observed at 1 μg pDNA loading. However, when 4 μg pDNA was loaded, the luciferase activity was drastically reduced.

It has been reported that the use of natural cationic peptides such as PS can improve both in-vitro and in-vivo gene transfection efficiency of both liposomal and polymeric nanoparticles.[27,28,31] It is also known that cell membranes are negatively charged (e.g. the zeta-potential of Kupffer cells in liver has a negative charge density of approximately –4 mV). Therefore, it is expected that positively charged nanosomes entrapping pDNA will exhibit increased adhesion to the cell surfaces, followed by sequential engulfment by the cells.[33] In this context, an interesting study by Aoki *et al.* has shown that cationic liposomes with suitable positive charges ranging from +15 mV to +25 mV can circulate in the blood and escape the reticuloendothelial system,[31] and can deliver the genetic materials into tissues. In our study, both cationic lipids (i.e. DOTAP) and cationic peptides (i.e. PS) provide positive charge to the particles. However, determination of the optimum ratio of lipids and peptides to pDNA is critical in designing a novel gene delivery system. Both B3 and B4 containing high-concentration nanosomes generated by HPH were shown to have large particle size and high surface charges. The high lipid content and high surface charge of these formulations were found to adversely affect cell viability as well as transfection capability. The B4 formulation had higher zeta-potential and higher gene transfection capability than B3. Thus, it appears that the comparatively lower particle size and significantly higher pDNA encapsulation efficiency of B4 (vs B3) were instrumental in achieving the higher gene transfection efficiency of the B4 formulation. The higher encapsulation efficiency ensured that high amounts of pDNA were available for transfection.

Using low-concentration nanosomes generated by HPH, the experimental B2 formulation attained a zeta-potential (+36.4 ± 1.2 mV) that is close to the range of zeta-potentials preferred for optimum gene delivery and gene transfection. B2 also showed significantly higher cell viability and transfection than B4, even though the same amount of PS was used for pDNA encapsulation in both formulations. The low-concentration nanosomes in B2 helped in achieving significantly lower particle size, least toxicity and significantly higher gene transfection. Therefore, by maintaining a favourable positive zeta-potential coupled with smaller particle size, B2 not only showed significant gene transfection efficiency compared with Fugene-6 and B4, but also showed less toxicity to the cells.

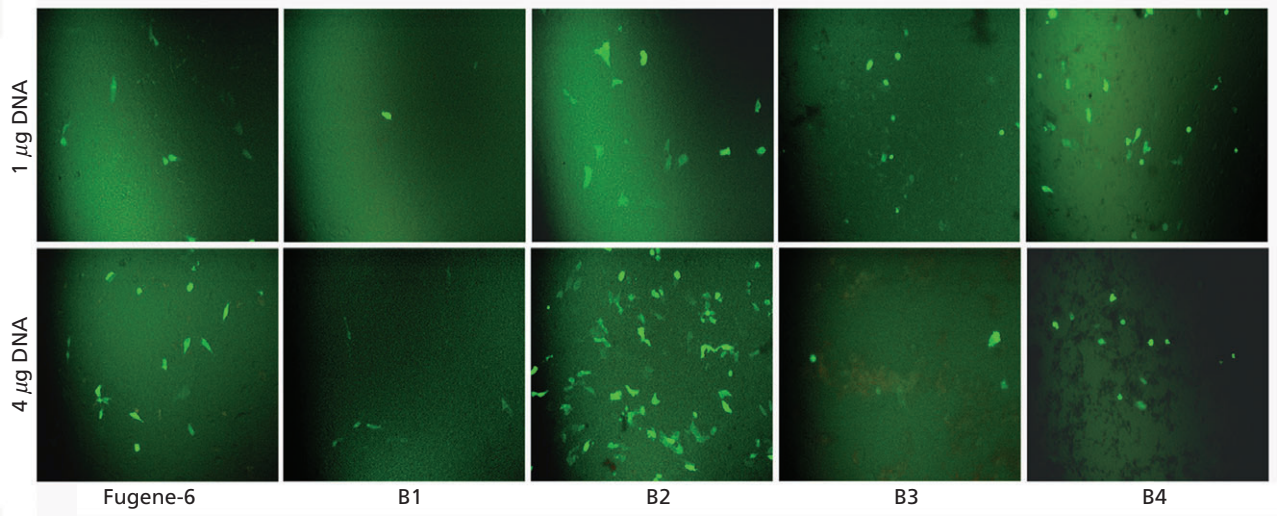

Figure 5 In-vitro transfection studies. 4T1 cells were transfected with different pDNA nanosomes (loaded with 1 and 4 μg pDNA) and with Fugene-6, and fluorescence photographs of green fluorescent protein expression were taken using a fluorescence microscope at 10× magnification.

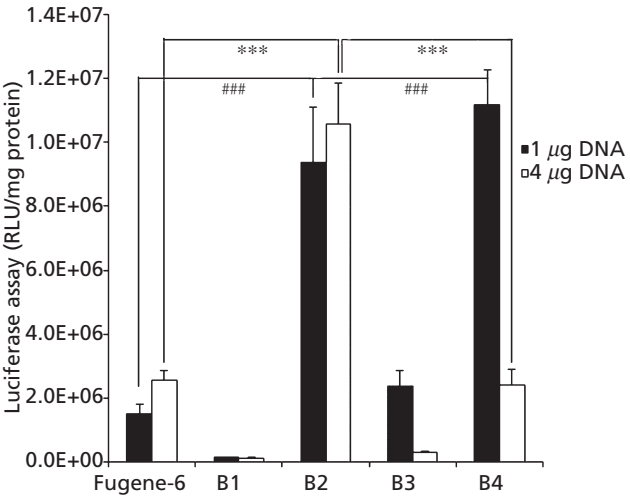

Figure 6 Luciferase activity of different pDNA nanosomes and Fugene-6 transfection normalized to total protein. $^{###}P < 0.0001$, luciferase activity of B2 and B4 batches with respect to Fugene-6 during 1 μg pDNA transfection; $^{***}P < 0.0001$, luciferase activity of B2 with respect to both Fugene-6 and B4 during 4 μg pDNA transfection.

Conclusions

This study investigates the use of HPH for generating nanosomes and the effect of varying formulation compositions on the properties of pDNA nanosomes. The physiochemical properties of pDNA nanosomes, such as particle size, zeta-potential, pDNA loading efficiency and in-vitro pDNA release, as well as biological properties, such as cell transfection and toxicity, were affected by lipid concentration and the presence (or absence) of cationic peptide (PS). In summary, the use of low-concentration nanosomes generated by HPH, combined with precondensed pDNA with PS, led to the formation of smaller particles with a favourable zeta-potential, which in turn significantly enhanced gene transfection efficiency as well as minimized cell toxicity. Optimal combinations of nanosomes, peptides and pDNA ratios can therefore be formulated to develop novel gene delivery systems and thus HPH will open a window for large commercial-scale production of gene delivery vehicles, which will be followed up in future study.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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References

1. Miller AD. Human gene therapy comes of age. *Nature* 1992; 357: 455–460.
2. Ross G *et al*. Gene therapy in the United States: a five-year status report. *Hum Gene Ther* 1996; 7: 1781–1790.
3. Otten G *et al*. Enhancement of DNA vaccine potency in rhesus macaques by electroporation. *Vaccine* 2004; 22: 2489–2493.
4. Doria-Rose NA, Haigwood NL. DNA vaccine strategies: candidates for immune modulation and immunization regimens. *Methods* 2003; 31: 207–216.
5. Stratov I *et al*. Vaccines and vaccine strategies against HIV. *Curr Drug Targets* 2004; 5: 71–88.
6. Dean HJ *et al*. Powder and particle-mediated approaches for delivery of DNA and protein vaccines into the epidermis. *Comp Immunol Microbiol Infect Dis* 2003; 26: 373–388.

7. Ilan Y. Technology evaluation: naked DNA. *Curr Opin Mol Ther* 1999; 1: 116–120.
8. Engelhardt JF *et al*. Adenovirus-mediated transfer of the CFTR gene to lung of nonhuman primates: biological efficacy study. *Hum Gene Ther* 1993; 4: 759–769.
9. Herz J, Gerard RD. Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice. *Proc Natl Acad Sci USA* 1993; 90: 2812–2816.
10. Gao X, Huang L. Cationic liposome-mediated gene transfer. *Gene Ther* 1995; 2: 710–722.
11. Felgner PL. Improvements in cationic liposomes for in vivo gene transfer. *Hum Gene Ther* 1996; 7: 1791–1793.
12. Wu GY, Wu CH. Receptor-mediated gene delivery and expression in vivo. *J Biol Chem* 1998; 263: 14621–14624.
13. Boussif O *et al*. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci USA* 1995; 92: 7297–7301.
14. Zhdanov RI *et al*. Cationic lipid-DNA complexes–lipoplexes–for gene transfer and therapy. *Bioelectrochemistry* 2002; 58: 53–64.
15. Brown MD *et al*. Gene delivery with synthetic (non viral) carriers. *Int J Pharm* 2001; 229: 1–21.
16. Driessen AJ *et al*. Mechanistic studies of antibiotic-induced permeabilization of phospholipid vesicles. *Biochemistry* 1995; 34: 1606–1614.
17. Mayer LD *et al*. Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochim Biophys Acta* 1986; 858: 161–168.
18. Lapinski MM *et al*. Comparison of liposomes formed by sonication and extrusion: rotational and translational diffusion of an embedded chromophore. *Langmuir* 2007; 23: 11677–11683.
19. Maulucci G *et al*. Particle size distribution in DMPC vesicles solutions undergoing different sonication times. *Biophys J* 2005; 88: 3545–3550.
20. Pupo E *et al*. Preparation of plasmid DNA-containing liposomes using a high-pressure homogenization–extrusion technique. *J Control Release* 2005; 104: 379–396.
21. Barbadas-Rodriguez R, Sabes M. Factors involved in the production of liposomes with a high-pressure homogenizer. *Int J Pharm* 2001; 213: 175–186.
22. Bachmann D *et al*. Preparation of liposomes using a Mini-Lab 8.30 H high-pressure homogenizer. *Int J Pharm* 1993; 91: 69–74.
23. Talsma H *et al*. The size reduction of liposomes with a high-pressure homogenizer (Microfluidizer). Characterization of prepared dispersions and comparisons with conventional methods. *Drug Dev Ind Pharm* 1989; 15: 197–207.
24. Liu Y *et al*. Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery. *Nat Biotechnol* 1997; 15: 167–173.
25. Liu F *et al*. Factors controlling the efficiency of cationic lipid-mediated transfection in vivo via intravenous administration. *Gene Ther* 1997; 4: 517–523.
26. Templeton NS *et al*. Improved DNA:liposome complexes for increased systemic delivery and gene expression. *Nat Biotechnol* 1997; 15: 647–652.
27. Dunne M *et al*. Encapsulation of protamine sulphate compacted DNA in polylactide and polylactide-co-glycolide microparticles. *J Control Release* 2003; 92: 209–219.
28. Li S. Characterization of cationic lipid-protamine-DNA (LPD) complexes for intravenous gene delivery. *Gene Ther* 1998; 5: 930–937.
29. Fischer D *et al*. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* 2003; 24: 1121–1131.
30. Berger N *et al*. Filter extrusion of liposomes using different devices: comparison of liposome size, encapsulation efficiency, and process characteristics. *Int J Pharm* 2001; 223: 55–68.
31. Aoki H *et al*. Effects of positive charge density on the liposomal surface on disposition kinetics of liposomes in rats. *Int J Pharm* 1997; 156: 163–174.
32. Perrie Y, Gregoriadis G. Liposome-entrapped plasmid DNA: characterisation studies. *Biochim Biophys Acta* 2000; 1475: 125–132.
33. Feng M *et al*. Intracellular uptake and release of poly(ethyleneimine)-co-poly(methyl methacrylate) nanoparticle/pDNA complexes for gene delivery. *Int J Pharm* 2006; 311: 209–214.