Original article

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EFFECTS OF SQUALANE ON THE SPERMINE-BASED CATIONIC NIOSOMES FOR GENE DELIVERY

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ABSTRACT

Gene therapy is a delivery of a defined therapeutic gene into specific cells of a patient to replace a defective gene. Lipid nanoparticles (LNPs) have been widely used as a carrier to improve the delivery efficiency. Of the LNPs delivery systems, niosomes which are formulated from non-ionic surfactants are generally cheaper and potentially more stable than liposomes which are formulated from phospholipids. However, it still has a storage stability problem. The aim of this study was therefore to investigate the effect of a helper lipid, namely squalane (Sq), on the physical stability, particle size, zeta potential, transfection efficiency and cytotoxicity of cationic niosomes. The cationic niosomes were composed of Span 20, cholesterol and spermine-based cationic lipid at a fixed molar ratio of 2.5:2.5:1, while the molar ratio of Sq was varied from 0.25 to 1. The zeta potential and the particle size of niosomes and niosome/DNA complexes were characterized. The results showed that the addition of Sq to the spermine-based niosomes reduced the particle size of the niosomes from 162.3 to 119.5 nm and increased the physical stability after kept at 4 °C for at least 4 weeks. *In vitro* transfection efficiency tested in HeLa cell revealed that niosome containing Sq at molar ratio of 1 (Sq1) exhibited comparable transfection efficiency to the niosome without Sq; however, lower amount of the Sq1 niosomes was required to form the complexes with DNA. None of the niosome formulations were toxic to cells at the niosome to DNA weight ratio which gave the highest transfection efficiency. These findings suggested that Sq may be used as a potential helper lipid in cationic niosomes for gene delivery.

Keywords: gene delivery, cationic niosomes, lipid nanoparticles, squalane, helper lipids

Introduction

Gene therapy, defined as the delivery of a therapeutic gene into specific cells of a patient to replace a defective gene, is a hopeful strategy to cure the inherited and acquired diseases caused by genetic deficiencies and abnormalities including cancers, cardiovascular diseases, and autoimmune disorders.[1] However, the large molecular size and hydrophilic property of nucleic acids, which is the main limitation of gene delivery, hinder their diffusion through the plasma membrane. Therefore, various strategies have been developed for transferring nucleic acids; for example, viral-based nanocarriers, polymer-based nanocarriers, lipid-based nanocarriers and also inorganic nano-carriers.[2-5]

Lipid nanoparticles (LNPs) such as liposomes and niosomes play an important role in genetic delivery, and they can be applied in various situations such as infectious diseases, metabolic diseases, cancers and regenerative medicines.[6,7] LNPs usually consist of lipid, helper lipid and surfactant. Niosomes, the LNPs used in this study, are vesicles formed by self-assembly of a non-ionic surfactant instead of a phospholipid, as in liposomes, which both have a similar bilayer structure, but niosomes are cheaper and more stable.[8] Cationic lipids are often added to a LNPs system to create the positive charge which is required for gene delivery. The positive charge on the surface of LNPs can bind with the negative charge of DNA and may electrostatically interact with the negatively charged molecules on the cell membrane (glycoproteins and proteoglycans) which may result in the enhancement of the cellular uptake.[9] Common cationic lipids used in gene delivery such as dioctadecylamidoglycylspermine (DOGS), dioleoyloxy-3-(trimethylammonio)propane (DOTAP) and dioleoxypropyl trimethylammonium chloride (DOTMA).[10] In this study, the spermine-based cationic lipid was used as cationic lipid in niosome formulations. The synthesis and characterization of this lipid was described in the published article by Niyomtham N., et al.[11] However, the stability of niosomes is still an important limitation for further use. For this reason, other lipid components in the formulation called "helper lipids" are usually added to provide better stability of LNPs and improve the transfection efficiency of LNPs/nucleic acid complexes.[12,13] Cholesterol (Chol) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) are generally used helper lipids because they promote gene transfer in LNPs, possibly by supporting bilayer stabilization.[14-16] Helper lipids also affect the electrostatic interaction between LNPs and DNA, the lipid self-assembly and secondary structure of DNA.[17,18] For *in vitro* delivery, the helper lipids facilitate the fusion of lipoplex to the cell membrane, release DNA to cytoplasm and promote endosomal escape.[13,19] Squalane (Sq), the neutral helper lipid, is also used to enhance the rigidity and stability of the niosome formulations. In addition, niosome with Sq showed high transfection efficiency and cellular uptake.[20] Thereby, in the present study, we used cholesterol (at a fixed molar ratio) combined with varied amount of Sq as the helper lipid in the niosome formulations containing the spermine-based cationic lipid. The particle size, zeta potential and physical stability of the niosomes formulated with different concentrations of Sq were evaluated. The binding ability of niosomes to DNA as well as the *in vitro* transfection efficiency and cytotoxicity of niosome/DNA complex at the different weight ratios were also investigated.

Materials and methods

Materials

DNA purification kit, QIAGEN® Plasmid Midi Kit, was purchased from Qiagen, Santa Clarita, CA, USA. Tissue culture reagents were purchased from GIBCO™ (Grand Island, NY, USA). Lipofectamine® 2000 and plasmid DNA (pDNA) were obtained from Invitrogen, NY, USA. Reagents for preparing niosomes including Sq, Span 20 and Chol were purchased from Sigma-Aldrich®, MO, USA. The

spermine-based cationic lipid, namely N^1,N^4-dimyristeroyloxyethyl-spermine (Figure 1a) was a gift from Dr. Boon-ek Yingyongnarongkul, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Ramkhamhaeng University (Bangkok, Thailand).

Plasmid preparation

The plasmid pEGFP-C2 encoding green fluorescent protein (4.7 kb) was amplified in *Escherichia coli* DH5-α and purified using the Qiagen endotoxin-free plasmid purification kit. The quantity and purity of the purified plasmid were evaluated by measuring the absorbance at 260 and the absorbance ratio at 260/280 nm, respectively, by Nanodrop spectrophotometer.

Preparation of cationic niosomes

The cationic niosomes were prepared using a thin film hydration with sonication method. In brief, solution of Span 20, Chol and Sq were separately prepared in a solvent mixture of ethanol/chloroform (1:1 v/v), and the spermine-based cationic lipid was dissolved in a chloroform/methanol mixture (2:1 v/v). The solution of spermine-based cationic lipid was then added to the mixture of Span 20 and Chol to obtain a mixture of Span 20:Chol:Spermine-based cationic lipids with the fixed molar ratio of 2.5:2.5:1, while the molar ratios of Sq were varied. Afterwards, the organic solvents were evaporated under N_2 gas flow to obtain the thin film, and the thin film was then kept in a desiccator overnight. The thin film was then hydrated by adding Tris buffer (20 mM Tris and 150 mM NaCl, pH 7.4) and sonicated using a bath sonicator for 30 min followed by a probe sonicator (Vibra-Cell™ Ultrasonic Processor, Sonics & Materials, Inc., USA) for 30 min (two cycles) in an ice bath. The prepared cationic niosomes were kept at 4°C until used.

Preparation of cationic niosome/DNA complexes

The niosome/DNA complexes were prepared by adding DNA solution into the cationic niosome solution at the niosomes to DNA weight ratio of 0.1 to 30. The mixture was gently mixed and the complexes were allowed to self-assemble for 30 min at room temperature.

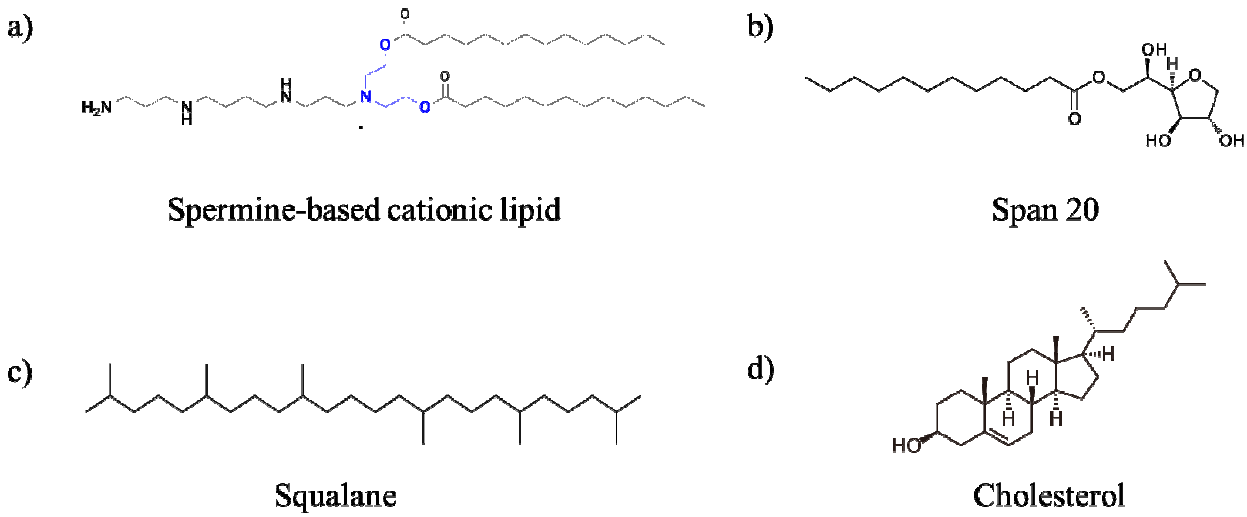

Figure 1 Chemical structures of a) spermine-based cationic lipid b) Span 20 c) squalane and d) cholesterol.

Size and zeta potential measurement

To measure the size and zeta potential of the niosomes and the niosome/DNA complexes, the niosomes and the niosome/DNA complexes were diluted 200 times with deionized water, and the particle size and zeta potential were determined using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). For physical stability study, niosome formulations were kept at 4 °C for 4 weeks. The particle size and zeta potential of the niosomes were measured every week. All measurements were done in triplicate.

Gel retardation assay

The binding ability of niosomes to DNA was evaluated by agarose gel retardation. The niosome/DNA complexes containing 0.2 µg DNA were prepared at the niosomes to DNA weight ratio of 0.1 to 30. Ten microliters of the complexes were mixed with 5 µL of 2X loading dye and loaded on 0.8% agarose gel. Gel electrophoresis was carried out for 30 min at 100 V in Tris acetate-EDTA (TAE) running buffer pH 8.3. The agarose gels were then stained with 0.5 µg/ml ethidium bromide for 10 min and destained with purified water for 5 min. The DNA bands were then visualized under a UV transilluminator using a GelDoc system. The 1kb DNA ladder (Invitrogen™) was used as a DNA marker.

In vitro transfection efficiency

In vitro transfection efficiency was performed in HeLa cells. The cells were seeded into 48-well plates at a seeding density of 1 x 10^4 cells/well in 0.25 mL growth medium and cultured at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. The cells were then incubated with cationic niosome/DNA complexes for 72 h at 37°C under 5% CO_2. Non-treated cells, the cells transfected with naked plasmid DNA and the cell transfected with Lipofectamine® 2000/DNA complex were used as non-treated control, negative control and positive control, respectively. All transfection experiments were performed in triplicate. The transfected cells (cells/cm^2) were inspected under fluorescent microscope, and the percentage of transfection efficiency was calculated using the Lipofectamine® 2000 (at carrier:DNA weight ratio of 2.5:1) transfected group as a reference.

Cytotoxicity of niosome/DNA complexes

The cytotoxicity of niosome/DNA complexes was determined in HeLa cell using MTT assay. The cells were seeded at a cell density of 8,000 cells per well in 96-well plates and incubated for 24 h prior to transfection. The complexes (at the same weight ratios of niosomes to DNA as *in vitro* transfection experiment) were then added and further incubated for 24 h. Subsequently, 25 µL of MTT solution (5 mg/ml) was added and the incubation was continued for 2 h. The medium was then removed, and the formazan crystals formed in the living cells were dissolved in 100 µl of DMSO. The relative cell viability (%) was calculated based on absorbance at 550 nm using a microplate reader (Victor Nivo™). The viability of non-treated control cells was defined as 100 % cell viability.

Statistical analysis

All experimental measurements were performed in triplicate. The data were expressed as the mean ± standard deviation (S.D.). Statistical analysis of the data was examined by F-test and t-test using Microsoft® Excel® 2016 MSO for Windows. The significance level was set at $p < 0.05$.

Results

Effect of Sq on particle size and zeta potential of the niosomes

The particle size and zeta potential of the niosomes containing Span 20, Chol, spermine-base cationic lipid at the fixed molar ratio of 2.5:2.5:1 and varied amounts of Sq are presented in Table 1. The addition of Sq in the formulation did not affect the charge of cationic niosomes, and all formulations had a positive surface charge which was suitable for

complexing with DNA by electrostatic interaction. Interestingly, the particle size of the niosomes significantly decreased from 162.27 ± 2.08 nm to 119.53 ± 0.51 nm ($p < 0.05$) as the amount of Sq in the formulation increased.

Effect of Sq on size and zeta potential of niosome/DNA complexes

In gene delivery, the size and surface charge of niosome/DNA complexes both influence the cellular uptake and transfection efficiency. Thus, the particle size and zeta potential of the niosome/DNA complexes were investigated, and the results are presented in Figure 2. The findings show that the positive charge of the niosome without Sq/DNA complex was observed at the weight ratio of 10 to 30 (Figure 2a). Positive charge of the Sq0.5/DNA and the Sq1/DNA complex were obtained at the weight ratio of 5 to 30 (Figure 2c, d). On the other hand, the Sq0.25/DNA complex showed a positive charge at the weight ratio 15 onward (Figure 2b). Therefore, the niosome formulations with different Sq contents affected the surface charge of the niosome/DNA complexes. The particle size of the niosome/DNA complexes in all formulations increased as the weight ratio of the niosomes was increased. The largest size was obtained when the zeta potential was closer to zero or slightly positive. The increase of the niosome/DNA ratio resulted in the smaller size of complexes because they were present as separated particles due to the electrostatic repulsion.

Binding ability of niosomes to DNA by gel retardation assay

As shown in Figure 3, all the niosome formulations were able to completely form complexes with DNA via electrostatic interaction at the niosome to DNA weight ratio of 15.

Effect of Sq on the physical stability of the niosomes

The physical stability of the niosomes was evaluated by the change of the particle size and zeta potential during the storage period, and the results are shown in Figure 4. The particle size of the niosome formulation without Sq was obviously increased (> 200 nm) after 3 weeks of storage. Interestingly, adding Sq to the formulation trended to improve the stability of cationic niosome up to 4 weeks. The addition of Sq slightly affected the charge of cationic niosomes, however all the niosome formulations still showed a positive surface charge after being kept at 4 °C for 4 weeks. Therefore, the addition of helper-lipid, Sq, in the formulation could preserve cationic niosomes.

Table 1 Particle size, polydispersity index (PDI) and zeta potential of the niosomes with different amounts of Sq. Each value represents the mean ± S.D. of three measurements.

Niosomes	Span 20:Chol:Cationic lipid:Sq (molar ratio)	Particle size (nm)	PDI	Zeta potential (mV)
No Sq	2.5:2.5:1:0	162.27 ± 2.08	0.317 ± 0.03	50.40 ± 4.00
Sq0.25	2.5:2.5:1:0.25	142.40 ± 1.31	0.338 ± 0.02	54.70 ± 2.31
Sq0.5	2.5:2.5:1:0.5	149.37 ± 2.20	0.311 ± 0.03	57.70 ± 1.45
Sq1	2.5:2.5:1:1	119.53 ± 0.51	0.190 ± 0.02	55.03 ± 1.86

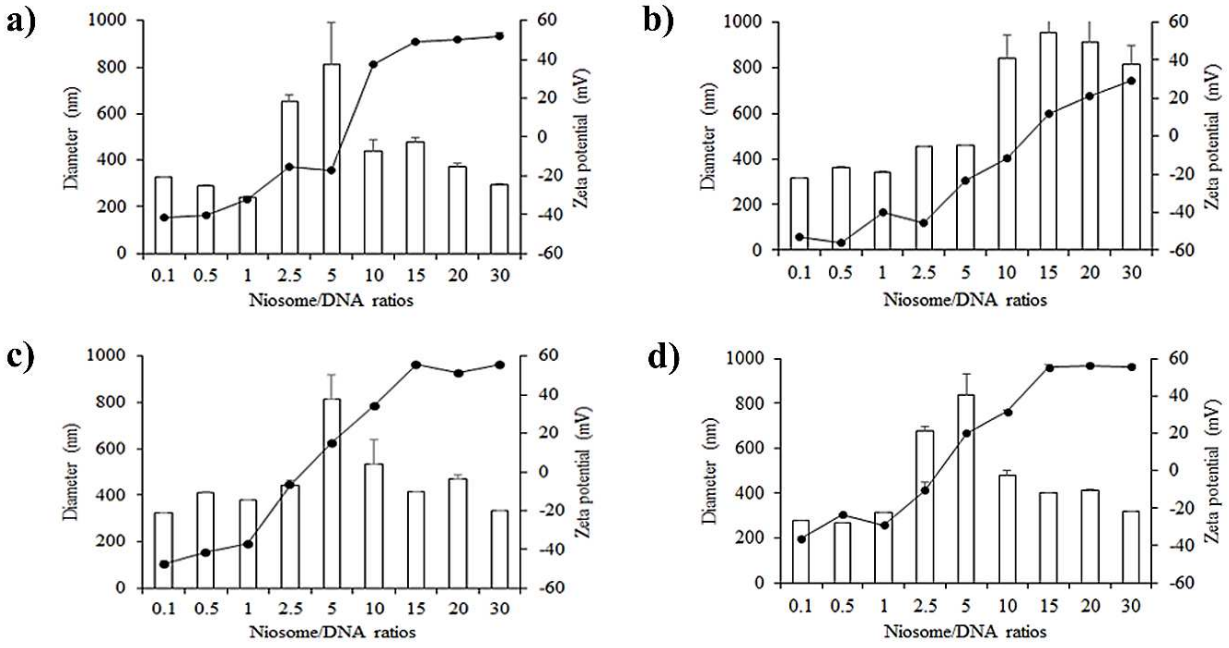

Figure 2 The particle size (bar) and zeta potential (line) of the niosomes to DNA complexes at the varied weight ratios ranging from 0.1 to 30. The niosome formulations were composed of a) niosome without Sq, b) Sq0.25, c) Sq0.5 and d) Sq1. Each value represents the mean ± S.D. of three measurements.

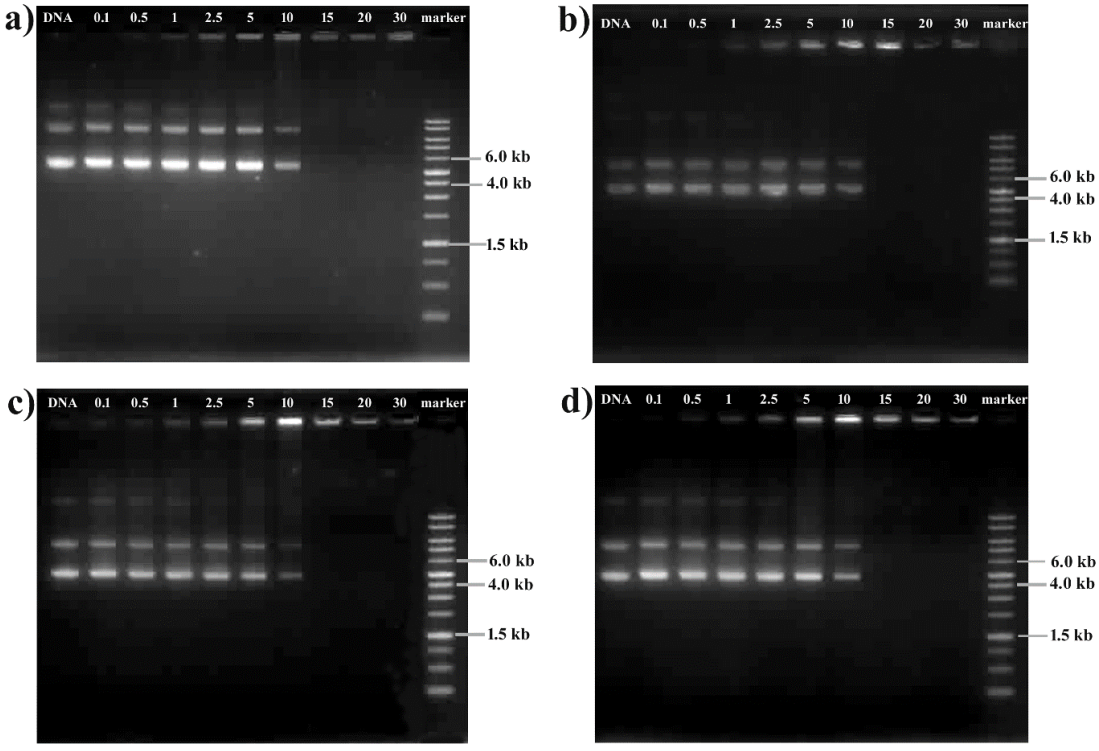

Figure 3 Gel retardation study for binding ability of niosomes to DNA, a) niosome without Sq b) Sq0.25 c) Sq0.5 and d) Sq1.

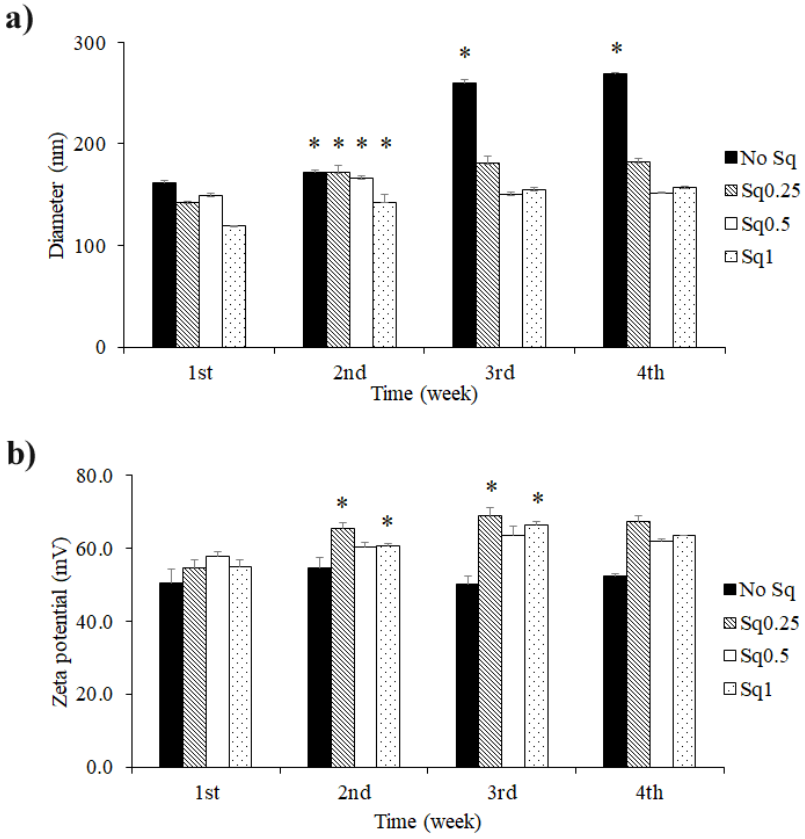

Figure 4 Physical stability of the cationic niosomes in terms of (a) particle size and (b) surface charge after being kept for 4 weeks at 4 ˚C. Each value represents the mean ± S.D. of three measurements. * Significantly different from the week before measurement ($p < 0.05$).

In vitro gene transfection efficiency

To compare the *in vitro* transfection efficiency of the niosome formulations with and without Sq, complexes of niosome/pEGFP-C2 were prepared, and the experiment was performed in HeLa cell. The percentage of transfection efficiency was calculated using Lipofectamine® 2000/DNA transfected group as positive control (100% transfection efficiency). The non-treated cells and transfected with naked DNA were a control (0 % transfection efficiency) and negative control, respectively. The transfection efficiency results were observed under a fluorescence microscope (Figure 5), and the percentage of transfection efficiency was calculated (Figure 6). The highest transfection efficiency of no Sq (96.04 ± 14%), Sq0.25 (62.71 ± 13%), Sq0.5 (57.00 ± 7%) and Sq1 (94.39 ± 19%) were obtained at the weight ratio of 5, 5, 2.5 and 2.5, respectively. The Sq1 formulation could be an effective gene delivery which no significant difference from no Sq formulation and Lipofectamine® 2000 was observed. Nevertheless, the optimal weight ratio with highest transfection efficiency of Sq1 formulation/DNA complex was less than niosome without Sq formulation.

Cytotoxicity of the niosome/DNA complexes

The cytotoxicity of the niosome/DNA complexes was evaluated in HeLa cell by MTT assay, and the result are shown in Figure 7. None of the niosome formulations had any toxicity (cell viability more than 90%) at the weight ratio which demonstrated the highest transfection efficiency. Therefore, these niosomes could be used as safe nanocarriers for DNA delivery.

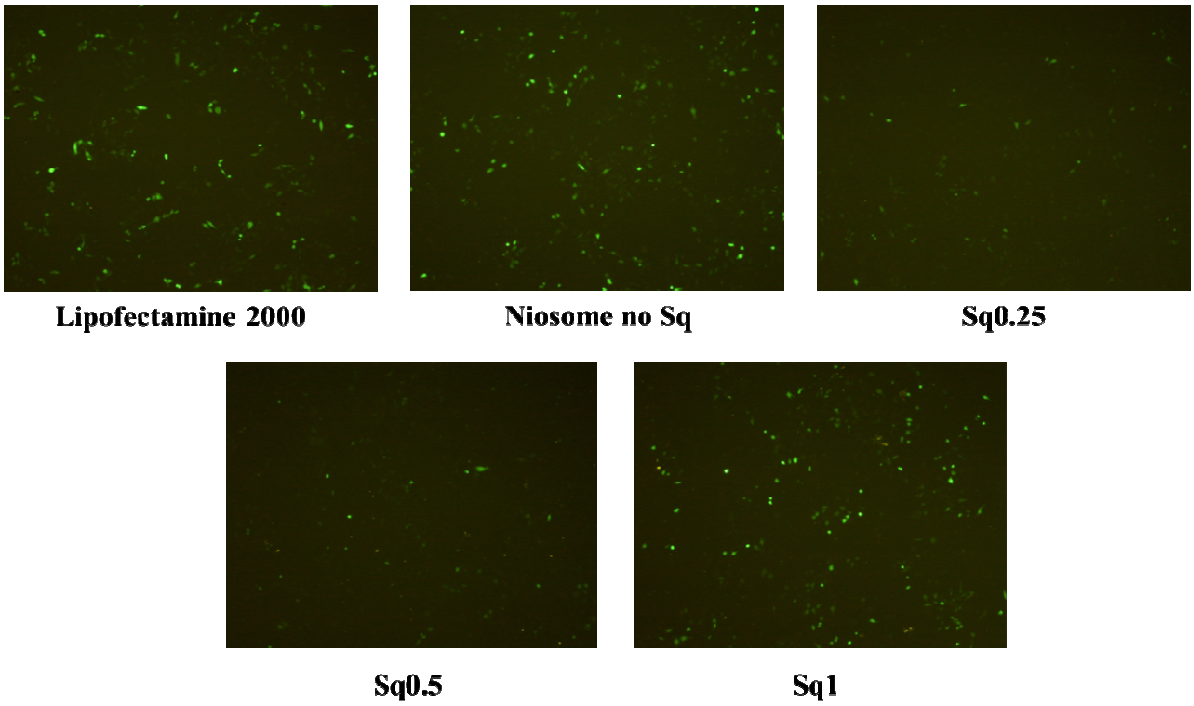

Figure 5 Fluorescence images of HeLa transfected with niosome/pEGFP-C2 complexes. The cell transfected with lipofectamine 2000/pEGFP-C2 was used as positive control.

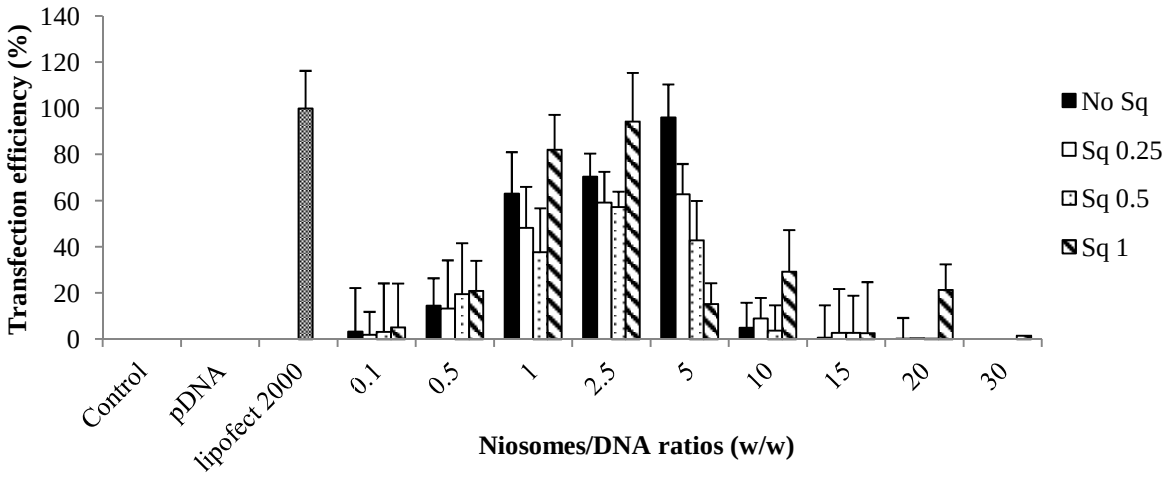

Figure 6 Percentage of transfection efficiency of niosome/pEGFP complexes in HeLa cells at different weight ratios. Each value represents the mean ± S.D. of three measurements.

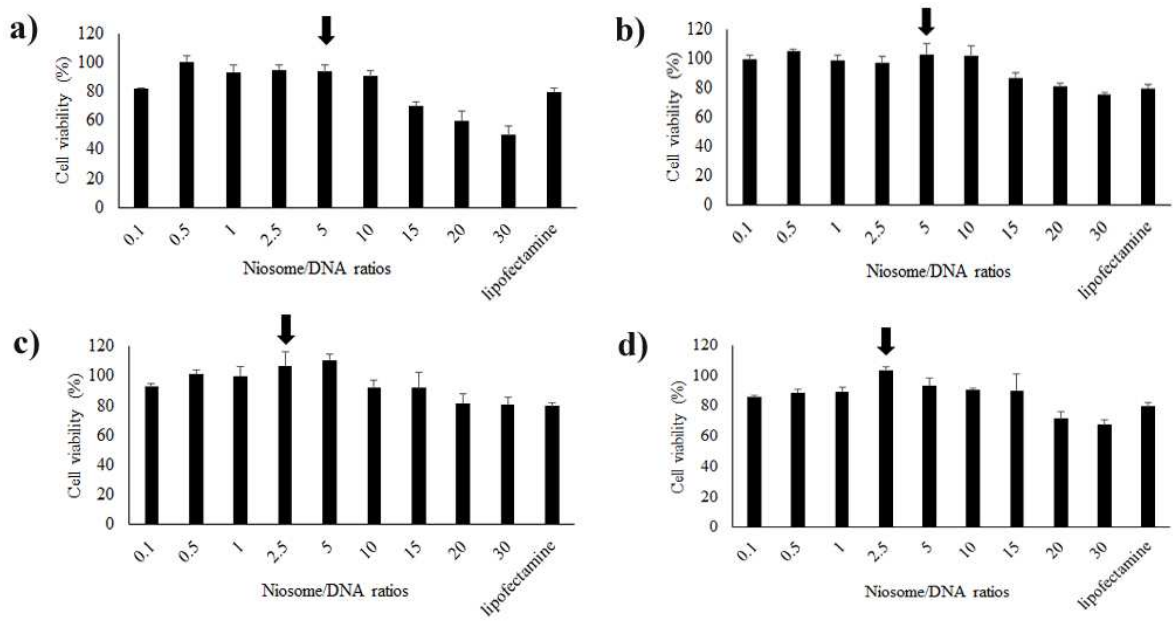

Figure 7 The cell viability of HeLa cell transfected with niosome/DNA complexes, a) niosome without Sq b) Sq0.25 c) Sq0.5 and d) Sq1. The arrows illustrated the ratios that provided the highest transfection efficiency. Each value represents the mean ± S.D. of three measurements.

Discussion

The addition of Sq in the formulation of cationic niosomes containing Span 20, cholesterol and spermine-based lipid provided good formulation properties and stability. The formulations containing Sq had smaller size than that the formulation without Sq. Furthermore, a higher amount of Sq in the formulations led to the smaller size of the niosome with narrow PDI. The surface charge of the niosome formulations with Sq was slightly more positive than that without Sq. This might be due to the hydrocarbon rich structure of Sq, which could be inserted into the hydrophobic part of Span20 and spermine-base cationic lipid in the niosomes. The interaction of these hydrophobic parts may tight up the lipid bilayers of niosome. For this reason, the niosomes containing Sq would have smaller size with slightly more positive surface charge than the formulation without Sq.[14,21] After the prepared niosomes were kept at 4 °C for 4 weeks, their size and zeta potential were measured every week to confirm whether the niosomes still had good physical stability. These parameters were considered because the suitable carrier for DNA delivery should be small and have positive charge, both of which are essential for complexing with DNA and delivery to the target cells. The findings revealed that the particle size of the formulations containing Sq did not change after the formulations was kept for 4 weeks. The surface charges of all the niosome formulations slightly changed, however their surface charges were still positive. From the results, the addition of Sq could fill the gap between the hydrophobic part of Span 20 and the spermine-based cationic lipid and promote the compaction of lipid bilayers of niosomes, thereby preserving the physical stability.[20,22]

The properties of the niosome/DNA complex were studied by zeta potential and particle size analysis. The cationic niosomes formed complexes with DNA via electrostatic interaction. The Sq0.5 and Sq1 formulations completely neutralized the negative charge of DNA at the weight ratio of cationic niosomes to DNA of 5. This revealed that niosomes containing Sq0.5 and Sq1 had higher binding ability than those without Sq (at weight ratio of 10). These niosomes containing high Sq content had smaller size and thus they facilitated

the binding with DNA. However, the binding ability assessed by gel retardation assay showed that the migration of DNA on agarose gel was completely retarded at the niosomes to DNA weight ratio of 15 for all the niosome formulations. These findings might be attributed to the fact that although the positive surface charge obtained at the lower niosome to DNA ratio, the interaction between DNA and niosomes in the complexes was not tight enough, and the DNA was therefore easily released from the complexes. The study of particle size of niosome/DNA complexes revealed that the largest size was obtained when the zeta potential was closer to zero. This might also be the reason why the DNA could be easily released from the complexes. Furthermore, the complexes with neutral zeta potential might not be stable and may form large complexes due to the easy aggregation or flocculation of the particles.[23,24] Increasing the weight ratio of the niosome to DNA until the zeta potential became more positive led to the smaller size of the complexes and increased complex integrity. In addition, the smaller size of the complexes is required for gene delivery because it could promote the transfection efficiency by enhancing the cellular uptake (endocytosis or pinocytosis) and also intracellular distribution.[25-27] Form the result, the optimal molar ratio of Sq in the cationic niosome formulation was found to be 1 mM (Sq1) as with the smallest size, good binding ability and good physical stability could be obtained. In term of the transfection efficiency, Sq1 formulation demonstrated equivalent transfection efficiency to no Sq formulation, but lower amount of the niosome was required. The cytotoxicity test indicated no toxicity of niosome/DNA complexes was found at the optimal weight ratio for all niosome formulations. Interestingly, although the positive surface charge of complexes is generally suitable for the interaction with negatively charged cell membranes for cellular uptake by endocytosis,[28] the highest transfection efficiency in the present study was achieved at the weight ratio which gave a slightly negative surface charge. This result was in agreement with previous reports.[29,30] This negative or neutral surface charge would be advantageous for gene delivery due to the lower binding to serum protein and other extracellular matrix, as well as the lower ability to disrupt the plasma membrane compared to the complexes with positive surface charge.[31,32] These finding implied that the spermine-based niosomes might be taken up into cells via different pathways from those in typical cationic niosomes and this issue should be therefore further investigated.

Conclusion

The effect of Sq on the spermine-based niosome formulations was investigated. The optimal molar ratio of Sq in the formulation was found to be 1 mM, Sq1. The smaller particle size and higher stability were obtained with the presence of Sq. The Sq1 formulation demonstrated equivalent transfection efficiency to formulation without Sq despite using the lower amount of the carrier. Consequently, Sq is an effective helper lipid to formulate the cationic niosome in order to enhance transfection efficiency and improve physical stability without toxicity for gene delivery.

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