การตั้งตำรับนีโอโซมประจุบวกสำหรับการนำส่งยีน : ผลของอัตราส่วนโดยโมลาร์ของลิปิดประจุบวกและอัตราส่วนโดยน้ำหนักของนีโอโซมต่อดีเอ็นเอ

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บทคัดย่อ

การตั้งตำรับนีโอโซมประจุบวกสำหรับการนำส่งยีน : ผลของอัตราส่วนโดยโมลาร์ของลิปิดประจุบวกและอัตราส่วนโดยน้ำหนักของนีโอโซมต่อดีเอ็นเอ

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บทนำ: นีโอโซมเป็นระบบนำส่งชนิดหนึ่งมีลักษณะเป็นถุงเล็กเตรียมจากสารลดแรงตึงผิวชนิดไม่มีประจุ ซึ่งใช้ทางด้านเภสัชกรรมกันอย่างกว้างขวาง ลิพิดประจุบวกจะทำให้เกิดประจุบวกบนพื้นผิวของนีโอโซมจึงสามารถนำไปใช้เป็นระบบนำส่งยีนได้ อย่างไรก็ตามการใช้ลิพิดประจุบวกในความเข้มข้นที่สูงอาจเป็นสาเหตุของความเป็นพิษต่อเซลล์และทำให้ประสิทธิภาพการนำส่งยีนต่ำลงได้ ดังนั้นในการศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของอัตราส่วนโดยโมลาร์ของลิพิดประจุบวกอนุพันธ์สเปอร์มีน (Ay) ในการตั้งตำรับนีโอโซมประจุบวกและอัตราส่วนโดยน้ำหนักของนีโอโซมประจุบวกต่อดีเอ็นเอต่อประสิทธิภาพในการนำส่งยีนและความเป็นพิษต่อเซลล์มะเร็งปากมดลูก (HeLa cells) โดยใช้พลาสมิดดีเอ็นเอที่สามารถแปลรหัสได้โปรตีนเรืองแสงสีเขียว (pEGFP-C2) **วิธีการดำเนินการวิจัย:** เตรียมตำรับนีโอโซมประจุบวกจาก Tween 61 คลอเลสเตอรอลและลิพิดประจุบวก (Ay) โดยให้มีอัตราส่วนโมลาร์ดังนี้ 2.5:2.5:1, 2.5:2.5:1.5 และ 2.5:2.5:2 เตรียมโดยวิธีทินฟิล์มไฮเดรชันร่วมกับการใช้คลื่นเสียงความถี่สูง ประเมินคุณลักษณะของสารประกอบเชิงซ้อนโดยวัดขนาดและประจุและหาอัตราส่วนโดยน้ำหนักของนีโอโซมประจุบวกต่อดีเอ็นเอที่สามารถเกิดสารประกอบเชิงซ้อนสมบูรณ์ด้วยวิธี gel retardation assay **ผลการศึกษาวิจัย:** จากการศึกษาพบว่าประสิทธิภาพในการนำส่งยีนเข้าเซลล์ของนีโอโซมประจุบวกเรียงลำดับดังนี้ นีโอโซม 2.5:2.5:1 > นีโอโซม2.5:2.5:1.5 > นีโอโซม2.5:2.5:2 โดยนีโอโซม 2.5:2.5:1 ที่อัตราส่วนโดยน้ำหนักของนีโอโซมประจุบวกต่อดีเอ็นเอเท่ากับ 10 นำส่งยีนเข้าเซลล์ได้ดีที่สุดและมีความปลอดภัยเมื่อทดสอบในหลอดทดลอง **สรุปผลการวิจัย:** อัตราส่วนโดยโมลาร์ของลิพิดประจุบวกในการตั้งตำรับนีโอโซมประจุบวกและอัตราส่วนโดยน้ำหนักของนีโอโซมประจุบวกต่อดีเอ็นเอมีผลต่อประสิทธิภาพในการนำส่งยีนและความเป็นพิษต่อเซลล์

คำสำคัญ: นีโอโซมประจุบวก, ลิพิดประจุบวกอนุพันธ์สเปอร์มีน, ทวีน 61, การนำส่งยีน

Abstract

Formulation of Cationic Niosomes for Gene Delivery: Effect of Molar Ratio of Cationic Lipid and Weight Ratio of Niosome/DNA Complexes

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Introduction: Niosomes, non-ionic surfactant vesicles, have been widely used in pharmaceutical field as a drug and biological delivery system. The cationic lipids can generate positive charge on the surface of niosomes that make niosomes suitable for gene delivery. However, usage cationic lipid in high concentration may cause of toxicity and low transfection

efficiency. The aim of this study was to investigate the effect of the molar ratio of spermine derivative-cationic lipid (Ay) in the cationic niosome formulations and the weight ratio of cationic niosomes/DNA complex on the transfection efficiency and the cytotoxicity in human cervical carcinoma cells (HeLa cells) using pDNA encoding green fluorescent protein (pEGFP-C2). **Materials and Method:** Various formulations of cationic niosomes were prepared from Tween 61, cholesterol and cationic lipid (Ay) at the molar ratio of 2.5:2.5:1, 2.5:2.5:1.5 and 2.5:2.5:2 using thin film hydration with sonication method. The measurements of size and zeta potential as well as gel retardation assay were performed in order to characterize the suitable weight ratio of cationic niosome/DNA complexes. **Results:** the transfection efficiency of these cationic niosomes was in the following order: niosomes (2.5:2.5:1) > niosomes (2.5:2.5:1.5) > niosomes (2.5:2.5:2). The highest transfection efficiency was observed in the formulation of niosomes at the molar ratio of (2.5:2.5:1) and the weight ratio of 10, moreover this formulation was safe *in vitro*. **Conclusion:** The molar ratios of cationic lipid in the cationic niosome formulations and weight ratios of cationic niosomes/DNA complexes are the important factor for transfection efficiency and cytotoxicity.

Keywords: Cationic niosomes, Spermine derivative cationic lipid, Tween 61, Gene delivery

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Introduction

Gene therapy is an alternative way to prevent or treat diseases. The aim of this approach is to transfer a genetic material such as DNA, RNA or antisense sequence into the cells that cause disease (Misra, 2013). Non-viral vector is a choice to deliver a gene that is a safe and effective way. In addition, the vector can be overcome the health risks and toxicity of viral vectors (Tros de Ilarduya *et al*, 2010). Niosomes, a self-assembly of non-ionic surfactant into the vesicles are a delivery system that have been widely used in pharmaceutical field and also recently used cationic niosomes as gene delivery systems (Marianecci *et al*, 2014; Pardakhty and Moazeni, 2013). Cationic lipids are comprised of hydrophilic and hydrophobic part in the molecules. In hydrophilic part usually have cationic (amine) groups that lead to the positive charge (Wasungu and Hoekstra, 2006). Hence, the niosomes that containing cationic lipid must be having a positive charge on the surface and can form electrostatic complexes with the negative charge of nucleic acid, i.e., DNA, RNA. However, usage cationic lipid in high concentration may cause of toxicity and low transfection efficiency (Lv *et al*, 2006; Zhdanov *et al*, 2002). Therefore, the objective of this study was to optimize of the molar ratio of cationic lipid (Ay) in the cationic niosome formulations and investigate the weight ratios of cationic niosomes/DNA complexes on transfection efficiency and cytotoxicity in HeLa cells.

Materials and Methods

Chemicals: Polyethylene glycol sorbitan monostearate

(Tween® 61), agarose and 3-(4,5-dimethlthaizol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich, MO, USA. Cholesterol was purchased from Carlo Erba Reagenti, MI, Italy. Spermine-based cationic lipid (Ay) (Fig. 1) was provided from Dr. Boon-ek Yingyongnarongkul, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Ramkhamhaeng University (Bangkok, Thailand). Lipofectamine® 3000 was obtained a gift from Invitrogen, NY, USA. HeLa cells, the human cervical cancer cell line, were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) Modified Eagle's medium (MEM), fetal bovine serum(FBS), Trypsin-EDTA and penicillin-streptomycin were purchased from GIBCO-BRL,

NY, USA. The pEGFP-C2 plasmid DNA, encoding green fluorescent protein (GFP) was obtained from Clontech, California, USA. All other chemicals were of cell culture and molecular biology grade.

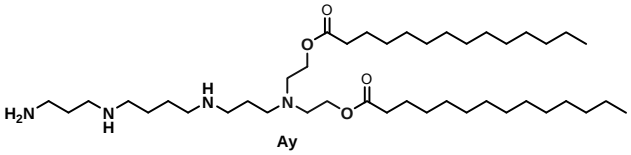

Figure 1 Chemical structure of cationic lipids (Ay)

Plasmid Preparation

pEGFP-C2 plasmid DNA was proliferated in *Escherichia coli* DH5-α and purified by using the Qiagen endotoxin-free plasmid purification kit (Qiagen, Santa Clarita, CA, USA).

Preparation of Cationic Niosomes

The cationic niosomes prepared by using thin film hydration with sonication method. In Brief, Tween 61 and cholesterol were dissolved in a mixture of ethanol/ chloroform (1:1 v/v ratio) and cationic lipid (Ay) was dissolved in a chloroform/methanol mixture (2:1 v/v ratio). The cationic lipid (Ay) solution was added to the solution of Tween 61 and cholesterol to obtained a mixture of Tween 61/cholesterol/cationic lipid at molar ratio of 2.5:2.5:1, 2.5:2.5:1.5 and 2.5:2.5:2. Next, the organic solvents were evaporated under N_2 gas flow to produce a thin film layer in the bottle. The thin film was kept in a desiccator to remove the remaining solvents. The thin film was hydrated by adding Tris buffer (20 mM Tris and 150 mM NaCl, pH 7.4). The dispersion was sonicated by using a bath sonicator for 30 min followed by a probe sonicator (Vibra-Cell™ Ultrasonic Processor, Sonics & Materials, Inc., USA) for 30 min (twice cycles).

Characterized Cationic Niosomes/DNA Complexes

The complexes of cationic niosomes-Ay/DNA were prepared by adding DNA solution into cationic niosome solution that prepared at different weight ratios. Then, gentle mixed by pipetting up and down and incubated the complexes for 30 min at room temperature for ensuring complexes are formed completely. Agarose gel electrophoresis was performed to confirm complex formation using 1% agarose gel (1 g agarose in 100 ml TAE buffer with 0.5 μg/ml ethidium bromide). The mixture of 15 μl of the complex comprising of 0.25 μg DNA was loaded per well. The electrophoresis was carried out for 45 minutes at 100 V. The DNA bands were visualized under an UV transilluminator using a GelDoc system.

Size and Zeta Potential Measurement

Particle size and zeta potential of cationic niosomes/DNA complexes were determined by photon correlation spectroscopy (PCS) using the Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK).

***In vitro* Transfection**

The day before transfection, HeLa cells were seeded into 48 well plates at a seeding density of 1 x 10^4 cells/well in 0.25 ml growth medium (MEM containing 10% fetal bovine serum and supplemented with % 1 non-essential amino acids, 1% glutamine, 100U/ml penicillin and 100 μg/ml streptomycin and were cultured at 3 7 ˚C in a humidified atmosphere of % 5 CO_2 for 24 h. The day of transfection, the cells were incubated with the cationic niosomes/DNA complexes at various weight ratios for 72 h at 37˚C under 5%CO_2. Non-treated cells and cells transfected with naked plasmid DNA and the complex of 0.75 μl Lipofectamine® 3000 with DNA were used as control, negative control and positive control, respectively. All transfection experiments were performed in triplicate.

Cytotoxicity Test

The evaluation of *in vitro* cytotoxicity of cationic niosomes/DNA complexes was performed by MTT assay. HeLa cells were seeded in a 96-well plate at a density of 1×10^4 cells/well in 100 μl of complete medium and incubated at 37˚C under 5% CO_2 overnight. Prior to testing, the cells were rinsed with phosphate-buffered saline (PBS, pH 7.4) and then loaded the cationic niosomes/DNA complexes at various weight ratios in the same concentrations as *in vitro* transfection experiment. After 24 h transfection, the complex solutions were replaced with 100 μl growth medium, which containing MTT (1 mg/ml) for

four h. Finally, the medium was removed, the cells were rinsed with PBS and formazan crystals formed in living cells were dissolved in 100 μl DMSO per well. Relative viability (%) was calculated based on the absorbance at 550 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA). Viability of non-treated control cells was arbitrarily defined as 100%.

Statistical Analysis

The statistical significance of differences in transfection efficiency and cytotoxicity were examined using one-way analysis of variance (ANOVA) followed by LSD *post hoc* test. The significance level was set at $p < 0.05$.

Results and Discussion

Cationic niosomes/DNA lipoplexes Formation

Agarose gel electrophoresis assay was to ascertain the optimal complexation conditions that cationic niosomes were formed complexes with DNA completely. In this experiment, the weight ratio of noisomes/DNA complexes varied from 0.5 to 30. Fig. 2 shows the naked DNA (Lane 2) and niosomes/DNA complexes at weight ratios of 0.5, 1, 2.5, 5, 10, 20 and 30 (Lanes 3–9). The naked DNA lane showed the DNA band, whereas the complexes were completely retained within the gel-loading well for all molar ratios of niosomes/DNA complexes at weight ratios above 10, illustrating that complete niosomes/DNA complexes were formed.

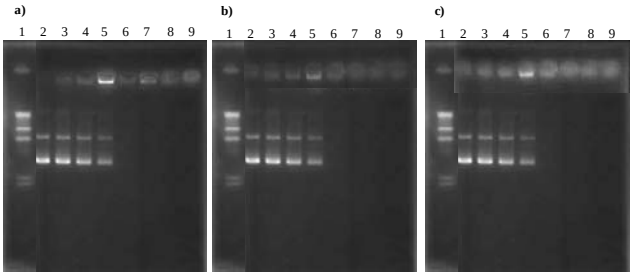

Figure 2 Gel retardation analysis of cationic niosomes/DNA complexes formulated with a) niosome 2.5:2.5:1 b) niosome 2.5:2.5:1.5 and c) niosome 2.5:2.5:2. Lane 1: λ*Hin*dIII DNA marker; lane 2: naked DNA, lane 3-9: niosomes/DNA complexes at weight ratios of 0.5, 1, 2.5, 5, 10, 20 and 30 respectively.

Size and Zeta Potential Measurement

Particle size and zeta potential of niosomes /DNA complexes are shown in Fig. 3. The particle sizes of niosomes/DNA complexes with weight ratio above 10 were in the range 200 to 1,000 nm and the particle sizes of the complexes was increased with the increasing molar ratio of cationic lipid (Ay). Zeta potential of all niosomes/DNA complexes were similar. The charge of the complexes was found to have increased with increasing in weight ratios and molar ratio of cationic lipid (Ay). The formulation of noisome 2.5:2.5:2 was showed the highest positive charge, followed by niosome 2.5:2.5:1.5 and niosome 2.5:2.5:1 respectively, that caused by the higher density of protonated amines on the surface of the vesicles.

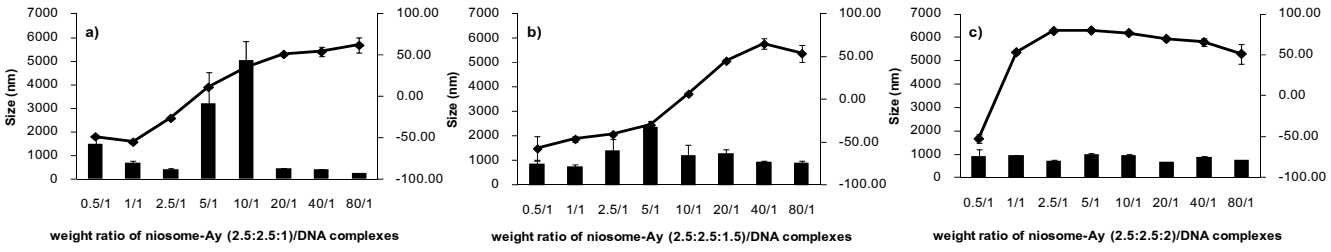

Figure 3 The particle size (bar graph) and zeta potential (◆) of cationic niosome/DNA complexes (a): niosome-Ay (2.5:2.5:1)/DNA, b): niosome-Ay (2.5:2.5:1.5)/DNA and c): niosome-Ay (2.5:2.5:2)/DNA). All values are mean ± S.D. (n=3).

In vitro Transfection

Transfection efficiency of niosomes/DNA complexes investigated in HeLa cells is shown in Fig. 4. The highest transfection efficiency for all molar ratio of cationic niosome formulation was achieved at weight ratio of 10. However, the efficiency of niosome 2.5:2.5:1/DNA complexes was higher than that of both noisome 2.5:2.5:1.5/DNA and niosome 2.5:2.5:2/DNA complexes. Furthermore, the efficiency of the complex was similar to Lipofectamine® 3000/DNA complex as a positive control. This result indicated that the transfection efficiency was decreased while increasing the molar ratio of cationic lipid in formulation. Increasing the cationic lipid in niosome formulation resulted in increase the surface charge of lipoplexes. Having high positive charge affected high toxicity to cells, therefore, influenced on the results of transfection.

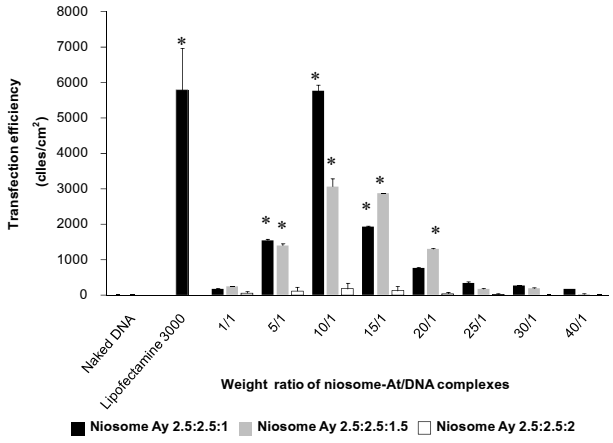

Figure 4 Transfection efficiency of cationic niosomes/DNA complexes using (■) noisome 2.5:2.5:1, (■) niosome 2.5:2.5:1.5 and (□) niosome 2.5:2.5:2 in HeLa cells. All values are mean ± S.D. (n=3). Difference values * were statistically significant ($p < 0.05$).

Cytotoxicity Test

As shown in Fig. 5, HeLa cells were incubated with noisome 2.5:2.5:1/DNA complexes at weight ratio of 1, the cell viability remained almost the same as that seen in the control group ($p > 0.05$). The cell viability of all niosomes/DNA complexes except the complex of noisome 2.5:2.5:1 at weight ratio of 1 was decreased when increasing the weight ratio of cationic niosomes. However, the viability of lipoplexes of niosome 2.5:2.5:1/DNA at weight ratio of 1 to 10 were over 80%, which the highest transfection efficiency was obtained. The result indicated the lipoplexes of niosome 2.5:2.5:1 at weight ratio of 1 to 10 were safe. The IC_{50} (the concentration of the complexes that cells were death 50%) of the complexes prepared using niosome 2.5:2.5:1, niosome 2.5:2.5:1.5 and noisome 2.5:2.5:2 were 73, 64 and 62 μg/ml, respectively. The average cell viability decreased when the increasing the molar ratio of cationic lipid. It could be concluded that the cationic niosomes with higher amount of cationic lipid (Ay) had higher toxicity to cells.

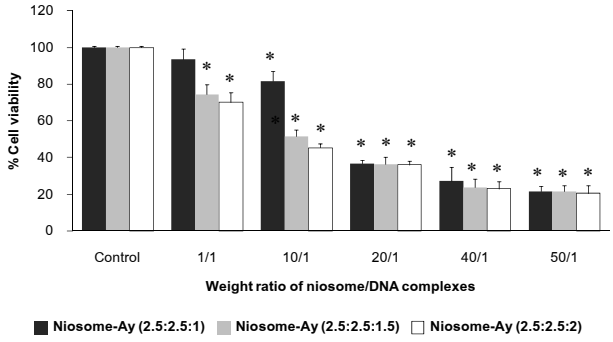

Figure 5 Cell viability of cationic niosomes/DNA complexes using (■) niosome 2.5:2.5:1, (■) noisome 2.5:2.5:1.5 and (□) niosome 2.5:2.5:2 in HeLa cells. All values are mean ± S.D. (n=5). Difference values * were statistically significant ($p < 0.05$).

Conclusion

The molar ratio of cationic lipid in the cationic niosome formulations and weight ratio of cationic niosomes/DNA complexes are an important factor for transfection efficiency and cytotoxicity. The noisome 2.5:2.5:1/DNA complexes at the weight ratio of 10 showed the highest transfection efficiency and not toxic *in vitro*.

Acknowledgements

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References

Lv H, Zhang S, Wang B, Cui S, Yan J. Toxicity of cationic lipids and cationic polymers in gene delivery. *J Control Release*, 2006, 114: 100-109.

Marianecci C, Di Marzio L, Rinaldi F, *et al*. Niosomes from 80s to present: the state of the art. *Adv Colloid Interfac Sci*, 2014, 205: 187-206.

Misra S. Human gene therapy: a brief overview of the genetic revolution. *J Assoc Physicians India*, 2013, 61: 127-133.

Pardakhty A, Moazeni E. Nano-niosomes in drug, vaccine and gene delivery; a rapid overview. *Nanomedicine J*. 2013, 1: 1-12.

Tros de Ilarduya C, Sun Y, DüzgüneŞ N. Gene delivery by lipoplexes and polyplexes. *Eur J Pharm Sci*, 2010, 40: 159-171.

Wasungu L, Hoekstra D. Cationic lipids, lipoplexes and intracellular delivery of genes. *J Control Release*, 2006, 116: 255-264.

Zhdanov RI, Podobed OV, Vlassov VV. Cationic lipid-DNA complexes-lipoplexes-for gene transfer and therapy. *Bioelectrochemistry*, 2002, 58: 53-64.