Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 356 (2008) 291–299
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Stability of luciferase plasmid entrapped in cationic bilayer vesicles

A. Manosroi [a,b,*], K. Thathang [a], R.G. Werner [c], R. Schubert [d], J. Manosroi [a,b]

[a] *Faculty of Pharmacy, Chiang Mai University, Chiang Mai 50200, Thailand*
[b] *Pharmaceutical-Cosmetic Raw Materials and Natural Products Research and Development Center (PCRNC), Institute for Science and Technology Research and Development, Chiang Mai University, Chiang Mai 50200, Thailand*
[c] *Boehringer Ingelheim International GmbH, 55216 Ingelheim am Rhein, Germany*
[d] *Department of Pharmaceutical Technology and Biopharmacy, University of Freiburg, Stefan-Meier-Str. 19, D-79104 Freiburg, Germany*

Received 4 October 2007; received in revised form 9 December 2007; accepted 2 January 2008
Available online 9 January 2008

Abstract

Characteristics and physical stability of luciferase plasmid (pLuc) entrapped in cationic bilayer vesicles prepared from various molar ratios of amphiphiles (DPPC, Tween61 or Span60), cholesterol (Chol) and cationic charge lipid (DDAB) were investigated. The cationic liposomes were composed of DPPC/Chol/DDAB in the molar ratio of 7:2:1. The cationic (Tween61 or Span60) niosomes were composed of Tween61/Chol/DDAB or Span60/Chol/DDAB in the molar ratio of 1:1:0.05. The maximum loading of pLuc was 15.29, 22.70, and 18.92 μg/mg of the total lipids or surfactants of liposomes, Tween61 and Span60 niosomes, respectively. The morphology of the vesicles showing multilamellar structure was characterized by transmission electron microscope (TEM). The particle sizes of the vesicles in nanosize range (160–850 nm) were determined by Photon Correlation Spectroscopy (PCS). Gel electrophoresis and gel documentation were modified to determine the entrapment efficiency of pLuc in cationic bilayer vesicles. The cationic bilayer vesicles gave the pLuc entrapment efficiency of 100%. The pLuc entrapped in cationic liposomes exhibited higher stability than pLuc in solution and pLuc entrapped in cationic Tween61 or Span60 niosomes, when stored at 4, 30 and 50 °C for 8 weeks. After 8 weeks at 4 °C, pLuc contents remained in cationic liposomes was 2 and 3 times higher than cationic Span60 and Tween61 niosomes, respectively. After 3 weeks, 50 and 2% of pLuc was remained in cationic liposomes at 30 and 50 °C respectively, whereas all pLuc in cationic Span 60 and Tween61 niosomes were degraded within 2 and 1 week, respectively. At 30 and 50 °C, pLuc in an aqueous solution or in bilayer vesicular formulations were not in a stable supercoil form. This study has indicated that the stability of pLuc can be enhanced by entrapping in cationic liposomes more than in niosomes. Higher temperature with increase storage time can affect the stability of pLuc even entrapped in bilayer vesicles.

Keywords: Bilayer vesicles; Liposomes; Niosomes; Luciferase plasmid; Stability; Entrapment efficiency

1. Introduction

Plasmid DNA has the limitation to use as therapeutic agents due to its less stability, especially susceptibility to heat and enzymatic degradation. DNA delivery systems are required not only to carry the gene to the target, but also for the protection of its stability as well. A number of approaches has been introduced to enhance the cytoplasmic and nuclear delivery of plasmid DNA, for examples, transfer of naked DNA by needle injection or electroporation (Zhang et al., 2002; Nishikage et al., 2004; Wolff and Vladimir, 2005), and the use of bilayer vesicles such as liposomes or niosomes (Kikuchi et al., 1999; Domashenko et al., 2000; Torchilin et al., 2003; Congiu et al., 2004; Jensen, 2004; Patil et al., 2004; Tokunaga et al., 2004). Liposomes are membrane bound spherical vesicles. Cationic liposomes represent as one of the most widespread non-viral transfection systems for gene delivery. Niosomes, non-ionic surfactant vesicles, are inexpensive alternatives of liposomes. Niosome possesses all the advantages of liposomes, but has superior of low cost, great stability, and ease of storage (Hao et al., 2002; Shahiwala and Misra, 2002; Manosroi et al., 2003). The transfer of specific genes into cells is a vital procedure for gene functions and therapies. Gene delivery by bilayer vesicles is convenient and safer than the viral methods. Some viral vectors may cause tumor in the recipients. Although the efficiency and specificity of bilayer vesicular systems are not high, they offer advantages over the viral delivery systems of being completely biodegrad-

* Corresponding author at: Faculty of Pharmacy, Chiang Mai University, Chiang Mai 50200, Thailand. Tel.: +66 53 894806; fax: +66 53 894169.
E-mail address: pmpti005@chiangmai.ac.th (A. Manosroi).

0378-5173/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2008.01.001

able, less toxic, less immunogenic and activating lower levels of complement than the viral vectors. Bilayer vesicles can transfer larger DNA molecules, simpler to use, less expensive and ease to produce, and can be administered efficiently and safely. These vesicles are expected to be one of the potential delivery system candidates for *in vivo* gene therapy. Development of reliable bilayer vesicular formulations is needed for DNA delivery. Kikuchi et al. (1999) reported a freeze dried empty liposomes (FDELs) method. This method does not expose DNA to high temperature of solvent evaporation and lyophilization, thereby reducing the risk of DNA degradation. Various sizes of DNA can be entrapped and stabilized in liposomes. Surfactant vesicle-mediated delivery of DNA vaccines have been reported (Perrie et al., 2004), suggesting that DNA can be effectively entrapped in a range of lipid and non-ionic based vesicular formulations. Luciferase plasmid (pLuc) has been widely used as the model gene for DNA delivery (Kikuchi et al., 1999; Congiu et al., 2004; Tokunaga et al., 2004). Although stability is one of the most concern parameters, only few stability studies of such plasmid have been reviewed. Hong et al. (1997) reported that condensing pLuc with polyamines prior to entrap in the liposome formulation DOPE/DDAB (1:1), resulting in the stable plasmid for 1 month at 4 °C. Sustained release of pLuc was also studied in lipid microtube-hydrogel delivery system for 50 days (Meilander et al., 2003). Concerning the stability of pLuc entrapped in bilayer vesicles, only few studies have reported on niosomes as gene delivery. Polyethyleneglycolated cationic niosomes, composed of DC-Chol, PEG2000-DSPE and the non-ionic surfactant-Span® have been studied for gene carrier in terms of their stable properties in storage as well as low cost materials (Huang et al., 2007). Niosomes encapsulating plasmid DNA encoding for HBsAg were prepared and characterized in comparing to liposomes (Vyas et al., 2005). They found that encapsulation of plasmid DNA within vesicular carriers (liposomes and niosomes) resulted in better immunological response in comparison to topically applied naked plasmid DNA because of the enhanced permeation of the encapsulated plasmid across the intact skin. However, study on thermal stability of the entrapped plasmid DNA in liposomes and niosomes has not been reported and compared. Our previous study has indicated that liposomes can protect the thermal labile drug, amphotericin B from high temperature storage (Manosroi et al., 2004a). Since DNA has the limitation for chemical and thermal stability and temperature is one of the important factors for DNA degradation (Bauer et al., 2003; Alexander et al., 2004), the thermal stability of the entrapped plasmid DNA in cationic liposomes and niosomes were investigated in this present study. The best cationic bilayer vesicles which provide the best stability of pLuc will be selected for further investigation of plasmid transfection efficiency in cell lines.

2. Materials and methods

2.1. Materials

pLuc was obtained from Boehringer Ingelheim (BII), Germany. It was propagated by transformation in *Escherichia coli* DH5α and purified using a QIAGEN purification kit (Qiagen GmbH, Hilden, Germany). The amount of pLuc was determined by gel electrophoresis using gel document apparatus and a spectrophotometry (Milton Roy spectronic Genesys 5). L-alpha-dipalmitoyl phosphaditylcholine (DPPC, 99.6%) was from Nikko Company, Japan. Cholesterol (Chol, 99.6%), and dimethyl dioctadecyl ammonium bromide (DDAB), a cationic lipid, were purchased from Sigma Co. (St. Louis, MO, USA) and used without further purification. Tween61 (polyoxyethylene sorbitan monostearate) and Span60 (sorbitan monosterate) were from Sigma Co. All other chemicals were analytical grade.

2.2. Preparation of cationic lyophilized empty bilayer vesicles by the FDEL method

The cationic liposomes were composed of DPPC/Chol/DDAB in the molar ratio of 7:2:1 (Manosroi et al., 2004a; Manosroi et al., 2004b). The cationic niosomes were composed of Span60/Chol/DDAB and Tween61/Chol/DDAB in the molar ratio of 1:1:0.05. The concentration of the lipids or surfactants mixture were 20 mM. All cationic bilayer vesicles were prepared by FDEL method (Kikuchi et al., 1999). Briefly, lipids or surfactants together with cholesterol and the cationic lipid (DDAB) were dissolved in chloroform. The solvent was removed by a rotary evaporator (R-124 Buchi, Switzerland) to get a thin film. The film was dried by evacuation in a desiccator under reduced pressure for over 12 h. The 9% sucrose aqueous solution was added to the film to obtain a dispersion, which was further swelled by swirling in a water bath at 60 ± 5 °C for 30 min. The resulting dispersion was sonicated by a microtip probe sonicator (Vibra Cell™, Sonics & Materials, Inc., Newtown, CT, USA) at pulse on 3.0 and pulse off 2.0, 33% amplitude, for 15 min. The dispersion was then lyophilized by a freeze-dryer (Alpha 1-2 LD model Christ, Germany), and kept at 4 °C until use.

2.3. Entrapment of pLuc in cationic bilayer vesicles

An aqueous solution of pLuc 10 μg was added to various amount of lyophilized cationic bilayer vesicle powder in order to obtained the maximum loading of pLuc. The lipoplex was formed by gently mixing with hands for 10 min at room temperature (30 °C). The resulting lipoplex was in white dispersion with no precipitation.

2.4. Determination of entrapment efficiency and maximum loading of pLuc in bilayer vesicles

The initial amount of pLuc was 0.1 μg. The entrapped pLuc contents in cationic bilayer vesicles were assayed by gel electrophoresis and gel documentation (Universal Hood, BioRad Laboratories, Milan, Italy). Five microliters of each lipoplex was loaded into each well of agarose gel using 1 kb ladder (NEB, Frankfurt, Germany) as a molecular weight laddder. The unentrapped pLuc determined from the band located the same level as the standard pLuc, while the entrapped pLuc remained

at the site of application. The amount of pLuc entrapped in cationic bilayer vesicles was extracted by phenol/chloroform (Kikuchi et al., 1999) with some modification. Briefly, the pLuc was extracted from the bilayer vesicular dispersion by adding 100 μl of phenol/cholroform/isoamyalcohol; P/C/IAA (25:24:1) into 100 μl of bilayer vesicular dispersion and then vortexed and pLuc in the water phase was collected and applied to gel electrophoresis, which was carried on a 0.8% agarose gel in 0.5X TBE (0.04 M Tris–borate pH 8.0) buffer at constant voltage (90 mV) for 30 min. The band density was determined by the Quantity One Program analysis by the gel documentation (Universal Hood, BioRad Laboratories, Milan, Italy). Both native close circular (supercoil) form at the lower band and rarely open circular form at the upper band were determined. The standard pLuc calibration curve was constructed and the band density was converted to the concentrations of pLuc. Percentages of pLuc entrapped in bilayer vesicles were calculated from the ratio of the amount entrapped in the vesicles to the initial amount of pLuc and multiplied by 100. The maximum loading of pLuc was the maximum amount (10 μg) of pLuc that can be entrapped or complexed with the vesicles (i.e. until the free pLuc band was observed when the vesicular dispersion without extraction was loaded in the gel) per mg total lipids or surfactants which were 0.6540, 0.4405 and 0.5285 mg for cationic DPPC liposomes, cationic Span60 niosomes and Tween61 niosomes, respectively.

2.5. Stability of pLuc entrapped in bilayer vesicles

An amount of 5 ml of bilayer vesicular formulation entrapped with pLuc was transferred to a clear glass-vial and kept at 4, 30, and 50 °C in a dark chamber for 8 weeks. Samples were withdrawn at predetermined time intervals (0, 1, 2, 3, 4, 6 and 8 weeks), and the remaining pLuc in the vesicles was determined by gel electrophoresis and gel documentation as previously described in Section 2.4. The characteristics of the cut and uncut pLuc extracted from the withdrawn samples were compared with the standard pLuc.

2.6. Morphology, particle size and ζ potential determination

A drop of bilayer vesicular dispersion was applied on a 300-mesh formvar copper grid on paraffin and allow the sample to adhere on the formvar for 10 min. The remaining dispersion was removed and a drop of 2% aqueous solution of uranyl acetate was applied for 5 min. The remaining solution was then removed. The sample was air dried and examined with a transmission electron microscope (TEM 1200S JEOL, JEOL Ltd., Tokyo, Japan). The morphology and lamellarity of the bilayer vesicles were observed. The diameter of bilayer vesicles containing and not containing pLuc was measured in five individual runs at 25 °C using the non-negative constrained least squares (NNLS) algorithm mode by the multimodal size distribution (MSD) analysis on a zeta plus zeta potential analyzer (Brookhaven Instruments Corporation, New York U.S.A.). The ζ (zeta) potential of all samples was obtained by the phase analysis light scattering (PALS) software for data analysis, in ten individual runs. The time-dependent correlation function on the scattered light intensity was measured at a scattering angle of 90°. All samples were diluted to 500 μg lipid/ml with freshly filtrated millipore water.

3. Results

3.1. pLuc preparation

The prepared pLuc with the size of 5.5 kb was identified by gel electrophoresis. The concentrations of the purified pLuc were in the range of 0.30–0.84 μg/μl. The standard uncut pLuc and that extracted from the bilayer vesicular formulations showed two bands of the native close circular (supercoil) form at the lower band and the rarely open circular form at the upper band. The purified cut plasmid when digested with *Eco*RI gave the three bands at 3.0, 1.8, and 0.7 kb which was identical to the standard pLuc from Boehringer Ingelheim (data not shown). The transformation efficiency of pLuc in *E. coli* was between 5.75×10^5 and 6.23×10^5 transformants/μg plasmid.

3.2. Gel electrophoresis and gel documentation validation for pLuc entrapment in bilayer vesicles

The density ratios of the lower band which was a stable (supercoil) form, to the upper band of pLuc were 5.34 ± 0.764, indicating about 5 times of higher density of the lower band than the upper band. Thus, the density of the lower band was used to construct the standard curve to determine the percentages of the entrapment of pLuc in all cationic bilayer vesicles. The good linear relationship ($r^2 = 0.9738$) between concentrations and density of the lower band was obtained.

3.3. Entrapment efficiency of pLuc in bilayer vesicles

For cationic liposomes (DPPC/Chol/DDAB at 7:2:1 molar ratio), the entrapped pLuc remained at the site of application in gel electrophoresis and no free pLuc band was observed since pLuc was bound tightly to the cationic lipid (DDAB) in the liposomes, which were too large size to move in the gel. This has indicated 100% entrapment efficiency of pLuc in cationic liposomes (Fig. 1A). Cationic niosomes (Tween61/Chol/DDAB and Span60/Shol/DDAB at 1:1:0.05 molar ratio) with the entrapped pLuc gave similar results to the cationic liposomes (Fig. 1B–C). The maximum loading of pLuc at 15.29 μg per mg of the total lipid was found in cationic liposomes, whereas 22.70 μg per mg and 18.92 μg per mg were observed in cationic Tween61 and cationic Span60 niosomes, respectively. The extracted pLuc from the cationic liposomes and cationic Tween61 niosomes showed three bands at 3.0, 1.8, and 0.7 kb which were identical to the standard pLuc when digested with *Eco*RI and had the total size of 5.5 kb (Fig. 2). This has indicated the stability of pLuc during the entrapment process and the solvent extraction of pLuc from the bilayer vesicles.

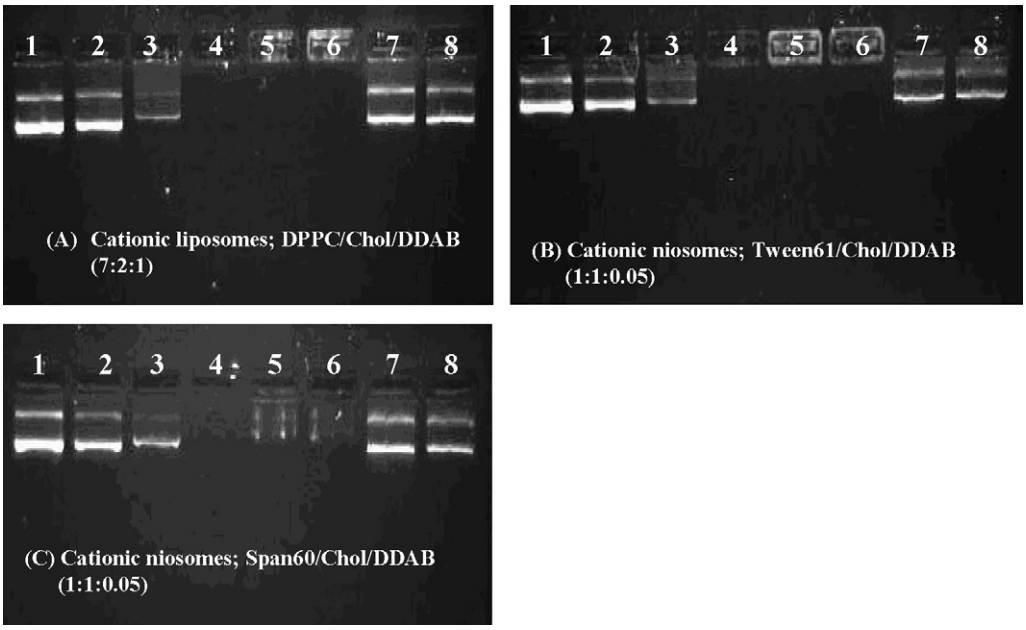


Fig. 1. Agarose gel electrophoresis of pLuc entrapped in cationic bilayer vesicles; **(A)** cationic liposomes; Lane 1–3 = standard pLuc 0.265, 0.1325, and 0.053 μg, respectively. Lane 4 = blank liposome. Lane 5–6 = initial pLuc-liposome without extraction. Lane 7–8 = extracted pLuc-liposomes using P/C/IAA. **(B)** cationic Tween61 niosomes and **(C)** cationic Span 60niosomes; Lane 1–3 = standard pLuc 0.265, 0.1325, and 0.053 μg, respectively. Lane 4 = blank niosome. Lane 5–6 = initial pLuc-niosome without extraction. Lane 7–8 = extracted pLuc-niosomes using P/C/IAA.

3.4. Morphology, particle size and ζ potential determination

All lyophilized empty cationic liposomes and niosomes were white powder with the diameter and the ζ potential listed in Table 1. All vesicles were large multilamellar vesicles. The particle size of DPPC and Span60 vesicles were 164.9 ± 1.8 and 595.4 ± 30.2 nm which were smaller than the Tween61 vesicles (769.4 ± 17.6 nm). The ζ potential is positive for all cationic bilayer vesicles of +73.20 ± 0.87, +41.90 ± 0.53 and +21.73 ± 0.49 mV for cationic DPPC liposomes, cationic Tween61 and Span60 niosomes respectively. The cationic vesicles entrapped with pLuc were not only had larger particle size than the unentrapped cationic vesicles, but also the ζ potential became negative. Fig. 3 showed the negative staining TEM images of the bilayer vesicles, showing the oligolamellar structure.

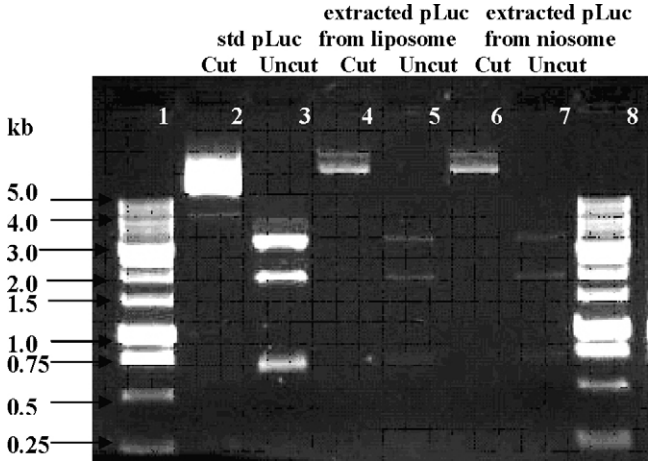


Fig. 2. Stability of the extracted pLuc from cationic bilayer vesicles using P/C/IAA. The pLuc was cut with *Eco*RI and compared to the standard pLuc from BII. Lane 1, 8 = DNA ladder. Lane 2–3 = standard pluc (1 μg/μl) uncut and cut, respectively. Lane 4–5 = pLuc extracted from cationic liposome uncut and cut, respectively. Lane 6–7 = pLuc extracted from cationic Tween61 niosomes uncut and cut, respectively.

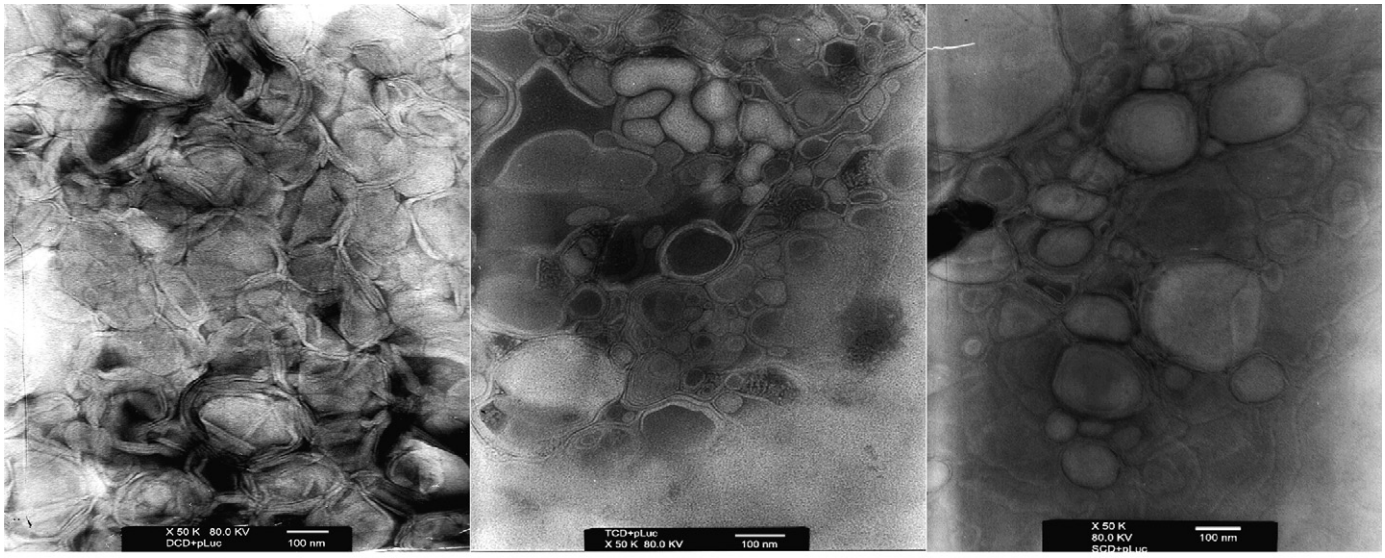
3.5. Stability of pLuc entrapped in cationic bilayer vesicles

Stability of pLuc entrapped in various cationic bilayer vesicles stored at 4, 30, and 50 °C for 8 weeks was shown in Figs. 4–6. At 4 °C, the pLuc in all cationic vesicles exhibited the stable

Table 1
Particle sizes in diameter (nm) and ζ potential of cationic bilayer vesicles entrapped and not entrapped with pLuc

Formulations (molar ratio)	Size[a] ± S.D. (nm)	ζ [b] ± S.D. (mv)
Without pLuc (blank bilayer vesicles)		
Cationic liposomes: DPPC/Chol/DDAB (7:2:1)	164.9 ± 1.8	+73.20 ± 0.87
Cationic niosomes: Tween61/Chol/DDAB (1:1:0.05)	769.4 ± 17.6	+41.90 ± 0.53
Cationic niosomes: Span60/Chol/DDAB (1:1:0.05)	595.4 ± 30.2	+21.73 ± 0.49
With pLuc		
DPPC/Chol/DDAB (7:2:1) + pLuc[b,c]	215.8 ± 2.5	−28.89 ± 2.87
Tween61/Chol/DDAB (1:1:0.05) + pLuc[b,c]	852.9 ± 8.6	−42.14 ± 0.35
Span60/Chol/DDAB (1:1:0.05) + pLuc[b,c]	632.2 ± 25.7	−71.25 ± 0.65

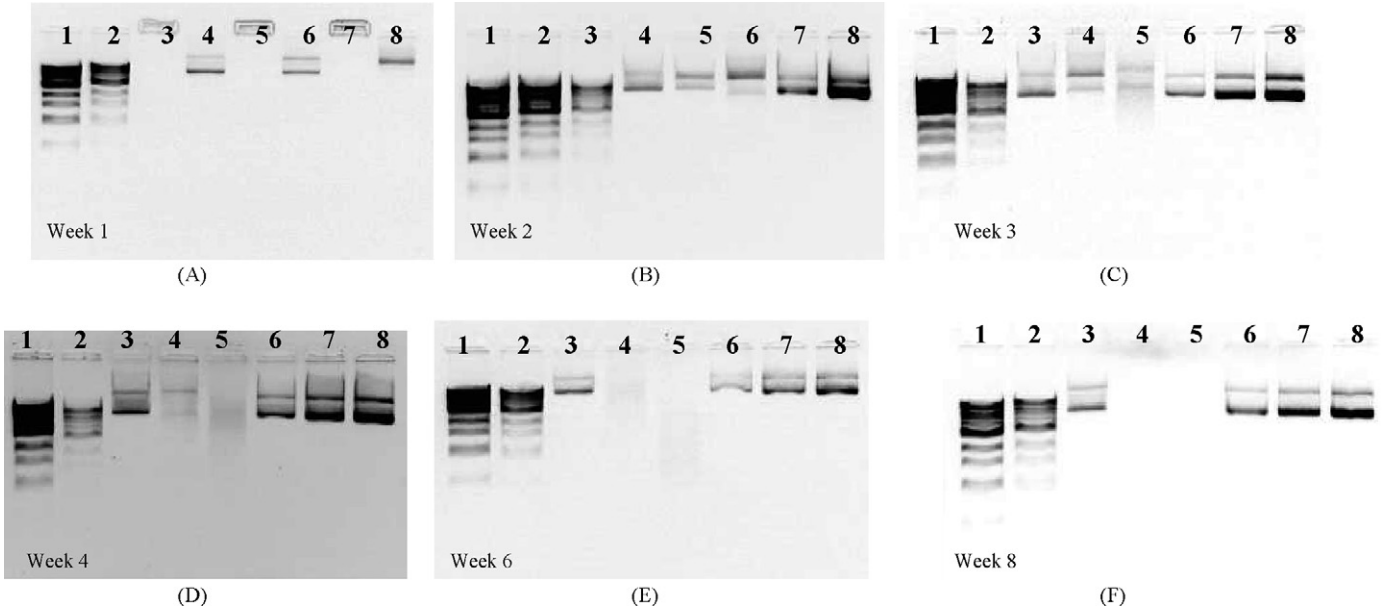
[a] PD, particle diameter (nm). The value is the average of 5 measurements.
[b] ZD, ζ, zeta potential (mV). The value is the average of 10 measurements.
[c] Data represented particle sizes of each formulation after incubation for 1 h at room temperature (30 ± 2 °C).



(A) (B) (C)

Fig. 3. The negative staining TEM images of the cationic bilayer vesicles containing pLuc prepared by FDEL method. Magnification at ×50 k **(A)** cationic DPPC liposomes **(B)** cationic Tween61 niosomes **(C)** cationic Span60 niosomes.

(supercoil) form during 8 weeks, while in an aqueous solution showed partial degradation with times even stored at 4 °C (Fig. 7). At higher temperature (30 and 50 °C), pLuc was in an open circular form which was the unstable form. This unstable form increased with times. Fig. 8A–C represented the remaining pLuc amount in cationic bilayer vesicles when stored at 4, 30, and 50 °C for 8 weeks. pLuc in cationic DPPC liposomes appeared to be more stable than pLuc in other bilayer vesicles. At 4 °C for 8 weeks, the amount of pLuc (30.41%) in cationic liposomes was the same as in an aqueous solution, and was about 2 and 3 times higher than in cationic Span60 (15.48%) and Tween61 niosomes (10.73%). At 30 °C, pLuc remaining in all bilayer vesicles was not intact after 3 weeks (Figs. 4–6). During 3 weeks stored at 30 °C, the remaining pLuc in cationic liposomes (53.65%) was higher than in aqueous solution (49.15%), in cationic Span60 niosomes (47.76%) and in cationic Tween61 niosomes (25.16%) (Fig. 8B). At 50 °C, pLuc in aqueous solution was completely degraded after 1 week, while at 2 and 3 weeks, none was found in cationic Span60 and Tween61 niosomes, respectively (Fig. 8C). However, the pLuc amount remaining in cationic DPPC liposome at 1, 2 and 3 weeks were 69.58, 43.33 and 2%, respectively.



(A) (B) (C)

(D) (E) (F)

Fig. 4. Agarose gel electrophoresis of pLuc entrapped in cationic DPPC liposomes at 4, 30, and 50 °C stored for 8 weeks. **(A)** week 1: lane 1,2 = DNA ladder, lane 3,5,7 = pLuc without extraction at 4, 30, 50 °C, lane 4,6,8 = extracted pLuc at 4, 30, 50° C. **(B)** week 2: lane 1,2,3 = DNA ladder, lane 4,5,6 = extracted pLuc at 4, 30, 50 °C, lane 7,8 = Std pLuc. **(C)** week 3: lane 1,2 = DNA ladder, lane 3,4,5 = extracted pLuc at 4, 30, 50 °C, lane 6,7,8 = Std pLuc. **(D)** week 4: lane 1,2 = DNA ladder, lane 3,4,5 = extracted pLuc at 4, 30, 50 °C, lane 6,7,8 = Std pLuc. **(E)** week 6: lane 1,2 = DNA ladder, lane 3,4,5 = extracted pLuc at 4, 30, 50 °C, lane 6,7,8 = Std pLuc. **(F)** week 8: lane 1,2 = DNA ladder, lane 3,4,5 = extracted pLuc at 4, 30, 50 °C, lane 6,7,8 = Std pLuc (at 0.05, 0.1, and 0.15 μg, respectively).

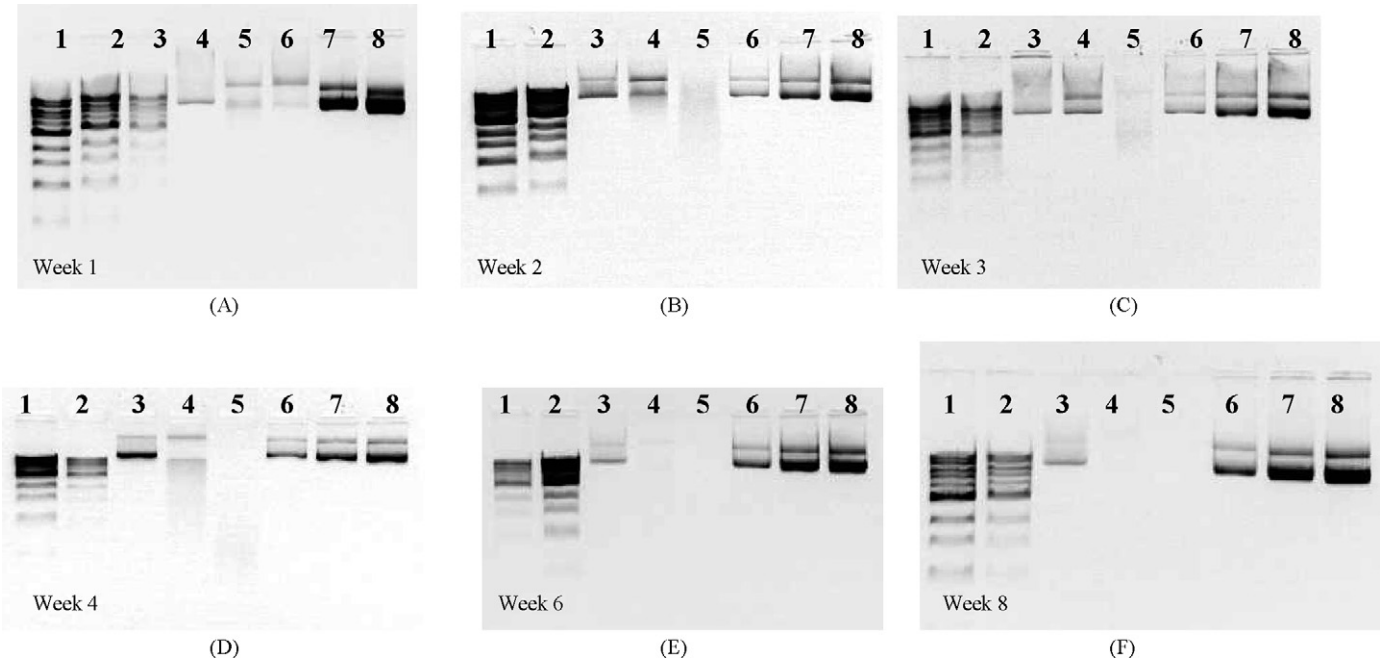


Fig. 5. Agarose gel electrophoresis of pLuc entrapped in cationic Tween61 niosomes at 4, 30, and 50 °C stored for 8 weeks. **(A)** week 1: lane 1,2,3 = DNA ladder, lane 4,5,6 = extracted pLuc at 4, 30, 50 °C, lane 7,8 = Std pLuc. **(B)** week 2: lane 1,2 = DNA ladder, lane 3,4,5 = extracted pLuc at 4, 30, 50 °C, lane 6,7,8 = Std pLuc. **(C)** week 3: lane 1,2 = DNA ladder, lane 3,4,5 = extracted pLuc at 4, 30, 50 °C, lane 6,7,8 = Std pLuc. **(D)** week 4: lane 1,2 = DNA ladder, lane 3,4,5 = extracted pLuc at 4, 30, 50 °C, lane 6,7,8 = Std pLuc. **(E)** week 6: lane 1,2 = DNA ladder, lane 3,4,5 = extracted pLuc at 4, 30, 50 °C, lane 6,7,8 = Std pLuc. **(F)** week 8: lane 1,2 = DNA ladder, lane 3,4,5 = extracted pLuc at 4, 30, 50 °C, lane 6,7,8 = Std pLuc (at 0.05, 0.1, and 0.15 μg, respectively).

4. Discussion

This present study has validated the pLuc amount determined by gel electrophoresis and gel documentation for percentages of entrapment determination. Cationic liposomes composed of the phospholipid (dipalmitoyl phosphaditylcholine; DPPC)/ Chol/ DDAB (the cationic lipid) were prepared in the molar ratio of 7:2:1 since we have demonstrated from our previous study

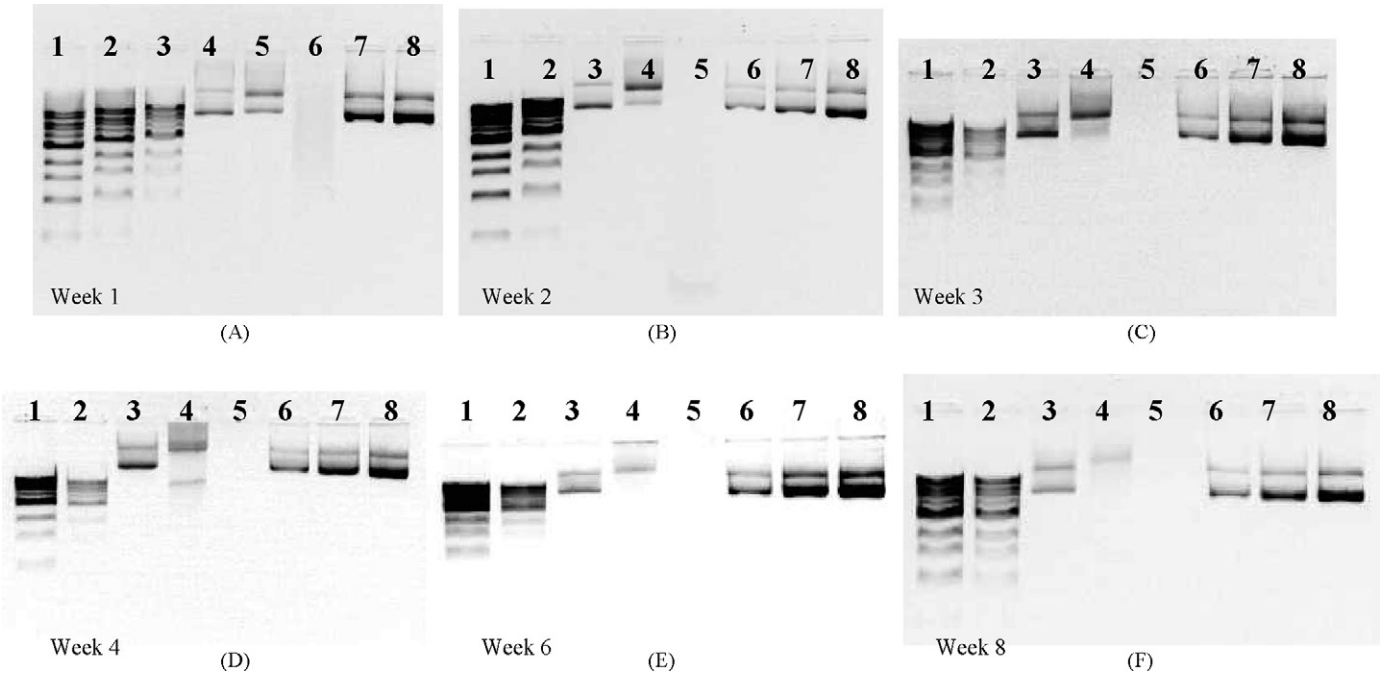


Fig. 6. Agarose gel electrophoresis of pLuc entrapped in cationic Span60 niosomes at 4, 30, and 50 °C stored for 8 weeks. **(A)** week 1: lane 1,2,3 = DNA ladder, lane 4,5,6 = extracted pLuc at 4, 30, 50 °C, lane 7,8 = Std pLuc. **(B)** week 2: lane 1,2 = DNA ladder, lane 3,4,5 = extracted pLuc at 4, 30, 50 °C, lane 6,7,8 = Std pLuc. **(C)** week 3: lane 1,2 = DNA ladder, lane 3,4,5 = extracted pLuc at 4, 30, 50 °C, lane 6,7,8 = Std pLuc. **(D)** week 4: lane 1,2,3 = DNA ladder, lane 4,5,6 = extracted pLuc at 4, 30, 50 °C, lane 7,8 = Std pLuc. **(E)** week 6: lane 1,2 = DNA ladder, lane 3,4,5 = extracted pLuc at 4, 30, 50 °C, lane 6,7,8 = Std pLuc. **(F)** week 8: lane1,2 = DNA ladder, lane 3,4,5 = extracted pLuc at 4, 30, 50 °C, lane 6,7,8 = Std pLuc (at 0.05, 0.1, and 0.15 μg, respectively).

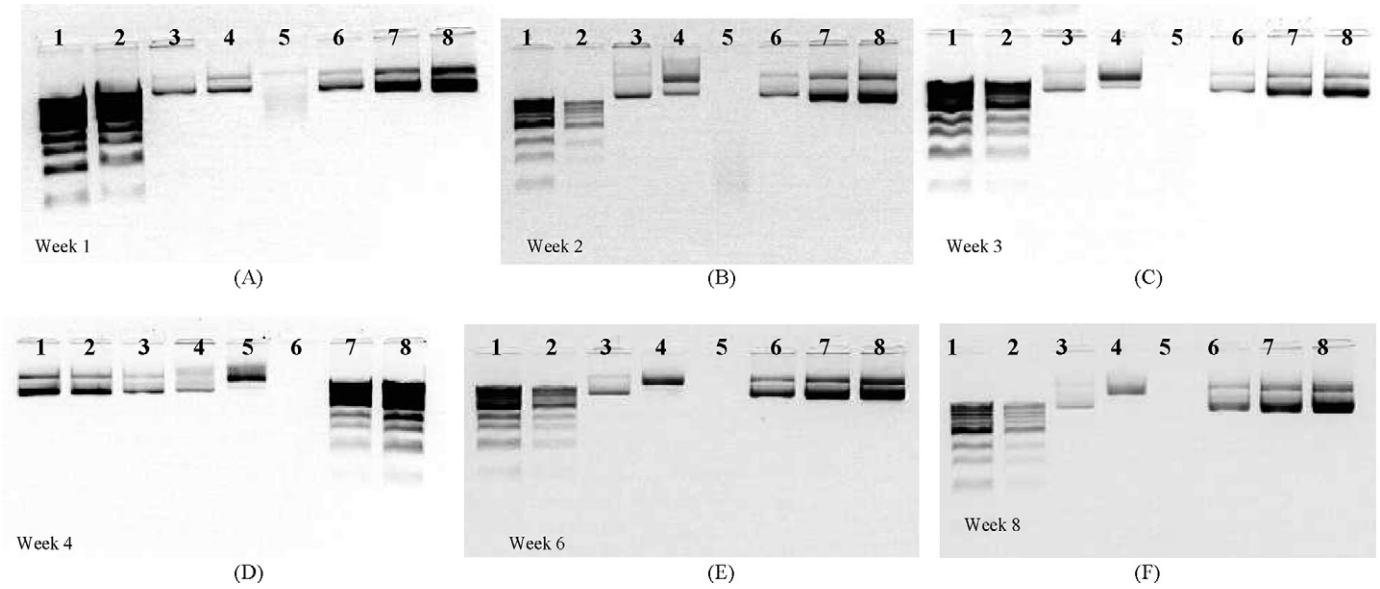


Fig. 7. Agarose gel electrophoresis of pLuc in an aqueous solution at 4, 30, and 50 °C stored for 8 weeks. **(A)** week 1: lane 1,2 = DNA ladder, lane 3,4,5 = pLuc in an aqueous solution at 4, 30, 50 °C, lane 6,7,8 = Std pLuc. **(B)** week 2: lane 1,2 = DNA ladder, lane 3,4,5 = pLuc in an aqueous solution at 4, 30, 50 °C, lane 6,7,8 = Std pLuc. **(C)** week 3: lane 1,2 = DNA ladder, lane 3,4,5 = pLuc in an aqueous solution at 4, 30, 50 °C, lane 6,7,8 = Std pLuc. **(D)** week 4: lane 1,2,3 = Std pLuc, lane 4,5,6 = pLuc in an aqueous solution at 4, 30, 50 °C, lane 7,8 = DNA ladder. **(E)** week 6: lane 1,2 = DNA ladder, lane 3,4,5 = pLuc in an aqueous solution at 4, 30, 50 °C, lane 6,7,8 = Std pLuc. **(F)** week 8: lane 1,2 = DNA ladder, lane 3,4,5 = pLuc in an aqueous solution at 4, 30, 50 °C, lane 6,7,8 = Std pLuc (at 0.05, 0.1, and 0.15 μg, respectively).

that the cationic liposomes composed of phosphatidylcholine/ cholesterol (Chol)/ cationic charged lipid (stearylamine) at the molar ratio of 7:2:1 gave high physical stability (Manosroi et al., 2004b), and showed deep penetration into the skin (Manosroi et al., 2004a). The same molar ratio of phosphatidylcholine/ cholesterol (Chol)/ cationic charged lipid (stearylamine) at 7:2:1 has also been studied by other group (Richardson et al., 1978; Morgan and Williams, 1980). For cationic niosomes, our previous study has also demonstrated that the vesicular membranes of Tween61 or Span60 to cholesterol at 1:1 molar ratio were in gel state and were able to efficiently entrap the water-soluble substance (calcein) (Manosroi et al., 2003). Furthermore, other study has also indicated that Tween61 in an equimolar combination with cholesterol exhibited great encapsulation capabilities of water soluble substances (Hood et al., 2003). Therefore, we have used this 1:1 molar ratio of Tween61 or Span60 to cholesterol to prepare the cationic niosomes. Hence, the cationic niosomes composed of Tween61/Chol/DDAB or Span60/Chol/DDAB at the molar ratio of 1:1:0.05 were investigated. The molar ratio of the cationic lipid (DDAB) in niosomes was only 2.5% of the total composition which was less than in the case of liposomes (10% of the total composition), since niosomes appeared to be more toxic than liposomes (Manosroi et al., 2007a; Manosroi et al., 2007b). So, DDAB was kept in minimal not only to reduce cost, but also to reduce the toxic side effect of such cationic lipid especially when in combination with the non-ionic surfactant (Tween61 and Span60).

This study has also demonstrated that the extraction process of pLuc from liposomes or niosomes by P/C/IAA gave identical bands to the standard pLuc when digested with *Eco*RI (Fig. 2), indicating the stability of pLuc during the entrapment and the extraction process of pLuc by organic solvent.

Cationic bilayer vesicles both liposomes and niosomes demonstrated entrapment efficiency of 100%. This was not only from the charge interaction between the negatively charged pLuc and the positively charged (DDAB) in the bilayer vesicles, but also the entrapment of pLuc in the aqueous layers of the vesicles. This charge interaction appeared to protect pLuc from vigorous conditions during the entrapment and extraction process since pLuc was not intact in neutral and anionic bilayer vesicles (data not shown). The maximum loading of pLuc were 15.29, 22.70 and 18.92 μg per mg of the total lipids or surfactants for cationic DPPC liposome, cationic Tween61 and cationic Span60 niosomes, respectively. The highest maximum loading of pLuc in cationic Tween61 niosomes may be due to the large polar head group of Tween61 which has higher hydrophilicity than the Span60 and DPPC molecule, thereby being able to load the higher amount of pLuc which has the hydrophilic nature. Span60 niosomes showed smaller particle size than the Tween61 niosomes. This may be from the larger head group of Tween61 than Span60 that can increase the repulsion interaction between the Tween61 head groups. So, the sizes of Tween61 vesicle was lager than Span60 and DPPC vesicles. In addition, DPPC contained double alkyl tails of C_{16}, whereas Span60 and Tween61 has single alkyl chain of C_{18}. The more bulky double alkyl chain of DPPC may hinder the condensation of the bilayer membrane (Manosroi et al., 2005). Even the more bulky double alkyl chain of DPPC may hinder the condensation of the bilayer membrane, when cholesterol is incorporated, the hydrogen bonding between cholesterol's hydroxyl and the lipid's carbonyl groups may enhance the stability of the bilayer (Presti et al., 1982) by condensing the vesicular membrane resulting in a smaller particle size. This may have more effect in liposomes than in niosomes. Cationic DPPC liposomes gave about 4.61 and 3.61

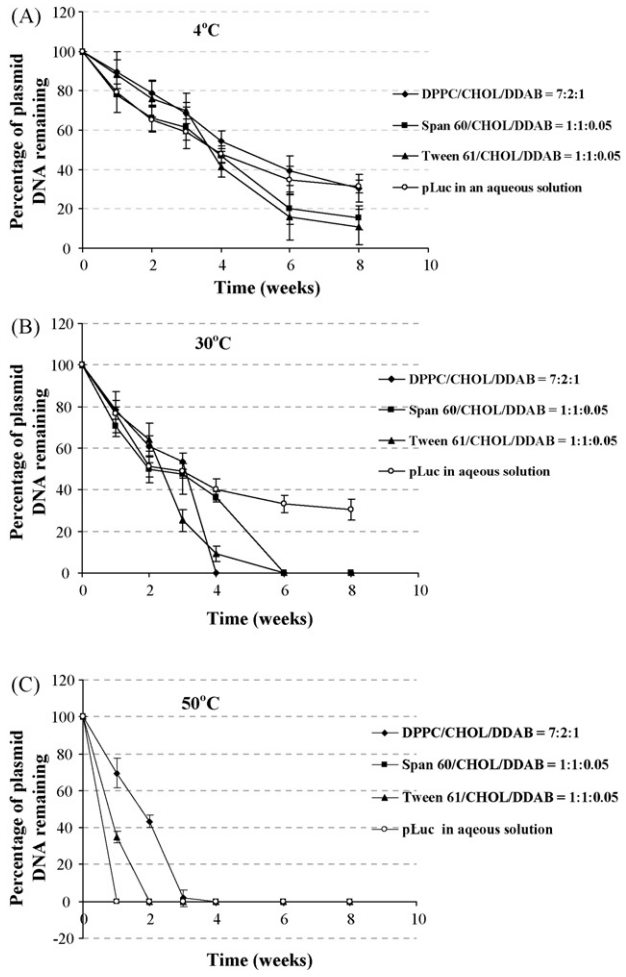


Fig. 8. The percentages of pLuc remaining in various cationic bilayer vesicles and in an aqueous solution after stored at 4 °C **(A)**, 30 °C **(B)**, and 50 °C **(D)** for 8 weeks.

times smaller particle size than cationic Tween61 and cationic Span60 niosomes, respectively.

All cationic bilayer vesicles entrapped with pLuc exhibited larger average particle size than the unentrapped cationic vesicles, since the negatively charged pLuc which adsorbed or entrapped in the vesicles may increase the aqueous phase volume resulting in an increase in particle sizes. The cationic bilayer vesicles such as liposomes, can form complexes with the negatively charged DNA (pLuc) via charge interaction, resulting in the multilamellar structures composed of DNA sandwiched between the lipid bilayers (Choosakoonkriang et al., 2001; Wiethoff et al., 2002). Thus, pLuc can both form complex with such cationic bilayer vesicles and entrap in the water channels between the vesicular membrane. This is supported by the work of Koltover et al. (1998).

The pLuc entrapped in all cationic bilayer vesicles still have stable (supercoil) form during 8 weeks, while pLuc in an aqueous solution degraded with times even stored at 4 °C. Cationic DPPC liposomes enhance the stability of pLuc 2 and 3 times higher than cationic Span60 and Tween61 vesicles at 4 °C for 8 weeks, respectively. At 30 °C, pLuc remaining in all bilayer vesicular formulations was not intact after 3 weeks. The remaining pLuc in cationic DPPC liposomes was the same as Span60 vesicles and 2 times more than cationic Tween61 vesicles. At 50 °C, pLuc in an aqueous solution was completely degraded after 1 week, while at 2 and 3 weeks, none was found in cationic Span60 and Tween61 niosomes, respectively. However, 2% of pLuc was found in cationic DPPC liposome at 3 weeks. The pLuc in cationic liposomes (DPPC/Chol/DDAB at 7:2:1 molar ratio) exhibited higher stability than in other bilayer vesicular formulations at all temperatures.

This study has showed that higher temperature facilitates the degradation of plasmid DNA in aqueous solution from the stable supercoiled form to the undesirable linear and open circular forms since high temperature as well as pH are important DNA degrading factors (Bauer et al., 2003; Alexander et al., 2004). DNA has the limitation for chemical and thermal stability. At 30 °C, pLuc entrapped in the vesicles was more stable than pLuc not entrapped in the vesicles. However, at high temperature at 50 °C, pLuc even entrapped in the vesicles was degraded the same as pLuc in the aqueous solution. Although plasmid DNA can prolong its stability when entrapped in vesicles in comparing to that in an aqueous solution, the plasmid both in an aqueous solution and in the vesicles at 30 and 50 °C was not in the desirable (supercoiled) form. At high temperature (50 °C) for a long times storage (8 weeks), the vesicular membrane may be destabilized, as well as the increase of hydrolytic degradation of the vesicular composition, especially the lipid product (Frrkjaer et al., 1982; Grit et al., 1989) resulting the leakage of the pLuc out of the liposomes and can be easily destroyed by heat. The increase of the hydroxyl radicals by high temperature were also involved in the damage to DNA in liposomes and in the solution.

It has been strongly indicated that the stability of the entrapped plasmid could have an impact on transfection efficiency. There is an understanding by many researchers that intact supercoil DNA is advantageous for efficient gene transfer over the open circular or linear DNA, from degradation process (Prazeres et al., 1999). The plasmid entrapped in cationic liposomes exhibited higher stability than plasmid in solution and plasmid entrapped in cationic Tween61 or Span60 niosomes. However, the remaining amount of plasmid DNA entrapped in cationic liposomes is lower than in aqueous solution, especially during a long time storage of 8 weeks at 30 °C. Nevertheless, pLuc remaining in an aqueous solution was all in the open circular form (Fig. 7) which is not a desirable form because of greater risk of the linear form integrating into the host genome (Nichols et al., 1995) and lower transfection efficacy of linear plasmids than the supercoiled form (Weintraub et al., 1986). On the contrary, pLuc remaining in liposomes was in the stable and desirable supercoiled form (Fig. 4.). So, it is still advantageous for entrapping the pLuc in liposomes for our further transfection study. In the case of niosomes, the sobilization properties of surfactants (Tween61 and Span60) which were the component of the cationic niosomal formulations (Manosroi et al., 2007a) may be another factor which can affect the integrity of the entrapped plasmid DNA, especially at high temperature and long time storage.

In summary, this study has suggested that the cationic liposome comprising of DPPC/Chol/DDAB at the molar ratio of

7:2:1 was the most suitable formulation for entrapping pLuc due to its higher protection of the plasmid against high temperature than other bilayer vesicles. The high gene transfection efficiency of plasmid entrapped in this formulation is also anticipated.

Acknowledgements

This work was supported by the Thailand Research Fund (TRF) under the RGJ-PhD program in Thailand, Pharmaceutical-Cosmetic Raw Materials & Natural Products Research and Development Center (PCRNC), Institute for Science and Technology (IST), Chiang Mai University, Thailand, Nanoscience and Nanotechnology Research Center Project, Faculty of Sciences, Chiang Mai University in Thailand, and Boehringer Ingelheim International GmbH, 55216 Ingelheim am Rhein in Germany.

References

Alexander, T.W., Sharma, R., Deng, M.Y., Whetsell, A.J., Jennings, J.C., Wang, Y., Okine, E., Damgaard, D., McAllister, T.A., 2004. Use of quantitative real-time and conventional PCR to assess the stability of the cp4 epsps transgene from Roundup Ready canola in the intestinal, ruminal, and fecal contents of sheep. J. Biotechnol. 112, 255–266.

Bauer, T., Weller, P., Hammes, W.P., Hertel, C., 2003. The effect of processing parameters on DNA degradation in food. Eur. Food Res. Technol. 217, 338–343.

Choosakoonkriang, S., Wiethoff, C.M., Smith, J.G., Russell, M.C., 2001. Infrared spectroscopic characterization of the interaction of cationic lipids with plasmid DNA. J. Biol. Chem. 276, 8037–8043.

Congiu, A., Pozzi, D., Esposito, C., Castellano, C., Mossa, G., 2004. Correlation between structure and transfection efficiency: a study of DC-Chol-DOPE/DNA complexes. Colloids Surf. B Biointerfaces 36, 43–48.

Domashenko, A., Gupta, S., Cotsarelis, G., 2000. Efficient delivery of transgenes to human hair follicle progenitor cells using topical lipoplex. Nat. Biotechnol. 18, 420–423.

Frrkjaer, S., Hjorth, E.L., Wrrts, O., 1982. Stability and storage of liposomes. In: Bundgaard, H., Bagger Hansen, A., Kofod, H. (Eds.), Optimization of Drug Delivery. Munksgaard, Copenhagen, p. 384.

Grit, M., de Smidt, J.H., Struijke, A., Crommelin, D.J.A., 1989. Hydrolysis of phosphatidylcholine in aqueous liposome dispersions. Int. J. Pharm. 50, 1–6.

Hao, Y., Zhao, F., Li, N., Yang, Y., Li, K., 2002. Studies on a high encapsulation of colchicine by a niosome system. Int. J. Pharm. 244, 73–80.

Hong, K., Zheng, W., Baker, Papahadjopoulos, D., 1997. Stabilization of cationic liposome-plasmid DNA complexes by polyamines and poly(ethylene glycol)-phospholipid conjugates for efficient *in vivo* gene delivery. FEBS Lett. 400, 233–237.

Hood, E., Leekumjorn, S., Bhethanabotla, V., VanAuker, M.D., 2003. Entrapment efficiency and membrane permeability of non-ionic surfactant vesicles. In: Proc. AIChE Annual Conference Proceeding CD-ROM, San Francisco, CA.

Huang, Y., Chen, J., Chen, X., Gao, J., Liang, W., 2007. PEGylated synthetic surfactant vesicles (Niosomes): novel carriers for oligonucleotides. J. Mater. Sci. Mater. M. (online first).

Jensen, T.G., 2004. Gene transfer into human epidermis as an experimental model for somatic gene therapy. Dan. Med. Bull. 51, 155–166.

Kikuchi, H., Suzuki, N., Ebihara, K., Morita, H., Ishii, Y., Kikuchi, A., Sugaya, S., Serikawa, T., Tanaka, K., 1999. Gene delivery using liposome technology. J Contr. Rel. 62, 269–277.

Koltover, I., Salditt, T., Ra¨dler, J.O., Safinya, C.R., 1998. An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery. Science 281, 78–81.

Manosroi, A., Kongkaneramit, L., Manosroi, J., 2004a. Stability and transdermal absorption of topical amphotericin B liposome formulations. Int. J. Pharm. 270, 279–286.

Manosroi, A., Kongkaneramit, L., Manosroi, J., 2004b. Characterization of Amphotericin B liposome formulations. Drug Dev. Ind. Pharm. 30, 535–543.

Manosroi, A., Panyosak, A., Rojanasakul, Y., Manosroi, J., 2007a. Characteristics and anti-proliferative activity of azelaic acid and its derivatives entrapped in bilayer vesicles in cancer cell lines. J. Drug. Target 15, 334–341.

Manosroi, A., Thathang, K., Werner, R.G., Schubert, R., Manosroi, J., 2007b. Cytoxicity of nanovesicular formulations on $B_{16}F_{10}$ cell lines by MTT assay. In: The 33rd Congress on Science and Technology of Thailand, October 18–21, 2007.

Manosroi, A., Wongtrakul, P., Manosroi, J., Sakai, H., Sugawara, F., Yuasa, M., Abe, M., 2003. Characterization of vesicles prepared with various non-ionic surfactants mixed with cholesterol. Colloids Surf. B Biointerfaces 30, 129–138.

Manosroi, A., Wongtrakul, P., Manosroi, J., Midorikawa, U., Hanyu, Y., Yuasa, M., Sugawara, F., Sakai, H., Abe, M., 2005. The entrapment of kojic oleate in bilayer vesicles. Int. J. Pharm. 298, 13–25.

Meilander, N.J., Pasumarthy, M.K., Kowalczyk, T.H., Cooperb, M.J., Bellamkonda, R.V., 2003. Sustained release of plasmid DNA using lipid microtubules and agarose hydrogel. J. Contr. Rel. 88, 321–331.

Morgan, J.R., Williams, K., 1980. Preparation and properties of liposome-associated gentamicin. Antimicrob. Agents Ch. 17, 544–548.

Nishikage, S., Koyama, H., Miyata, T., Ishii, S., Hamada, H., Shigematsu, H., 2004. *In vivo* electroporation enhances plasmid-based gene transfer of basic fibroblast growth factor for the treatment of ischemic limb. J. Surg. Res. 120, 37–46.

Nichols, W.W., Ledwith, B.J., Manam, S.V., Troilo, P.J., 1995. Potential DNA vaccine integration into host cell genome. Ann. NY Acad. Sci. 27, 30–39.

Patil, S.D., Rhodes, D.G., Burgess, D.J., 2004. Anionic liposomal delivery system for DNA transfection. AAPS J. 6, 1–10.

Perrie, Y., Barralet, J.E., McNeil, S., Vangala, A., 2004. Surfactant vesicle-mediated delivery of DNA vaccines via the subcutaneous route. Int. J. Pharm. 284, 31–41.

Prazeres, D.M., Ferreira, G.N.M., Monteiro, G.A., Cooney, C.L., Cabral, J.M.S., 1999. Large-scale production of pharmaceutical-grade plasmid DNA for gene therapy: problems and bottlenecks. Trends Biotechnol. 17, 169–174.

Presti, F.T., Pace, R.J., Chen, S.I., 1982. Cholesterol–phospholipid interaction in membranes. Stoichiometry and molecular packing of cholesterol-rich domains. Biochemistry 21, 3831–3835.

Richardson, V.J., Jeyasingh, K., Jewkes, R.F., Ryman, B.E., Tattersall, M.H.N., 1978. Possible tumor localization of Tc-99m-labeled liposomes: effects of lipid composition, charge, and liposome size. J. Nuel. Med. 19, 1049–1054.

Shahiwala, A., Misra, A., 2002. Studies in topical application of niosomally entrapped Nimesulide. J Pharm Pharmaceut Sci. 5, 220–225.

Tokunaga, M., Hazemoto, N., Yotsuyanagi, T., 2004. Effect of oligopeptides on gene expression: comparison of DNA/peptide and DNA/peptide/liposome complexes. Int. J. Pharm. 269, 71–80.

Torchilin, V.P., Levchenko, T.S., Rammohan, R., Volodina, N., Sternberg, B.P., D'Souza, G.M., 2003. Cell transfection *in vitro* and *in vivo* with non-toxic TAT peptide-liposome–DNA complexes. PNAS 100, 1972–1977.

Vyas, S.P., Singh, R.P., Jain, S., Mishra, V., Mahor, S., Singh, P., Gupta, P.N., Rawat, A., Dubey, P., 2005. Non-ionic surfactant based vesicles (niosomes) for non-invasive topical genetic immunization against hepatitis B. Int. J. Pharm. 296, 80–86.

Weintraub, H., Cheng, P.F., Conrad, K., 1986. Expression of transfected DNA depends on DNA topology. Cell 46, 115–122.

Wiethoff, C.M., Gill, M.L., Koe, G.S., Koe, J.G., Russell, M.C., 2002. The structural organization of cationic lipid-DNA complexes. J. Biol. Chem. 277, 44980–44987.

Wolff, J.A., Vladimir, B., 2005. The Mechanism of Naked DNA Uptake and Expression. Adv. Gen. 54, 1–20.

Zhang, L., Nolan, E., Kreitschitz, S., Rabussay, D.P., 2002. Enhanced delivery of naked DNA to the skin by non-invasive *in vivo* electroporation. Biochim. et Biophys. Acta 1572, 1–9.