약제학회지(2005), 제 35 권 제 5 호
J. Kor. Pharm. Sci., Vol. 35, No. 5, 337-341 (2005)

Encapsulation of Plasmid DNA in Pegylated Liposome

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(Received August 14, 2005 · Accepted October 17, 2005)

ABSTRACT – The purpose of the study was to prepare the pegylated liposome carrying plasmid DNA with optimal encapsulation efficiency. Plasmid DNA (pCEP4 clone 790, 10.6 kb) was entrapped in the pegylated liposome composed of neutral lipid, POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), cationic lipid, DDAB (dimethyl dioctadecyl ammonium bromide) and anionic lipids, DSPE-PEG 2000 (distearoyl phosphatidyl ethanolamine polyethylene glycol 2000) and DSPE-PEG 2000-maleimide by freezing/thawing method. Free plasmid DNA was separated from the encapsulated one by Sepharose CL-4B column chromatography. The DNA amount encapsulated into the pegylated liposome was increased as cationic lipid concentration, initial amount of plasmid DNA and total lipid amount were increased.

Key words – pCEP4 clone 790 DNA, Pegylated liposome, Encapsulation

Gene therapy promises to prevent, treat or cure both acquired and inherited genetic diseases such as cancer, cystic fibrosis and autoimmune disease. The successful gene therapy is depending on the delivery system which can efficiently and selectively deliver the gene to the target site with minimal toxicity. Recombinant viruses have been demonstrated as useful vectors for transducing cells both *in vitro* and *in vivo*. However, the use of viral vectors is limited due to immunogenicity, pathogenicity, size limitation of gene carried and high production expense.[1-5] In addition, viral vectors are rapidly cleared from the circulation, limiting gene delivery to first pass organs such as the liver, spleen and lung.[6,7]

Due to these problems of viral vectors, non-viral vectors, such as cationic lipid or cationic polymer, have been investigated as the alternatives to the viral system.[8,9] Although non-viral vectors are suitable for large-scale production, not limited by gene size and have low immunogenicity, there are still some drawbacks to overcome. The transfection sites by cationic lipid/DNA complexes (lipoplexes) are limited to the lung and liver due to the large size and excessive positive charge. In addition, lipoplexes have relatively low transfection efficiency compared with viral vectors and may result in toxicity *in vitro* and *in vivo*.[9-11] Although cationic polymers can condense DNA efficiently, form a relatively small and stable DNA/polymer complexes, and conjugate various ligands for targeted gene delivery, there are some shortcomings as follows: poor solubility and biodegradability, rapid plasma protein binding and clearance from the circulation, and toxicity such as lung infarction and activation of complement.[10,12-14)]

Therefore, it is necessary to develop the gene delivery system that has long systemic circulation time, sufficient safety *in vivo*, and greater gene expression in the target site. For this purpose, the present study was performed to prepare the pegylated liposome[15] containing pCEP4 clone 790 DNA which can enhance the systemic circulation time and to investigate the effects of various factors on the plasmid DNA amount encapsulated within the pegylated liposome to optimize the encapsulation efficiency.

Methods

Materials

POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) and DDAB (dimethyl dioctadecyl ammonium bromide) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). DSPE-PEG 2000 (distearoyl phosphatidyl ethanolamine polyethylene glycol 2000) and DSPE-PEG 2000-maleimide were obtained from Shearwater Polymers (Huntsville, AL, USA). [α-^{32}P] dCTP (3000 Ci/mmol) was purchased from Perkin Elmer Life Sciences (Boston, MA, USA), Microscint 40 from Packard (Meriden, CT, USA) and Nick translation system from Invitrogen (Carlsbad, CA, USA). Pancreatic DNase I and Exonuclease III were supplied by Promega (Madison, WI, USA). QIAfilter Plasmid Maxi kit was purchased from Qiagen (Chatsworth, CA, USA), Sepharose CL-4B from Amersham Pharmacia (Uppsala, Sweden) and ampicillin from Sigma-Aldrich (St. Louis, MO, USA). HEPES, Tris/base and LB media were obtained from USB (Cleveland, OH, USA). Econo chro-

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matography column (1.5 × 10 cm) was purchased from Bio-Rad (Hercules, CA, USA) and Sephadex G-25 quick spin column from Roche Diagnostics Co. (Indianapolis, IN, USA). Liposofast extruder and polycarbonate filters were supplied by Avestin (Ottawa, Canada). Plasmid DNA (pCEP4 clone 790) was kindly gifted by Dr. William M. Pardridge (UCLA, LA, USA).

Plasmid DNA preparation and radio-labeling

E. coli transformed with plasmid DNA (pCEP4 clone 790, 10.6 kb) was inoculated into 4 mL of LB media containing 100 μg/mL ampicillin for 8 hrs at 37°C with vigorous shaking. This culture was poured into 150 mL of LB media and incubated for 18-20 hrs at 37°C with shaking. Plasmid DNA was purified from *E. coli* using QIAfilter Plasmid Maxi kit, dissolved in TE buffer [10 mM Tris/1 mM EDTA (pH 8.0)], and stored at 4°C. Plasmid DNA concentration was determined by measuring UV absorbance at 260 nm using UV/VIS Spectrophotometer DU-18 (Beckman Co., Fullerton, CA, USA). The purity of plasmid DNA was examined by the measurement of the absorbance at 260 nm (A_{260}) and at 280 nm (A_{280}). The ratio of A_{260} to A_{280} was over 1.7.

Plasmid DNA (1 μg) was radio-labeled with [α-^{32}P] dCTP and purified using Sephadex G-25 quick spin column as described previously.[16] The purity of radio-labeled plasmid DNA was over 95%. The radio-labeled plasmid DNA was used to calculate the amount of plasmid DNA encapsulated into the pegylated liposome.

Pegylated liposome synthesis and plasmid DNA encapsulation

Neutral lipid, POPC (9.2-27.6 μmole), cationic lipid, DDAB (0.4-1.6 μmole), anionic lipids, DSPE-PEG 2000 (0.3-0.9 μmole) and DSPE-PEG 2000-maleimide (0.1-0.3 μmole) were dissolved in chloroform, and then evaporated under nitrogen gas stream for 10 min to form a thin layer lipid film. The dried thin layer lipid film was incubated at room temperature (R.T.) for 30 min and dispersed in 200 μL of 0.05 M Tris/HCl (pH 7.0). Subsequently, the pegylated liposome dispersion was sonicated for 2 min using a Branson 3210 sonicator (Branson Ultrasonic Co., Danbury, CT, USA) and vortexed for 90 sec. An appropriate amount of plasmid DNA (10-300 μg) and radio-labeled plasmid DNA (1 μCi) were added to the pegylated liposome. Then, the pegylated liposome was frozen in ethanol/dry ice for 5 min and thawed at R.T. for 25 min.[17] This freezing/thawing cycle was repeated six times. The pegylated liposome was diluted with 0.05 M HEPES (pH 7.0) and final volume was adjusted to 500 μL, followed by extrusion 5 times through each of two stacked polycarbonate filters with pore size of 200 and 100 nm using a Liposofast extruder (Avestin, Ottawa, Canada). The non-encapsulated plasmid DNA was digested by adding pancreatic DNase I (10 U), Exonuclease III (50 U) and 5 mM $MgCl_2$. After incubating at 37°C for 1 hr, the reaction was stopped by the addition of 20 mM EDTA. The sample was applied to a 1.5 × 10 cm Sepharose CL-4B column, and the digested plasmid DNA was separated from the DNA encapsulated into the pegylated liposome. The amount of DNA encapsulated into the pegylated liposome was determined by measuring [^{32}P]-radioactivity using a Microplate scintillation & Luminescence counter (TopCount NXT, Packard Instrument Co., Meriden, CT, USA).

Liposome size measurement

Liposome size was measured using a zeta potential/particle sizer, NICOMP™ 380 ZLS (PSS·NICOMP, Santa Barbara, CA, USA) in the volume-weighted mode. The liposome was composed of 92 mole% POPC, 4 mole% DDAB, 3 mole% DSPE-PEG 2000 and 1 mole% DSPE-PEG 2000 maleimide, and was extruded through two stacked polycarbonate filters with pore size of 100 nm. Then, the liposome was diluted to the appropriate volume with distilled water for the size measurement.

Results and Discussion

Plasmid DNA (pCEP4 clone 790, 10.6 kb) was encapsulated into the pegylated liposome composed of POPC (9.2-27.6 μmole), DDAB (0.4-1.6 μmole), DSPE-PEG 2000 (0.3-0.9 μmole) and DSPE-PEG 2000-maleimide (0.1-0.3 μmole) by freezing/thawing method (Figure 1). As shown in Figure 2, the separation between plasmid DNA entrapped in the pegylated

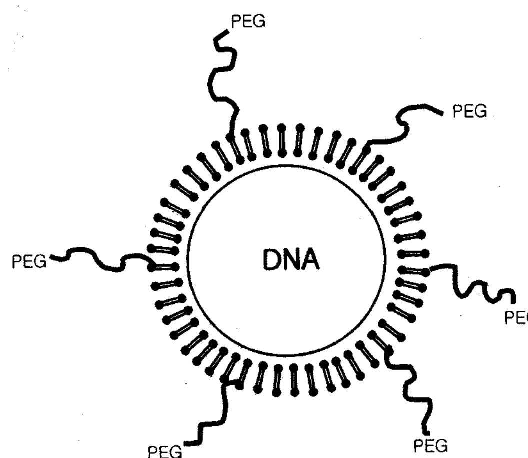

Figure 1–Structure of the pegylated liposome containing pCEP4 clone 790 DNA.

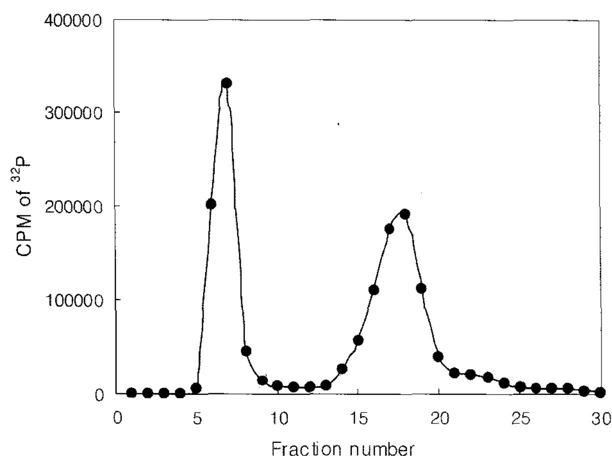

Figure 2–Sepharose CL-4B gel filtration chromatography. Plasmid DNA entrapped in the pegylated liposome (first peak) was separated from the exteriorized nuclease-digested plasmid DNA (second peak).

liposome (first peak) and the exteriorized nuclease-digested DNA (second peak) was accomplished by Sepharose CL-4B column chromatography based on the size.[16-18)]

The study was conducted with various concentrations of DDAB (0.4, 0.8, 1.2 and 1.6 μmole) to examine the effect of cationic lipid (DDAB) concentration on DNA amount entrapped in the pegylated liposome. In the study, anionic lipid concentrations (DSPE-PEG 2000 and DSPE-PEG 2000-maleimide) were kept constant while neutral lipid (POPC) concentration was decreased with increasing DDAB concentration to maintain total lipid amount as 20 μmole. As DDAB content was increased, DNA amount encapsulated was also increased (Figure 3). DNA amount entrapped in the pegylated liposome using 8 mole% DDAB were 7-fold greater than that using 2 mole% DDAB. This result corresponds to the finding of Sarabolac *et al.* that 2 mole% increase in DODAC (N,N-dioleoyl-N,N-dimethylammonium chloride) concentration resulted in 40% increase in the encapsulation efficiency of plasmid (pCMVLuc) in stabilized plasmid-lipid particles (SPLP).[19)]

The effects of initial amount of plasmid DNA and total lipid amount on DNA amount encapsulated into the pegylated liposome were also examined. As initial DNA amount was increased from 10 to 300 μg, DNA amount entrapped in the pegylated liposome was also gradually increased (Figure 4 (A)). For DNA encapsulation efficiency, there were no clear changes from 10 to 100 μg of initial DNA amount whereas the efficiency was clearly reduced at 200 and 300 μg of initial DNA amount (Figure 4(B)). It has been reported that entrapment yields were not dependent on the initial amount of DNA

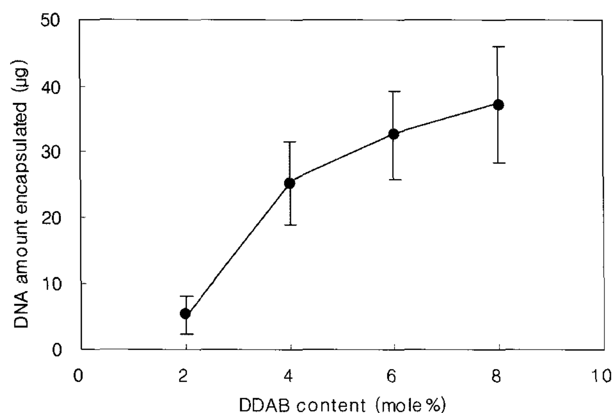

Figure 3–Effect of DDAB concentration (2-8 mole%) on DNA amount encapsulated into the pegylated liposome. pCEP4 clone 790 DNA (100 μg) and [^{32}P]-DNA (1 μCi) were encapsulated into the pegylated liposome composed of POPC, DDAB, DSPE-PEG 2000 and DSPE-PEG 2000-maleimide. Each data point represents the mean±S.D. from triplicate experiments.

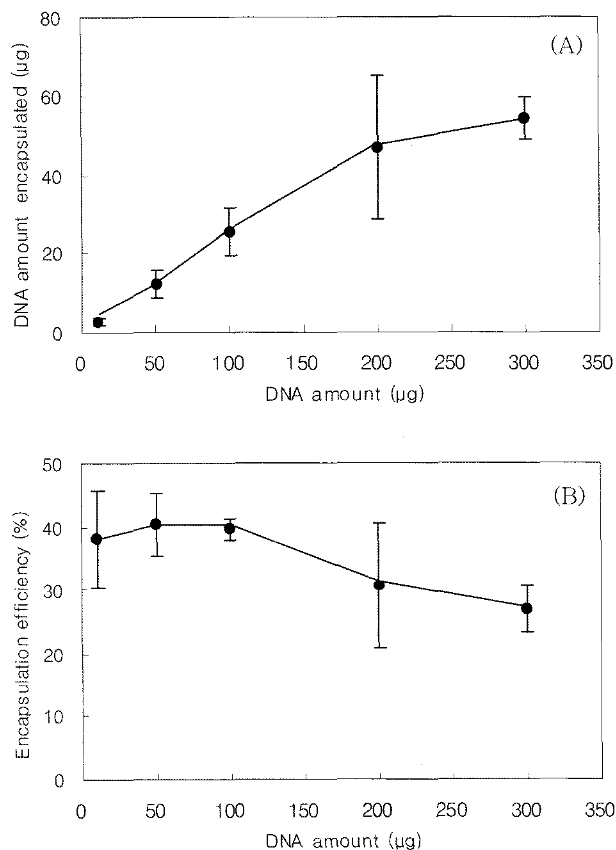

Figure 4–Effect of initial DNA amount (10-300 μg) on DNA amount encapsulated into the pegylated liposome (A) and encapsulation efficiency (B). The total lipid amount was maintained as 20 imole and the liposome was composed of 92 mole% POPC, 4 mole% DDAB, 3 mole% DSPE-PEG 2000 and 1 mole% DSPE-PEG 2000-maleimide. Each data point represents the mean±S.D. from triplicate experiments.

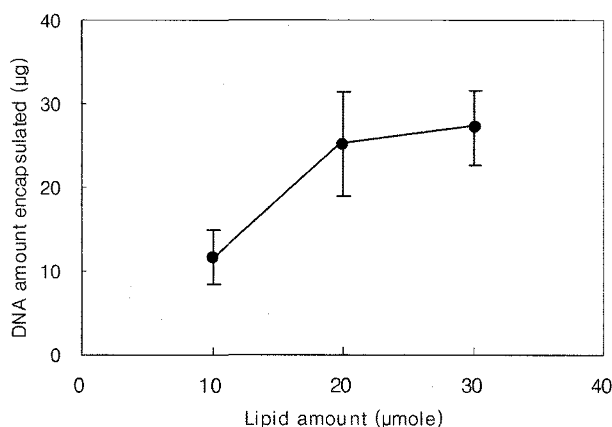

Figure 5–Effect of total lipid amount (10-30 μmole) on DNA amount encapsulated into the pegylated liposome. The liposome was composed of 92 mole% POPC, 4 mole% DDAB, 3 mole% DSPE-PEG 2000 and 1 mole% DSPE-PEG 2000-maleimide using 100 μg of plasmid DNA. Each data point represents the mean±S.D. from triplicate experiments.

between 0.8 and 100 μg.[17] However, DNA encapsulation efficiency was reduced with increasing plasmid DNA concentration from 100 to 1000 μg/mL.[19] Keeping initial DNA amount at 100 μg, increase in total lipid amount from 10 to 30 ìmole resulted in an increase of total DNA amount encapsulated within the pegylated liposome (Figure 5). However, in the case of 30 μmole lipid, the extrusion of liposome dispersion through polycarbonated filters was not easy due to its high viscosity. According to Sarabolac *et al.,* as lipid concentration was increased from 1 to 10 mg/mL, encapsulation efficiency was also increased, and at high encapsulation efficiency (>70%), aggregation of the formulations was observed.[19] Therefore, it is suggested that there are optimal initial amount of DNA and total lipid amount to promote encapsulation efficiency.

After extrusion through polycarbonate filters with pore size of 100 nm, the liposome size was determined to 113.4±12.2 nm using a Zeta Potential/Particle Sizer. The small liposomes ranging from 100 to 200 nm in diameter could take advantage of transfection to target site efficiently.[20]

In the present study, we prepared the pegylated liposome containing pCEP4 clone 790 DNA (10.6 kb) and investigated several factors that can affect encapsulation amount and efficiency. The pegylated liposome has been shown to accumulate into various form of solid tumor. However, there was no specific gene expression with it.[20] Therefore, to deliver gene to target site more effectively and selectively, the ligands such as monoclonal antibody, epidermal growth factor, transferrin and insulin should be conjugated to the pegylated liposome in the future study.

Conclusions

The pegylated liposome containing pCEP4 clone 790 DNA was prepared by freezing/thawing method. The DNA amount entrapped in the pegylated liposome was increased as cationic lipid concentration, initial amount of plasmid DNA, and total lipid amount were increased.

Acknowlegements

This work was supported by the Intramural Research Grant of Ewha Womans University (2004) and the Korea Research Foundation Grant (KRF-2004-005-C00090).

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