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Immunoliposomes Carrying Plasmid DNA : Preparation and Characterization

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The objective of this study was to characterize immunoliposomes carrying plasmid DNA with optimal encapsulation efficiency and antibody density. Plasmid DNA was encapsulated by the freezing/thawing method into liposomes composed of POPC (1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine), DDAB (didodecyl dimethyl ammonium bromide), DSPE-PEG 2000 (distearoyl phosphatidyl ethanolamine polyethylene glycol 2000) and DSPE-PEG 2000-maleimide. The liposomes carrying plasmid DNA were extruded through two stacked polycarbonate filters, of different pore size, to control the liposome size. Then, rat IgG molecules were conjugated to the liposomes. The immunoliposomes containing plasmid DNA were separated from the free plasmid DNA and unconjugated IgG by Sepharose CL-4B column chromatography. The DNA amount encapsulated was affected by DDAB (cationic lipid) concentration, the initial amount of plasmid DNA between 10 μg and 200 μg, the total lipid amount and plasmid DNA size, but not significantly by liposome size. By varying the ratio of DSPE-PEG 2000-maleimide to IgG, the number of IgG molecules per liposome was changed significantly.

Key words: Plasmid DNA, IgG, Immunoliposome, Encapsulation, Conjugation

INTRODUCTION

Gene targeted therapy has become a viable possibility for the treatment of diseases such as cancer. The efficacy of gene therapy places considerable dependence on the delivery system which can efficiently and selectively deliver the gene to the target site with minimal side effect.

Although the virus has been a useful vector for transducing cells, viral systems are rapidly cleared from the circulation, limiting delivery to first-pass organs such as the liver and spleen (Miller, 1992; Worgall, 1997). In addition, viral vectors may induce immune responses and nonspecific inflammation in humans (Boris-Lawrie and Temin, 1993; Smith *et al.*, 1993; Setoguchi *et al.*, 1994; Yang *et al.*, 1995a, b). Therefore, non-viral delivery systems such as cationic lipid or cationic polymer, which are suitable for large-scale production, not limited by vector size and have low immunogenicity, have been studied as an attractive alternative to the viral system (Lamb *et al.*, 1993; Nabel *et al.*, 1993; Li and Huang, 2000). However, numerous studies have shown that these systems are also cleared rapidly by the reticuloendothelial system and that the transfection sites are limited to the lung and liver due to the large size and positively charged characteristics (Li and Huang, 2000; Schatzlein, 2001). In addition, plasmid DNA-cationic lipid complexes can result in toxic side effect *in vitro* and *in vivo* (Harrison *et al.*, 1995; Li and Huang, 1997). Although much longer circulation times and lower toxicities can be achieved by liposomes composed of neutral lipid, reduced encapsulation efficiency and gene expression level are disadvantages of these neutral liposomes compared with cationic liposomes. Many research groups have tried to overcome these problems (Bailey and Sullivan, 2000; Stuart and Allen, 2000; Pastorino *et al.*, 2001).

Therefore, it is necessary to develop a gene delivery system which has a longer blood circulation time, sufficient safety for human use and greater gene expression in the target site. For this purpose, we prepared pegylated liposomes carrying plasmid DNA, and then investigated the effect of the following various parameters on the DNA amount encapsulated into the liposomes: cationic lipid concentration, initial DNA amount, total lipid amount, DNA size and liposome size. Furthermore, to prepare immunoliposomes capable of both gene delivery and targeting, we conjugated rat IgG to the liposomes carrying plasmid

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DNA and examined the effect of the ratio of DSPE-PEG 2000-maleimide to rat IgG on the number of IgG molecules conjugated per liposome.

MATERIALS AND METHODS

Materials

POPC (1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine) and DDAB (didodecyl dimethyl ammonium bromide) were purchased from Avanti-Polar Lipids Inc. (Alabaster, AL, USA), and DSPE-PEG 2000 (distearoyl phosphatidyl ethanolamine polyethylene glycol 2000) and DSPE-PEG 2000-maleimide from Shearwater Polymers (Huntsville, AL, USA). [α-^{32}P]dCTP (3000 Ci/mmol) was obtained 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, Exonuclease III and Wizard® Plus Midipreps DNA Purification System were obtained from Promega (Madison, WI, USA). *N*-succinimidyl[2,3-^{3}H]propionate ([^{3}H]-NSP, 101 Ci/mmol) and Sepharose CL-4B were purchased from Amersham Biosciences (Uppsala, Sweden). Ampicillin and LB broth powder were supplied by USB (Cleveland, OH, USA). Econo chromatography column (1.5×10 cm) was from Bio-Rad (Hercules, CA, USA), Sephadex G-25 quick spin column was from Roche Diagnostics Co. (Indianapolis, IN, USA), and Liposofast extruder was from Avestin (Ottawa, Canada). Traut's Reagent (2-imminothiolane·HCl) was purchased from Pierce Chemical Co. (Rockford, IL, USA). Centricon YM-30 (molecular weight cut-off: 30,000) was obtained from Amicon Co. (Beverly, MA, USA). Clone 753, 756, and 790 plasmids were generously gifted by Dr. William M. Pardridge (University of California, Los Angeles, USA). Rat IgG was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plasmid DNA preparation and radio-labeling

E. coli transformed with plasmid DNA (pGL2 clone 753, pSV clone 756 or pCEP4 clone 790) were inoculated into LB media with 100 μg/mL ampicillin for 16 - 20 h at 37°C. Plasmid DNA was purified by Wizard® Plus Midipreps DNA Purification System and dissolved in TE buffer [10 mM Tris/1 mM EDTA (pH 8.0)]. Plasmid DNA concentration was determined by measuring UV absorbance at 260 nm using UV/VIS Spectrophotometer DU-18 (Beckman Co., Fullerton, CA, USA).

Plasmid DNA was labeled with [α-^{32}P]dCTP by Nick translation method using DNA polymerase I and DNase I, as described previously (Shi and Pardridge, 2000). Unincorporated plasmid DNA was removed by G25 Sephadex column. The purity of [^{32}P]-plasmid DNA was over 95%. [^{32}P]-plasmid DNA was used as a marker to measure the DNA amount encapsulated within the liposomes.

Liposome formation and plasmid DNA encapsulation

Neutral lipid, POPC (9.2 μmole - 27.6 μmole), cationic lipid, DDAB (0.2 μmole - 1.4 μmole), anionic lipid DSPE-PEG 2000 (0.3 μmole - 0.9 μmole) and DSPE-PEG 2000-maleimide (0.1 μmole - 0.3 μmole) were firstly dissolved in chloroform, and then evaporated under N_2 gas stream to produce a thin layer lipid film. The dried lipid film was dispersed in 200 μL of 0.05 M Tris-HCl buffer (pH 7.0) and sonicated for 2 min using a Branson 3210 sonicator (Branson Ultrasonic Co., Danbury, CT, USA), followed by 2 min of vortexing. An appropriate amount of plasmid DNA and 1 μCi of [^{32}P]-plasmid DNA were added to the liposome dispersion. The liposome dispersion was frozen in ethanol/dry ice for 5 min and thawed at room temperature for 25 min. This freeze-thaw cycle was repeated 6 times. The liposome dispersion was diluted to a lipid concentration of 40 mM by adding 0.05 M HEPES buffer (pH 7.0), followed by extrusion 5 times through each of two stacked polycarbonate filters with pore size of 200 nm and 100 nm. For liposome size measurement, 400 nm pore size filters were used as the first extrusion step. 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 for 1 h at 37°C, the reaction was stopped by adding 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 liposomes. Usually 26 - 30 elutes of about 1 mL each were collected. The fractions 7 - 9 were usually turbid, indicating that they contained a great number of liposomes, while the others did not show visible turbidity (Huwyler *et al.*, 1996; Monnard *et al.*, 1997; Shi and Pardridge, 2000). The analysis of plasmid DNA encapsulated in the liposomes was accomplished by measuring [^{32}P]-radioactivity using a liquid scintillation counter (TopCount NXT, Packard Instrument Co., Meriden, CT, USA).

Liposome size measurement

Liposome size was determined by a Zeta Potential/Particle Sizer, NICOMP™ 380 ZLS (PSS·NICOMP, Santa Barbara, CA, USA), operated in the volume-weighted mode. The liposomes extruded through 400 nm, 200 nm or 100 nm pore size filters were diluted to the appropriate volume with distilled water.

Conjugation of rat IgG to the liposomes carrying plasmid DNA

The rat IgG was radio-labeled with *N*-succinimidyl[2,3-^{3}H]propionate ([^{3}H]-NSP) as described previously (Pardridge *et al.*, 1992). Briefly, 1 mCi of [^{3}H]-NSP was added to 100 μg of rat IgG dissolved in 100 μL of 0.1 M sodium borate

(pH 8.5)/0.5 M NaCl. The mixture was then shaken at 4°C for 45 min. [^{3}H]-IgG was separated from unreacted [^{3}H]-NSP by elution through a 0.7×28 cm Sephadex G25 column with 0.01 M Na_2HPO_4/0.15 M NaCl (pH 7.4). [^{3}H]-IgG had a specific activity of 0.11 μCi/μg and trichloroacetic acid (TCA) precipitability of 98%.

An appropriate amount of rat IgG (3.0 mg or 1.5 mg) was thiolated with 40:1 molar excess of 2-imminothiolane (Traut's reagent), as described previously (Huwyler *et al.*, 1996). In brief, rat IgG and 1 μCi of [^{3}H]-IgG were dissolved in 0.15 M sodium borate/0.1 mM EDTA (pH 8.5), followed by the addition of Traut's reagent. After incubation at room temperature for 60 min, rat IgG solution was concentrated using Centricon YM-30 and the buffer was exchanged with 0.05 M HEPES/0.1 mM EDTA (pH 7.0). The thiolated rat IgG containing [^{3}H]-IgG was conjugated to the liposomes carrying plasmid DNA overnight at room temperature. Unconjugated IgG was separated by Sepharose CL-4B column chromatography (Shi and Pardridge, 2000). Each column elute was analyzed by liquid scintillation counting. The number of IgG molecules conjugated per liposome was calculated from the total [^{3}H]-IgG cpm in the column elutes containing liposomes and the specific activity of the labeled IgG, assuming 10^5 lipid molecules per liposome, as described previously (Huwyler *et al.*, 1996).

RESULTS

Encapsulation of plasmid DNA in liposomes and synthesis of immunoliposomes

Plasmid DNA was encapsulated within neutral liposomes composed of 92 mole% POPC, 4 mole% DDAB, 3 mole% DSPE-PEG 2000 and 1 mole% DSPE-PEG 2000-maleimide by freezing/thawing method. Then the thiolated rat IgG was conjugated to the linker lipid, DSPE-PEG 2000-maleimide.

By Sepharose CL-4B column chromatography, immunoliposomes were separated from exteriorized nuclease-digested DNA and from unconjugated IgG (Fig. 1). The peak of [^{3}H]-IgG conjugated to the liposomes was overlapped with the peak of [^{32}P]-DNA encapsulated within the liposomes, suggesting that the plasmid DNA and the IgG were incorporated in the same structure.

Effect of cationic lipid concentration on DNA amount encapsulated

To investigate the effect of cationic lipid (DDAB) concentration on the DNA amount encapsulated within the liposomes, a series of studies were performed by varying DDAB concentration. DDAB content used in this study was 1, 3, 4, 5, and 7 mole% (0.2, 0.6, 0.8, 1.0, and 1.4 μmole). To maintain the total lipid amount as 20 μmole, neutral lipid (POPC) concentration was decreased according to increasing DDAB concentration. DSPE-PEG 2000 and DSPE-PEG 2000-maleimide concentrations were kept constant at 3 mole% and 1 mole%, respectively. Also, 100 μg of plasmid DNA (pGL2 clone 753) and 1 μCi of [^{32}P]-DNA were used. As shown in Fig. 2, increasing DDAB content resulted in an increase in the DNA amount encapsulated into the liposomes. Using 7 mole% DDAB, the amount of DNA encapsulated into the liposomes was about 8-fold greater than that using 1 mole% DDAB.

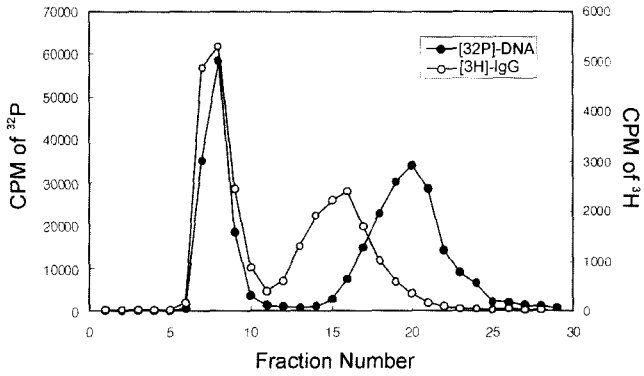

Fig. 1. Sepharose CL-4B gel filtration chromatography. Immunoliposomes (left-hand peaks) were separated from the exteriorized nuclease-digested plasmid DNA and unconjugated IgG (right-hand peaks).

Effect of initial plasmid DNA and total lipid amount on DNA amount encapsulated

The influence of the initial amount of plasmid DNA on the DNA amount encapsulated into the liposomes was investigated with the liposomes composed of 92 mole% POPC, 4 mole% DDAB, 3 mole% DSPE-PEG 2000 and 1

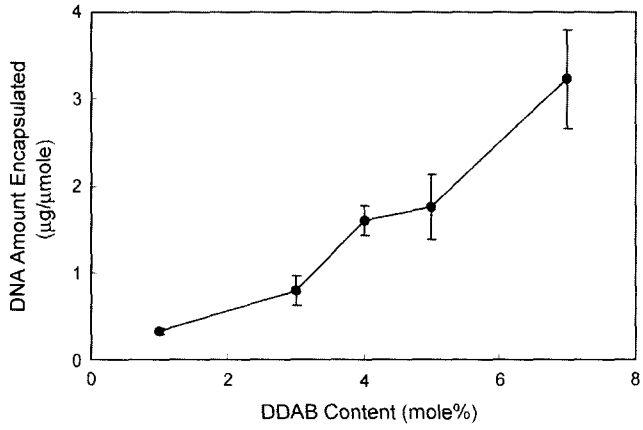

Fig. 2. Effect of cationic lipid concentration on DNA amount encapsulated into the liposomes. Plasmid DNA (pGL2 clone 753, 100 μg) and [^{32}P]-DNA (1 μCi) were encapsulated into the liposomes composed of DDAB (1 to 7 mole%), POPC, DSPE-PEG 2000 and DSPE-PEG 2000-maleimide. Each data point represents the mean ± S.D. from triplicate experiments.

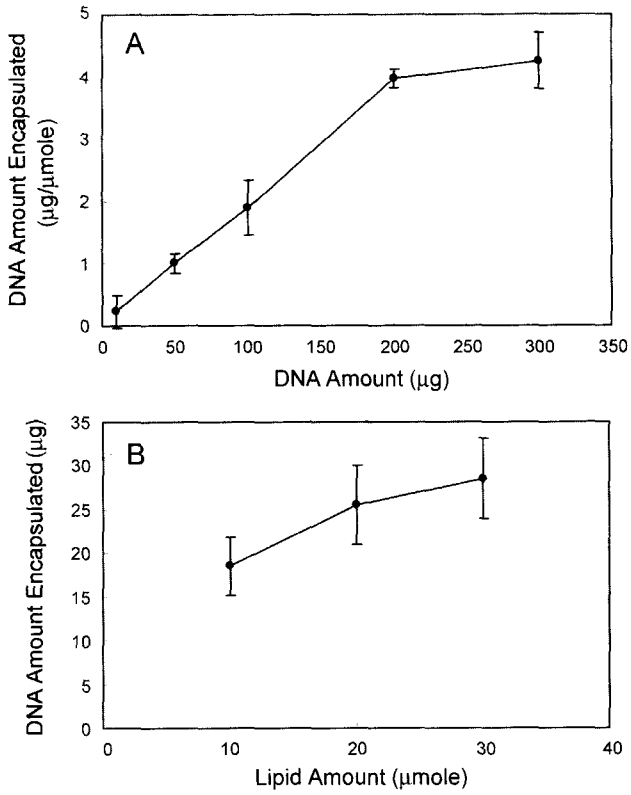

Fig. 3. Effect of initial DNA and total lipid amount on DNA amount encapsulated into the liposomes. The liposomes were composed of 92 mole% POPC, 4 mole% DDAB, 3 mole% DSPE-PEG 2000 and 1 mole% DSPE-PEG 2000-maleimide. (A) Effect of the initial DNA amount on the DNA amount encapsulated into the liposomes. The total lipid amount was kept at 20 μmole and 10 to 300 μg of plasmid DNA (pGL2 clone 753) and 1 μCi of [^{32}P]-DNA were used for the encapsulation. (B) Effect of the total lipid amount on the DNA amount encapsulated into the liposomes. The initial DNA amount was kept at 100 μg and the total lipid amount was varied between 10 μmole and 30 μmole. Each data point represents the mean ± S.D. from 3-5 experiments.

mole% DSPE-PEG 2000-maleimide. When the DNA amount was varied between 10 μg and 300 μg at a constant total lipid amount of 20 μmole, the DNA amount encapsulated within the liposomes increased linearly as the initial DNA amount increased up to 200 μg (Fig. 3A). There were no clear differences in DNA encapsulation efficiency from 10 μg to 200 μg initial DNA amount but the efficiency was clearly reduced at 300 μg initial DNA amount (Park *et al.*, 2003).

While maintaining the initial plasmid DNA amount at 100 μg, increasing the total lipid amount from 10 μmole to 30 μmole resulted in an increase of the total DNA amount encapsulated within the liposomes (Fig. 3B). However, in the case of 30 μmole lipid, the extrusion of liposome dispersion through the polycarbonated filters was difficult due to its high viscosity.

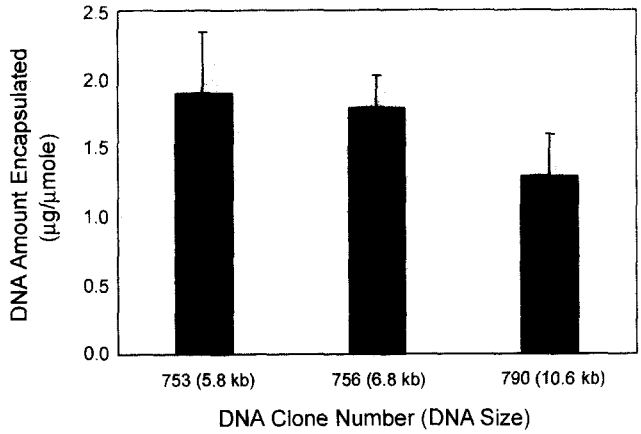

Fig. 4. Comparison of DNA amount encapsulated with different size of plasmid DNA. The liposomes were composed of 92 mole% POPC, 4 mole% DDAB, 3 mole% DSPE-PEG 2000 and 1 mole% DSPE-PEG 2000-maleimide. Also, 100 μg of plasmid DNA and 1 μCi of [^{32}P]-DNA were used. Data represent the mean ± S.D. from 3-5 experiments.

Effect of plasmid size on DNA amount encapsulated

The effect of plasmid size on the DNA amount encapsulated into the liposomes was examined for the liposomes composed of 92 mole% POPC, 4 mole% DDAB, 3 mole% DSPE-PEG 2000 and 1 mole% DSPE-PEG 2000-maleimide (Fig. 4). The plasmids used in this study were pGL2 clone 753 (5.8 kb), pSV clone 756 (6.8 kb) and pCEP4 clone 790 (10.6 kb). The DNA amount encapsulated into the liposomes was reduced with increasing plasmid DNA size. An approximate 2-fold increase in plasmid DNA size resulted in an approximate 30% decrease in the DNA amount encapsulated in the liposomes.

Effect of liposome size on DNA amount encapsulated

We also investigated the effect of liposome size on the DNA amount encapsulated into the liposomes (Table I). Lipid composition was kept at 92 mole% POPC, 4 mole% DDAB, 3 mole% DSPE-PEG 2000 and 1 mole% DSPE-PEG 2000-maleimide, and 100 μg of plasmid DNA (pGL2 clone 753) was used. To control liposome size, the liposomes carrying plasmid DNA were forced through 2

Table I. Liposome size and DNA amount encapsulated into the liposomes after extrusion through polycarbonate filters with pore size of 400 nm, 200 nm, and 100 nm

Pore size of polycarbonate filters	100 nm	200 nm	400 nm
Liposome size (nm)	113.4 ± 12.2	140.9 ± 8.2	176.0 ± 19.4
DNA amount encapsulated (μg/μmole)	1.60 ± 0.17	1.78 ± 0.42	1.80 ± 0.45

Values are presented as the mean ± S.D. from triplicate experiments.

stacked polycarbonate filters with pore size of 400 nm, 200 nm or 100 nm. The liposome size was measured by a Zeta Potential/Particle Sizer (NICOMP™ 380 ZLS, PSS·NICOMP, USA) in the volume-weighted mode. The liposome size extruded through the 400-nm-sized filters was reduced from 1906.7 ± 411.7 nm (unextruded size) to 176.0 ± 19.4 nm (extruded size). After extrusion through the 200- and 100-nm-sized filters, mean diameters of the liposomes were decreased to 140.9 ± 8.2 nm and 113.4 ± 12.2 nm, respectively. Although the liposome size was reduced, the DNA amount encapsulated into the liposomes was not changed remarkably. This result suggests that the liposome size can be controlled by extrusion through polycarbonate filters of a certain pore size, but that the liposome size does not significantly influence the DNA amount encapsulated within the liposomes.

Effect of the ratio of DSPE-PEG 2000-maleimide to rat IgG on the number of IgG molecules conjugated per liposome

To determine if the number of IgG molecules conjugated per liposome was affected by the ratio of DSPE-PEG 2000-maleimide to IgG, a series of studies were performed with variations in the ratio of 5 to 1, 10 to 1 and 20 to 1 (Fig. 5). For the ratios of 10 to 1 and 20 to 1, 0.02 μmole (3.0 mg) and 0.01 μmole (1.5 mg) IgG were used, respectively, and the liposomes were composed of 18.4 μmole (92 mole%) POPC, 0.8 μmole (4 mole%) DDAB, 0.6 μmole (3 mole%) DSPE-PEG 2000 and 0.2 μmole (1 mole%) DSPE-PEG 2000-maleimide. When preparing the immunoliposomes with the ratio of 5 to 1, 0.7 μmole (3.5 mole%) DSPE-PEG 2000 and 0.1 μmole (0.5 mole%) DSPE-PEG 2000-maleimide were used and the amount of POPC and DDAB were kept at 18.4 μmole (92 mole%) and 0.8 μmole (4 mole%), respectively, to maintain the total lipid amount as 20 μmole and liposome charge as neutral. The adjusted amount of IgG was 0.02 μmole (3.0 mg).

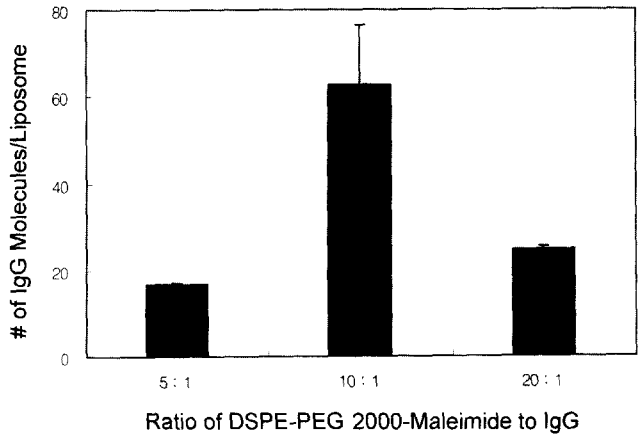

Fig. 5. The number of IgG molecules conjugated per liposome depending on the ratio of DSPE-PEG 2000-maleimide to IgG. Rat IgG, containing 1.23 μCi of [^{3}H]-IgG, was conjugated to the liposomes composed of 92 mole% POPC, 4 mole% DDAB, 3 mole% or 3.5 mole% DSPE-PEG 2000 and 1 mole% or 0.5 mole% DSPE-PEG 2000-maleimide. Also, 100 μg plasmid DNA (pGL2 clone 753) was used for the encapsulation. Data represent the mean ± S.D. from 3-4 experiments.

The highest number of IgG molecules conjugated to the liposome was observed in the immunoliposomes with the ratio of 10 to 1. The number of IgG molecules conjugated per liposome was reduced from 62.6 ± 13.9 to 16.8 ± 0.3 as the ratio was decreased from 10 to 1 to 5 to 1. At the ratio of 20 to 1, the number of IgG molecules conjugated per liposome (24.7 ± 0.6) was also less than that at the ratio of 10 to 1. This result suggests that the ratio of 10 to 1, for DSPE-PEG 2000-maleimide to IgG, is optimal for conjugating the maximum number of IgG molecules to liposomes.

DISCUSSION

In this study, we prepared and characterized immunoliposomes which can be more stable in the blood stream by polyethyleneglycol (PEG)-derivatized lipids and target the effect site by conjugating IgG (monoclonal antibody) to the tips of the PEG strands. In Sepharose CL-4B column chromatography, the co-location of the two peaks (Fig. 1), the encapsulated [^{32}P]-DNA and the conjugated [^{3}H]-IgG, demonstrated that the plasmid DNA and the IgG were incorporated in the same structure (Shi and Pardridge, 2000).

To characterize the liposomes carrying plasmid DNA, we investigated the effect of several parameters on the DNA amount encapsulated into the liposomes. As cationic lipid concentration increased, the DNA amount encapsulated also increased significantly (Fig. 2). The amount of DNA encapsulated within the liposomes containing 7 mole% DDAB was approximately 8-fold higher than that containing 1 mole% DDAB. This observation corresponds to the finding of Saravolac *et al.* (2000) that a 2 mole% increase in DODAC (*N,N*-dioleoyl-*N,N*-dimethylammonium chloride) concentration resulted in a 40% increase in the encapsulation efficiency of plasmid (pCMVLuc) in stabilized plasmid-lipid particles (SPLP). Our result confirms that encapsulation efficiency is considerably dependent on cationic lipid concentration and that electrostatic interactions are important for DNA encapsulation. Furthermore, this result suggests that it is possible to encapsulate a considerable amount of DNA into liposomes containing a low concentration of cationic lipid (≤ 7 mole%), thereby decreasing the toxic side effect both *in vitro* and *in vivo*.

Increasing the initial amount of plasmid DNA from 10 to 200 μg at a fixed lipid amount of 20 μmole resulted in a

linear increase in the DNA amount encapsulated into the liposomes, while the DNA amount encapsulated was not significantly increased with an initial plasmid DNA of 300 μg (Fig. 3A). This result suggests that it may not be efficient to use an initial amount of plasmid DNA over 200 μg. The total DNA amount encapsulated was increased with increasing total lipid amount (Fig. 3B), but at 30 μmole lipid there was a greater possibility for experimental error during the extrusion step due to its high viscosity. According to Monnard *et al.* (1997), entrapment yields were independent of variations in the initial amount of DNA between 0.8 and 100 μg. Also, Saravolac *et al.* (2000) reported that increasing plasmid concentration from 100 to 1000 μg/mL decreased the plasmid encapsulation efficiency. In addition, encapsulation efficiency was increased with increasing lipid concentration (1 - 10 mg/mL), and at high encapsulation efficiency (>70%) aggregation of the formulations was remarkably observed (*Saravolac et al.*, 2000). Based on these results, it is suggested that there are optimal initial amount of DNA and total amount of lipid to promote encapsulation efficiency.

Plasmid DNA size also influenced the DNA amount encapsulated within the liposomes (Fig. 4). The amount of encapsulated DNA was reduced with increasing size of plasmid DNA, suggesting that there is a limit to the size of plasmid required to be encapsulated within the liposomes. This suggestion is supported by the finding of Saravolac *et al.* (2000) that when using plasmid ranging from 2.9 kb to 15.6 kb and 50 kb λ-phage DNA, the encapsulation efficiency was decreased with increasing plasmid size at two DODAC concentrations, 7 and 24 mole%.

However, the size of liposome did not play an important role in the DNA amount encapsulated in the liposomes (Table I). By extruding the liposomes through polycarbonate filters of different pore sizes, we were able to prepare liposomes with appropriate size. The DNA amount encapsulated was not clearly reduced with decreasing liposome size. As the size of the liposomes was not changed dramatically, it appears that the DNA amount encapsulated was only slightly affected.

The conjugation of ligand-like antibody to the liposome surface can improve targeting, cell binding, cellular uptake and treatment effect. It was reported that the antibody density of the immunoliposomes was an important factor for target binding; liposomes containing 74 antibody molecules per liposome were mainly accumulated in the lung, whereas liposomes conjugated with small numbers of antibody molecules were accumulated in the blood at a higher level than in the lung (Maruyama *et al.*, 1999). According to Huwyler *et al.* (1996), conjugation of higher amounts of OX26 monoclonal antibody (MAb) to liposomes (197 OX26 MAb per liposome) resulted in relatively low brain targeting and accumulation of immunoliposomes. Also, doxorubicin delivery was proportional to the amount of Fab' incorporated in the immunoliposomes up to 30 - 40 MAb fragments per liposome (Mamot *et al.*, 2003). Therefore, in order to optimize immunoliposome binding to the target cells, we investigated the number of IgG molecules per liposome by varying the ratio of DSPE-PEG 2000-maleimide to IgG (Fig. 5). The highest number of IgG molecules conjugated per liposome was observed at the ratio of 10 to 1. Based on this result, we are continuing our research to find the optimal number of IgG molecules per liposome for efficient gene expression in the target cells.

The immunoliposomes prepared and characterized here may be a useful gene delivery vector to increase blood circulation time and gene expression level while decreasing toxicity. In addition to antibodies, other ligands such as epidermal growth factor, transferrin and insulin can be conjugated to the liposomes for targeting and various plasmid DNA can be entrapped within the liposomes. Therefore, it is expected that an efficient gene delivery system for treating a diverse range of diseases will be developed by future *in vitro* and *in vivo* study.

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