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Mediated Liposome for Gene Delivery to Mice Brain Part I. Design and Characterization of Liposome-DNA Complexes

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Abstract - The purpose of this research is to develop a novel liposome-mediated system for delivery of expression plasmid into specific regions in the rat brain. Complexes of plasmid DNA and different liposome were prepared in phase 1 of the study. The composition, method of preparation were varied and the physico-chemical characterization of the different systems were investigated two different methods of preparation were used, in the first method the liposome were prepared simultaneously with the DNA entrapped into the liposome and in the second method, the liposome were prepared first and then complexes with the DNA were performed. The liposome formulations were composed of DOTAP: Cholesterol; and DC-Chol: DOPE and different lipid helpers. The particle size of liposomes prepared with DNA entrapped into the liposome was larger than those prepared with liposome-DNA complexation. All liposome formulations were spherical, uniform in size and have smooth surface. In vitro DNase digestion experiments demonstrated that liposome protects 60-80% plasmid DNA from DNase digestion. The plasmid: DNA lmediated liposome can be widely prepared, have less risk than the use of viral vectors, can protect DNA from DNase digestion, none toxic and therefore can be used repeatedly in vivo.

Keywords : mediated liposomes; gene delivery; targeting; gene expression; plasmid-DNA expression; targeting to mice brain.

I. Introduction

Liposomes are self-closed spherical particles where one or several lipid membranes encapsulate (s) part of the solvent in which they freely float into their interio (1-5). Liposome (6) is distinguished by large multilamellar vescicles (MLV) and unilamellar vesicles which can be small (SUV), large (LUV) or giant unilamellar vesicles (GUV).

The major purpose of gene therapy is to deliver genetic material into target cells to rproduce specific therapeutic proteins needed to correct or or to modulate disease. However, developing appropriate biotherapeutics, such as plasmid-based gene expression vectors delivered successfully to the target cell is one of the major practical problem in gene therapy today (7). Approaches available for introduction of DNA into cells include viral transduction or plasmid transfection. These systems are effective for the expression of a variety of trangenes in brain issues. However, several technical problems are associated with issues such as immunogenicity, scale up, random integration and cellular tropism, which may limit them as therapeutic agents (8).

Many efforts have been devoted to the development of non-viral delivery due to the disadvantages of viruses used for gene delivery (9-12). Cationic liposomes have several attractive features as as vectors for gene transfer: They are non-immunogenic and non-toxic; cationic liposome as DNA carriers can transfect postmitotic, non-dividing cells including neurons; cationic liposome can deliver multiple genes of any type (linear or super coiled) nucleic acid and finally, cationic liposome are relatively simple to prepare and can be administered to the body by different several routes.

Gene therapy is potentially powerful method for treatment of neurological diseases for which classical pharmacotherapy is unavailable or not easily applicable (13-14). Transfection within the brain has distinct advantages over other administration sites. The post-mitotic stage of nature neurons may prolong transgene expression. Moreover, the liquid volume in which in which the delivery vectors needs to be distributed and the metabolism of the plasmid can be limited because of the lack of major clearance mechanisms, such as those in the liver or kidney. In addition the cerebrospinal fluid has limited nuclease activity as compared to plasma and thus provides for longer half-life of the administered DNA in the nervous system (15).

The hypothesis of this investigation is that mediated liposome-DNA complex may protect the DNA from degeneration by DNase; use of cationic mediated liposome for targeting plasmid DNA is more safe, non-toxic compared to the use of viral vector. Also, the intra-hipocampus infusion of liposome-DNA complex may leads to DNA expression in specific brain region. The overall goal of this research is to develop a novel liposome mediated system for delivery of expression plasmids into specific brain regions in the rat. The specific objectives of this study are to prepare different

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liposome formulations; use B-galactosidase reporter plasmid construct; determine complexation between cationic liposome formulations and the plasmid-DNA; and examine the efficiency of the mediated liposome to protect DNA plasmid from DNase digestion.

II. Experimental and Methods

a) Materials

DOTAP, N-[1{2,3-deoxypropyl]-N, N, N trimethyl ammonium., lot No. 181TAP-65, Avanti Polar Lipids, AL, USA.; DOPE, dioleoyl phosphatidylethanolamine, lot No. 181PE-228, Avanti Polar Lipids, AL, USA.; DC-Chol., 3B-(N,N,N,-dimethylaminoethane}-carbimol}cholesterol, lot No. 017H8476, Sigma, MO, USA.; Cholesterol, lot No. 96H8476, Sigma, MO, USA; Protamine sulfate, lot No. 02K7400, Sigma, MO, USA; pSV-B-Galactosidase control vector, lot No. 12706113, Promoga Corporation, Madison, USA; XL-10-Gold^R , ultracomponent cellls, Stratagene Services, USA; Turbo DNase, Ambion Inc., USA; Wizard^R PlusMaxipreps DNA Purification System, lot No. 241531, Promega Corporation, Madison, USA. All other ingredients are of chemical grade.

b) Methods

i. *Plasmid Preparation*

a. Transformation of XL-10-Gold^R ultracompetent cells with plasmid pSV-pSV-B-Galactosidase. Plasmid psV-B-Galactosidase contains SV40 early promoter and enhancer drive transcription of the lacZ gene, which encodes the B-Galactosidase enzyme. The plasmid was propagated in XL-10-Gold^R ultracompetent cells (Stratagene) following the manufacturer's instruction.

b. Production and purification of plasmid pSV-Galactosidase. The pSV-B-Galactosidase was purified by Wizard^R PlusMaxipreps DNA purification system following the manufacturer's instruction.

ii. *Design of Liposome and Preparation of Liposome-pSV-B-Galactosidase Complex*

a. *Preparation of liposomes containing DOTAP and cholesterol with DNA entraped in the liposome*

A mixture of DOTAP and cholesterol at 1:1 molar ratio (8.38 mg DOTAP and 4.64 mg of cholesterol was dissolved in 12 ml chloroform. The organic solvent was removed using rotary evaporator at 40^{0}C and vaccum for 2 hours. The thin layer of lipid film formed on the wall of the flask was hydrated using 1 ml of 5% dextrose solution containing 1 mg of DNA and 0.6 mg of protamine sulfate. The mixture of the hydrated thin film of the lipids and DNA was agitated by vortexing for 30 seconds and then incubated at 37^{0}C for 30 seconds. This process was repeated 8 times (n=8). The liposome suspension was sonicated for 20 seconds and vortexed for 30 seconds.

b. *Preparation of liposome containing DOTAP and cholesterol*

The cationic DOTAP was mixed with cholesterol at equimolar concentration (8.38 mg DOTAP with 4.64 mg cholesterol). The mixture of lipid was dissolved in HPLC grade chloroform using 1 litre round bottom flask. The organic solvent was evaporated using rotary evaporator at 30^{0}C for 30 minutes and then dried under vacuum for 15 minutes. The dried thin film was hydrated in 5% dextrose solution to give a final concentration of 20 mM DOTAP and 20 mM cholesterol (20 mM DOTAP-Cholesterol). The hydrated lipid film was agitated in a water bath at temperature of 50^{0}C for 45 minutes and then at 35^{0}C for 10 minutes. The mixture was covered and kept overnight at room temperature. After 24 hours, the mixture was sonicated for 10 minutes at 50^{0}C. DOTAP-cholesterol liposome (75 ul) was mixed with protamine sulfate (30 ug) and the mixture was kept at room temperature for 10 minutes before use. 75 ul of plasmid DNA (0.6 ug/ul) was slowly added while stirring and the mixture was incubated at room temperature for 10 minutes before use. The final concentration of DNA was 0.3 ug/ul.

c. *Preparation of liposomes containing DC-Chol and DOPE*

DC-chol-DOPE cataionic liposomes were prepared by mixing DC-Chol and DOPE at 1.5:1 molar ratio (6.37 mg DC-Chol and 6.13 mg DOPE). The mixture of the two lipids was dissolved in HPLC grade chloroform. The organic solvent was evaporated in a rotary evaporator at temperature of 55^{0}C for 60 minutes, then dried under vacuum for 30 minutes. The film was hydrated in 5% dextrose solution to give a final concentration of DC-Chol:DOPE liposome (1.25 mg/ml). The hydrated film was agitated in a water bath at 55^{0}C for for 45 minutes and at 35^{0}C for an additional 10 minutes and the mixture was kept overnight at room temperature. After 24 hours, the mixture was sonicated for 5 minutes at 50^{0}C, transferred to a tube and heated at 50^{0}C for 10 minutes. Then the mixture, was extruded through a Millipore filters in a descending order of 1 um, 0.45 um, 0.2 um, 0.1 um using syringe. Portion of the liposome that did not pass through 0.1 um filter was heated again at 50^{0}C for 5 minutes before passing through a new 0.1 um filter. The filtered fractions were stored under argon gas at 4^{0}C. 75 ul of Plasmid DNA (0.6 ug/ul) was added slowly while stirring to equal amount of DC-Cho:DOPE liposome and the mixture is incubated at room temperature for 10 minutes before use. The final concentration of DNA is 0.3 ug/ul.

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iii. *Characterization of Liposome and Plasmid DNA Complexes*

a. *Particle size distribution*

The particle size was determined by Malvern particle size diffraction analyzer using a scale constant of 300 nm, the laser beam passed through the liposome dispersion and the light scattered was measured in 19 to 30 seconds. A blank of distilled water was used.

b. *Morphology of liposome and plasmid DNA complex using scanning electron microscope (SEM)*

The liposomes were coated with conducted film into pin mount and tighten. The image appeared on the screen after clicking on beam and high voltage /xKV buttoms. The brightness and the magnification were adjusted and the image was saved.

iv. *DNase digestion study*

Four reactions were performed:

Components	Reaction 1	Reaction 2	Reaction 3	Reaction 4
pSV-B-Gal	N/A	N/A	1.5 ug	N.A
pSV-B-Gal lipos.complex	N/A	5 ul	N/A	5 ul
DNase reaction buffer	100 ul	100 ul	100 ul	100 ul
DNase	3 ul	3 ul	N/A	N/A

All mixtures were incubated for 2.5 hours at 37^0C and 100 um of 1:1 phenol:chloroform mixture was added, mixed gently and centrifuged at 14,000 rpm for 4 minutes at room temperature. The supernatant was transferred to tube and mixed with 100 ul chloroform and centrifuged at 4,000 rpm for 4 minutes at room temperature. The supernatant was removed and the pellets were washed with 70% alcohol and dried. The DNA was dissolved in 50 ul buffer. Aliquots of plasmid DNA were analyzed using agarose gel electrophoresis and UV spectrophotometer.

III. Results and Discussion

In phase 1 study, the plasmid DNA was successfully encapsulated into different liposome formulations and or formed DNA:liposome complexes. The liposome formulations prepared were containing DOTAP and cholesterol with entrapped DNA; DOTAP and cholesterol and DC-Chol:DOPE liposomes complexes with DNA. All liposomes were cationic, DOTAP and DC-Chol are two cationic lipids and they provided a positive charge for the liposome. They are considered to be as a lipids helper. Cholesterol was also used as an alternative lipid helper that resulted in more stable complexes than those containing DOPE.

Two manufacturing methods were also used to prepare the liposomes. In the first method, plasmid DNA solution was used to hydrate the lipid film and the lipids formed the bilayers membrane while the DNA was encapsulated into the liposomes. In the second method, after preparation of the liposome, a complex is formed between the DNA and the liposome. The first method gave better entrapment efficiency of DNA and better protection of the DNA.

The particle size of the different mediated liposome-DNA systems are shown in Table 1. The particle sizes for all formulations were larger than expected (50 nm – 200 nm). Liposome prepared with DOTAP-Cholesterol have the largest particle size while liposome prepared with DC-Chol:DOPE were of the smallest particle size.

Figures 1 and 2 show the surface morphology of liposome prepared with DOTAP:Cholesterol and entrapped DNA. The liposomes appear to be spherical and of smooth surface.

Figure 3 shows the electrophoresis spectra of free liposome; DNA s and DNA encapsulated liposome after exposure to DNase enzyme. Adding DNase to free DNA resulted in complete degradation of DNA while DNA encapsulated liposome was not affected by DNase, indicating that the liposome was able to protect the DNA. Tables 2 to 4 show that the recovery of efficiency of the DNA from DNA entrapped liposome was between 67% to 83% using UV spectrophotometer. The lost quantity of DNA was due to the difficulty of avoiding loss during the extraction and the precipitation processes. The particle size was large when DNA was entrapped into the liposome. This may be due to that the hydration of lipid with DNA solution resulted in formation of very heterogeneous population with possible large size. On the other hand when liposome was first made and then complexed with DNA, it is assumed DNA and cationic liposome aggregate because of electrostatic attractive forces and formation of small stoichiometric complexes.

IV. Conclusions

Mediated liposomes with entrapped DNA lipid or liposome complexes with DNA plasmid were successfully prepared. Liposome prepared with DC-Chol: DOPE complexes with DNA were the smallest in size while liposome prepared with DOTAP:Cholesterol and entraped DNA plasmid into liposome gave the highest efficiency entrapment and best protection of DNA against DNase digestion. The composition of the liposome and the method of preparation have an effect

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on the physico-chemical properties of the liposome. All mediated liposome formulations showed spherical particles and smooth surface. The mediated liposomes were able to protect DNA against DNase digestion and degradation.

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Table 1 : Mean Particle Size of the Different Mediated Liposome-DNA Systems

Formulations	Mean Particle Size ib nm (n=3)
DOTAP:Cholesterol with DNA Entrapped in the Liposome	512.5
DOTAP:Cholesterol Liposome Complexes with DNA	723.7
DC-Chol:DOPE Liposome Complexes with DNA	309.8

Table 2 : Quantitative Analysis of DNA in DOTAP:Cholesterol with Entraped DNA to the Liposome Using Ultra Violet Spectrophotometer

Samples	Initial Quantity (ug)	Mean Final Quantity in ug (n=3)	Percent Efficiency
Free DNA	1.50	1.15	83.33
Free DNA Treated with DNase	1.50	0.04	2.89
DNA Entraped in Liposome	1.50	1.06	70.7
DNA Entraped in Liposome treated with DNase	1.50	0.91	60.9

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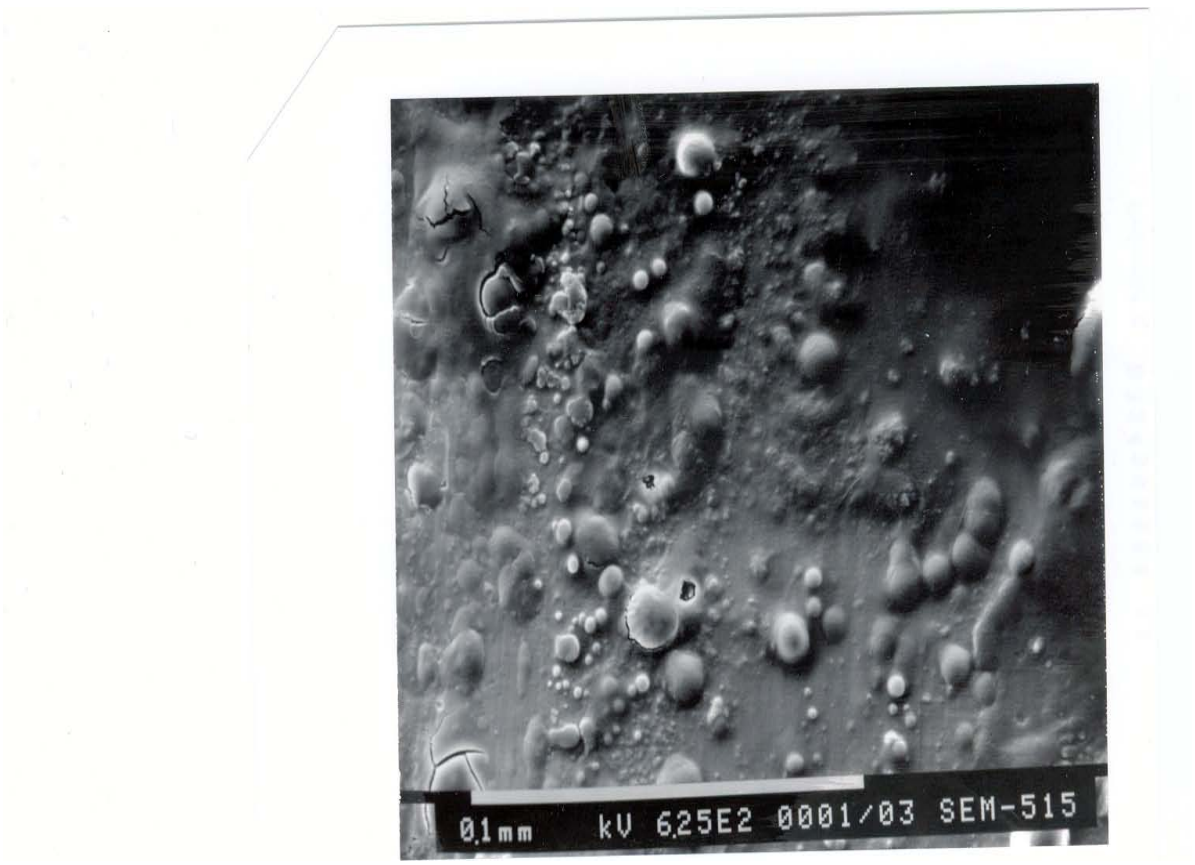
Table 3 : Quantitative Analysis of DNA in DOTAP:Cholesterol Liposome Complexes with DNA Using Ultra Violet Spectrophotometer

Samples	Initial Quantity (ug)	Mean Final Quantity in ug (n=3)	Percent Efficiency
Free DNA	1.50	1.18	78.5
Free DNA Treated with DNase	1.50	0.03	2.0
DOTAP:Cholesterol Liposome Complexes with DNA	1.50	0.83	66.1
DOTAP:Cholesterol Liposome Complexes with DNA and Treated with DNase	1.50	0.78	52.3

Table 4 : Quantitative Analysis of DNA in DC-Cholesterol:DOPE liposome Complexes with DNA Using Ultra Violet Spectrophotometer

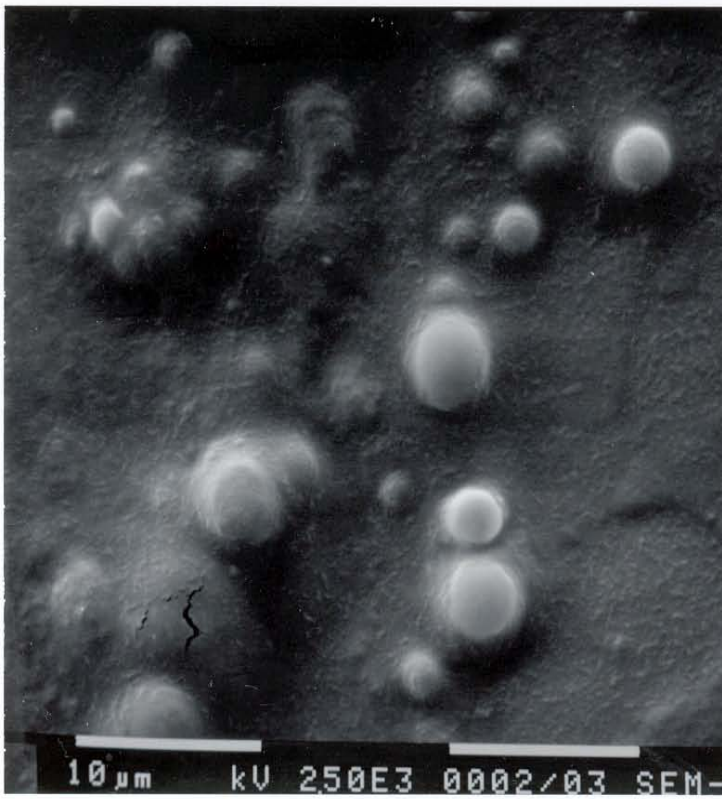
Samples	Initial Quantity (ug)	Mean Final Quantity in ug (n=3)	Percent Efficiency
Free DNA	1.50	1.15	76.5
Free DNA Treated with DNase	1.50	0.00	0
DC-Chol:DOPE Liposome Complexes with DNA	1.50	0.042	70.2
DC-Chol:DOPE Liposome Complexes with DNA and Treated with DNase	1.50	0.033	55.2

Figure 1 : Scanning Electron Microscope for DOTAP:Cholesterol Liposome Complexes with DNA at low Magnification



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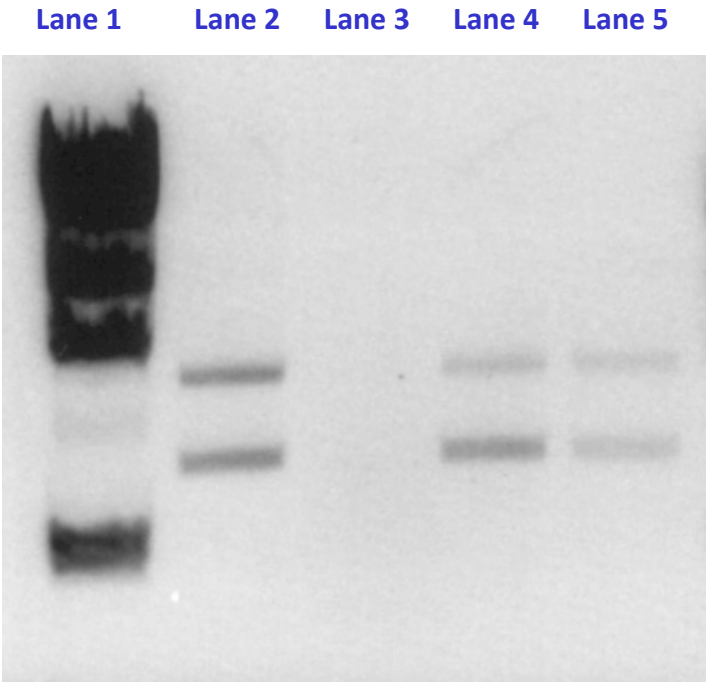
Figure 2 : Scanning Electron Microscope for DOTAP:Cholesterol Liposome Complexes with DNA at High Magnification



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Figure 3 : Agarose Gel Electrophoresis Analysis

Lane 1 : Molecular Weight Marker
Lane 2 : Free DNA
Lane 3 : DNA Treated With DNase
Lane 4 : Liposome:DNA Complexes
Lane 5 : Liposome:DNA Complexews Treated with DNase



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