To the Graduate Council:

I am submitting herewith a dissertation written by Xiaohuai Zhou entitled "Liposome Mediated Gene Transfer in Mammalian Cells." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

Leaf Huang

Leaf Huang, Major Professor

We have read this dissertation
and recommend its acceptance:

John W. Koontz

Daniel G. Blair

David A. Brian

Accepted for the Council:

Lewinkel

Associate Vice Chancellor
and Dean of The Graduate School

LIPOSOME MEDIATED GENE TRANSFER IN MAMMALIAN CELLS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Xiaohuai Zhou

May, 1992

ACKNOWLEDGMENTS

I would like to express the most sincere gratitude to my major professor, Dr. Leaf Huang, who gave me the opportunity to work in his laboratory and trained me patiently. I would also like to thank the other committee members, Dr. David Brian, Dr. John Koontz and Dr. Daniel Roberts, and the program director, Dr. Jeffrey Becker, for their comments and guidance. My thanks also go to Dr. Alexander Klibanov and Mr. Dick Williams for their assistance in this project. I appreciate the many helpful suggestions from my fellow lab members. I am especially grateful that it has been made possible for me to study in this country.

ABSTRACT

Studies described in this dissertation include the following four parts: 1) the development of a freeze-thaw method for an improved DNA encapsulation in pH-sensitive liposomes, 2) the development of lipopoly-L-lysine (LPLL) for an efficient DNA transfection, 3) the development of LPLL/dioleoylphosphatidylethanolamine (DOPE) liposomes for an efficient DNA transfection, and 4) an investigation of the mechanism by which LPLL/DOPE liposomes mediate DNA delivery.

To overcome the problem of inefficient DNA encapsulation in anionic pH-sensitive liposomes, a freeze-thaw method for DNA entrapment was developed. Three cycles of freeze-thawing a mixture of DNA and empty sonicated liposomes resulted in an efficient DNA entrapment. Smaller plasmids were entrapped more efficiently than the larger ones. Unlike the reverse-phase evaporation method, this protocol did not cause significant damage to the plasmids. DNA-containing liposomes prepared by this method mediated transfection in cultured L929 cells. The transfection activity depended on the pH-sensitivity of liposomes and could be modulated by protein kinase C activators incorporated in the liposome membrane. These findings may help to improve our liposome system for further studies on gene therapy.

In an effort to construct a novel targetable and efficient DNA carrier other than the anionic pH-sensitive immunoliposomes, a cationic lipid was synthesized by conjugating poly-L-lysine with a lipid. The resulting conjugate was termed LPLL. The synthesis of LPLL represents the first step in the construction of such a DNA carrier. LPLL if tested effective for

DNA delivery would be incorporated with a targeting ligand to promote target-specific delivery. LPLL contained an average of two lipids per poly-L-lysine molecule (MW ~3,000). It indeed mediated efficient DNA transfection. Under the optimized conditions, LPLL had a 3-fold higher activity than Lipofectin which is a widely used commercial preparation of cationic liposomes. Moreover, compared to Lipofectin the action of LPLL was more resistant to the inhibitory effect of serum. However, scraping of the treated cells was necessary for the transfection activity of LPLL.

The mechanical treatment required for the transfection activity of LPLL could be avoided by mixing LPLL with a helper lipid, DOPE. The activity of LPLL/DOPE liposomes was systematically optimized with respect to the lipophilicity of LPLL, LPLL/DOPE ratio, DNA/liposome ratio, and the concentration of DNA/liposome complexes. Under the same conditions, liposomes containing lipopoly-D-lysine had a similar activity to those containing LPLL, indicating that the degradation of polylysine chain which should take place in lysosomes is not required for the release of DNA into the cytoplasm.

The mechanism by which LPLL/DOPE liposomes mediated transfection was studied in more detail. It was found that cellular factors were involved in the formation of DNA/liposome complexes. The complexes entered the cytoplasm by either penetrating through the plasma membrane or destabilizing endosomes after being endocytosed. Our results posed questions on a previous hypothesis which suggested that cationic liposomes mediated DNA translocation via a fusion mechanism. These studies shall pave the way to the ultimate goal of targeted delivery of DNA for gene therapy.

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LIST OF ABBREVIATIONS

CAT	chloramphenicol acetyl transferase
DCC	1,3-dicyclohexylcarbodiimide
DEPE	dielaidoylphosphatidylethanolamine
DMDOPE	N-dimethyldioleoylphosphatidylethanolamine
DOG	dioleoylglycerol
DOPC	dioleoylphosphatidylcholine
DOPE	dioleoylphosphatidylethanolamine
DOPS	dioleoylphosphatidylserine
DOSG	dioloeylsuccinylglycerol
DOTMA	N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride
DPSG	dipalmitoylsuccinylglycerol
EDTA	ethylene diamine tetraacetic acid
EM	electron microscopy
HBS	hepes buffered saline
LPDL	lipopoly-D-lysine
LPLL	lipopoly-L-lysine
MGDG	monogalactosyldiglyceride
MMDOPE	N-monomethyldioleoylphosphatidylethanolamine
NGPE	N-glutarylphosphatidylethanolamine
NHS	N-hydroxysuccinimide
PBS	phosphate buffered saline
PDL	poly-D-lysine
PKC	protein kinase C

PLL	poly-L-lysine
PMA	phorbol 12-myristate 13-acetate
SUV	small unilamellar vesicle
Z-Gly-Phe	carbobenzoxy-glycyl-L-phenylalanine
Z-Phe-Phe-Gly	carbobenzoxy-D-phenyl-L-phenylglycine

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PART I

INTRODUCTION

1. Background

Introduction of exogenous genetic material into living cells or organisms, i.e. gene transfer, is an essential component of recombinant DNA technology. Currently as the concept of gene therapy is developed to a clinical reality (1 - 5), gene transfer becomes the key step of this new therapeutic strategy. Accordingly, much effort have been made on the development of suitable methods for DNA delivery (see ref. 6 for a review). Existing techniques include calcium phosphate, DEAE-dextran, polybrene, microinjection, red cell ghost, electroporation, laser, liposome, viral vectors, particle bombardment, polylysine conjugate methods (6).

Retroviral vectors, one of the most widely used delivery systems (7 - 9), have attracted serious attention in the field of gene transfer and therapy. Retroviruses are single-stranded RNA viruses. Upon infection, the RNA genome is reverse transcribed into a double-stranded DNA which is then stably integrated into the host genome. Thus, the strategy of using retroviral vectors for gene transfer is to replace retroviral genes with a gene(s) of interest and to encapsidate the hybrid genome in viral envelopes with the help of packaging cells. (Packaging cells produce envelope glycoproteins encoded by genes which have been deleted from the vectors.) Retroviral vectors have two major advantages for gene transfer and therapy: 1) they infect a variety of cells with high frequency, and 2) the viral genome is stably integrated into the host genome. To date, a great number of genes have been introduced into various hosts by this method (9). Moreover, applications of this delivery system are currently undergoing clinical trials of gene therapy. However, retroviral vectors have several

drawbacks. 1) cell-specific targeting is impossible; while targeted delivery is desirable for gene therapy. 2) the vectors may induce insertional mutagenesis because of their random integration. Possible consequences include deregulated expression of protooncogenes and house-keeping genes. 3) due to technical difficulties vector preparations are usually contaminated with replication-competent helper virus which may cause clinical complications. 4) procedures for preparation of vectors are complicated and laborious. 5) inconvenient in vitro or ex vivo protocol is required for efficient infection. 6) there still exist a number of technical problems, e.g. size limitation of the insert (< 7 kb), instability of vector genome, low titre of vector preparations, instability of high titre producer lines if available. In contrast, liposomal delivery system can potentially avoid all the above problems and is therefore gaining an increasing level of enthusiasm.

Liposomes are composed of lipids arranged in concentric bilayers encapsulating an aqueous space. Use of liposomes as a delivery vehicle was realized soon after the discovery by Bangham and his colleagues in the mid 1960s (10). However, applications for gene transfer were not started until the late 1970s (11). Since then, liposome mediated transfection has been accomplished in mycoplasmas (12), bacteria, fungi, plant protoplasts, cultured mammalian cells (13) and intact animals (14 - 22). This methodology involves three processes which will be discussed below: 1) loading of nucleic acids into liposomes, 2) uptake of the loaded liposomes by target cells, and 3) functional expression of the delivered genes in the cells.

2. Methods for Loading of DNA into Liposomes

Liposomes can be formulated to contain negative, positive, or no charge. To achieve gene transfer, DNA molecules are encapsulated into neutral or negatively charged liposomes. For positively charged (cationic) liposomes, no encapsulation of DNA is necessary.

Loading of DNA into Neutral and Negatively Charged Liposomes:

DNA molecules to be delivered are usually more than 5 kb. They can only be encapsulated in liposomes with relatively large diameter (>100 nm)such as mutilamellar vesicles (MLVs) and large unilamellar vesicles (LUVs). A number of methods have been used for the entrapment of DNA (see also Table I.A.).

Lipid hydration method. The original protocol for liposome preparation, i.e. lipid hydration method, is also the first protocol demonstrated suitable for DNA encapsulation (23). Liposomes are prepared simply by hydrating phospholipids in a buffer containing DNA with hand-shaking. Size of the resulting liposomes is very large and heterogeneous, ranging from 0.5 to 7.5 um (24). They can accommodate DNA molecules of more than 10 kb (23). DNA also adsorbs to the external liposome surface non-specifically but can be removed by nuclease treatment. The encapsulation efficiency for this method is very high; up to 40% of input DNA has been entrapped. This technique is very simple. However, liposomes thus produced are too large (0.5 ~ 0.7 um) to be taken up efficiently via an endocytosis mechanism.

Ca^{++}-EDTA-chelation method. This protocol involves addition of EDTA to a mixture containing DNA and cochleates of Ca^{++}-phosphatidylserine (PS) which is spontaneously formed from PS liposomes in the presence of Ca^{++} (25). DNA encapsulation efficiency is reversely proportional to the molecular weight of linear DNA fragments (26). For 5 kb molecules, only 1% of input is entrapped yielding a specific encapsulation efficiency of 0.05 ug DNA/umol lipid. This protocol has been modified (27). A mixture of DNA and Ca^{++}-PS cochleates is dialyzed against the buffer containing a low concentration of EDTA instead of direct addition of the chelator. Encapsulation efficiency can be enhanced by 10 fold. The Ca^{++}-EDTA-chelation technique is only applicable to liposomes containing negatively charged lipids such as PS which form cochleates with Ca^{++}. Furthermore, acid-sensitive liposomes although negatively charged may not be prepared by this method, because they contain a hexagonal phase forming lipid, dioleoylphosphatidylethanolamine (DOPE). No bilayer structure would exist when the negatively charged lipids complex with Ca^{++}. Therefore, application of this method is limited by the liposome composition.

Ether injection method. According to this method LUVs are prepared by slowly injecting an ether solution of lipids into an aqueous buffer containing DNA (28, 29). Although LUV produced has a larger aqueous compartment or captured volume (up to 35 l/mol lipid) than SUV and MLV (<10 l/mol lipid), this technique did not give efficient entrapment. Encapsulation efficiencies of 2~6% (29) and 15% (12) have been reported. The low entrapment may be due to the high extent of dilution of DNA which is necessary for this method.

Reverse-phase evaporation method. Liposomes prepared with this method are termed reverse-phase evaporation vesicles (REVs). The protocol involves sonicating a mixture of DNA and liposomes in a large volume of organic solvent (30, 31). Removal of the organic solvent from the resulting water-in-oil emulsion leads to the formation of DNA-containing liposomes. This method gives a high encapsulation efficiency; up to 40% of input DNA is entrapped (31). The obvious drawback of this technique is that DNA is subject to sonication. As short as 5 seconds of the treatment causes significant fragmentation of the plasmids (32). Furthermore, the protocol is time-consuming because a dialysis step is needed for the removal of residual organic solvent.

Dialysis method. Liposome formation and spontaneous DNA entrapment occur during removal by dialysis of detergent from a mixed micellar solution containing phospholipids and DNA (33, 34). This method is also applicable for loading DNA to reconstituted viral envelopes (34). Drawbacks of this technique are inefficient encapsulation and time-consuming procedures. However, it allows a convenient and effective incorporation of the targeting ligands to liposome membrane. Liposomes for targeted DNA delivery have been prepared with this protocol (20).

As can be seen from above, reverse-phase evaporation method allows efficient DNA entrapment. Effective encapsulation can also be achieved by other means. It is found that inclusion of cholesterol and Sendai viral glycoproteins in liposome membrane significantly enhance the amount of DNA entrapped (27). The enhancement is related to the increase in size and stability of the liposomes. Lysozyme has been shown to improve DNA entrapment by 100 fold (35). It is speculated that the basic protein acts to

neutralize the charge and to condense the dimension of DNA, and also to serve as an amphipathic template for lipid bilayer formation. Condensation of linear DNA molecules by spermine or bacteriophage proteins also improves DNA entrapment (36). Recently, a number of polycations including polylysines have been reported to induce condensation of plasmid DNA (37). Condensed plasmids could be more readily encapsulated into liposomes.

Complexation of DNA with Cationic Liposomes:

Due to charge interaction, cationic liposomes associate with DNA spontaneously (38). When cationic lipids are incorporated to negatively charged or neutral liposomes, a 5~70-fold increase in the amount of liposomal DNA is obtained (39). Under optimized conditions, 100% of DNA becomes liposome-associated (38). However, DNA association efficiency for the various cationic liposomes does not correlate with the transfection efficiency (39). Although liposomal DNA is not susceptible to DNase treatment it is believed that nucleic acids bind to the outer surface of cationic liposomes instead of being entrapped inside the aqueous compartment (39).

3. Cell Uptake and Fate of Liposomes

After the realization that liposomes can be a drug carrier, the liposome-cell interaction has been rigorously studied (for reviews see ref. 40, 41). Evidence has been presented that negatively charged and neutral liposomes are taken up by cells via adsorption, endocytosis, and fusion

mechanisms. Endocytosed liposomes are destined to lysosomes for degradation. On the other hand, liposomes fused with the cell membrane discharge their contents into the cytoplasm. Therefore, fusion but not endocytosis or adsorption will result in an effective gene transfer. Unfortunately, fusion is not the major mechanism by which liposomes interact with cells.

Papahadjopoulos' group concluded that DNA-containing liposomes are endocytosed by cells (42). Interestingly they found that chloroquine, a weak base which interfered with the endocytosis process by increasing the intravesicular pH of endosomes and lysosomes, enhanced the transfection activity of liposomal DNA. They speculated that the enhancement was due to the fact that chloroquine disabled the degradative function of the lysosomes or it prevented the fusion of endocytotic vesicles with the lysosomes. In either case, endocytosed liposomal DNA would have a higher possibility to escape into the cytoplasm either by fusing directly with or causing some non-specific breakdown of the phagolysosomal membrane. To prove their speculation, they have studied the cellular fate of liposome-encapsulated fluorescently labeled macromolecules (43). They found that the macromolecules remained confined to the intracellular vesicles. Therefore, they were unable to define the specific mechanism by which liposomal DNA escaped the endocytotic vesicles.

Using techniques including fluorescence and electron microscopy (44, 45), electron microscopic autoradiography and subcellular fractionation (46), Nicolau's group has identified that liposomal DNA is transported to lysosomes, mitochondria and nuclei directly from the endocytotic vesicles. They have also shown that DNA-containing liposomes

fuse with cell membrane. Inhibition of endocytosis process only caused a partial reduction in the amount of DNA delivered to the nuclei by the negatively charged liposomes (47). Thus, the fusion mechanism can partially account for the intracellular delivery of DNA.

Base on the understanding of the three uptake mechanisms of liposomes, two approaches have been taken to improve the efficiency of intracellular delivery. The first approach involves the use of liposomes coated with fusion-promoting molecules, e.g. Sendai virus (16, 34) and influenza virus (16, 36, 48) glycoproteins. Sendai virus fusion protein F_0 upon binding of the virus to permissive cells is cleaved into two polypeptides, F_1 and F_2 (49). The exposed hydrophobic N terminus of F_1 induces membrane fusion between the virus and the cell. Hemagglutinin (HA) of influenza virus is responsible for the viral fusion activity (49). When the virus is taken up into the endosomes, HA_0 is proteolysed into HA_1 and HA_2 by a trypsin-like enzyme at pH 5.0 - 5.5. The N terminus of HA_2 then interacts with the endosomal membrane resulting in virus-cell fusion (49). Incorporation of these proteins to the liposome membrane triggers liposome-cell fusion and hence induces the cytoplasmic discharge of liposome contents. Fusion of liposomes with cells can also be induced by polyethylene glycol (50). However, such treatment is unlikely to be suitable for improvement of <u>in vivo</u> gene transfer. The second approach to enhance intracellular delivery involves the use of pH-sensitive liposomes (for reviews, see 54, 55). These liposomes become destabilized and fusion-competant upon taken up into the endosomes which have an acidic pH of 5.0 to 6.5 (52, 53). They may also induce the rupturing of endosomes. As a result, pH-sensitive liposomes release their contents into the cytoplasm.

Various ligands have been conjugated to liposomes to achieve the targeted delivery of the encapsulated drugs (56). Ligand-bearing liposomes bind to the cell surface receptors and are often taken up via the receptor-mediated endocytosis (57). Incorporation of endocytosable ligands to the pH-sensitive liposomes gives rise to a targeted and efficient cytoplasmic delivery of the liposomal contents.

Recently, cationic liposomes have been shown to transfer effectively anionic macromolecules such as DNA (38), RNA (58), and some proteins (59). It is generally agreed that free cationic liposomes are taken up by cells mainly via adsorption; fusion and endocytosis also take place (60, 61). However, opinions on the uptake mechanism of complexes of DNA with cationic liposomes are controversial. Felgner's group suggests that the complexes fuse with the plasma membrane to enter the cytoplasm (62). On the other hand, Huang's (63) and Silvius' (64) groups hypothesize that the complexes are endocytosed and induce rupturing or destabilization of the endosomal membrane thereby releasing DNA into the cytoplasm. Further study is necessary to understand the nature of the delivery process and to improve the performance of cationic liposomes.

4. Functional Expression of Delivered Genes

Use of liposomes as a vehicle for gene transfer was first demonstrated by Wilson et al. who had shown that liposome-encapsulated poliovirus was infectious to otherwise non-permissive cells (11). The infectivity was resistant to antiserum against the virus, confirming that the infection was mediated by liposomes. Similarly, Fraley et al. had shown

that E. coli cells treated with liposomal pBR322 in the presence of DNase conferred tetracycline resistance whereas treatment with free plasmids did not result in transformation (29). Following these initial observations, Papahadjopoulos' group had systematically studied the conditions for liposome mediated transfection (31, 42). It was found that liposomes composed of PS : cholesterol = 1 : 1 (molar ratio) had the optimal delivery efficiency compared to other neutral or negatively charged liposomes. Moreover, exposure of cells to polyethylene glycol or glycerol and chloroquine further enhanced the efficiency (see I.B. for interpretation). However, under the optimized conditions, the transformation frequency was still only comparable to that of the DEAE-dextran method. Despite the relatively low transfection efficiency, it had been reported that these liposomes mediated both short and long term transformation in a number of cell lines including both adherent and suspension cells (65 - 67).

In vivo transfection using PS based liposomes has also been demonstrated (14). Nicolau and coworkers had succeeded in the transfer and expression of a plasmid carrying preproinsulin I gene in rat. Intravenously injected liposomes were taken up almost entirely by liver and spleen of the treated animal. The amount of insulin detected in these two organs as well as in the blood for rats received liposomal DNA were significantly higher than for rats received free liposomes plus free DNA. However, the elevated levels of insulin diminished about 10 hours after injection. Recently, a similar study has been reported (15). pRSVneo plasmid carrying a neomycin phosphotransferase II gene had been encapsulated in liposomes and injected to rat intravenously. Expression of the exogenous gene was observed for at least 7 days.

Chimerasomes:

All of the studies described above used liposomes composed of PS or PC with or without cholesterol. The efficiency of cytoplasmic delivery was relatively low. Thus improvements have been made by engineering the composition of the liposomes. One of the approaches involves the incorporation of viral fusion proteins to liposomes (16, 34, 36, 48). The resulting vesicles referred to as chimerasomes have a higher fusogenic activity, hence a higher efficiency of cytoplasmic delivery.

Loyter and coworkers have first shown that liposomes coated with Sendai viral proteins mediated efficient gene transfer (34). About 20% of the treated cells were transfected. They (48) and Tikchonenko et al. (36) have recently found that liposomes containing influenza virus glycoproteins had a similar delivery efficiency to those containing Sendai viral proteins.

Mannino and coworkers have compared transfection frequencies for chimerasome and Ca-phosphate precipitation methods (16). Stable transfection of a bovine papilloma virus-based plasmid in cultured cells was performed using both techniques. The number of foci/ug DNA obtained for chimerasome method was 10^5 times higher than that of the Ca-phosphate precipitation method. The investigators have also encapsulated into the chimerasomes a plasmid carrying polyoma virus early genes (16). Expression of these genes may lead to tumorigenic transformation. When the DNA-containing chimerasomes were injected subcutaneously, 4 out of 12 treated mice developed tumors which express polyoma virus early proteins and contain the delivered DNA sequence. In contrast, none of the mice injected with free chimerasomes mixed with unencapsulated plasmid DNA developed tumors.

As described above, chimerasomes show an improved efficiency of delivery. However, due to their non-specific fusion activity, chimerasomes may not be ideal for in vivo gene transfer since it usually requires tissue specific delivery and expression. Use of targeting ligand coated influenza chimerasomes (plus the use of tissue specific promoters) may be more appropriate for such treatment because they would remain non-fusogenic until being taken up into the acidic endosomes (48). Incorporation of targeting ligands may hopefully direct a major portion of the chimerasomes to the desired target before they discharge their contents non-specifically. However, injection of liposomal viral proteins may elicit immunoresponses in the host.

Uchida and coworkers have developed a delivery system similar to chimerasomes (17 - 19). They have fused intact Sendai virions to ganglioside-bearing liposomes encapsulating the exogenous DNA. DNA encapsulated in such fusion hybrid effectively transfected cultured cells and intact animals. However, no pathogenicity of the carrier has been examined so far.

pH-Sensitive Liposomes:

Another approach to enhance the cytoplasmic delivery is by the use of pH-sensitive liposomes (51). These liposomes release their contents in response to an acidic environment such as that in the interior of an endocytotic vesicle. Thus, lysosomal degradation of the content material can be reduced. Targeted delivery is also possible by conjugation of antibodies to the liposomes (51). The resulting vesicles are termed pH-sensitive immunoliposomes.

Gene transfer mediated by pH-sensitive immunoliposomes has been explored by Wang and Huang (20, 68, 69). Transfection of cultured Ltk^- mouse cells ($H\text{-}2K^k$ positive) with liposomes coated with anti-$H\text{-}2K^k$ antibody and encapsulating plasmids carrying a thymidine kinase gene of the Herpes Simplex Virus gave a 47% efficiency for the short term transformation and 2% for the long term transformation (69). These values are comparable to the highest ones reported for Sendai virus modified liposome method (70). It should be noted that the latter technique does not allow cell-specific transfection. Targeted gene transfer in vivo using the pH-sensitive immunoliposome system has also been demonstrated (20). The plasmids used in the study contained the E. coli chloramphenicol acetyltransferase (CAT) gene under the control of an inducible promoter. Target tumor cells grown in the mice injected with pH-sensitive immunoliposomal DNA expressed a significantly higher level of CAT activity than those in the control groups. Moreover, expression of the exogenous gene depended on the injection of its inducer in the treated mice. CAT activities were also detected in other organs. However, the levels were substantially lower and they were not inducer regulated. It was shown later that the target tumor cells contained an inducer responsive protein which is required for the transcription driven by the inducible promoter (71). These results all together showed a great promise of this model system for gene therapy since targeted delivery and controlled gene expression can both be achieved at the same time.

Cationic Liposomes:

Recently liposomes bearing positive charges, i.e. cationic liposomes have been reported to be effective for transfection (38, 63, 64, 72). Five to more than one hundred-fold improvement in the transfection frequency over the Ca-phosphate or DEAE-dextran method has been obtained (38). In some cases, at least 95% of the treated cells were transfected (73). Furthermore, cationic liposomes are convenient to use since no DNA encapsulation is required. Thus, these liposomes are a powerful tool for the DNA mediated transfection. Although most studies involving cationic liposomes are carried out in cultured cells, Brigham et al. (21) and Hazinski et al. (22) have found that intratracheally or intravenously injected liposome/DNA complexes transfected lung tissues of mice and rat. Controlled gene expression by use of an inducible promoter has also been demonstrated (22). Therefore, this delivery system is also potentially applicable for in vivo gene transfer. Since the action of cationic liposomes is non-specific, modification of this system toward the targeted delivery is necessary in order to achieve the ultimate goal of gene therapy.

5. Scope of the Project Described in This Dissertation

Liposome mediated DNA delivery as reviewed above show a great promise as an alternative for gene transfer other than the commonly used viral vectors. Chimerasomes, pH-sensitive immunoliposomes and cationic liposomes are effective DNA carriers. It can also be seen from the review that research in the field of liposomes for gene transfer has become increasingly active but is still at a stage of development. Although our

laboratory has made a major contribution by demonstrating that pH-sensitive immunoliposomes mediate efficient transfection both in vitro and in vivo, further studies with this model system have been hampered by the low efficiency of DNA encapsulation. To overcome this problem, this study was initiated with the following specific aims: 1) to develop a new method for an improved DNA entrapment in the pH-sensitive liposomes, and 2) to construct a novel targetable and efficient DNA vehicle. In this effort, a cationic lipid, LPLL, was synthesized from polylysine as the first step in construction of such a DNA carrier. Polylysine contains multiple primary amino groups which would facilitate conjugation of a targeting ligand to the cationic lipid. The resulting vehicle is assumed to be able to promote target-specific delivery and effective translocation of DNA into the cytoplasm of cells of interest. LPLL alone mediate efficient transfection; however, its activity depended on scraping of the treated cells. Therefore, this study was continued with the following specific aims: 3) to develop a cationic liposome which does not depend on scraping for activity, and 4) to investigate the mechanism by which the cationic liposome mediates DNA delivery.

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Table I: Methods for DNA encapsulation in liposomes

Method	Liposome composition	Liposome size (um)	DNA (dalton)	DNA marker	Removal of free DNA	DNA encapsulation input%	DNA encapsulation ug/umol [1]	Nonspecific association [2]	Nuclease treatment	Reference
lipid hydration	ePC	?	mouse ascite (10~20xE6)	prelabeled [3H] DNA	sedimentation	30~40	1.2~1.6	yes	yes	23
lipid hydration	ePC, ePC:chol (7:2, w/w)	0.5~0.7	pBR322, pCR1 (2.6, 8.6xE6)	prelabeled [3H] DNA	sedimentation	30~40	1.2~1.6	yes	yes	24
chelation	PS	?	HpaI, MboI-T7 (0.3~14.2xE6)	prelabeled [32P] DNA	sedimentation	0.3~10	0.02~0.5	no	yes	26
chelation	PS	?	pMB9 (3.5xE6)	nick tmaslated [32P] DNA	sedimentation	6~8	?	no	yes	74
chelation	PS, PS:chol (4:1, w/w)	?	HaeIII-T7	nick translated [32P] DNA	sedimentation	20~27	?	no	no	27
ether injection	PC:PG (10:1, m/m)	0.1~1.5	pBR322 (2.6xE6)	prelabeled [32P] DNA	chromotagraphy (Sepharose 4B)	2~6	1.2~3.0	no	yes	29
ether injection	PC:PS:chol (8:2:10, m/m)	?	pBR322 (2.6xE6)	nick translated [32P] DNA	chromotagraphy (Sepharose 4B)	15	0.15	?	yes	12
reverse-phase evaporation	PS:chol (1:1, m/m)	0.4	SV40 DNA	prelabeled [3H] pBR322, poly(A)	flotation	30~40	0.39	no	yes	31, 42
dialysis	Sendai virus envelope	?	pBR322 (2.6xE6)	prelabeled [3H] DNA	sedimentation	10	?	yes	yes	34
dehydration-rehydration	PC:chol (1:1, m/m)	<1	?	[3H] DNA	?	72	?	?	?	75

1. Amount of DNA in micrograms that became encapsulated into one micromole of liposomes

2. DNA adsorbed non-specifically to the outer surface of liposmes

PART II

IMPROVED ENCAPSULATION OF DNA IN pH-SENSITIVE LIPOSOMES FOR TRANSFECTION

1. Abstract

A simple method has been developed to prepare liposomes containing large amounts of DNA. It is an improvement on the efficiency and speed of DNA encapsulation over our previous dialysis method [Proc. Natl. Acad. Sci. USA 84 (1987) 7851 - 7855]. The procedure consisted of three cycles of freeze-thawing a mixture of sonicated liposomes and DNA. The encapsulation efficiency depended on the size of DNA. For a small plasmid (2.7 kb), approximately 40% of input DNA was entrapped with an efficiency of 16 ug DNA/umol lipid. For larger plasmids (4.8 kb), the encapsulation efficiency decreased to about 17%. Transfection of cultured mouse L929 cells mediated by the DNA-containing liposomes was assayed with a plasmid containing the E. coli chloramphenicol acetyl transferase gene. The transfection activity of the liposome was primarily determined by its pH-sensitivity. Acid-sensitive liposomes transfected cells efficiently, whereas pH-insensitive liposomes were much less active. The level of the expression of the exogenous gene in the treated cells could be further modulated by protein kinase C (PKC) activators which were incorporated into the liposomal membrane as a minor lipid component. Transfection conditions were optimized with respect to DNA, lipid and PKC activator concentrations. The results of the current study may help the use of liposomal delivery system for applications in gene therapy.

2. Introduction

The technique of introducing exogenous genes into cells is a key step for gene therapy which is recognized for its potential importance in the prevention or treatment of viral, cancer, genetic or metabolic disorders (1, 2). Schemes of gene therapy usually require efficient delivery of DNA in vivo; in many cases target specific delivery are desirable. Most of the existing gene transfer methods such as calcium phosphate precipitation (3), electroporation (4), microinjection (5), high velocity microprojectile (6), viral vector infection (7, 8, 9, 10), and cationic liposomes (11) cannot fulfill all of the requirements. We have previously developed a pH-sensitive immunoliposome system which can deliver efficiently and specifically to target cells fluorescent dye (12), diphtheria toxin A fragment (13), and anti-cancer drugs (14). By using the same system, we have also demonstrated that plasmid DNA can be delivered to specific targets at a relatively high efficiency in both cultured cells (15) and a mouse model (16). However, due to the low entrapment of DNA into liposomes, the therapeutic application of the system was not fully developed. We have now developed a simple method for preparing liposomes encapsulating larger amounts of DNA. Factors affecting the encapsulation efficiency were studied. Conditions for DNA delivery and subsequent gene expression mediated by liposomes prepared by this method were also optimized.

3. Materials and Methods

Materials:

Dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylcholine (DOPC) and dioleoylsuccinylglycerol (DOSG) were purchased from Avanti. Ficoll (type 400), calcein, acetyl coenzyme A, dioleoylglycerol (DOG), and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma. Chloramphenicol-D-threo-[1,2-^{14}C] (57 mCi/mmol) was obtained from ICN. Plasmids pUC18, pUCSV2CAT (constructed by Dr. Mark Magnuson of Vanderbilt University), and pCH110 (purchased from Pharmacia) were amplified in E. coli and purified by CsCl gradient centrifugation method (17).

Liposome Preparation:

Solvent free lipid films of typically DOPE:DOSG = 1:1 (molar ratio) were hydrated over night in filtered Hepes buffered saline (HBS, 10 mM Hepes, 130 mM NaCl, pH 8.0) at concentration of 50 mM. The lipid suspension was sonicated to small unilamellar vesicles (SUV) (diameter approximately 80 nm) in a bath type sonicator (Laboratory Supplies Co. Inc., Hicksville, NY). The pH of the suspension was maintained at 8.0. Plasmid DNA in equal volume of HBS was mixed with the suspension. For calcein-containing liposomes, buffer of 0.2x HBS containing 50 mM calcein (pH 8.0) was used instead of HBS. The mixture was frozen in liquid nitrogen, thawed at 0°C, and then vortexed for typically three cycles. To facilitate quantitation of lipid and DNA, a trace amount of hexadecyl [^{3}H]cholestanyl ether was included in the lipid mixture as a lipid marker;

plasmid was labeled with α-[^{32}P] dCTP using nick translation (17). Precautions were taken to keep the nick translated plasmid intact. Unencapsulated calcein was separated from liposomes by chromatography on Bio-Gel A0.5M column.

Ficoll-gradient Flotation:

Unencapsulated DNA was separated from liposomes by a modified method of ficoll-gradient flotation (18). Briefly, liposome preparation in 100 ul of HBS was mixed with 400 ul of 30% ficoll in HBS (pH 8.0) in a 5 ml cellulose nitrate tube. Three ml of 15% ficoll (pH 8.0) and 0.5 ml of HBS (pH 8.0) were sequentially overlaid. The tube was centrifuged at 36,000 rpm at 15°C for 1 hour in a SW50.1 rotor. Fractions of 200 ul each were collected from top of the tube and counted for radioactivity.

Electrophoresis of DNA:

Liposomal DNA was extracted with chloroform:methanol (7:3) and electrophoresed on an 1% agarose gel slab and visualized by ethidium bromide staining (17).

Acid-induced Leakage:

Liposomes containing calcein were diluted and assayed for acid-induced calcein release. The percent leakage of liposomes was calculated from the dequenching of calcein as described (19).

Transfection:

L929 cells were grown to about 60% confluency in 100 mm plates i.e., $5x10^6$ cells/dish in McCoy's medium containing 10% calf serum. The cells were washed and then incubated with DNA-containing liposomes in 5 ml serum-free medium at 37°C in 5% CO_2 for 3 hours. At the end of incubation, cells were washed three times. Growth medium containing 10% serum was added to the plate. The cells were further cultured for two days before assayed for chloramphenicol acetyl transferase (CAT) activity.

CAT Assay:

CAT activity was assayed with a thin layer chromatography method according to a modified method of Gorman et al. (20). The amount of CAT activity in the cell extract was calculated by defining one unit of CAT activity as the conversion of one nanomole chloramphenicol to its acetylated derivatives per minute at 37°C.

4. Results

The composition of pH-sensitive liposome used in this study was DOPE:DOSG (1:1, molar ratio). Liposomes of the same composition with a molar ratio of DOPE:DOSG = 4:1 had been shown to be stable in plasma (21). However, higher mol% of DOSG than previously reported was used because it was necessary for the formation of stable liposomes prepared by the freeze-thaw method employed here.

Determination of DNA Encapsulation Efficiency:

To facilitate the tracing and quantitation, liposome was labeled with a ^{3}H marker and the plasmid was labeled with ^{32}P by nick translation. Conditions for nick translation had been optimized to maintain the integrity of the plasmid. Unencapsulated DNA could be separated from liposomes by ficoll-gradient flotation method (Fig. 1). Fig. 1C and D showed the total separation of free DNA and empty liposomes which had been mixed together before centrifugation. Since no significant amount of DNA associated with liposomes, it was not necessary to treat the liposomes with DNase, a procedure previously used to remove DNA adsorbed to liposome surface (22). The result suggested that DNA co-floated with liposomes at the top of the gradient represented the DNA entrapped in the liposomes.

Encapsulation of DNA by Freeze-thaw Method:

Liposomes can be prepared by a freeze-thaw method. The procedure has been used to entrap small molecules (23, 24). Freeze-thawing a mixture of free DNA and empty liposomes resulted in 43.2% and 17.3 ug/umol lipid entrapment of a plasmid of 2.7 kb in size (Fig. 1A). However, the encapsulation efficiency was reduced to 9.4% and 3.8 ug/umol lipid by extruding the liposomes through a 0.4 um filter (Fig. 1B). This result again suggested that liposome-associated DNA was entrapped inside the liposomes and not simply adsorbed on the surface. Because unextruded liposomes could be efficiently taken up by cells (see below) and three cycles of freeze-thaw completely sterilized the liposome preparations, extrusion of liposomes was not carried out in the remaining studies. The size distribution of the unextruded liposomes was quite heterogeneous (390 - 850

nm) as revealed by negative-stain electron microscopy, with the average diameter being 660 ± 158 nm.

The number of freeze-thaw cycles affected the entrapment of DNA (Table I). Maximum trapping was achieved with three cycles; additional cycles resulted in slightly decreased encapsulation efficiencies. The size of plasmid also determined the entrapment efficiency (Table II). DNA molecules of greater base pair number were encapsulated less efficiently. However, increase of DNA molecular weight did not correspond linearly to the decrease of encapsulation efficiency (Table II). For a small plasmid of 2.7 kb, an average of 40% original DNA was entrapped in the liposomes with an efficiency of approximately 16 ug DNA/umol lipid.

Since the conditions for preparing liposomes involved dramatic changes in temperature, we investigated its effect on the integrity of plasmid (Fig. 2). The physical state of the DNA was analyzed by agarose gel electrophoresis. Liposome entrapped DNA was extracted from liposomes purified by ficoll-gradient flotation and analyzed by gel electrophoresis. It can be seen that three cycles of freeze-thaw reduced the extent of supercoiling of a portion of the plasmids and converted a very small percentage of supercoiled circular plasmids into relaxed circular structures (lane 2, 4-7). The presence of lipids (lane 4-7) and extrusion procedure (lane 7) did not cause any further change in the physical state of the plasmid DNA.

Acid Sensitivities of Liposomes:

The acid-sensitivities of liposomes of three different lipid compositions are shown in Fig. 3. A water-soluble fluorescent dye, calcein,

was entrapped in liposomes using the freeze-thaw method. The percent leakage of the entrapped fluorescent dye from the liposomes were measured at different pH values. DOPE:DOSG (1:1, molar ratio) liposomes released 90% of their content at pH 5.0. In comparison, DOPC:DOSG (1:1, molar ratio) and DOPC liposomes only released less than 50% and 10%, respectively, of their contents at the same pH. Thus, the release of the contents from liposomes at acidic conditions depended on the lipid composition of the liposomes.

Transfection of L929 Cells with DNA-Containing Liposomes:

The transfection activities of liposomes were measured by their abilities to mediate the entry and subsequent expression in treated cells of a reporter plasmid pUCSV2CAT which carried the gene of E. coli CAT under the control of SV40 early promoter. Production of the exogenous enzyme in transfected cells were detected by assaying for the CAT activity in the cell extracts. Data in Table III showed that the acid-sensitivity of the liposomes was directly related to the transfection activity. DOPE:DOSG liposomes, which were the most acid-sensitive, transfected cells with the highest efficiency. DOPC:DOSG liposomes although released about 50% of their contents (Fig. 3) at pH 5.0 had only 1/8 activity of DOPE:DOSG liposomes. pH-insensitive liposomes composed of DOPC alone hardly transfected cells. It was also shown in Table III that the transfection activities for extruded DOPE:DOSG liposomes was significantly reduced as compared to the unextruded preparation. This is probably due to the fact that extrusion of liposomes significantly reduced the trapping efficiency of DNA (Fig. 1). Free DNA mixed with empty DOPE:DOSG liposomes did not transfect cells.

Since the unencapsulated plasmids did not show transfection activity, they were not removed from the DNA-containing liposome preparations for routine transfection experiments.

Optimization for Transfection Mediated by pH-sensitive Liposomes:

The optimal concentration of DNA for liposome encapsulation was determined by mixing various amounts of pUCSV2CAT DNA with a fixed amount (0.8 umole) of lipid (DOPE:DOSG = 1:1). Liposomes thus prepared were used to transfect L929 cells. Fig. 4 shows that initial increase of DNA concentration elevated the transfection activity of the liposomes. Further increase of DNA concentration beyond 16 ug did not further enhance the activity. The optimal condition was 16 ug DNA/0.8 umol lipid. Similarly, the optimal concentration of lipid was determined with respect to transfection activity (Fig. 5). The result showed a saturatable pattern with 0.6 umol lipid/16 ug DNA being the optimal. The results of these two experiments agreed closely with each other.

It is known that transcription of SV40 DNA can be induced by tumor promoting phorbol esters (25) which probably modulates the transcription factors such as AP-1 through the activation of protein kinase C (PKC) (26). The PKC activators are lipophilic and can be easily incorporated into liposome membrane. Fig. 6 showed that inclusion of 2.4 mol% of PMA and DOG in liposomes could enhance the extent of gene expression by 158% and 141%, respectively. Liposomes containing higher mol% of the PKC activators failed to further enhance the gene expression. This could be due to the fact that high concentrations of PMA down regulate PKC activity (27, 28). The result demonstrated that liposomes can co-deliver to target cells

their aqueous contents and lipophilic drugs which were incorporated into the liposomal membrane.

5. Discussion

We have reported that pH-sensitive immunoliposomes deliver DNA specifically to target cells with high efficiency (15, 16). However, the procedure used for DNA entrapment was a detergent dialysis method which was inefficient and time-consuming. In an effort to improve such a system for gene therapy, we developed a freeze-thaw method for preparing liposomes containing larger amounts of DNA.

Freeze-thaw conditions, especially the fast freeze (liquid N_2) and slow thaw (0°C) used in the present study, promote the aggregation and fusion of lipid membranes (29). This is primarily due to the dehydration conditions imposed by the freeze and thaw (29). Apparently, bilayer destabilization during the repeated freeze-thaw cycles had generated transient "holes" in the membrane to allow the penetration of DNA. It is not surprising that the encapsulation efficiency for DNA in the liposomes depended on the size of the plasmid DNA (Table II). DNA of a smaller size could penetrate the "holes" more efficiently than DNA of larger sizes. Thus, plasmids of 2.7 kb showed a encapsulation efficiency which was 2.4 and 2.8 fold higher than that of the plasmids of 4.8 and 7.1 kb in size, respectively (Table II). It is possible that only larger liposomes in the heterogeneous population of the freeze-thawed liposomes could entrap the larger plasmid DNA. The efficiency of encapsulation even for the larger plasmids was much improved compared with our previous method of detergent dialysis (15, 16,

30). In those experiments most of the liposome-associated DNA were adsorbed on the outer surface which are potentially sensitive to nuclease digestion and other non-specific inactivation factors (22, 30).

Another advantage of the present method is the speed and the simplicity of the protocol. Large amount of SUV can be produced in bulk and stored at 4°C for prolonged periods of time. Three cycles of freeze-thaw of the SUV-DNA mixture are easily done in a standard laboratory setting. No separation of the unentrapped DNA from liposomes is necessary since the free DNA shows no transfection activity. The entire process of DNA encapsulation can be done in approximately 3 hours. This is compared with the detergent dialysis method which takes approximately 2 to 3 days. Furthermore, the encapsulated DNA preparation is sterile as the freeze-thaw cycles have efficiently killed the contaminating bacteria. Only minimal level of damage of DNA was observed after the freeze-thaw cycles (Fig. 2).

Data in Table III and Fig. 3 clearly demonstrated the importance of acid-sensitivity of the liposomes for an efficient DNA delivery to cells. Partially pH-sensitive liposomes composed of DOPC:DOSG showed a transfection efficiency intermediate between those of pH-sensitive (DOPE:DOSG) and pH-insensitive (DOPC) liposomes. Liposomes of different compositions entrapped DNA with similar efficiencies under the same condition (data not shown). Thus, acid-induced destabilization of liposome was closely related to the high efficiency of cytoplasmic delivery. It has been proposed (30) that pH-sensitive liposomes destabilize and become fusion competent when they encounter the acidic environment in the endosomes, thereby releasing their entrapped DNA into the cytoplasm of the cells. In

comparison, pH-insensitive liposomes do not destabilize in the endosome and would be less efficient in the cytoplasmic delivery of the entrapped DNA.

Our results also demonstrated an unique feature of using liposomes as a drug carrier. In addition to deliver their hydrophilic contents, liposomes could at the same time deliver hydrophobic agents via their membrane compartments. In the present study, PKC activators, PMA and DOG, were delivered to cells to modulate the expression of the co-transfered gene. These PKC activators are lipids which can be easily incorporated as part of the liposome membrane. The action of PKC activators may not be limited to the activation of gene expression. It had been shown that PMA induces the internalization of CD4 molecules on the lymphoid cells (31). A variety of other lipophilic drugs could also be delivered to cells together with the entrapped nucleic acids in liposomes. This would facilitate a combination therapy of DNA and conventional drugs which could be more effective in treating cancer and viral diseases. While this manuscript was being prepared, a report appeared which also demonstrated the stimulation of transfection by PKC activators (32).

In summary, we have developed a simple method for efficient encapsulation of DNA into liposomes, including the pH-sensitive liposomes. The transfection activities of these liposomes have been demonstrated. These results should be helpful for future development of gene therapy.

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Table I. Effect of freeze-thaw cycle on DNA encapsulation efficiency [1]

Number of freeze-thaw cycles	DNA encapsulation efficiency	
	input DNA% [2]	ug DNA/umol lipid
0	2.0	0.8
1	9.7	3.9
2	10.6	4.2
3	16.5	6.6
4	14.7	5.9
5	12.4	5.0

1. A mixture of pUCSV2CAT DNA and SUV composed of DOPE:DOSG (1:1) was freeze-thawed for various cycles. DNA encapsulation efficiency was determined by ficoll-flotation centrifugation method. Data were collected from a single experiment with no replicate.
2. Percentage of total input DNA that became encapsulated into liposomes.

Table II. Effect of plasmid size on encapsulation efficiency [1]

Plasmid	Size (kb)	DNA encapsulation efficiency	
		input DNA%[2]	ug DNA/umol lipid
pUC 18	2.7	43.2 , 36.0	17.3 , 14.4
pUCSV2CAT	4.8	17.1 , 16.5	6.8 , 6.6
pCH110	7.1	14.2	5.7

1. Plasmid DNA of different sizes were encapsulated into liposomes composed of DOPE : DOSG (1 : 1) by three cycles of freeze-thaw. DNA encapsulation efficiency was determined by ficoll-flotation centrifugation method. Data of two independent experiments are shown for pUC18 and pUCSV2CAT plasmids.
2. Percentage of total input DNA that became encapsulated into liposomes.

Table III. Efficiency of liposome mediated transfection [1]

Transfection reagent			CAT activity
Lipid composition	DNA	Extrusion[2]	(mU/mg protein)
DOPE:DOSG[3]	entrapped	no	5.48
DOPC:DOSG[3]	entrapped	no	0.71
DOPC	entrapped	no	0.07
DOPE:DOSG[3]	entrapped	yes	0.56
DOPC	entrapped	yes	0.04
DOPE:DOSG[3]	free	no	0.06
DOPC	free	no	0.05

1. pUCSV2CAT DNA was either encapsulated into liposomes of different compositions or mixed with empty liposomes. Transfection efficiency of each preparation was determined by expression of CAT activity in treated cells. Data were collected from a single experiment with no replicate.
2. Liposomes were extruded 3 times through polycarbonate filters of 0.4 um pore size.
3. DOPE or DOPC:DOSG = 1:1

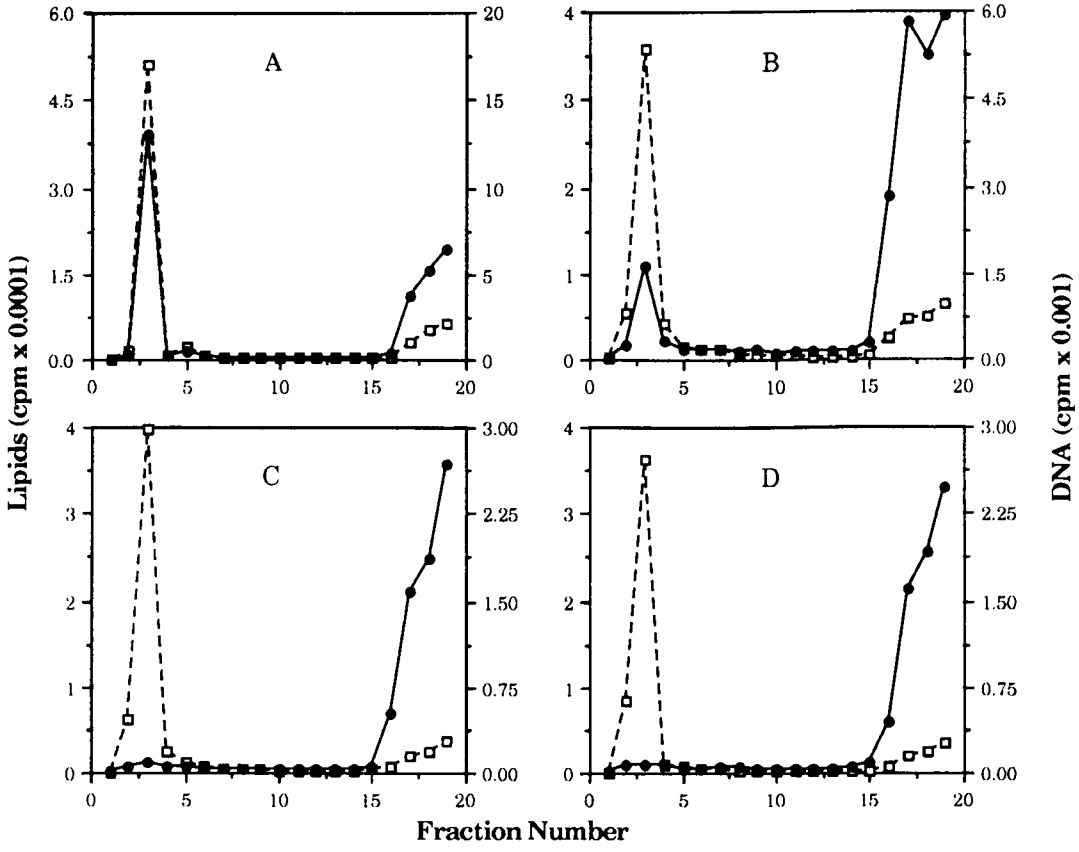


Figure 1. Effect of extrusion on DNA encapsulation efficiency. Free pUC18 plasmid DNA was separated from liposomes composed of DOPE:DOSG (1:1) by ficoll-gradient flotation to determine the encapsulation efficiencies for various liposome preparations. A. unextruded DNA-containing liposomes; B. DNA-containing liposomes extruded three times through 0.4 um polycarbonate filters (Nucleopore Corp.); C. unextruded empty liposomes mixed with free plasmid DNA; D. extruded empty liposomes mixed with free plasmid DNA. Lipid was labeled with ^{3}H (open square) to a specific activity of $5.6x10^{4}$ cpm/umol and DNA was labeled with ^{32}P (closed circle) to specific activities of 510 cpm/ug (A and B) and 130 cpm/ug (C and D). Data were collected from a single experiment without replicate.

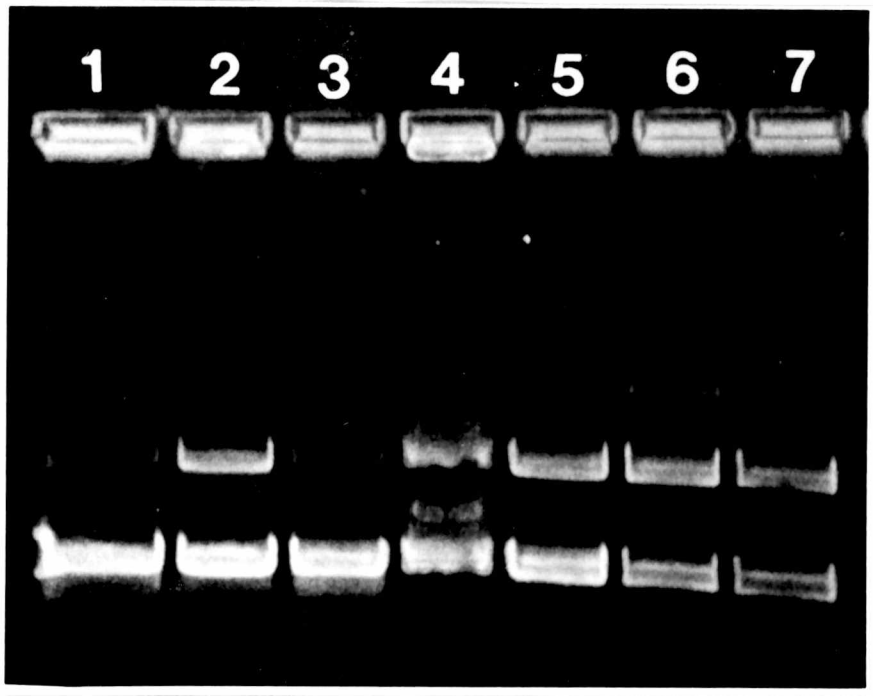


Figure 2. Effect of freeze-thaw on the integrity of pUCSV2CAT plasmid DNA. Lane 1, untreated plasmid; Lane 2, free plasmid freeze-thawed three times; Lane 3, untreated plasmid extracted with chloroform/methanol; Lane 4, Unencapsulated plasmid isolated by sedimentation from liposome preparation; Lane 5, unencapsulated plasmid from liposome preparation extracted with chloroform/methanol; Lane 6, plasmid from unextruded liposomes extracted with chloroform/methanol; Lane 7, plasmid from extruded liposomes extracted with chloroform/methanol. Liposomes were composed of DOPE:DOSG (1:1). 0.5 ug DNA was applied to each lane. The stain in the wells (top of the gel) was not due to DNA. They probably resulted from contaminations in the ethidium bromide staining solution. The lower and middle bands represented plasmids in two different extents of supercoiling. The upper band represented relaxed plasmids.

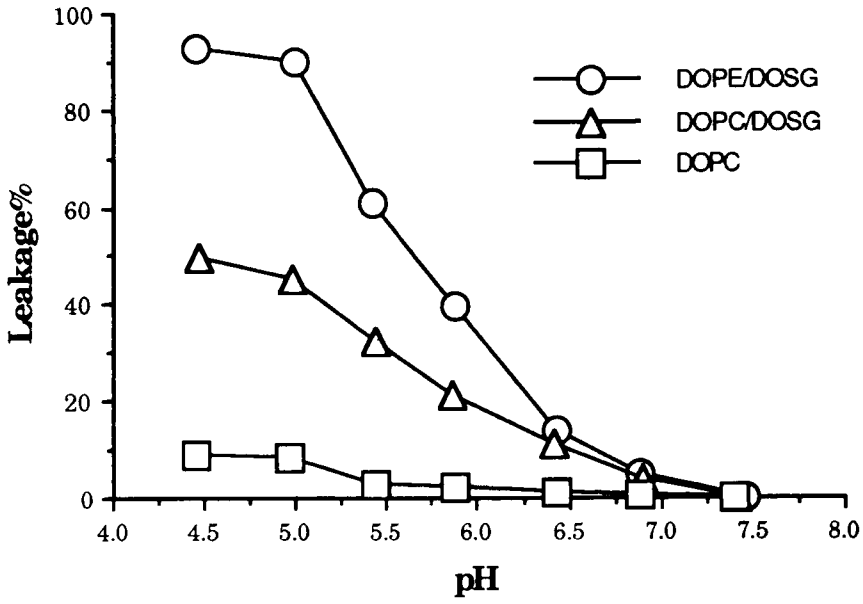


Figure 3. Effect of acidification on liposome stability. Liposomes of various composition were assayed for acid-induced leakage of the entrapped calcein as described in Methods. Data were collected from a single experiment without replicate.

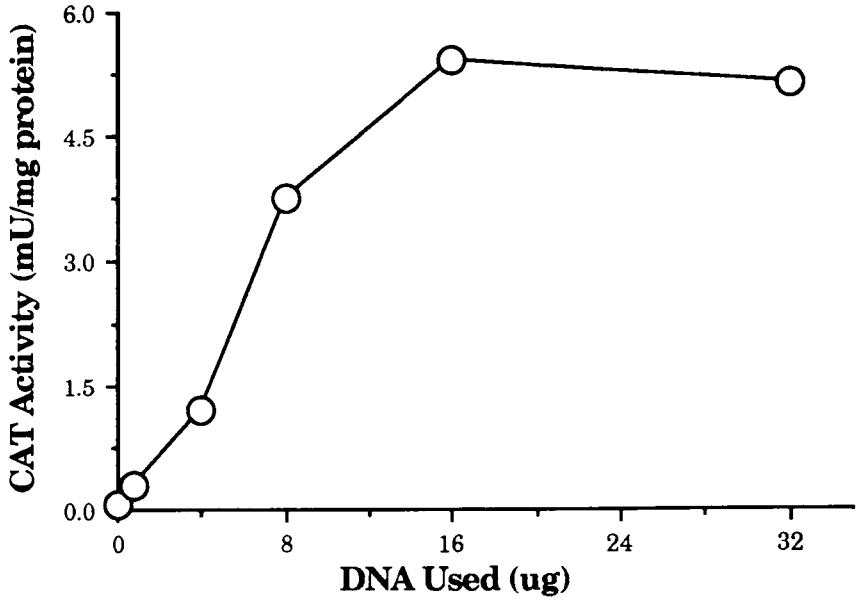


Figure 4. Effect of DNA dose on transfection efficiency. Various amounts of pUCSV2CAT DNA mixed with 0.8 umol lipids (DOPE:DOSG = 1:1) were used for liposome preparation and subsequent transfection of L929 cells. CAT activities in treated cells were assayed as described in Methods. Data were collected from a single experiment without replicate.

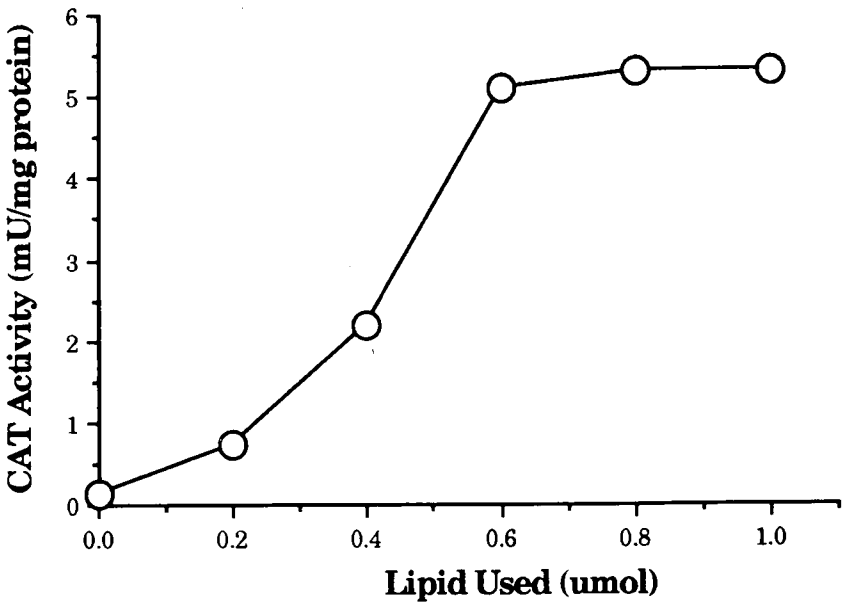


Figure 5. Effect of lipid dose on transfection efficiency. Various amounts of lipid (DOPE:DOSG = 1:1) mixed with 16 ug pUCSV2CAT DNA were used for liposome preparation and subsequent transfection of L929 cells. CAT activities in treated cells were assayed as described in Methods. Data were collected from a single experiment without replicate.

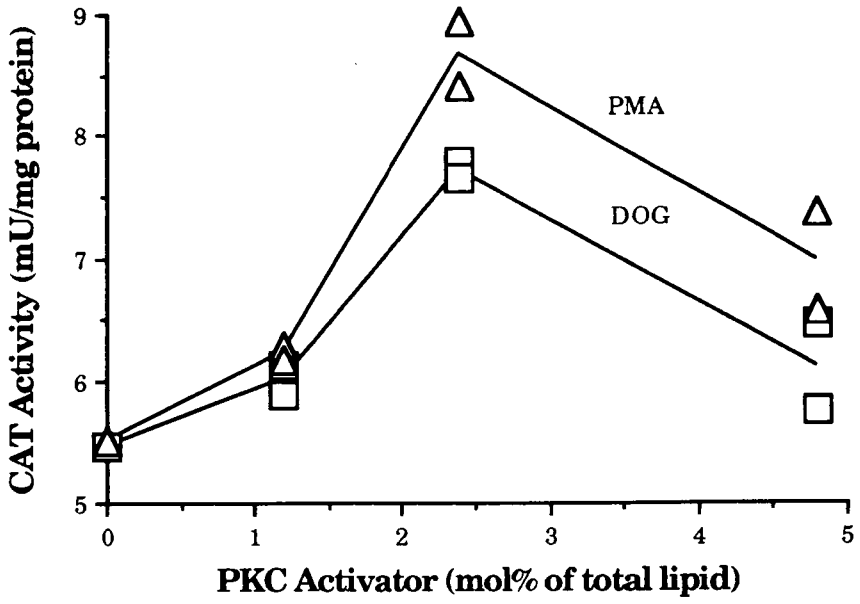


Figure 6. Effect of PKC activator concentration on transfection efficiency. Various amounts of PMA (open triangle) or DOG (open square) were incorporated into the membranes of liposomes. 16 ug pUCSV2CAT DNA and 0.8 umol lipid (DOPE:DOSG = 1:1) were used to prepare liposomes. Transfection activities of the preparations were determined by using L929 cells as described in Methods. Data were collected from a single experiment with duplicates for each point.

PART III

LIPOPHILIC POLYLYSINES MEDIATE EFFICIENT DNA TRANSFECTION IN MAMMALIAN CELLS

1. Abstract

Low molecular weight (MW ~ 3000) poly(L-lysine) (PLL) conjugated to N-glutarylphosphatidylethanolamine is an effective carrier to promote DNA-mediated transfection in cultured mammalian cells. The conjugates, named "lipopoly-L-lysine (LPLL)", contained an average of two phospholipid groups per molecule of PLL. Similar conjugates of the non-degradable poly(D-lysine) also had a similar transfection activity, indicating that the degradation of the carrier is not required for the activity. Unconjugated polylysines had little activity. The transfection activity of the LPLL has been optimized with respect to the DNA concentration, DNA/carrier ratio, incubation time and the presence of serum in the incubation medium. The binding of LPLL with DNA was measured by the degree of retardation of DNA in agarose gel electrophoresis. It was found that at the optimal DNA/LPLL ratio for transfection, all DNA was in large complexes which did not enter the gel. The transfection activity of the LPLL, under optimal conditions, was approximately 3-fold higher than that of Lipofectin, a widely used commercial reagent. Moreover, LPLL mediated transfection even in the presence of 10% calf serum; whereas the Lipofectin lost about 70% of its activity under the same conditions. However, unlike Lipofectin the transfection activity of the LPLL depended on scraping the treated cells. Furthermore, LPLL only transfected attached monolayer cells, and not suspension cells.

2. Introduction

Delivery and the subsequent expression of exogenous DNA into living cells, i.e. DNA-mediated transfection, have become increasingly important for both basic research in cell biology and clinical studies of genetic therapy. Reagents which facilitate the transfection process have been developed, among which the cationic lipids or liposomes have received much attention (1). These reagents readily complex with DNA and mediate the entry of DNA into cells. The protocol is relatively simple and non-toxic to the cells. For example, liposomes composed of mixtures of phospholipid and a cationic amphiphile such as DOTMA (2), cationic cholesterol derivatives (3), alkyl ammonium (4), and other double-chain amphiphiles (3), belong to this category of reagents. Particularly, liposomes containing DOTMA, i.e. "Lipofectin", have received a wide range of acceptance as a convenient and efficient reagent for transfection of mammalian cells (2,5). Recently, it has been shown that Lipofectin is also effective in the delivery of RNA (6), and proteins (7) into living cells.

Recently, another lipophilic molecule has been reported as an efficient transfection reagent, i.e. lipopolyamine (8). This reagent mediates the transfection by itself; it does not require any phospholipid to form liposomes. In other words, a micellar complex of lipopolyamine and DNA is sufficient for transfection. The cationic polyamine is an essential part of the reagent since it complexes with the anionic DNA. In this regard, polylysine molecules conjugated to the asialoorosomucoid (9) or transferrin (10) are also effective in the target-specific delivery of DNA to the cells which

express the appropriate receptor. The role of the polylysine is again to serve as an efficient carrier for DNA.

It thus seems reasonable to assume that a target-specific, efficient DNA vehicle can be constructed with three components: a target-specific ligand, a polycation, and a lipophilic moiety. A relatively simple way to construct this carrier would be to conjugate the ligand to the lipopolyamine. However, there are only four primary amino groups on the lipopolyamine. Consumption of any one of these for ligand conjugation would probably reduce the DNA complexing activity of the molecule. We have therefore decided to use a larger polycation, i.e. the polylysine. As the first step of the project, we have prepared a polylysine-phospholipid conjugate, i.e. the LPLL, and show in this report that it is an efficient transfection regent. We have also optimized the transfection protocol and show that the transfection activity of the LPLL is about 3-fold higher than that of the Lipofectin.

3. Materials and Methods

Materials:

N-Glutaryl-1,2-dioleoylphosphatidylethanolamine (NGPE), dioleoylphosphatidylethanolamine (DOPE), and dioleoylphosphatidylcholine (DOPC) were obtained from Avanti Polar Lipids, Inc. 1,3-Dicyclohexylcarbodiimide (DCC) and triethylamine were purchased from Aldrich Chemical Company. N-Hydroxysuccinimide (NHS), poly(L-lysine) (PLL) hydrobromide (mol. wt. 3340), poly(D-lysine) (PDL) hydrobromide (mol. wt. 3800), dimethyl sulfoxide, and acetyl coenzyme A lithium salt were obtained from Sigma Chemical Co.

Lipofectin was purchased from Bethesda Research Laboratories Inc. Chloramphenicol, D-threo-[1,2-^{14}C] (57 mCi/mmol) was purchased from ICN Biomedicals, Inc. pSV2CAT plasmid DNA was grown in E. coli and purified by the CsCl gradient centrifugation method (11).

Synthesis of Lipopolylysines:

NGPE was dialyzed against 0.001 M HCl and then lyophilized and redissolved in chloroform. NGPE was activated as the N-hydroxysuccinimide ester by mixing with DCC at 1.2 equivalent in $CHCl_3$ and with NHS at 1 equivalent in dimethylformamide. The reaction proceeded at room temperature for 48 h. The crystal reaction byproduct, dicyclohexylisourea, was removed by filtration through glass wool. LPLL or LPDL was prepared as follows: Activated NGPE was added to PLL or PDL in dimethyl sulfoxide at a molar ratio of polylysine : NGPE = 1 : 2. Triethylamine at equal weight with PLL or PDL was added to the above mixture. The reaction was completed in a few min. (The chemical synthesis of LPLL is illustrated in Appendix 1.) Reaction products were analyzed on a silica gel plate using a solvent system of chloroform : methanol = 65 : 30. Polylysine and the phospholipid were stained by ninhydrin and Zinzade's solution, respectively (12). Since all of the NHS ester of NGPE was consumed in the reaction, the stoichiometry of lipopolylysine was assumed to be polylysine : NGPE = 1 : 2. Lipopolylysines were used without any further purification.

Electrophoresis of DNA:

LPLL suspension was prepared in 20 mM phosphate buffer (pH 7.4) at a concentration of 0.66 ug/ml. Various amounts of LPLL were mixed with 0.5 ug of DNA in serum free medium. The final volume was 20 ul. The mixture was incubated at room temperature for 15 min before loading onto an 1% agarose gel slab. Electrophoresis was carried out in TAE (0.04 M Tris-acetate, 0.001 M EDTA) buffer according to the standard technique (11).

Cell Culture:

L929 and Vero cells were grown in McCoy's medium containing 10% calf serum. RDM4 and CEM cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum. HeLa cells were grown in Dulbecco's minimal essential medium supplemented with non-essential amino acids and 10% fetal bovine serum. For the attached cells, cultures of approximately 80% confluency were used for the transfection experiments.

Transfection:

Polylysines, lipopolylysines or lipopolylysine/lipid mixtures were suspended in 200 ul phosphate buffer (20 mM, pH 7.4) and sonicated to clarity. (The micellular structure of LPLL is illustrated in Appendix 2.) The suspension and pSV2CAT DNA were diluted separately to 2.5 ml with serum-free medium and then mixed. The complexed DNA was then added to cells. After incubation at 37^{o}C, cells were washed three times with serum-free medium. Serum containing growth medium was added back to the culture plate. Cells were then detached by scraping with a S/P cell scraper and divided into two new plates. Two days later, cells were

harvested to assay for CAT activity. The transfection protocol for Lipofectin was provided by the manufacturer. Forty ug of Lipofectin was used to deliver 10 ug of DNA.

Assay of Chloramphenicol Acetyl-transferase Activity:

Chloramphenicol acetyl-transferase (CAT) activity was determined by the method of Gorman et al. (13). Cell extract was prepared by the freeze-thaw method. 0.5 to 1 mg of cellular protein was heat-treated at 65^{o}C for 10 min, and then added with 20 ul of 4 mM acetyl-CoA and 0.2 uCi of [^{14}C]chloramphenicol. After 1.5 h incubation at 37^{o}C, chloramphenicol and its derivatives were extracted with ethyl acetate and separated by thin-layer chromatography on a silica gel plate. The plate was autoradiographed and the appropriate spots were scraped for radioactivity counting to analyze the percentage of chloramphenicol acetylation. One unit of CAT activity is defined as conversion of one nanomole chloramphenicol into its acetylated derivatives per min.

4. Results

Comparison of Different Transfection Reagents:

We have prepared several different transfection reagents and compared their transfection activities on L929 mouse fibroblast cells using a plasmid DNA (pSV2CAT) containing a standard marker gene coding for the E. coli CAT. These experiments were done by mixing the LPLL or other reagents with a constant amount (10 ug) of pSV2CAT DNA and added to cells in a serum-free medium. After 3 h of incubation at

37°C, cells were washed, scraped and recultured in fresh serum-containing medium for 2 days before the CAT activity of the cells was assayed. Table I shows the results of the experiment. LPLL transfected the cells very efficiently; the expression of the CAT activity was approximately 6.4 mU/mg protein. Similarly efficient was the LPDL, a non-biodegradable polymer conjugate. This result indicates that the degradation of the carrier is not essential for efficient transfection. The native PLL or PDL without the phospholipid conjugation was not active, indicating the importance of the lipophilic moiety for an efficient transfection reagent. It has been reported (2, 4) that DOTMA and other cationic lipids exhibit their maximal transfection activities when they are mixed with a phospholipid, particularly an unsaturated phosphatidylethanolamine, to form cationic liposomes. To test if mixing with phospholipids enhances the transfection activity, we have used DOPC and DOPE to mix with the LPLL (DOPE or DOPE/LPLL = 13 : 1, molar ratio) and tested the transfection activity. It was found that mixing with either DOPC or DOPE the activity of LPLL had decreased to about 50% of that shown by the LPLL alone (Table I). This result indicates that LPLL works more efficiently as a solo reagent; no liposomal formulation is needed. It is interesting to note that the transfection activity of LPLL was approximately 3-fold higher than that of the Lipofectin, a cationic liposome formulation widely used for DNA transfection (2, 14, 15).

Effect of LPLL Dose on the Transfection Efficiency:

To test if the transfection activity of LPLL was dose-dependent, an experiment was done in which various amounts of LPLL were used with a

constant amount (10 ug) of pSV2CAT DNA. Fig. 1 shows a bell-shaped dose-response curve with the optimal dose of LPLL occurring at approximately 60 - 70 ug. The decrease of CAT activity at higher doses was not due to the cytotoxicity of LPLL because cells treated with up to 132 ug LPLL per plate for 6 h still had more than 90% viability after 24 h post incubation.

Effect of DNA Dose on the Transfection Efficiency:

We then fixed the dose of LPLL at 66 ug and varied the DNA concentration. Again, a bell-shaped dose-response curve was obtained (Fig. 2). The optimal DNA concentration was 10 ug. For the subsequent experiments, we have used 66 ug LPLL and 10 ug DNA as a standard condition. The ratio of positive-to-negative charges of the LPLL-DNA complex under this optimal condition is approximately 6. At this ratio, slightly turbid complexes could be seen, but no precipitates were formed.

Electrophoresis of DNA-LPLL Complexes:

To examine the complex formation of DNA with LPLL, various amounts of LPLL were added to DNA and the complexes were electrophoresed in an agarose gel. The gel was stained with ethidium bromide to visualize DNA. As can be seen in Fig. 3, when 0.5 ug of DNA complexed with as little as 1.1 ug LPLL a retardation of the DNA bands in the gel (lane 2) had resulted. It indicates that the DNA-LPLL complex is larger in size and/or less negatively charged (hence less movement toward the anode) than the native DNA. At 3.3 ug LPLL, when the positive-to-negative charge ratio was 6, complete retardation of the DNA bands

occurred (lane 4). Thus, the optimal DNA-LPLL complex of the charge ratio 6 was fairly large in size and/or not negatively charged.

Effect of Incubation Time on the Transfection Efficiency:

The efficiency of the transfection was incubation time-dependent. Little transfection was detectable for incubation times shorter than 1 h. Maximal efficiency was observed at 3 h of incubation (Fig. 4). Although the cells had been incubated with the LPLL-DNA complex in a serum-free medium, no gross morphological changes or cell death was observed at the end of 6 h incubation.

Effect of Serum Concentration on the Transfection Efficiency:

Although the transfection incubation was routinely done in a serum-free medium, we have tested the effect of serum addition to the incubation medium on the efficiency of transfection. It is clear from the data in Fig. 5 that the calf serum produced a concentration-dependent inhibition effect on the transfection efficiency. Almost no transfection was detectable at 40 - 50 % serum concentration. However, considerable (90% or greater) amount of the transfection activity was still observed at 10% serum concentration. In contrast, little transfection activity was observable if 10% serum was added to the Lipofectin reagent (Fig. 5). This result indicates that LPLL can tolerate the inhibitory effect of the serum better than the Lipofectin. Most mammalian cells are healthy in a medium containing 10% serum. Thus, the lipopolylysine reagent may be more useful than the Lipofectin for those cells which require serum for viability.

Effect of Cell Scraping on the Transfection Efficiency:

We routinely transfer the transfected cells into new plates at a lower cell density to avoid cell overgrowth during the 2-day postincubation period. The transfer was done by mechanically scraping the cells off the plate and reculturing them in new plates. It turns out that the scraping step is important for the maximal transfection activity, because cells transferred by using either trypsin or EDTA showed only low levels of activity (Table II). Cells not transferred to the new plates also showed a low activity. Interestingly, the transfection activity of Lipofectin was also enhanced by the scraping procedure by approximately 3-fold (Table II).

Effect of Cell Type on the Transfection Activity of LPLL:

L929 cells are monolayer cells. It was interesting to see if LPLL is effective in other monolayer cells and suspension cells. Table III shows that two other monolayer cells, Vero (monkey cells) and HeLa (human cells), could be effectively transfected by LPLL using a protocol which was optimized for the mouse L929 cells. However, two suspension cell lines, mouse RDM4 and human CEM, showed little transfection activity. Both RDM4 and CEM cells are lymphoid cells which are known to be resistant to transfection (16, 17).

5. Discussion

The long-term goal of the project is to develop a target-specific and efficient vehicle for DNA transfection. Such a vehicle would be very useful for genetic therapy treatment currently under active investigation.

Polylysines are suitable for this purpose for the following reasons. First, the polymer readily complexes with DNA of different sizes, both linear, circular and supercoiled (18, 19). A vehicle composed of polylysine would be a general carrier for DNA. Secondly, polylysine contains many primary amino groups which can be conveniently used for conjugation with lipids or ligands. The cationic oligomer or polymer itself is not a good carrier for DNA transfection; a lipophilic derivative significantly enhances the transfection activity (Table I and Ref. 8). Polylysine-conjugated drugs, although potent in drug action, are usually non-specific. Additional conjugation with ligands, such as antibody or hormones, is needed for the target specificity of the vehicle (9, 10). The third advantage of using polylysine as a DNA carrier is its relatively low toxicity to cells. This is particularly the case for PLL which is degradable by the target cells. Lastly, the polymer is commercially available in different polymeric sizes and is relatively inexpensive. We have thus chosen polylysine as the starting point of the project.

As shown in Table I, the native polylysine, no matter L- or D-isomer, showed very little activity for DNA transfection; only the phospholipid conjugates exhibited a high level of activity. This result clearly demonstrates the importance of lipophilicity of the transfection reagent. While the mechanism by which the lipophilic polylysine mediates the DNA transfection is not known, it is unlikely that the large macromolecular complex can actually enter the cytoplasm by directly penetrating the plasma membrane. A more plausible mechanism is that the DNA-lipopolylysine complexes bind to the cell surface and are internalized to the cellular endosomes or lysosomes. The phospholipid moiety of the complex

could conceivably cause a perturbation in the membranes of the endosomes or lysosomes, leading to a release of the complex from these organelles into the cytoplasm. Liposomes containing cationic lipids mediate the delivery of DNA into cells probably with a similar mechanism (4). Although there is not enough experimental data at present to support the hypothesis, the mechanism is consistent with the observation that both PLL and PDL are similarly effective in transfection (Table I). Since PDL is not biodegradable, it implies that the release of the DNA-polymer complex does not require the degradation of the polymer, which takes place in the lysosome compartment. Since PLL is less toxic than the PDL (20), we have chosen to perform the detailed investigations with the PLL conjugates.

The dose-response curves for LPLL (Fig. 1) and for DNA (Fig. 2) are both bell-shaped. This is not a surprise in view of the polyvalent nature of DNA and polylysine. Optimal levels of complex formation between the polyvalent binders requires a balance in the concentrations of both binders. The presence of excess of either binder would result in fewer and/or smaller complexes. The optimal amounts of LPLL and DNA are such that the positive-to-negative charge ratio of the complex is approximately 6. This means that the complex contains a net positive charge (Fig. 3) which would allow an efficient binding of the complex to the negatively charged cell surface. Furthermore, the complex can probably tolerate the presence of other negatively charged macromolecules such as serum proteins. This is probably the reason why the action of LPLL transfection reagent was only slightly inhibited in the presence of 10% calf serum (Fig. 5). The Lipofectin reagent, on the other hand, was very sensitive to the presence of serum; little activity could be detected when 10% calf serum was added (Fig. 5).

The data in Table II clearly demonstrate the importance of mechanical scraping for the maximal transfection of LPLL. It has been reported that scraping alone can help load the cells with macromolecules (21). Mechanical shearing during the scraping procedure could produce transient membrane damage to allow a more efficient entry of the complex into the cytoplasm. Alternatively, the cells could be stimulated to undergo more active endocytosis as a part of the membrane repair mechanism. Interestingly, the transfection activity of Lipofectin was also enhanced by scraping the cells, suggesting that the two reagents share a common mechanism of action. The enhancement effect of cell scraping does not imply that an efficient transfection requires an intermittent period of time during which the cells must be in suspension. This is because the cells which were detached from the plate by a treatment of trypsin or EDTA did not show a high level of transfection (Table II). In fact, suspension cells such as mouse RDM4 and human CEM cells were not transfected well with the LPLL reagent (Fig. 5). These lymphoid cells are known to be relatively resistant to transfection; they are usually transfected with the electroporation method (22 - 24).

The present LPLL reagent contains an average of 16 lysine residues and 2 phospholipid groups. While this reagent works well for DNA transfection, it is possible that polylysines of greater or shorter length may also work. Conjugates with more or fewer phospholipid groups could also be active in transfection. Furthermore, the location of the phospholipid groups on the polylysine chain is probably random for the present LPLL reagent. Specific location such as at either or both ends of the polylysine

molecule could possible give a different transfection activity. These possibilities are currently under investigation.

In summary, we have described a new transfection reagent which is composed of a simple conjugate of a cationic polymer and phospholipids. The reagent works well for the DNA transfection of a number of monolayer cells, even in the presence of 10% serum. The transfection protocol is relatively simple and non-toxic to cells. The transfection activity of the new reagent is about 3-fold higher than that of the Lipofectin, a commonly used reagent for DNA transfection. This new reagent should also be useful in the construction of a target-specific delivery vehicle for DNA.

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Table I: Comparison of transfection efficiency for different transfection reagents [1]

Transfection reagent	CAT activity (% of max)
Lipofectin	31.7
PLL	1.3
LPLL	100
LPLL/DOPC	50
LPLL/DOPE	41.5
DNA	0.1
PDL	2.9
LPDL	87.8
E. coli CAT [2]	50

1. L929 cells were transfected with pSV2CAT (10 ug) alone or complexed with Lipofectin (40 ug), PLL (44 ug), LPLL (66 ug), LPLL/DOPC (66 ug/138 ug), LPLL/DOPE (66 ug/130 ug), PDL (44 ug) or LPDL (66 ug). Cells were washed and scraped after a 3 h incubation with the mixtures and harvested 2 days later for CAT activity determination. Data were collected from a single experiment with no replicate.
2. 10 mU of E. coli CAT was used as a positive control of the CAT assay

Table II: The effect of post-treatment on transfection efficiency [1]

Transfection reagent	Post-treatment	CAT activity (% of max)
Lipofectin	none	10.4
	scraping	31.4
LPLL	none	0.8
	scraping	100
	trypsin	2.7
	EDTA	2.0

1. Mouse L929 cells (approximately 40% confluent) were incubated with complexes of LPLL/DNA (66 ug/10 ug) or Lipofectin/DNA (40 ug/10 ug) for 3 h. Cells were washed and either scraped or detached by trypsin or EDTA treatment and recultured in a new plate for 2 days. CAT activity of the cell extract was determined as described in Methods. Data were collected from a single experiment without replicate.

Table III: Comparison of transfection efficiency for different cell lines [1]

Cell line	CAT activity (% of max)
L929	100
Vero	71.3
HeLa	43.2
RDM4	0.7
CEM	0.5

1. L929, Vero, HeLa, RDM4 and CEM cells were transfected with LPLL/ pSV2CAT (66 ug / 10ug) complexes and assayed for CAT activities following the standard procedures described in Methods. Data were collected from a single experiment without replicate.

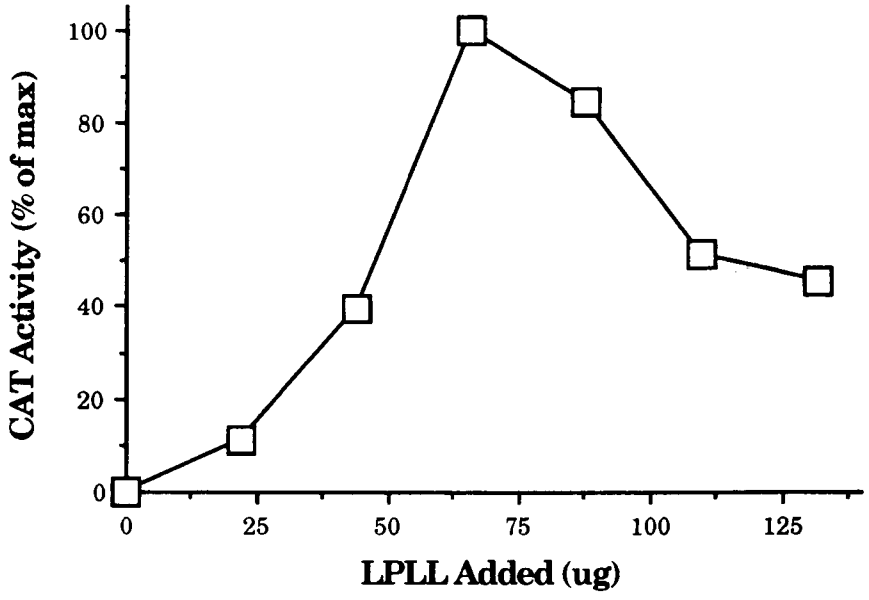


Figure 1. The effect of LPLL dose on transfection efficiency. Various amounts of LPLL were complexed with 10 ug of pSV2CAT DNA. L929 cells were transfected with the mixtures and assayed for CAT activities following the standard procedures described in Methods. Data were collected from a single experiment without replicate.

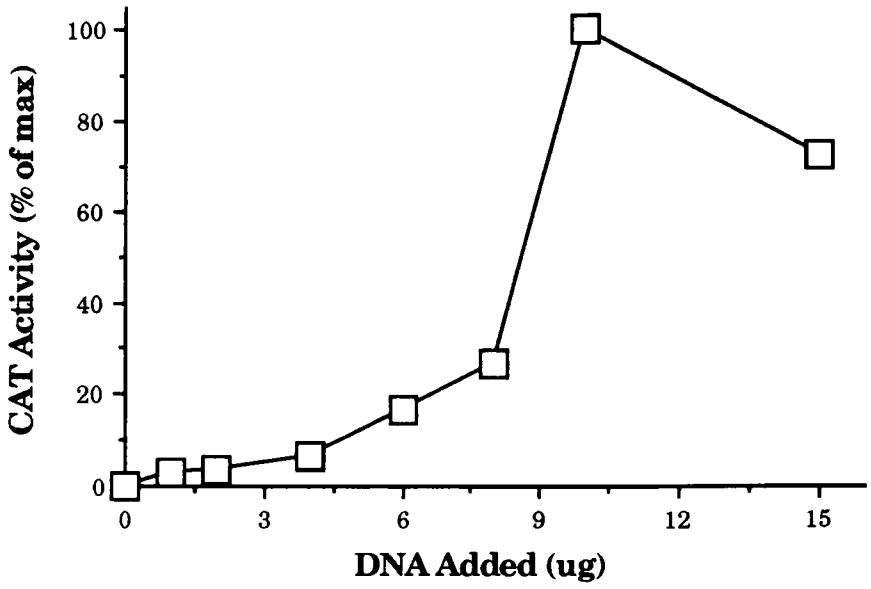


Figure 2. The effect of DNA dose on transfection efficiency. Various amounts of pSV2CAT DNA were complexed with 66 ug of LPLL. L929 cells were transfected with the mixtures and assayed for CAT activities following the standard procedures described in Methods. Data were collected from a single experiment without replicate.

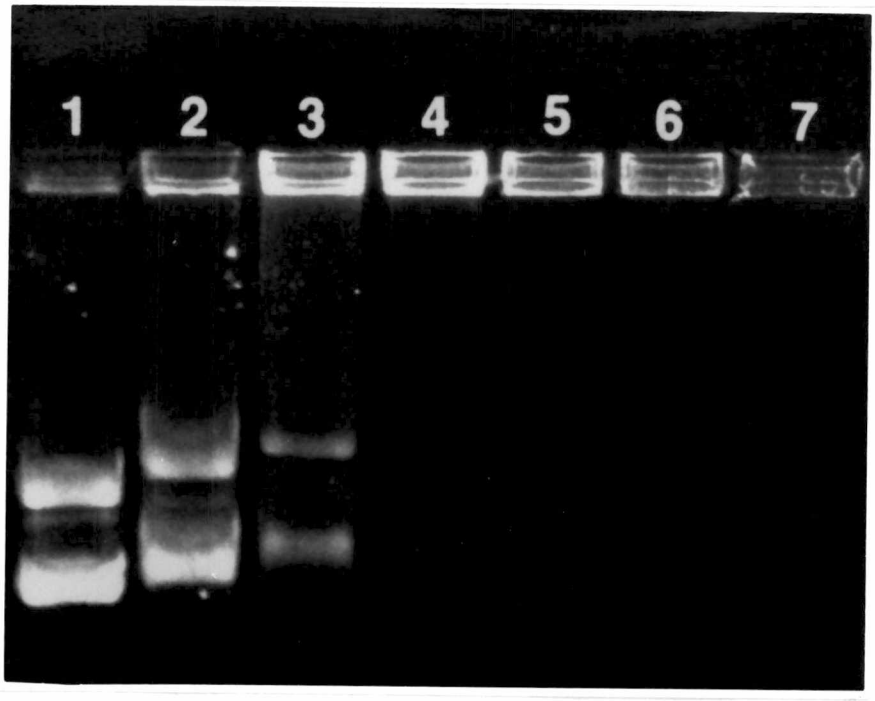


Figure 3. Dose-dependent retardation of DNA electrophoretic mobility by LPLL. 0.5 ug of pSV2CAT was complexed with 0, 1.1, 2.2, 3.3, 4.4, 5.5, and 6.6 ug (lane 1 to 7, respectively) of LPLL in serum free medium. The complexes were electrophoresed on 1% agarose gel. DNA was visualized with ethidium bromide. The upper and lower bands in lane 1 represented free plasmids in two different extents of supercoiling. Data were collected from a single experiment without replicate.

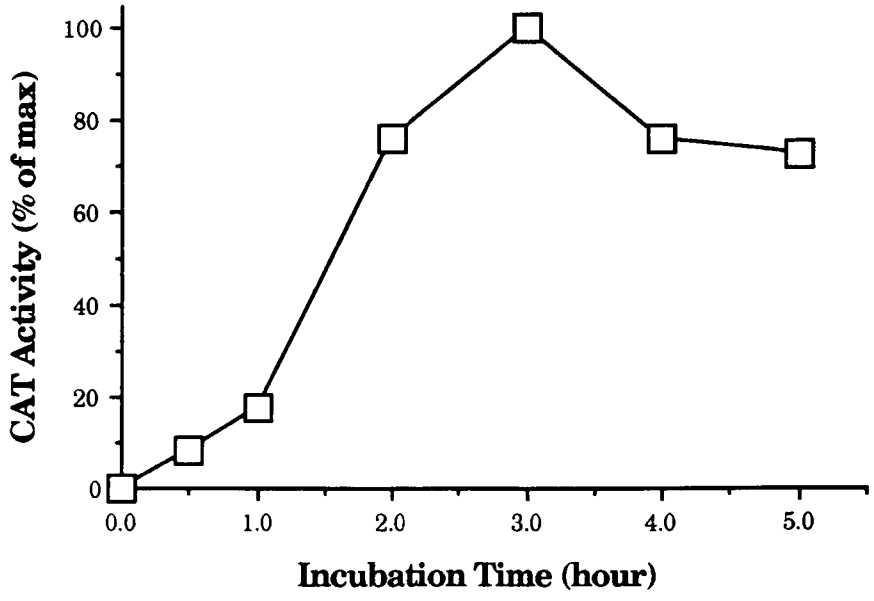


Figure 4. The effect of incubation time on transfection efficiency. L929 cells were incubated with mixtures of 10 ug pSV2CAT DNA and 66 ug LPLL for various lengths of time. At the end of incubation, cells were washed and scraped and harvested 2 days later for CAT activity assay. Data were collected from a single experiment without replicate.

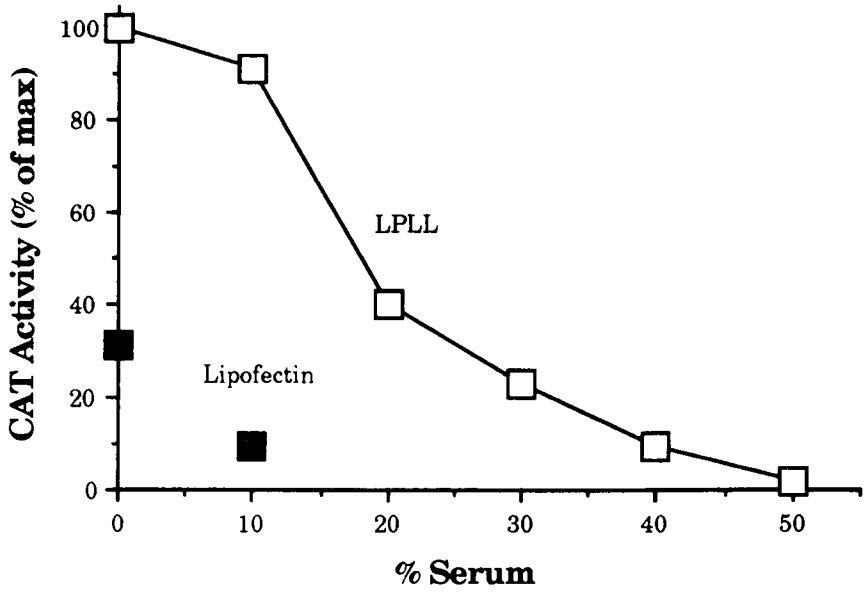


Figure 5. The effect of serum concentration on transfection efficiency. 10 ug pSV2CAT DNA and 66 ug of LPLL were complexed and then mixed with various amounts of serum. The mixtures were added to L929 cell monolayers. After a 3 h incubation, cells were washed, scraped and harvested 2 days later for CAT activity assay. Data were collected from a single experiment without replicate.

PART IV

LIPOSOMES CONTAINING LIPOPOLYLYSINE AS A VEHICLE FOR GENE TRANSFER I: CHARACTERIZATION AND OPTIMIZATION

1. Abstract

The ability of a polycationic lipid, lipopoly-L-lysine (LPLL), to mediate efficient DNA transfection depended on scraping of the treated cells [Biochim. Biophys. Acta, 1065 (1991) 8 - 14]. It was found that the mechanical treatment could be avoided by including a helper lipid to the liposome composition. Among the helper lipids tested, a hexagonal phase forming lipid, dioleoylphosphatidylethanolamine (DOPE), gave rise to the highest activity. The optimal liposome composition contained LPLL : DOPE = 1 : 8 (molar ratio). The transfection efficiency was further optimized by varying the lipophilicity of the LPLL, the ratio of the cationic liposome to DNA, and the concentration of the liposome/DNA complex. Under the optimized conditions, liposomes containing lipopoly-D-lysine (LPDL) had a similar transfection activity to those containing LPLL, indicating that the cytoplasmic delivery and expression of the exogenous gene do not require the degradation of the polylysine chain.

2. Introduction

Recent years have seen a rapid development of cationic liposomes (1 - 8). This type of reagent provides a simple and efficient means of introducing DNA (1 - 8), RNA (9), and proteins (10, 11) into living cells. The liposomes also modulate the infectivity of viruses (12, 13). Thus, they are useful tools for studying gene expression and regulation, and protein functions. Cationic liposomes are usually used only for in vitro studies because of their sensitivity to the serum components. However, a recent study showed that

i.v. injected DNA complexed with Lipofectin, a cationic liposome preparation, was capable of mediating DNA transfection in mouse (14). It is also reported that exogenous DNA can be detected in organs of mice injected with DNA/cationic liposome complexes (15). It thus appears that cationic liposomes can be used for DNA delivery in animals.

Target-specific DNA carriers are desirable for studies of gene transfer. This is especially the case for human gene therapy. We have previously reasoned (7) that the cationic liposome system can be incorporated with a target-specific ligand for the construction of a targetable and efficient DNA vehicle. To develop such a carrier, we first synthesized a cationic lipid, LPLL (7), which contains multiple primary amino groups for a convenient conjugation of the targeting ligands. LPLL mediates efficient transfection in mouse L929 cells. Its activity is about 3-fold higher than that of Lipofectin under the optimal conditions. Moreover, LPLL is more resistant to the neutralizing effect of serum than Lipofectin. However, the transfection activity depends on the mechanical scraping of the treated cells. This requirement severely limits its application in vivo. We have now discovered that this undesirable treatment can be avoided by including a helper lipid to the liposome composition. The transfection condition for this new reagent has been optimized with respect to the transfection activity in mouse L929 cells.

3. Materials and Methods

Materials:

Dipalmitoylsuccinylglycerol (DPSG), DOPE, monomethyl DOPE (MMDOPE), dimethyl DOPE (DMDOPE), dielaidoylphosphatidylethanolamine (DEPE), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylserine (DOPS), and monogalactosyldiglyceride (MGDG) were purchased from Avanti Polar Lipids. Poly(L-lysine) (PLL, MW 3300), poly(D-lysine) (PDL, MW 3700), dimethyl sulfoxide, acetyl coenzyme A, cholesterol, and dipalmitoyl glycerol were purchased from Sigma. N-hydroxysuccinimide, 1,3-dicyclohexylcarbodiimide and triethylamine were from Aldrich. D-threo [1,2-^{14}C] chloramphenicol (57 mCi/mmol) and [^{14}C] succinic anhydride were from ICN Biomedicals. Ethyl acetate was obtained from Fluka. [^{14}C] DPSG was synthesized from dipalmitoyl glycerol and [^{14}C] succinic anhydride as described (16). Plasmid pUCSV2CAT (4.8 kb, constructed by Dr. Mark Magnuson of Vanderbilt University) was amplified in E. coli and purified by CsCl gradient centrifugation method.

Cell Culture:

L929 cells were grown in McCoy's medium supplemented with 10% calf serum. For transfection experiments, cells were seeded one day earlier in 100 mm plates to reach approximately 60% confluency at time of treatment.

Synthesis of Lipopolylysines:

LPLL and LPDL were synthesized according to a protocol described previously (7) with modifications: DPSG was activated to the N-hydroxysuccinimide ester with 1 molar equivalent each of 1,3-dicyclohexylcarbodiimide and N-hydroxysuccinimide in ethyl acetate. The reaction proceeded overnight at room temperature. Crystals of dicyclohexylurea, a reaction byproduct, was removed by filtration through glass wool. The filtrate was dried under a stream of nitrogen gas and desiccated. N-hydroxysuccinimide ester of DPSG was recrystallized from methanol:ethanol:chloroform (1:2:2) at $0^{o}C$. LPLL and LPDL were synthesized by mixing PLL or PDL with two molar equivalent of N-hydroxysuccinimide ester of DPSG in dimethyl sulfoxide. The reaction proceeded at $50^{o}C$ for 10 min in the presence of triethylamine which was added at equal weight with the polylysines. Triethylamine was removed by evaporation under a stream of nitrogen gas and desiccation. LPLL and LPDL were dissolved in chloroform and stored at $-20^{o}C$. (The chemical synthesis of LPLL is illustrated in Appendix 1.)

Transfection:

LPLL/DOPE mixture in $CHCl_3$ was dried under a stream of nitrogen gas and desiccated. The lipid film was hydrated briefly in 100 ul sterile distilled water and sonicated in a bath sonicator to clarity. (The bilayer structure of LPLL/DOPE is illustrated in Appendix 3.) The liposome suspension was combined with pUCSV2CAT DNA diluted to 100 ul with sterile distilled water. The mixture was incubated at room temperature for

15 min before added to cells in 5 ml serum-free medium. After 3 h incubation at 37°C, cells were washed and cultured for 2 days. CAT activity in treated cells was assayed as described (7). One unit of CAT activity is defined as the conversion of one nanomole chloramphenicol to its acetylated derivatives per min. Most of the data are expressed as relative activity with 100% activity being that of the cells treated with 285 ug of LPLL/DOPE (1 : 8) liposomes and 10 (Fig. 1 - 3) or 15 (Fig. 4 - 7) ug of DNA for 3 hours at 37°C. Under these conditions, the cells usually showed a CAT activity of 26 to 36 miliunits/mg protein.

4. Results

Previously, LPLL was synthesized by conjugating N-glutaryl-1,2-dioleoylphosphatidylethanolamine (NGPE) to PLL (7). The negatively charged phosphate group on NGPE was later found to interact non-specifically with PLL which is positively charged at the physiological pH. This non-specific interaction interfered with the characterization of the reaction product, LPLL. It also contributed to the problem of aggregation during the formation of DNA/cationic liposome complex. Therefore, NGPE was replaced by DPSG. Like NGPE, DPSG has a terminal carboxylic group which can be activated to react with the amino groups of PLL. Unlike NGPE, DPSG does not carry any other charged groups on the molecule. LPLL and LPDL used in the following studies were synthesized from DPSG and PLL or PDL. The new lipopolylysines still required scraping for efficient DNA delivery. L929 cells receiving scraping or not after incubation

with LPLL/DNA complexes expressed 217% and 5% CAT activities, respectively.

Dependency of the Transfection Activity of LPLL on the Helper Lipids:

LPLL mediated efficient DNA transfection. However, its activity depended on scraping of the treated cells (7). It had been shown that other cationic lipids such as N[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA) (1) and 3β[N-(N', N'-dimethyl-aminoethane)-carbamoyl] cholesterol (8) required a helper lipid, DOPE, for transfection activity. Helper lipids required for optimal results varied from one cationic lipid to another (5). To investigate whether the requirement of scraping for the transfection activity of LPLL could be substituted by inclusion of another lipid to the liposome composition, a group of lipids were screened for the helper lipid activity. Liposomes composed of LPLL and various helper lipids (1 : 8, molar ratio) were tested for their abilities to delivery a marker plasmid, pUCSV2CAT, to the cultured mouse L929 cells. Transfection activity was measured by the level of expression of the exogenous gene coding for the chloramphenicol acetyl-transferase (CAT). Fig. 1 shows that combination of DOPE with LPLL gave the highest activity. Interestingly, the increasing extent of methylation on the amino group of DOPE, i.e. MMDOPE, DMDOPE, and DOPC progressively decreased the activity. This result suggests that the head group structure of the helper lipid is important for the efficient delivery of DNA. DEPE, which is a trans isomer of DOPE, also gave a reduced activity, indicating that the structure of lipid tail of the helper lipid also plays a role in the determination of transfection activity. Other neutral lipids, MGDG and cholesterol, and

negatively charged lipids, DOPS and DPSG, in combination with LPLL were all inactive for transfection.

Effect of the Lipophilicity of LPLL on Transfection Activity:

While structure of the helper lipid affected transfection activity of the cationic liposomes, the structure of LPLL might also be a determining factor. We had speculated that the lipophilicity of LPLL would influence the efficiency of DNA delivery (7). Thus a series of LPLL containing up to about three DPSG units per PLL moiety were synthesized and tested for their activities. Fig. 2 shows that conjugates containing an average of about two DPSG molecules per molecule of PLL had the highest activity. Fewer or more lipid units on each PLL chain had resulted in a reduced activity. This result suggests that the conformation of the PLL chain on the liposome surface may affect the transfection efficiency. Unconjugated PLL alone was inactive for transfection (Fig. 2), demonstrating the importance of the lipophilic moiety on PLL for the translocation of plasmid DNA into the cytoplasm of the treated cells. It should be noted that PLL used in this study contained an average of 16 lysine residues and 17 amino groups (16 ε- and 1 α-amino groups). The optimal conjugate contained about 2 lipid groups per PLL chain, thus leaving 15 free amino groups for binding with the anionic DNA. Also shown in Fig. 2 is the lack of transfection activity of LPLL of various coupling ratios in the absence of DOPE. Thus, the requirement of a helper lipid is independent of the conjugate structure.

Optimization of the DOPE/LPLL Ratio:

The transfection activity of the cationic liposomes was further optimized by varying the ratio of the optimal helper lipid, DOPE, to LPLL. Fig. 3 shows that liposomes with a molar ratio of DOPE/LPLL between 6 to 8 transfected cells most efficiently. Thus, the concentration of DOPE in the liposome bilayer is important for the cytoplasmic delivery of DNA. It is interesting to note that this optimal ratio is much higher than that of Lipofectin (DOPE/DOTMA = 1) (1).

Optimization of the DNA/liposome Ratio:

We had previously found that there was an optimal ratio of DNA to free LPLL for transfection (7). Change of this ratio dramatically decreased the transfection efficiency. Since the DNA carrier used here was LPLL/DOPE liposomes instead of free LPLL, it was important to redetermine the optimal ratio of DNA to the cationic liposome.

We first used a fixed amount of 285 ug liposomes to complex with various amounts of pUCSV2CAT DNA. Transfection activity of the complexes was assayed. Data in Fig. 4 show a bell-shaped DNA dose-response curve, with the optimal amount of plasmid DNA being 15 ug. The decline of activity at high DNA doses was not due to cytotoxicity to the treated cells; all complexes tested had no detectable cytotoxicity as measured by the trypan blue exclusion test (not shown).

Next, we used 15 ug of pUCSV2CAT DNA to complex with various amounts (up to 342 ug) of liposomes. Fig. 5 shows the transfection efficiency of the complexes. Again, a bell-shaped dose-response curve was obtained. The optimal ratio equaled to 15 ug DNA/285 ug liposome, confirming the

result shown in Fig. 4. We had decided to use this optimal ratio for the routine transfection experiments.

Finally, we determined the effect of dose of the optimal DNA/liposome complex on the transfection efficiency. Data in Fig. 6 show that the transfection activity of the complex progressively increased with the dose of the complex added to the cells. Even at the highest dose used in the experiment no detectable cytotoxicity of the treated cells was observed. Furthermore, under the same conditions cationic liposomes composed of LPDL/DOPE mediated transfection with a similar activity to that of LPLL/DOPE liposomes except for the highest dose (Fig. 6). This result suggests that no degradation of the polylysine chain is required for the delivery and the subsequent expression of the exogenous DNA. LPDL is not degradable due to the D-isomeric form of the lysine residues.

Effect of Serum on the Transfection Activity:

Serum components inhibited transfection activity of cationic lipids or liposomes (7). To determine the serum sensitivity of the LPLL/DOPE liposomes, transfection experiments were carried out in a medium containing various concentrations of bovine calf serum. Data in Fig. 7 show that 20% serum reduced the activity by 70%. Very little activity was detected at 40% or higher concentration of serum.

5. Discussion

In an effort to develop a targetable and efficient DNA carrier, we had synthesized a polycationic lipid, LPLL (7). Free LPLL required scraping of the treated cells for its transfection activity. The mechanical treatment was thought to produce transient membrane damage to the cells to facilitate the entry of the LPLL/DNA complexes into the cytoplasm. In comparison to the free LPLL, some LPLL containing liposomes by themselves mediated efficient transfection. In other words, these liposomes translocated the plasmid DNA into the cytoplasm without the use of mechanical scraping. LPLL mixed with DOPE gave rise to the optimal activity (Fig. 1). Interestingly, a number of other cationic lipids when combined with DOPE also transfected cells efficiently (1, 2, 4 - 8). This suggests that cationic liposomes may share a similar mechanism of action.

It was clear from Fig. 2 that the lipophilicity of LPLL was important for the optimal transfection activity. LPLL with fewer or more lipid moieties on each conjugate were weaker bilayer stabilizers for DOPE (unpublished observation). Therefore, it is possible that a high DOPE content in the liposome bilayer contributed to the high transfection activity. Data in Fig. 3 also show that the concentration of DOPE in the liposome bilayer was linearly proportional to the transfection activity when the molar ratio of DOPE to LPLL was below 6. Since DOPE is a hexagonal phase forming lipid under the physiological conditions, the process of transfection mediated by the LPLL/DOPE liposomes may involve an event of hexagonal phase formation (4). This notion is consistent with the observation that helper lipids having less potential to promote the hexagonal phase transition, e.g.

DEPE and MMDOPE, etc., had weaker transfection activities when combined with LPLL (Fig. 1).

At the optimal ratio of liposome to DNA (Fig. 4 and Fig. 5), the complexes had a positive-to-negative charge ratio of about 9. This net positive charge possibly provides the high affinity of the complexes to cell surface which is negatively charged. In this view, it is not difficult to understand why serum (Fig. 7 and ref. 7) and purified polyvalent negatively charged molecules such as salmon sperm DNA (unpublished results) and sulfated proteoglycans (18) are potent inhibitors of transfection mediated by cationic liposomes. In comparison to the current reagent, complexes of DNA with other cationic liposomes such as Lipofectin (1) and DC-chol/DOPE (8) liposomes only require positive-to-negative charge ratios of approximately 3 ~ 4 to obtain optimal results. This is probably because the charge density on LPLL/DOPE liposomes is higher than those on other cationic liposomes. For example, LPLL/DOPE liposomes and Lipofectin contain 1.7 and 0.5 umol positive charges per umol lipid, respectively. Consequently, LPLL/DOPE liposomes are less efficient in neutralizing the negative charges of DNA molecules than other cationic liposomes reported in literatures.

Like LPLL/DOPE liposomes, LPDL/DOPE liposomes also mediated efficient transfection (Fig. 6), indicating that the degradation of the polylysine chain which takes place in lysosomes is not necessary for the cytoplasmic delivery of DNA. It is therefore reasonable to assume that the abilities of LPLL and LPDL to stabilize DOPE and to associate with DNA and cell membrane are the essential and sufficient requirements for the transfection activity. The mechanism by which these cationic liposomes

mediate DNA delivery have been further investigated as described in the accompanying paper.

The current LPLL reagent differs from other cationic liposomes in that it carries multiple primary amino groups which would facilitate chemical modifications such as conjugation with carbohydrates, proteins, and other targeting ligands. This is especially important if targeted gene transfer is desired. PLL derivatized with antibody mediated target-specific delivery of plasmid DNA in both cultured cells and in a mouse model (unpublished results). It is therefore possible that LPLL/DOPE liposomes coated with targeting molecules could mediate DNA transfection in a target-specific manner. Another advantage offered by the LPLL reagent is its low cytotoxicity. Preparations containing LPLL (7) or other biodegradable cationic lipids (5, 8) had no significant cytotoxicity when cells were subjected to prolonged exposure at or above optimal concentrations. Whereas under the same conditions cationic detergents (2) and ether bond containing lipids (1), which are non-biodegradable, caused immediate cell death.

Cationic liposomes in general mediate efficient transfection. The condition of treatment is relatively mild and the protocol is simple. In addition to in vitro transfection (1 - 8), cationic liposomes have been reported to mediate gene transfer in intact animals by intratracheal instillation (14, 19), site directed (20) and intravenous (14) injection. Topical and ex vivo applications may also be possible. Therefore, cationic liposomes offer a reasonable alternative to the viral vectors for gene therapy.

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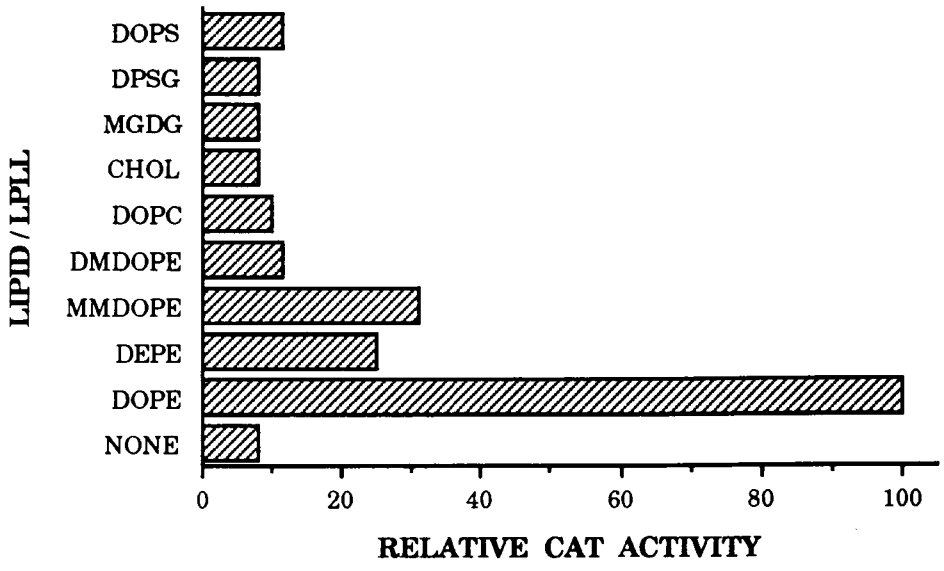


Figure 1. Dependency of the transfection activity of LPLL on helper lipids. Lipid dispersions were prepared from 122 ug LPLL alone or mixed with eight molar excess of DOPE, DEPE, MMDOPE, DMDOPE, DOPC, cholesterol, MGDG, DPSG or DOPS. Each of the dispersions was mixed with 10 ug of pUCSV2CAT DNA. The mixtures were used to transfect L929 cells. CAT activity in the treated cells was assayed as described in Methods. Data were collected from a single experiment with duplicates for each point.

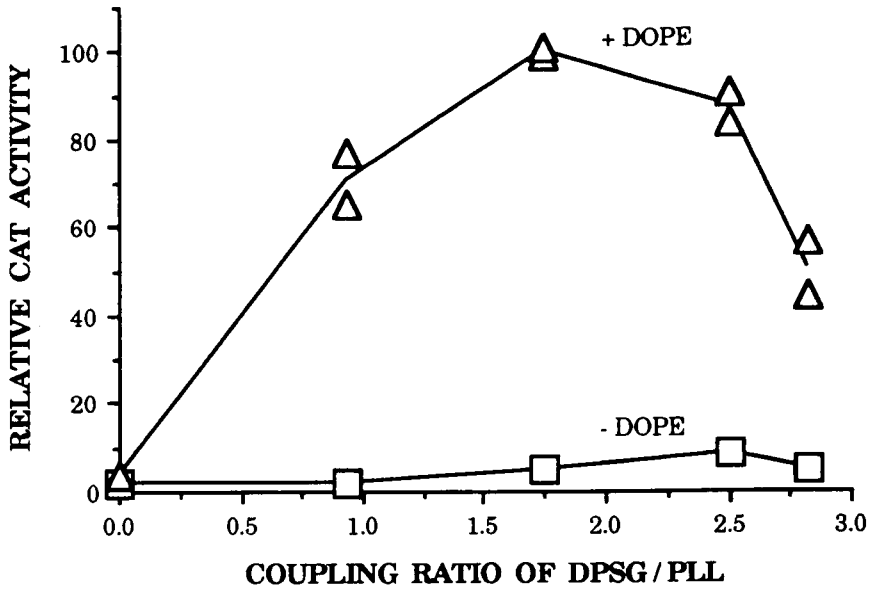


Figure 2. Effect of coupling ratio of DPSG/PLL on transfection activity. PLL was derivatized with DPSG to different degrees. For the purpose of quantitation, PLL was trace labeled with fluorescamine, DPSG with ^{14}C. Liposomes composed of LPLL alone or mixed with 8 molar excess of DOPE were assayed for their transfection activities. Data were collected from a single experiment with duplicates for each point.

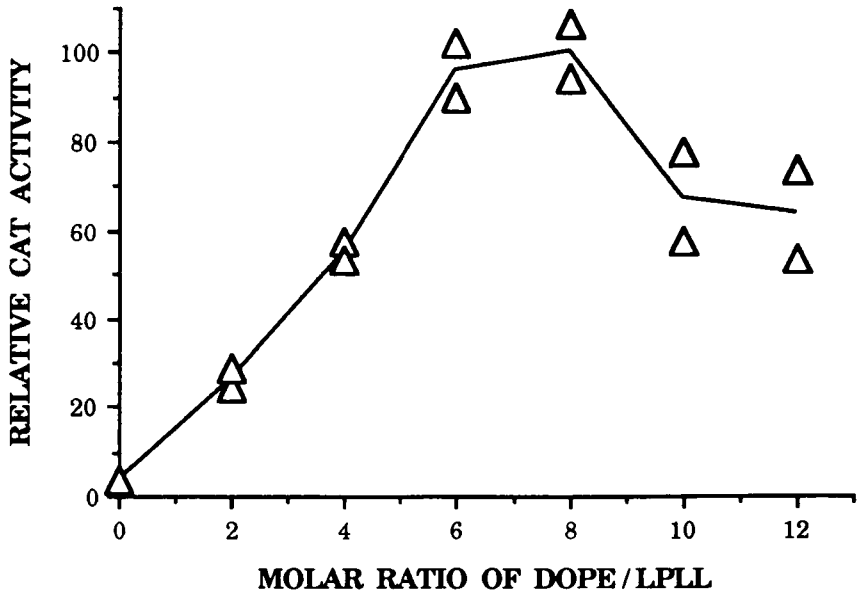


Figure 3. Effect of DOPE/LPLL ratio on transfection activity. Liposomes were prepared from mixtures of DOPE/LPLL with different molar ratios. Transfection activity of the liposome preparations was determined. Data were collected from a single experiment with duplicates for each point.

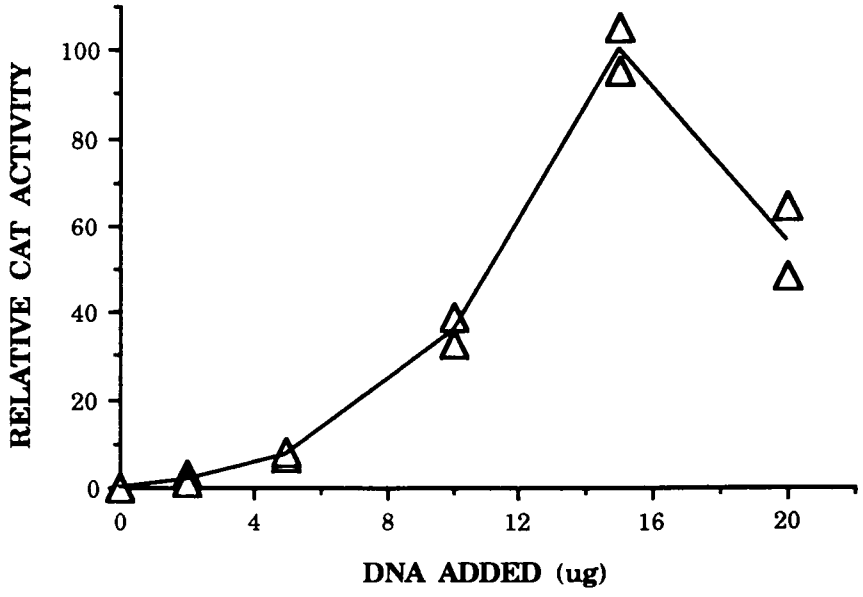


Figure 4. Effect of DNA dose on transfection activity. Various amounts of PUCSV2CAT DNA was mixed with a fixed amount of 285 ug LPLL/DOPE liposomes. Transfection activity of the complexes was determined. Data were collected from a single experiment with duplicates for each point.

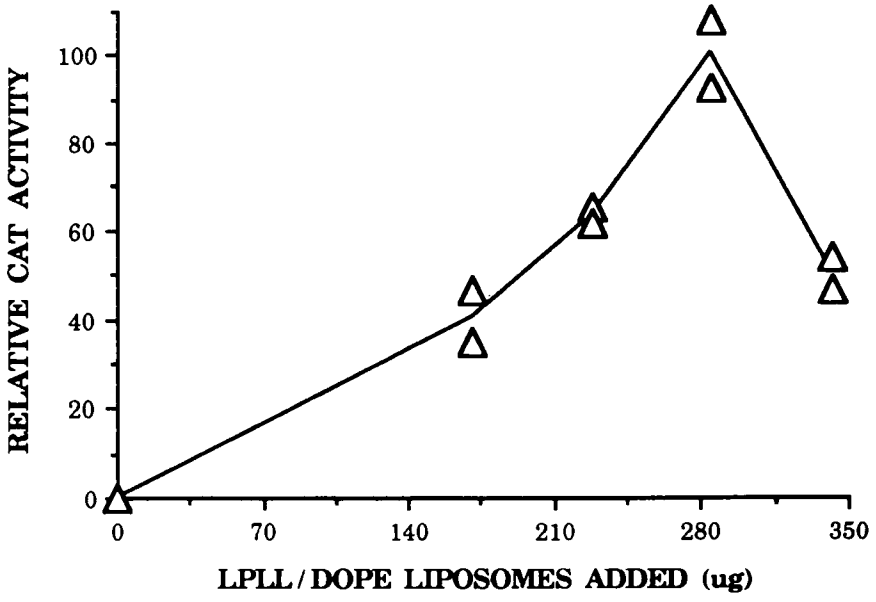


Figure 5. Effect of liposome dose on transfection activity. Various amounts of liposomes was mixed with a fixed amount of 15 ug pUCSV2CAT DNA. Transfection activity of the complexes was determined. Data were collected from a single experiment with duplicates for each point.

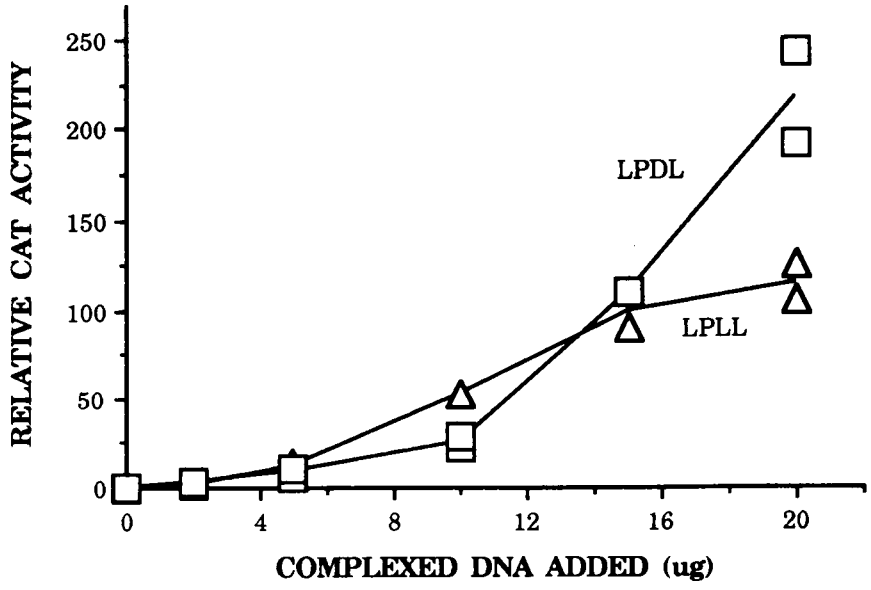


Figure 6. Effect of the dose of DNA/liposome complexes on transfection activity. Liposomes were prepared from LPLL/DOPE or LPDL/DOPE and complexed with pUCSV2CAT DNA at the optimal ratio. Various amounts of the complexes was used to transfect L929 cells. Data were collected from a single experiment with duplicates for each point.

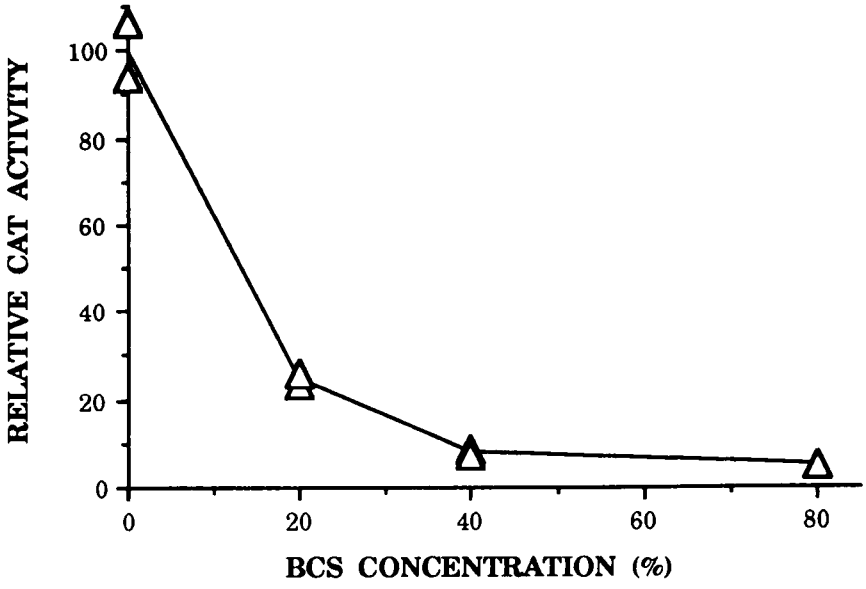


Figure 7. Effect of concentration of serum on transfection activity. L929 cells were transfected with DNA/liposome complexes in medium containing various concentrations of bovine calf serum (BCS). CAT activity in the treated cells was determined. Data were collected from a single experiment with duplicates for each point.

PART V

LIPOSOMES CONTAINING LIPOPOLYLYSINE AS A VEHICLE FOR GENE TRANSFER II: MECHANISM OF ACTION

1. Abstract

Interaction of cationic lipopolylysine liposomes with plasmid DNA and of the resulting DNA/liposome complexes with cultured cells were studied. DNA readily bound to liposomes resulting in a complete retardation of the DNA in agarose gel electrophoresis. However, negative-stain electron microscopy has revealed that the optimal mixture of DNA and liposomes for transfection contained approximately 73% free liposomes. The free and DNA-associated liposomes in the mixture formed larger complexes in the presence of cultured cells, suggesting that some cellular factor(s) may be involved in the formation of the active complexes. Transfection activity of the complexes was enhanced by up to 6-fold if cells were pretreated with agents interfering with the process of endocytosis. Meanwhile, pretreatment of cells with a peptide which inhibits membrane fusion decreased about 60% of the activity. These results indicated that DNA/liposome complexes are taken up by an endocytosis mechanism and that cytoplasmic delivery of DNA involves a fusion-related event probably in the endosome compartment. The transfection process was visualized by thin-section electron microscopy. It was found that the complexes entered the cytoplasm mainly by destabilizing endosomes and occasionally by penetrating through the plasma membrane. Therefore, our findings disagree with a previous hypothesis which suggests that transfection mediated by the cationic liposomes is via fusion of the liposomes with the plasma membranes of the treated cells.

2. Introduction

It is well established that cationic liposomes mediate efficient DNA transfection in mammalian cells; however, the mechanism of transfection remains obscure. It has been hypothesized that plasmid DNA interacts with cationic liposomes such that one single plasmid molecule is trapped in the interior of a complex consisting of four intact liposomes (1). The DNA/liposome complexes then fuse with the plasma membrane of the treated cells, resulting in the delivery of the exogenous DNA into the cytoplasm (1). It has also been proposed that DNA/liposome complexes after being endocytosed destabilize the endocytic vacuole membrane resulting in the release of the exogenous DNA into the cytoplasm (2, 3). The fusion hypothesis is based on the observations that free and DNA-associated cationic liposomes fuse with negatively charged liposomes and plasma membrane of the treated cells, respectively (1). However, it has been found that the fusion activity of cationic liposomes is abolished by preincubation with DNA (3). Furthermore, fusion activities of several different cationic liposomes in the absence or presence of DNA do not correlate with their transfection activities (3).Thus, the original hypothesis has not gained a broad spectrum of experimental support.

We have prepared a new type of cationic liposomes which consist of a lipopeptide, lipopoly-L-lysine (LPLL), and a hexagonal phase forming phospholipid, dioleoylphosphatidylethanolamine (DOPE) (4). These liposomes differ from other cationic liposome formulations by its low cytotoxicity and the unusually high positive-to-negative charge ratio of the optimal liposome/DNA complex for transfection (4). To understand the

mechanism of DNA delivery by these new liposomes, we have studied the morphology of the complexes and their interactions with mouse L929 cells by electron microscopy. The results have provided some insight about the mode of entry of DNA into the cytoplasm of the treated cells.

3. Materials and Methods

Materials:

Cytochalasin B, chloroquine, Z-Phe-Phe-Gly, Z-Gly-Phe, poly-L-lysine and acetyl CoA were purchased from Sigma. Dioleoylphosphatidylethanolamine (DOPE) and dipalmitoylsuccinylglycerol (DPSG) were purchased from Avanti Polar Lipids. D-threo [1,2-^{14}C] chloramphenicol (57 mCi/mmol) were from ICN Biomedicals. Plasmid pUCSV2CAT was amplified in E. coli and purified by CsCl gradient centrifugation method. Lipopoly-L-lysine (LPLL) was synthesized from poly-L-lysine and DPSG as described (4).

Liposome Preparation:

Preparation of LPLL/DOPE (1 : 8, molar ratio) liposomes was performed as described in the accompanying paper (4).

Agarose Gel Electrophoresis:

Half ug of pUCSV2CAT DNA alone or mixed with LPLL/DOPE liposomes was loaded onto an 1% agarose mini-gel slab. Samples were

electrophoresed in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) at 100 V for 45 min and then visualized by staining with ethidium bromide.

Liposome Aggregation Assay:

Liposomes were mixed with various amounts of DNA and then diluted with free phosphate buffered saline (PBS) or conditioned PBS. Turbidity of the mixtures was measured as absorbance at 400 nm with a spectrophotometer. Conditioned PBS was prepared by the following protocol: L929 cells with 60% confluency in a 100 mm plate were washed 3 times with PBS and then incubated with 5 ml PBS for 3 h at 37°C. The supernatant was centrifuged at 2,000 rpm in a table top centrifuge for 10 min to remove large particles and then stored at 0°C. Conditioned PBS was prepared and used in the same day.

Transfection:

Cultured L929 cells, transfection procedures and assay for CAT activity in the transfected cells were described in the accompanying paper (4). In some transfection experiments, cells were also treated with 10 mM NH_4Cl, 25 ug/ml cytochalasin B, 100 uM chloroquine, 100 mM carbobenzoxy-glycyl-L-phenylalanine (Z-Gly-Phe) or carbobenzoxy-D-phenyl-L-phenylglycine (Z-Phe-Phe-Gly) 30 min before the addition of liposome/DNA complexes.

Electron Microscopy:

Samples for negative-stain electron microscopy (EM) were stained with 1% uranyl acetate. Samples of the liposome/DNA complex were

embedded in 2% agar and processed for thin-section EM following protocols described elsewhere (5, 6). L929 cells treated with the complexes were similarly processed except without using agar embedding. All EM examinations were carried out in a Hitachi H-600 TEM/STEM microscope.

4. Results

The cationic liposomes used in this study were composed of LPLL : DOPE = 1 : 8 (molar ratio). They had been shown in the accompanying paper to mediate optimal DNA transfection in mouse L929 cells (4).

Electrophoretic Mobility of DNA Mixed with Liposomes:

Association of DNA with cationic liposomes was studied using a gel retardation technique. In this experiment, increasing amounts of cationic liposomes were added to a constant amount of plasmid DNA. The mixtures were electrophoresed on an agarose gel slab. DNA was visualized by ethidium bromide staining. As shown in Fig. 1, mixing 0.5 ug plasmid DNA with as little as 0.2 ug cationic liposomes (lane 3) resulted in a significant retardation of the DNA. Lane 1 showed the mobility of free plasmids. With 1.0 ug cationic liposomes (lane 5), i.e. when positive-to-negative charge ratio of the mixture equaled to 1, plasmid DNA was completely retarded, suggesting that under this condition negative charges of the DNA were quantitatively neutralized by the cationic liposomes. Further addition of liposomes led to the appearance of some DNA complexes at the cathode side of the wells (lane 6 to 8). This might be because the complexes had become positively charged. At the optimal ratio

of DNA/liposome for transfection (lane 8), plasmid DNA was also completely retarded; however, it was not stained well by ethidium bromide. The decreased fluorescent intensity was not due to loss of DNA as determined by quantitation of radioactivity in an experiment using ^{32}P-labeled DNA (not shown). The DNA might had been coated with cationic liposomes such that ethidium bromide could not intercalate between the stacked nucleotide bases.

Electron Microscopic Examination of Liposomes and DNA/liposome Mixtures:

Free liposomes and DNA/liposome mixtures were visualized by negative-stain electron microscopy. Fig. 2A shows the free cationic liposomes which were heterogeneous with an average diameter of 148 ± 38 nm. When added to the plasmid DNA at the optimal ratio for transfection, about 27% of the liposomes became electron dense (arrowheads in Fig. 2B). The remaining population of liposomes were morphologically indistinguishable from free liposomes. Thus, the electron dense particles represented liposomes associated with DNA. Each complex appeared to contain a single liposome because the size of the complexes (99 ± 21 nm) was similar to that of the free liposomes. The DNA/liposome complexes were also visualized by thin-section EM using an agar-embedding technique. As can be seen in Fig. 2C, the complexes were electron dense and displayed some double-lined structures (arrowheads). Similar structures were also observed in the complexes added to the cells (Fig. 5). Excess amount of DNA caused aggregation of liposomes (Fig. 2D).

Aggregation of The Optimal DNA/liposome Mixture Induced by Cellular Factors:

The optimal DNA/liposome mixture for transfection contained approximately 73% free liposomes (Fig. 2B). However, in samples of cells treated with the optimal mixture we had never observed any free liposome structures either within or outside the cells (Fig. 5). We speculated that the free and DNA-associated liposomes in the mixture could interact with each other to form complexes when they were added to the cells. To test this possibility, the mixture as well as free liposomes were incubated in PBS or conditioned PBS which had been incubated with cells. Turbidity of the samples was determined at various time points. Fig. 3 shows that components in the conditioned PBS significantly and rapidly enhanced the turbidities of suspensions containing free or DNA-complexed liposomes. Addition of DNA also increased the turbidity of free liposomes. No aggregation was observed when liposomes were incubated in PBS for up to 60 min. Thus, factors produced by cells and secreted into the conditioned PBS promoted the complexation and aggregation of free liposomes with DNA/liposome mixtures such that the final complex seen by cells contained no free liposomes.

Effect of Drugs on Transfection Mediated by DNA/liposome Complexes:

To investigate the cell uptake and the fate of DNA/liposome complexes, L929 cells were treated with chemical agents interfering with the endocytosis process before exposed to the transfection reagent. Data in Table 1 show that treatment with cytochalasin B enhanced the transfection activity by about 4-fold. The drug blocks endocytosis by inhibiting the

assembly of microfilaments from free actin. Endocytic vesicles containing the DNA/liposome complexes were rarely seen in cells pretreated with cytochalasin B as revealed by thin-section EM observation (not shown), confirming that the inhibitor effectively prevents the internalization of the complexes. NH_4Cl and chloroquine, which increase the intravesicular pH of endosomes, also enhanced the transfection activity by 1.6 and 6 fold, respectively. These results demonstrated that interruption of the endocytosis pathway increased the efficiency of cytoplasmic DNA delivery.

The DNA/liposome complexes might be translocated into the cytoplasm via a fusion or fusion-related process. Accordingly, an experiment was performed in which cells were exposed to a fusion inhibiting peptide, Z-Phe-Phe-Gly (7), before or after the addition of DNA/liposome complexes. Fig. 4 shows that this peptide inhibited the transfection activity in a time-dependent manner. Pretreatment of cells with Z-Phe-Phe-Gly for fifteen minutes or longer reduced the activity of the complexes by about 60%. Peptide added together with the complexes yielded only about 40% inhibition of the activity; while no significant inhibition was observed if the peptide was added at fifteen min or later. A control peptide, Z-Gly-Phe which has no inhibition activity (7), had no effect on transfection at all time points tested. These results indicate that the action of Z-Phe-Phe-Gly was not instantaneous; it required at least approximately 15 min preincubation with cells. Furthermore, the step inhibited by the peptide occurred soon after the addition of the DNA/liposome complex since delayed addition of peptide showed no inhibitory effect.

Electron Microscopic Study of Complex-Cell Interaction:

To further define the mechanism of DNA delivery, the transfection process in cells treated with the DNA/liposome complexes was visualized by thin-section EM. The DNA/liposome complexes were endocytosed (Fig. 5A) into endosomes (Fig. 5B). Interaction of complexes with cells was also seen at the coated pits (not shown). Thus, internalization of the complexes was probably not solely via the receptor-mediated endocytosis. The complexes were identified with their high electron density and doubled-lined structures (arrowheads) as seen in Fig. 2C. Fig. 5C and D show that the complexes destabilized endosomes. Arrowheads in the micrographs indicate the part of the complex which had intruded into the cytoplasm and was not surround by the endosome membrane, while the remaining portion of the complex stayed in the endosome. In 23% of the cells which bore complex-containing endosomes, 15% of the organelles were disrupted. Occasionally, complexes were seen penetrating directly through the plasma membrane (Fig. 5E). This event occurred much less frequently (only observed in 0.7% of the cells) compared to the endosome disruption. Complexes were also found to be free and not membrane bound in the cytoplasm (Fig. 5F). These results suggested that the DNA/liposome complexes enter the cytoplasm by destabilizing either the endosome or plasma membrane and that disruption of endosome is the major pathway of entry. Control complexes of DNA with LPLL/DOPC (1 : 8, molar ratio) liposomes which showed no transfection activity (4) were only found associated with the coated pits (Fig. 5G). Thus, they were likely taken up via the receptor-mediated endocytosis pathway. These control complexes mostly remained confined in the endosomes (Fig. 5H); less than 1% of the complex-

containing endosomes were destabilized. Pretreatment of cells with chloroquine enhanced the transfection activity of DNA/liposome complexes (Table 1). The drug was assumed to disable the degradative function of the lysosomes or to prevent the fusion of endosomes with the lysosomes (8). Therefore, it should increase the possibility of the DNA/liposome complexes to escape the endocytic vacuoles. To test this hypothesis, cells were pretreated with chloroquine before exposed to the complexes. Three representative micrographs of the sample (Fig. 5I - K) showed the process of discharge of the complexes from the swollen endocytic vacuoles into the cytoplasm. In Fig. 5I, the complex appeared to be forced to one side of the endosome probably due to osmotic swelling of the organelle resulted from the chloroquine treatment. Endosome membrane in close contact with the complex could be distinguished although not as visible as the remaining portion. Disruption of the membrane would release the complex into the cytoplasm. The complexes shown in Fig. 5J and K (arrowhead) had obviously destabilized the endosome and had partially and almost completely entered the cytoplasm, respectively. Fifty-seven percent of the endosomes observed had been destabilized. This is to be compared with only 15% of endosome destabilization observed in cells not pretreated with chloroquine. Cells pretreated with cytochalasin B were also examined. Only about 2% of the cells showed direct penetration of the complexes through the plasma membrane. Thus, this event still took place infrequently even under the condition in which internalization of the complex was blocked. While this low frequency does not fully explain the enhancement effect of cytochalasin B, it indicates that cytochalasin B enhanced the possibility of the complexes to translocate the plasma membrane at least partially by

blocking the endocytosis process. This drug inhibits the assembly of microfilaments in the cytoskeleton beneath the plasma membrane which might facilitate the penetration of the complexes into the cytoplasm.

5. Discussion

It is shown in Fig. 1 that DNA readily binds to cationic liposomes. DNA in the optimal mixture for transfection, which has a positive-to-negative charge ratio of approximately 9, is completely adsorbed to the liposomes. However, under this condition only a portion of liposomes interacts with DNA (Fig. 2B). In other words, the optimal mixture contains a significant portion of free liposomes. It has been suggested that initial interaction of cationic liposomes with cells depends on the charge attraction with the negatively charged cells surface (9). Thus, a role of the free liposomes is perhaps to provide additional positive charges to the complexes to increase their uptake by the treated cells. This assumption is valid only if the free and DNA-associated liposomes interact with each other to form complexes. Indeed, this was the case when the optimal DNA/liposome mixture was incubated in the conditioned PBS (Fig. 3). It should be noted that the association of free liposomes is not so efficient in the absence of cellular factor(s), indicating that the factor(s) acts to bridge the free and DNA-complexed liposomes and are likely important for the optimal transfection activity. Preincubation of the optimal DNA/liposome mixture in the conditioned medium decreased the transfection activity to 78% (unpublished data), demonstrating that the factor(s) had influenced the property, e.g. the negative-to-positive charge ratio of the complexes. The

nature of the cellular factor(s) remains unknown and will be characterized in future experiments.

Thus, the structure of the complexes is similar to what has been proposed for the DNA/Lipofectin complexes in which the DNA molecule is entrapped in the interior space between intact liposomes (1). It would be of interest to investigate whether cellular factors are also involved in the formation of DNA/Lipofectin complexes. Aggregation of cationic liposomes and DNA/liposome complexes in the presence of cells had been observed but was not investigated further (3, 9). The phenomenon was thought to be an artifact resulting from the procedures of sample preparation (9). However, our data clearly indicate that the cells play an important role in the formation of the active DNA/liposome complexes, at least for the LPLL/DOPE liposomes.

A model for the DNA delivery mediated by LPLL/DOPE liposomes is summarized in Fig. 6. DNA/liposome complexes are formed in the presence of cellular factors. The complexes either directly penetrate into the cytoplasm through the plasma membrane or are endocytosed into the endosomes. Delivery from the endocytotic vacuoles to the lysosomes results in the degradation of the complexes. Alternatively, the complexes can escape into the cytoplasm by destabilizing the endosomal membrane. Disruption of endosomes is probably the major mechanism by which the complexes enter the cytoplasm, because this event was observed much more frequently than the penetration of the complexes through the plasma membrane in the EM observation. The mechanism by which the cytoplasmically discharged complexes enter the nuclei is not clear. However, they could diffuse into the nuclei during mitosis at which event

the nuclear envelope is disassembled. They could also be actively transported into the nuclei because polylysine contained in the complexes might serve as a signal for nuclear targeting. A sequence element, Lys-Lys-Lys-Arg, has been found in a number of nuclear protein (14).

Data in Table 1 and Fig. 5A clearly show that DNA/liposome complexes are taken up via an endocytosis pathway. Delivery of the complexes from endosomes to lysosomes are not expected to promote transfection. Thus, inhibition of this pathway by chloroquine or NH_4Cl improves the transfection activity (Table 1). We had speculated previously that the complexes destabilized endosome membrane to enter the cytoplasm (2, 10). We have now presented direct evidence to support our speculation (Fig. 5C, D, J, K). The complexes also destabilize plasma membrane to enter the cytoplasm although it only takes place infrequently (Fig. 5E). This is probably a partial reason why an inhibition of endocytosis by cytochalasin B enhanced the transfection activity (Table 1). Although the fusion-inhibiting peptide decreased the activity of DNA/liposome complexes (Fig. 4), it does not necessary mean that cytoplasmic discharge of DNA is exclusively a result of membrane fusion between the complexes and the treated cells. The peptide has been shown to inhibit the conversion of lipid bilayer to the hexagonal phase by stabilizing the bilayer structure (11). Both membrane fusion and destabilization involve an intermediate step of hexagonal phase formation (12). Thus, this peptide may inhibit the complex-induced destabilization of endosome and plasma membrane, thereby preventing the cytoplasmic entry of the complexes. As a result, it decreases the transfection activity.

Our current model for the mechanism of transfection mediated by cationic liposomes is in contrast to a previous hypothesis which suggested membrane fusion is responsible for the cytoplasmic DNA delivery (1). We have pointed out that a fusion event between the liposome and the plasma membrane will not result in the translocation of DNA which is bound to the outer surface of the liposomes (2). Furthermore, we were not able to detect membrane fusion between the FITC labeled complexes and the treated cells by fluorescence microscopy (unpublished observation). Additional evidences against the fusion model come from the facts that preincubation with DNA abolishes the fusion activity of cationic liposomes and that the fusion activities of various cationic liposomes do not correlate with their transfection activities (3). It is interesting to note that similar to LPLL/DOPE liposomes, anionic pH-sensitive liposomes also promoted cytoplasmic delivery of DNA via the destabilization of the endosome membrane (13). Substitution of DOPE with DOPC, a phospholipid which does not form hexagonal phase, in either the cationic or the anionic liposomes diminished the transfection activity (4, 13). Both type of liposomes, although carry opposite type of charge, share a similar mechanism of action.

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Table 1. Effect of cytochalasin B, chloroquine and NH4Cl on transfection activity [1]

Inhibitor	Relative CAT activity
none	94 , 106
cytochalasin B	410 , 445
chloroquine	565 , 670
NH4Cl	160 , 165

1. L929 cells were pretreated with the drugs for 30 min and exposed to mixtures of 10 ug DNA / 190 ug liposome for 3 h. At the end of incubation, cells were washed and cultured for 2 days then harvested and assayed for CAT activity. Data were collected from a single experiment with duplicates for each point.

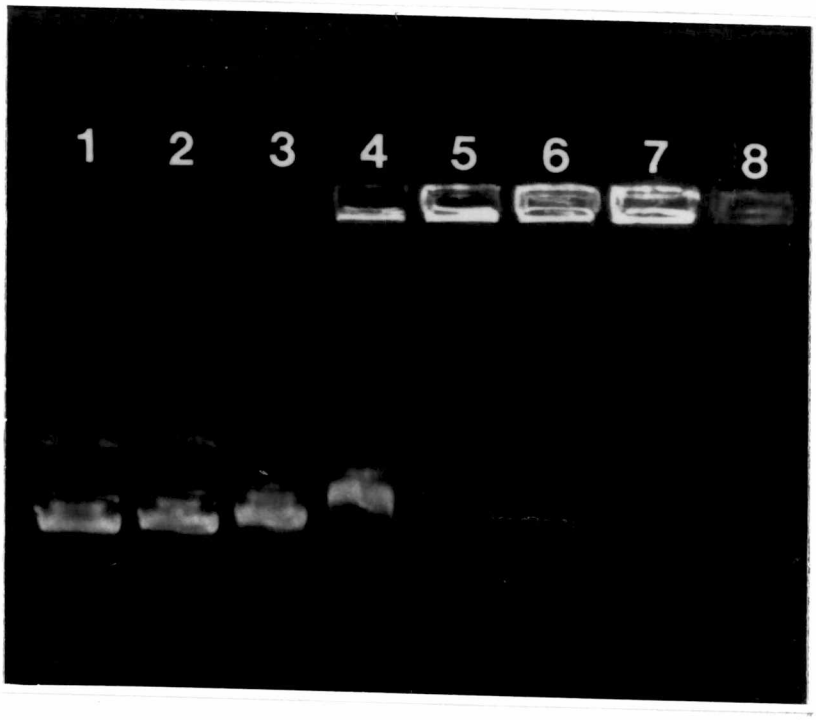
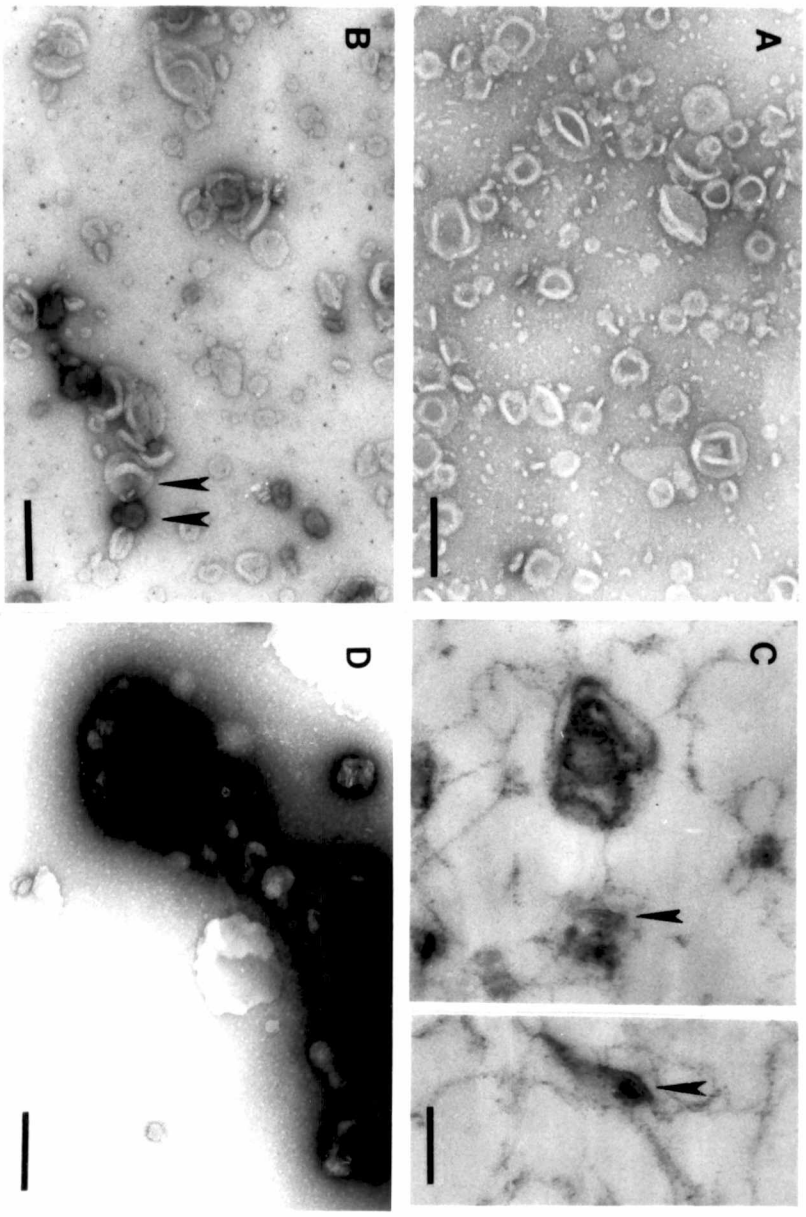


Figure 1. Effect of dose of LPLL/DOPE liposomes on the electrophoretic mobility of DNA. Half ug of pUCSV2CAT DNA was mixed with 0, 0.1, 0.2, 0.5, 1.0, 1.5, 1.9, or 9.5 ug of LPLL/DOPE liposomes, respectively (lane 1 - 8). Electrophoresis and staining of the DNA samples were carried out as described in Methods. The upper and lower bands in lane 1 represented free plasmids in two different extents of supercioling. Data were collected from a single experiment without replicate.

Figure 2. Structure of LPLL/DOPE liposomes and DNA/liposome mixtures. Liposomes (190 ug) alone (Fig. 2A) or complexed with 10 or 40 ug DNA (Fig. 2B and D), respectively, were stained with 1% uranyl acetate. A DNA/liposome mixture (10 ug/190 ug) was embedded in agar and processed for thin-section EM (Fig. 2C, both panels). The reticular structure seen in Fig. 2C represents the agar polymer which was observed in samples without DNA/liposome complex (micrograph not shown). (A - D) magnification: x50,000; bar represents 0.2 um. Data were collected from a single experiment without replicate.


A
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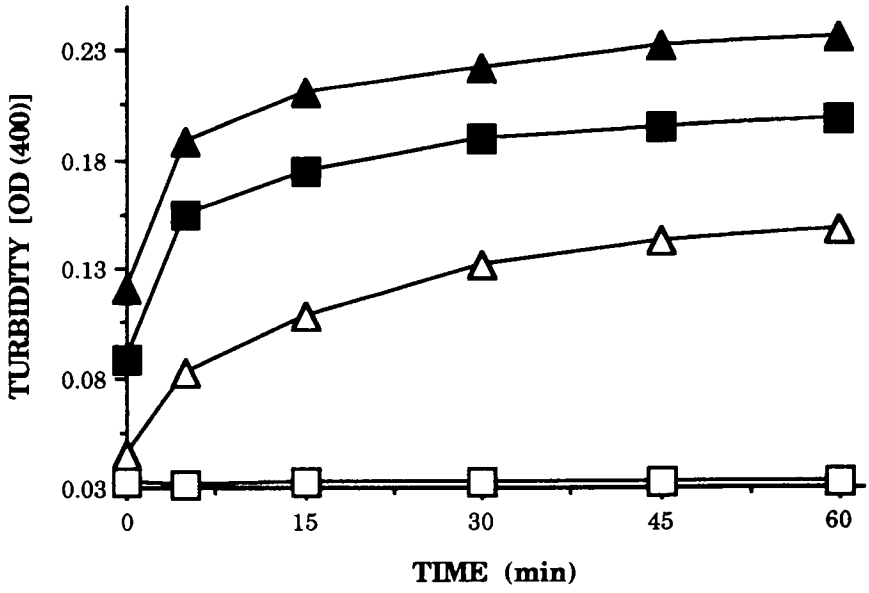


Figure 3. Effect of cellular factor(s) on the aggregation of LPLL/DOPE liposomes and DNA/liposome mixture. Liposomes alone (squares) or mixed with DNA at the optimal ratio for transfection (triangles) were incubated in PBS (open symbols) or conditioned PBS (closed symbols). Optical densities at 400 nm for the suspensions were determined at various time points. Data were collected from a single experiment without replicate.

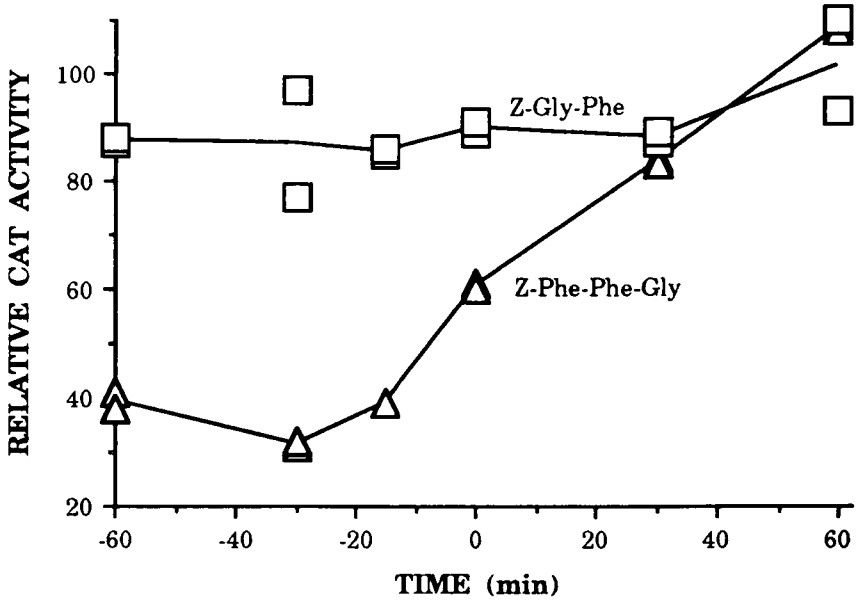
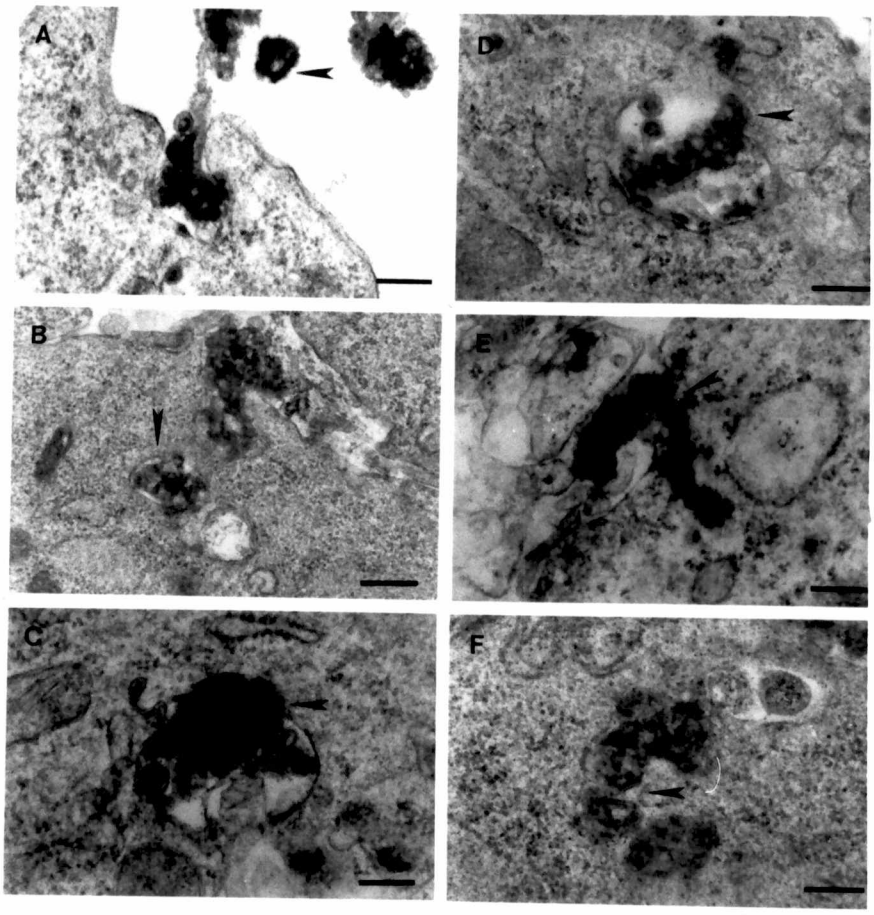
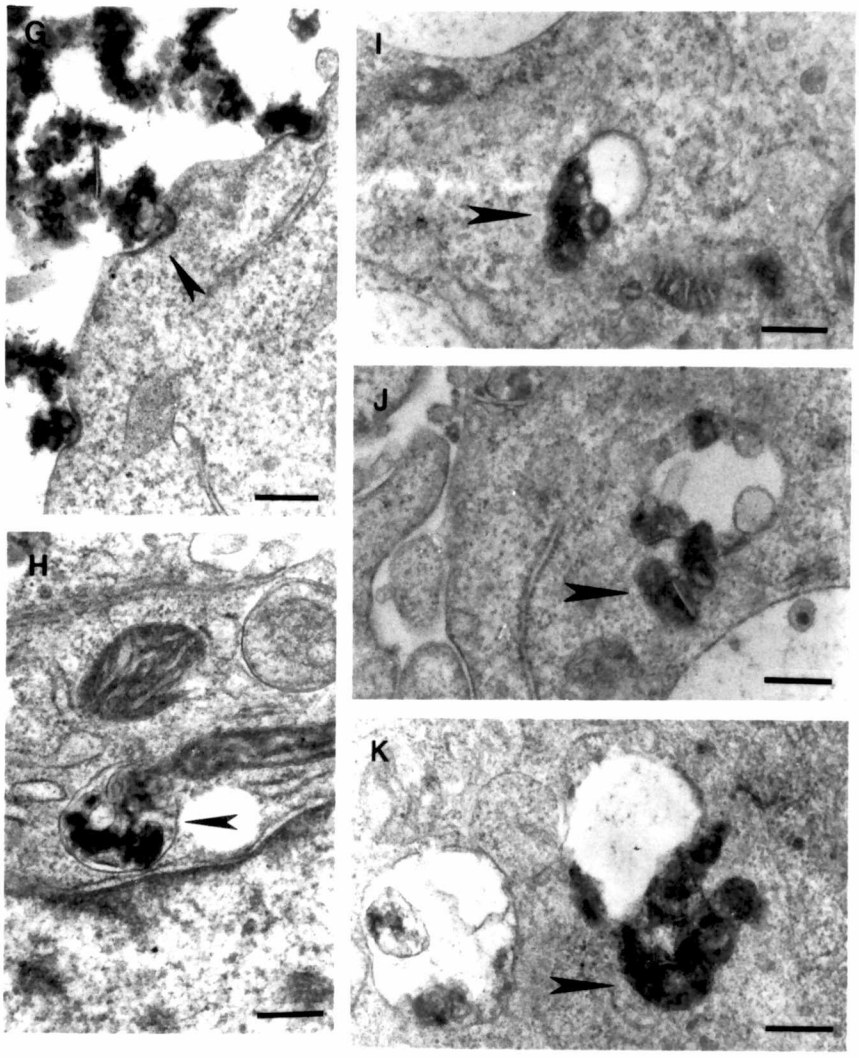


Figure 4. Effect of a fusion-inhibiting peptide on transfection activity. L929 cells were exposed to mixtures of 10 ug DNA/190 ug liposome for 3 h and 100 mM peptides added at various time points. At the end of incubation, cells were washed and cultured for 2 days then harvested and assayed for CAT activity. Data were collected from a single experiment with duplicates for each point.

Figure 5. Thin section electron micrographs of the L929 cells treated with various DNA/liposome complexes. L929 cells were incubated with DNA/liposome complexes in serum-free medium for 60 min then harvested and prepared for electron microscopy. (A - F) Cells were exposed to the optimal complexes of DNA with LPLL/DOPE (1 : 8, molar ratio) liposomes. (A) Endocytosis of the complexes. magnification: x40,000; bar represents 0.25 um. (B) Internalization of the complexes into endosomes. magnification: x30,000; bar represents 0.33 um. (C, D) Complex-induced destabilization of endosomes. (C) magnification: x50,000; bar represents 0.20 um. (D) magnification: x40,000; bar represents 0.25 um. (E) Direct penetration of the complexes through the plasma membrane. magnification: x40,000; bar represents 0.25 um. (F) Localization of the complexes in the cytoplasm. magnification: x50,000; bar represents 0.20 um. (G, H) Cells were exposed to the control complexes of DNA with LPLL/DOPC (1 : 8, molar ratio) liposomes. (G) Interaction of the control complexes with the coated pits. magnification: x30,000; bar represents 0.33 um. (H) Confinement of the control complexes in the endosomes. magnification: x30,000; bar represents 0.33 um. (I - K) Cells were pretreated with chloroquine for 30 min before exposed to the optimal complexes of DNA with LPLL/DOPE (1 : 8, molar ratio) liposomes. Micrographs showed the process of complex-induced destabilization of endosomes. magnification: x30,000; bar represents 0.33 um. Data were collected from a single experiment without replicate.


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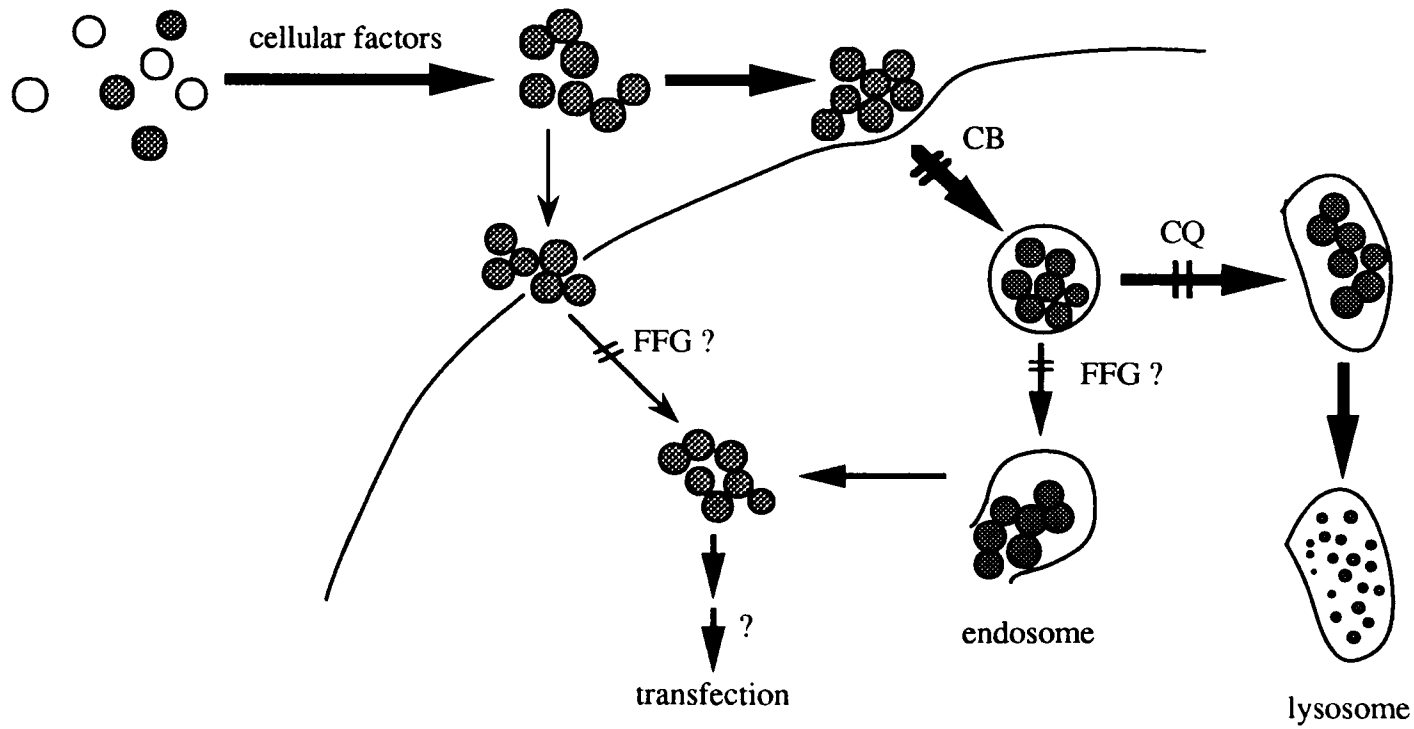


Figure 6. A model for the DNA delivery mediated by LPLL/DOPE liposomes. Steps with heavy arrows represent the major pathway. CB: cytochalasin B; CQ: chloroquine; FFG: Z-Phe-Phe-Gly.

PART VI

SUMMARY AND PERSPECTIVE

1. Summary and Perspective

Previously, results of our laboratory showed that pH-sensitive immunoliposomes mediated target-specific transfection both in vitro and in vivo. These findings demonstrated an exciting potential of the liposomes as a vehicle for gene transfer. Therapeutic application of this delivery system, however, was hampered by the inefficient DNA entrapment in liposomes. We have now overcome this problem by developing a new method of DNA encapsulation.

The new protocol involves freeze-thawing a mixture of empty liposomes and DNA to be entrapped. The freeze-thawing process is thought to generate transient "holes" in the membrane to allow the penetration of DNA into the aqueous compartment of liposomes. Since both DNA and empty liposomes can be prepared at high concentration, a large portion of aqueous volume is captured in the liposomes. Consequently, DNA molecules are encapsulated efficiently. Consisting with the proposed mechanism, smaller plasmids are entrapped in liposomes at higher percentages than the larger ones probably because they can more readily penetrate a given size of "holes" in the liposome membrane. Separation of free DNA from liposomal DNA is achieved by using a ficoll-gradient flotation technique. This assay shows that the association of DNA with liposomes is not due to non-specific surface adsorption. While the freeze-thaw method allows an efficient DNA encapsulation, it does not cause fragmentation of the DNA. This is to be compared with the REV method which often produces fragments. Furthermore, our new method is simpler and less time-consuming. It would facilitate studies of liposome mediated

DNA delivery. Functional expression of DNA encapsulated in liposomes prepared by the freeze-thaw method is demonstrated. DNA contained in the pH-sensitive liposomes has a higher transfection activity than the pH-insensitive liposomes. The activity can be modulated by PKC activators, PMA and DOG, incorporated in the liposome membrane.

pH-Sensitive liposomes are attractive vehicles for in vivo gene therapy. The development of freeze-thaw method for the efficient DNA entrapment is an effort to improve this system for therapeutic applications. However, before this final goal is achieved, the stability of liposomes in blood has to be improved. Large pH-sensitive liposomes are destabilized in serum due to the removal of the stabilizer lipids from the membrane (1, 2). One way to avoid the problem is to reduce the size of liposomes to less than 200 nm (1, 2). Unfortunately, we have found that liposomes extruded through less than 400 nm pore size membrane loose a significant amount of the entrapped plasmid DNA possibly due to rupturing of liposomes. Since condensation of the plasmid DNA has recently been demonstrated (3), it would be important to determine if condensed plasmids can be entrapped and retained in small liposomes. Viral particles are also likely to be entrapped in the small liposomes because of their compactness. Use of liposomes to deliver recombinant viruses would combine the advantages of the two gene transfer systems. Liposomal viruses could probably mediate target-specific gene transfer and highly efficient transduction in vivo. These possibilities are very exciting but await testing at the present time.

In this project, a new cationic DNA vehicle, lipopoly-L-lysine (LPLL), was also developed. LPLL is synthesized by conjugating lipids with low molecular weight poly-L-lysine (PLL). LPLL as well as lipopoly-D-lysine

mediated efficient DNA transfection. In contrast, PLL and poly-D-lysine are much less active. This is not a surprising result. We have reasoned that an efficient DNA delivery vehicle can be constructed with a polycation and a lipophilic moiety. Polycations such as PLL readily complex with different sizes of DNA; thus, LPLL would be a simple and universal transfection reagent. Our main purpose of developing LPLL is that it contains multiple primary amino groups which is important for the convenient coupling of targeting ligands such as antibodies or hormones. We assume that the resulting polylysine conjugates would promote a target-specific and efficient gene transfer. As also described in Chapter III, LPLL/DNA complexes transfect a number of cultured monolayer cells. The activity depends on the ratio of LPLL to DNA and incubation time and is inhibited by high concentration of serum. LPLL compared favorably to Lipofectin, a commercial cationic liposome preparation, with respect to the transfection activity, cytotoxicity and resistance to the inhibitory effect of serum. However, efficient transfection mediated by LPLL requires scraping of the treated cells. The mechanical treatment is thought to produce membrane damage to facilitate the entry of LPLL/DNA complexes into the cytoplasm.

In Chapter IV, we have described that the undesirable scraping requirement can be avoided by mixing LPLL with DOPE. Transfection activity of LPLL/DOPE liposomes is examined in relation to the lipophilicity of LPLL, DOPE/LPLL ratio, DNA/liposome ratio, and concentration of DNA/liposome complexes. The results indicate that cytoplasmic DNA delivery mediated by the complexes may involve the process of hexagonal phase formation. It is also shown that the degradation of polylysine

backbone is not necessary for the transfection activity of the cationic liposomes.

A more detailed study on the action of the DNA/liposome complexes is described in Chapter V. We have unexpectedly found that although DNA readily binds to LPLL/DOPE liposomes, a significant portion of liposomes in the optimal mixture for transfection does not complex with DNA. Furthermore, the free and DNA-associated liposomes in the mixture form larger complexes in the presence of cellular factors, probably proteins. A model for the DNA delivery mediated by LPLL/DOPE liposomes is summarized in Fig. V-6. DNA/liposome complexes are endocytosed and delivered to the lysosomes for degradation. Meanwhile, they can escape endosomes by destabilizing the vacuole membrane. Occasionally, complexes also enter the cytoplasm by directly penetrating through the plasma membrane. This model is based on the following results: 1) EM observations that DNA/liposome complexes destabilize both endosomal and plasma membrane to enter the cytoplasm and that chloroquine and cytochalasin B enhance the frequency of complex-induced endosome rupturing and direct penetration of the complexes through the plasma membrane, respectively, 2) both chloroquine and cytochalasin B increase the transfection activity of the DNA/liposome complexes, and 3) a tripeptide which inhibits destabilization of lipid bilayer decreased the transfection activity.

DNA/liposome complex-mediated destabilization of membrane bilayer is thought to be promoted by DOPE in the liposomes, because substitution of DOPE with DOPC does not resulted in membrane disruption. In this regard, it is interesting to note that anionic pH-sensitive liposomes

composed of DOPE : Chol : OA (4 : 4 : 2, molar ratio) mediated cytoplasmic delivery of DNA bound on the outer surface of the liposomes; whereas DOPC : Chol : OA (4 : 4 : 2, molar ratio) were inactive in transfection (4). It was speculated that the DOPE, but not DOPC, containing liposomes promoted disruption of endosome membrane (4). DOPE prefers to aggregate into the hexagonal-phase, i.e. a non-bilayer structure under the physiological conditions; therefore, destabilization of membrane bilayer mediated by both the cationic and anionic DOPE containing liposomes may involve a common event of hexagonal-phase formation. Anionic DOPE containing liposomes are taken up by cells less efficiently due to their low affinity to the negatively charged cell surface. Incorporation of a cell-specific ligand to the liposomes was required to enhance the delivery efficiency (7). In contrast, bare cationic, DOPE containing liposomes by themselves bind to cells spontaneously through charge interaction. Consequently, they mediate highly efficient cytoplasmic DNA delivery; up to 95% of the treated cells can be transfected (5). Characteristics of DNA delivery mediated by pH-sensitive immunoliposomes and cationic LPLL/DOPE liposomes were further compared in Table VI-1.

Since it has been established that LPLL/DOPE liposomes are efficient DNA carriers, studies on the coupling of targeting ligands to the cationic liposomes and gene transfer mediated by the resulting conjugates should be proceeded. The new vehicles are expected to deliver DNA to target cells via a specific ligand-receptor interaction. Thus, it is important to determine whether excess liposomes is still required for the optimal transfection activity. If the answer is yes, it would then be necessary to investigate whether the DNA/carrier mixture to be injected into blood stream should be

complexed with cellular factors before injection. Furthermore, serum components may partially neutralize the positive charges of the complexes; the ratio of DNA to carrier should be re-optimized. In the meantime, it would be of interest to characterize the nature of the cellular factors, e.g. their isoelectrical points and cellular functions. There is a possibility that they might also be involved in the uptake of the DNA/liposome complexes. It is reported very recently that conjugates of avidin/biotinylated oligonucleotides are taken up more efficiently than the less positively charged conjugates of strepavidin/biotinylated oligonucleotides and the free biotinylated oligonucleotides (6). Furthermore, the uptake is inhibited by the addition of other polycations such as protamine. This observation is relevant to our current study. It suggests that the enhanced uptake is probably promoted by some polycation-binding cellular factor(s).

References

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Table I: A comparison of pH-sensitive immunoliposomes and cationic LPLL/DOPE liposomes

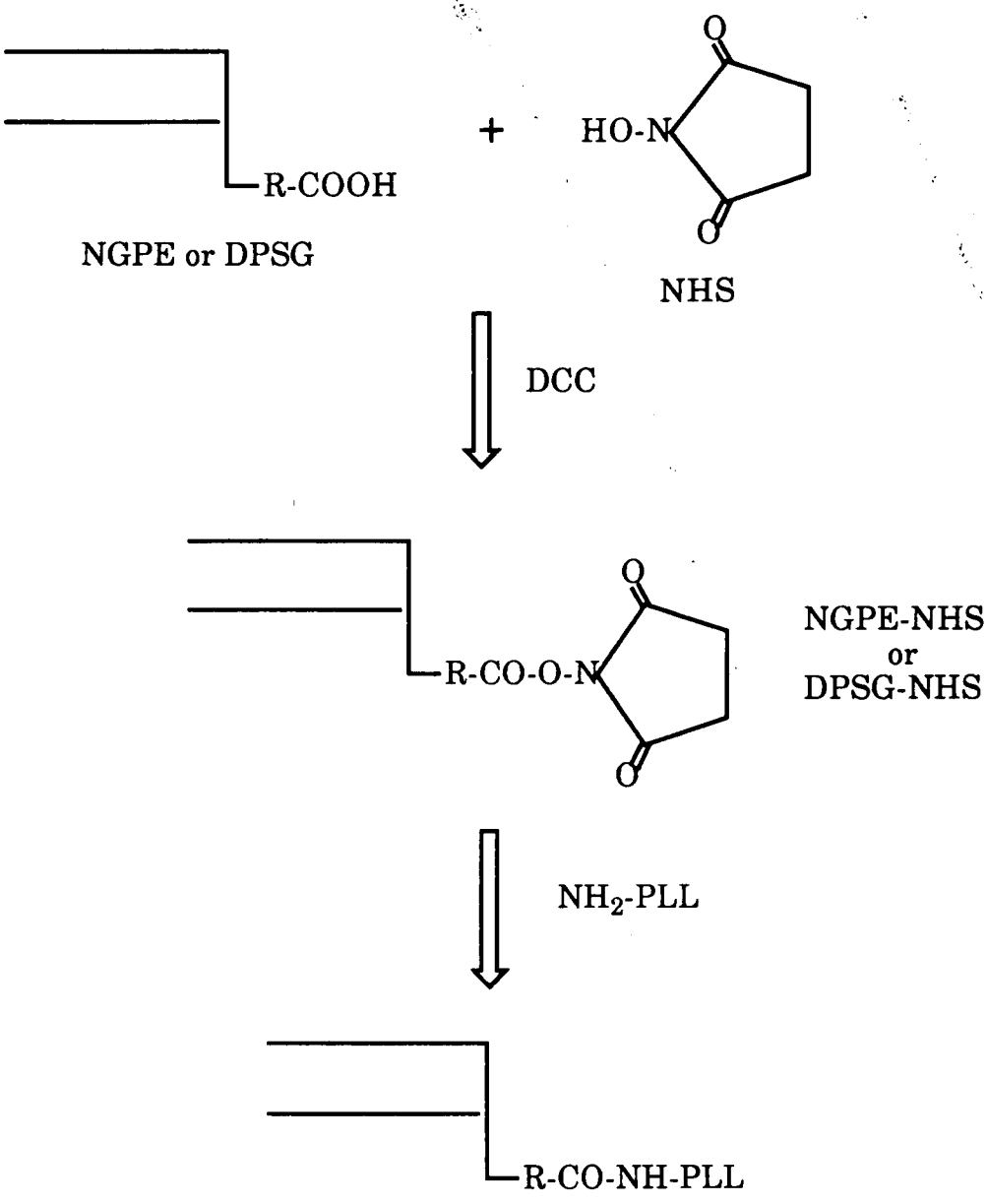
pH-sensitive immunoliposomes	cationic LPLL/DOPE liposmes
negatively charged, bind to cells via ligand-receptor interaction	positively charged, bind to cells spontaneously via charge interaction
DNA delivery efficiency inhibited by chloroquine which acts to prevent acid-induced destabilization of the liposomes in endocytic vacuoles	DNA delivery efficiency enhanced by chloroquine which acts to prevent degradation of the liposome/DNA complexes and to increase the entry of the complexes into the cytoplasm

Both types of liposmes require DOPE for the optimal DNA delivery efficiency. Substitution of DOPE with DOPC in the liposome composition decreases the transfection activity.

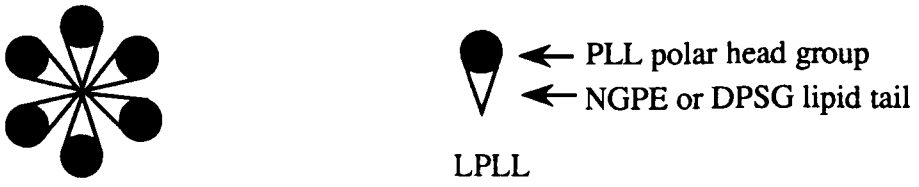
Both types of liposomes translocate DNA adsorbed to the outer surface of the liposomes into the cytoplasm.

Both types of liposomes destabilize endosomal membrane to discharge DNA into the cytoplasm. Membrane destabilization is probably promoted by the DOPE, a hexagonal-phase forming lipid.

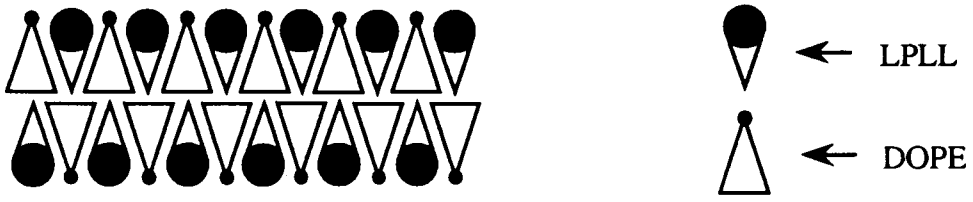
APPENDIX



Appendix I: Chemical systhesis of LPLL



Appendix II: LPLL in the micellular structure



Appendix III: The bilayer structure of LPLL/DOPE

(molar ratio of the lipids not correctly represented)

VITA

Xiaohuai Zhou was born in Shantou City, China on July 30, 1964. He attended Xinhua Elementary School and graduated from Shantou High School for Overseas Chinese in July 1981. The following September he entered Jinan University of Guangzhou and in July, 1985 received the degree of Bachelor of Science in Biology. He participated in an extensive training program in the Guangzhou English Language Center between September, 1985 and July, 1986. In September, 1986 he entered The University of Tennessee, Knoxville and in December, 1991 received the Doctor of Philosophy degree in Life Sciences.

He is currently employed as a post-doctoral fellow in the Department of Microbiology at the State University of New York at Stony Brook.