## INFLUENCE OF CALCIUM ON THE TRANSFECTION PROPERTIES OF LIPID-BASED GENE DELIVERY SYSTEMS

by

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We accept this thesis as conforming

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#### ABSTRACT

This thesis is focused on examining the influence of calcium on the transfection potencies and the intracellular processing of two lipid-based gene delivery systems: plasmid DNA-cationic lipid complexes, and stabilized plasmid-lipid particles (SPLP).

Results in Chapter 2 demonstrate that calcium can increase the in vitro transfection potency of plasmid DNA-cationic liposome complexes up to 20-fold. The effect is  $Ca^{2+}$ -specific: other cations such as  $Mg^{2+}$  and  $Na^+$  did not give rise to enhanced transfection and the presence of EGTA inhibited the stimulatory effect. It was shown that  $Ca^{2+}$  increased cellular uptake of the DNA-lipid complexes, suggesting that increased transfection potency arose from increased intracellular delivery of both cationic lipid and plasmid DNA in the presence of  $Ca^{2+}$ . In particular, the levels of intact intracellular plasmid DNA were significantly enhanced when  $Ca^{2+}$  was present. The generality of the  $Ca^{2+}$  effect for enhancing complex-mediated transfection is demonstrated for a number of different cell lines and different cationic lipid formulations.

The influence of Ca<sup>2+</sup> on SPLP, a system in which plasmid DNA is encapsulated within unilamellar lipid vesicles, is investigated in Chapter 3. It was shown that the transfection potency of SPLP in baby hamster kidney cells could be enhanced several hundred-fold by the presence of Ca<sup>2+</sup>. Interestingly, Ca<sup>2+</sup>enhanced transfection did not result from enhanced uptake of SPLP into the baby hamster kidney cells. Evidence from fluorescent microscopy studies employing rhodamine-labeled SPLP, Southern blots analysis of delivered DNA, and <sup>31</sup>P NMR

ii

studies of appropriate model membrane systems suggested that  $Ca^{2+}$  enhances the transfection potency of the encapsulated system by assisting in the destabilization of the endosomal membrane. This work was extended to encapsulated systems that contained higher amounts of the cationic lipid and that contained a cationic pegylated lipid on its surface. Increases in potency of 3 to 5 orders of magnitude at the optimal  $Ca^{2+}$  concentrations were observed, indicating that  $Ca^{2+}$  and cationic lipid act synergistically to increase the transfection potency of lipid-based gene transfer systems. In summary, results of this work show that  $Ca^{2+}$  acts as an efficient cofactor for enhancing the transfection properties of lipid-based gene delivery systems.

# TABLE OF CONTENTS

ABSTRACT		ii
TABLE OF CONTE	ENTS	iv
LIST OF TABLES.	·	vii
LIST OF FIGURES	5	viii
ABBREVIATIONS		<b>x</b>
ACKNOWLEDGME	ENTS	xiv
DEDICATION		xv
CHAPTER 1: INTI		1
1.1 Project over 1.1.1 Viral 1.1.2 Nonv 1.1.2.1 1.1.2.2	rview: Gene therapy and current DNA delivery methods -based vectors /iral-based vectors Gene delivery by physical or chemical methods Gene delivery employing liposomes	1 2 3 3
1.2 Lipids 1.2.1 Cher 1.2.1.1 1.2.1.2 1.2.1.3 1.2.1.4 1.2.1.5 1.2.2 Struc 1.2.2.1 1.2.2.2 1.2.2.3	mistry and physics of lipids. Phospholipids Sphingolipids Cholesterol Cationic lipids Poly(ethylene glycol) (PEG) lipids ctural behavior of lipids. Gel and liquid crystalline phase transition Lipid polymorphism.	7 9 11 11 12 14 14 15 17
1.3 Liposomes 1.3.1 Clas 1.3.1.1 1.3.1.2 1.3.1.3 1.3.2 Prep 1.3.2.1	ssification of liposomes Multilamellar vesicles (MLVs) Large unilamellar vesicles (LUVs) Small unilamellar vesicles (SUVs) paration of LUVs Extrusion techniques	22 22 24 24 24 24 25

1.3	3.2.2 Detergent dialysis techniques	25
1 4 Lipid-	mediated DNA transfection	26
141	DNA-cationic linid complexes	26
1.4.1	Stabilized Plasmid-Linid Particles	
1.4.2	Cefectere employed in linid based mediated transfection	23
1.4.3	Collactors employed in lipid-based mediated transfection	31
1.5 Calcii	um (Ca <sup>2+</sup> )	33
1.5.1	Biological activities of Ca <sup>2+</sup>	33
1.5.2	Influence of Ca <sup>2+</sup> on lipid polymorphism	34
1.5.3	Roles of Ca <sup>2+</sup> in transfection	36
1.6 Thesi	is objectives	38
CHAPTER	2: CALCIUM ENHANCES THE TRANSFECTION POTENCY	OF
	PLASMID DNA-CATIONIC LIPOSOME COMPLEXES	39
2.1 Intro	duction	39
2.2 Mate	rials and methods	42
2.2.1	Plasmids	42
2.2.2	Cell lines	42
2.2.3	Lipids and Chemicals	42
2.2.4	Preparation of LUVs	43
2.2.5	In vitro transfection in the presence of Ca <sup>2+</sup>	43
2.2.6	Determination of transgene expression	44
227	Intracellular uptake studies employing <sup>14</sup> C-radiolabeled lipids	45
2.2.8	Dot blot and Southern blot analyses	46
		47
2.3 Resu		4/
2.3.1	The transfection potency of plasmid DNA-lipid complexes is increas	sed
	in the presence of Ca <sup>2+</sup>	47
2.3.2	The proportion of cells transfected is increased in the presence of C	Ca²⁺
		49
2.3.3	Cation-dependent transfection enhancement is Ca <sup>2+</sup> specific	51
2.3.4	Ca <sup>2+</sup> increases the rate of transfection by complexes	51
2.3.5	Increased intracellular delivery of lipid is observed in the presence	of
	Ca <sup>2+</sup>	53
236	Increased intracellular delivery of intact plasmid DNA is observed in	
2.0.0	the presence of $Ca^{2+}$	
7 2 7	The level of transfection onbancement is dependent on the time of	
2.3.1	The level of translection enhancement is dependent on the time of $Co^{2+}$ addition	EO
		38 af
2.3.8	Ca <sup>-</sup> -ennanced transfection for complexes is observed in a variety	
	cell lines	60

v

•

2.3.9 Ca <sup>2+</sup> enhances the transfection potency of complexes containing a variety of cationic lipids62
2.4 Discussion65
CHAPTER 3: CALCIUM DRAMATICALLY ENHANCES THE TRANSFECTION POTENCY OF STABILIZED PLASMID-LIPID PARTICLES
3.1 Introduction69
3.2 Materials and methods       71         3.2.1 Materials       71         3.2.2 Preparation of SPLP       72         3.2.3 Transfection in the presence of Ca <sup>2+</sup> 72         3.2.4 Determination of SPLP uptake into cells       73         3.2.5 Fluorescence microscopy studies       74         3.2.6 Intracellular processing of plasmid DNA       74         3.2.7 <sup>31</sup> P NMR spectroscopy       75         3.2.8 Entrapment of Ca <sup>2+</sup> inside SPLP       76         3.2.9 Insertion of CPL       77
<ul> <li>3.3 Results</li></ul>
CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS

.

<ul><li>4.1 Summary</li><li>4.2 Future directions</li></ul>	
REFERENCES	

# LIST OF TABLES

Table 1	Polymorphic phase preferences of common lipids in biological	
	membrane	18
Table 2	Types and actions of cofactors commonly employed in lipid-based	
	mediated transfection	32
Table 3	Effect of Ca <sup>2+</sup> in various mixed lipid systems	36

# LIST OF FIGURES

Figure 1.1	Schematic illustration of the cellular barriers encountered during	
	liposome-mediated transfection in vitro6	
Figure 1.2	Lipids as components of membrane bilayer8	
Figure 1.3	Structures of naturally occurring lipids used in this work	
Figure 1.4	Structures of synthetic cationic lipids DOTMA and DODAC13	
Figure 1.5	Polymorphic phase behavior of lipids16	
Figure 1.6	Proposed models of membrane fusion and intermediate structures 20	
Figure 1.7	Classification of liposomes23	
Figure 1.8	Schematic representation of plasmid DNA-cationic liposome	
	complexes	
Figure 1.9	Schematic structure of stabilized plasmid-lipid particles	
Figure 2.1	Effect of increasing Ca <sup>2+</sup> concentrations on the transfection activities of	
	plasmid DNA-cationic liposome complexes48	
Figure 2.2	Fluorescence microscopy of BHK cells transfected with pCMVGFP	
	using complexes in the presence of Ca <sup>2+</sup> 50	
Figure 2.3	Specificity of Ca <sup>2+</sup> for enhancing transfection	
Figure 2.4	Effect of incubation time on transfection employing control DNA-lipid	
	and Ca <sup>2+</sup> -enhanced DNA-lipid complexes	
Figure 2.5	Kinetic analysis of the intracellular delivery of lipids in the presence of	
	Ca <sup>2+</sup>	
Figure 2.6	Analysis of plasmid DNA uptake using dot blot and intracellular	
	integrity using Southern blot	

Figure 2.7	Effect of the order of Ca <sup>2+</sup> addition on transfection potency
Figure 2.8	Effect of Ca <sup>2+</sup> on transfection for different cell lines61
Figure 2.9	Effect of Ca <sup>2+</sup> on transfection properties of different liposomal
	formulations63
Figure 3.1	Ca <sup>2+</sup> enhances the transfection potency of SPLP in a specific manner
Figure 3.2	Stability of SPLP in the presence of Ca <sup>2+</sup> 82
Figure 3.3	Influence of Ca <sup>2+</sup> on the cellular uptake of SPLP84
Figure 3.4	Influence of Ca <sup>2+</sup> on cell morphology following uptake of LUV labelled
	with Rh-DOPE as detected using fluorescence microscopy
Figure 3.5	Influence of Ca <sup>2+</sup> on the integrity of SPLP plasmid following uptake of
	SPLP into BHK cells88
Figure 3.6	<sup>31</sup> P NMR spectra of various model membrane systems in the presence
	of Ca <sup>2+</sup> 90
Figure 3.7	External Ca <sup>2+</sup> was responsible for stimulating transfection
Figure 3.8	Effect of Ca <sup>2+</sup> on SPLP systems containing cationic PEG lipids or
	higher amounts of DODAC94

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# ABBREVIATIONS

β- <b>gal</b>	β-galactosidase
ВНК	baby hamster kidney
BCA	bicinchoninic acid
Chol	cholesterol
CL	cardiolipin
cmc	critical micellar concentration
CMV	cytomegalovirus
CPL	cationic pegylated lipid
CPRG	chlorophenol red galactopyranoside
DC-CHOL	3-β-[N-(N',N'-dimethylaminoethyl)carbamoyl]-cholesterol
DDAB	dimethyldioctadecylammonium bromide
DEAE-Sepharose	diethylaminoethyl-Sepharose
DMEM	Dulbecco modified Eagle medium
DMTAP	1,2-dimyristoyloxy-3-trimethylamminopropane
DNA	deoxyribonucleic acid
DODAB	N,N-dioleoyl-N,N-dimethylammonium bromide
DODAC	N,N-dioleyl-N,N-dimethylammonium chloride
DODMA-AN	N-[2,3-(dioleyloxy)propyl]-N,N-dimethyl-N-
	cyanomethylammonium chloride
DOPC	1,2-dioleoyl-3-phosphatidylcholine
DOPE	1,2-dioleoyl-3-phosphatidylethanolamine
DOPS	1,2-dioleoyl-3-phosphatidylserine

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DOTAP	1,2-dioleoyl-3-(oxytrimethylammino)propane	
DOTMA	N-[2,3-(dioleyloxy)propyl]-N,N,N-trimethylammonium chloride	
DSC	differential scanning calorimetry	
DSDAC	N,N-distearyl-N,N-dimethylammonium chloride	
EDTA	ethylenediaminetetraacetic acid	
EGTA	ethylene glycol-bis( $\beta$ -aminoethylether)- $N$ , $N$ , $N$ ', $N$ '-tetraacetic acid	
EM	electron microscopy	
ESR	electron spin resonance	
FBS	fetal bovine serum	
FID	free induction decay	
GFP	green fluorescence protein	
HBS	HEPES buffered saline	
HEPES	N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid	
H <sub>II</sub>	hexagonal	
HMG	high mobility group	
i.v.	intravenous	
IMI	inverted micellar intermediates	
LBPA	lysobisphosphatidic acid	
Luc	luciferase	
LUVs	large unilamellar vesicle system	
MLVs	multilamellar vesicles	
NP-40	Nonidet P40	
OGP	n-octyl-β-D-glucopyranoside	

PA	phosphatidic acid
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEG	polyethyleneglycol
PEG-CerC <sub>20</sub>	1-O-(2'-(ω-methoxypolyethyleneglycol <sub>(2000)</sub> )succinoyl)-2-N-
1	arachidoylsphingosine
Pen-Strep	pencillin-streptomycin
PG	phosphatidylglycerol
PI	phosphatidylinositol
<sup>31</sup> P-NMR	phosphorus-31 nuclear magnetic resonance
PS	phosphatidylserine
QELS	quasi-elastic light scattering
Rd	rhodamine
RES	reticuloendothelial system
Rh-PE	<i>N</i> -(lissamine rhodamine B sulfonyl)-1,2-dioleoyl- <i>sn</i> -
	phosphatidylethanolamine
SDS	sodium dodecyl sulfate
SK-OV3	human ovary adenocarcinoma
SPLP	stabilized plasmid-lipid particles
SSC	sodium chloride, sodium citrate
SUVs	small unilamellar vesicles
Т <sub>Н</sub>	transition temperature

TMCtrans-monolayer contactTRC-TEMtime-resolved cryo-transmission electron microscopyTristris(hydroxymethyl)aminomethaneTriton X-100t-octylphenoxypolyethoxyethanol

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xiv

To my parents:

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And my siblings:

Charles, Salina, and Stella

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#### CHAPTER 1

#### INTRODUCTION

#### **1.1 Project overview: Gene therapy and current DNA delivery methods**

More than 4500 human genetic diseases result from a defect in a single gene (Mckusick, 1992). With the growing knowledge on the genetic basis of diseases and the advance in engineering gene transfer vectors, gene therapy is emerging as a method of treatment for both genetic and acquired diseases. The principle involves the correction of genetic disorders by adding, deleting, or replacing genes. A major limitation, however, lies in the inability to deliver the gene of interest in a safe and effective manner. The current gene transfer methods consist of both viral-based and nonviral-based vectors. This section will present an overview of these gene delivery systems and discuss their associated problems.

## 1.1.1 Viral-based vectors

Viral vectors are replication-defective viruses that contain therapeutic genes within the viral genome. Common viral vectors include retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus. Viral vectors are highly efficient, as they possess the machinery necessary for gene delivery. Retroviruses utilize viral enzymes to copy their genomes into DNA and integrate into the host chromosome (Varmus, 1988). Long-term gene expression employing retroviral vectors can be achieved but their infection is limited to dividing cells (Anderson, 1992). However, the ability to integrate into the host genome contains a risk of

insertional mutagenesis. Although the adenoviral genome is not integrated into the host DNA, adenovirus has the advantage that it can infect both dividing and nondividing cells (Grunhaus and Horwitz, 1992). Adeno-associated virus can also infect both dividing and non-dividing cells, but its activity requires the presence of a helper virus such as adenovirus or herpes virus (Muzyczka, 1992). Herpes simplex virus is a double-stranded DNA virus with a genome measuring more than 150 kb (Roizman and Sears, 1990). Although herpes simplex virus can infect a variety of dividing and non-dividing cells, it also causes some cytotoxicity (Glorioso et al., 1994). Despite being efficient gene transfer mediators, a number of disadvantages are associated with viral vectors. A high viral titer can be difficult to produce for many of the viral vectors. Furthermore, viral vectors have a limitation in gene packaging: adeno-associated viral vectors can only carry 5 kb, retroviral vector can carry up to 7 kb, and adenoviral vectors can package around 10 to 12 kb. Most importantly, this system has the complication of becoming immunogenic, cytopathic, or recombinogenic. Such drawbacks have prompted the development of nonviral gene carrying systems.

#### 1.1.2 Nonviral-based vectors

Nonviral vectors have advantages over the viral vectors as they have low acute toxicity and are nonimmunogenic. They can also be produced on a large scale and do not have a gene packaging limitation. Drawbacks with the nonviral vectors include their lower transfection efficiency than viral vectors and their

transient gene expression, as they do not contain the machinery capable to mediate gene delivery or gene integration.

## 1.1.2.1 Gene delivery by physical or chemical methods

Plasmid DNA containing therapeutic genes can be introduced into cells by either physical (such as microinjection and electroporation) or chemical (such as DEAE-dextran, calcium phosphate (CaPO<sub>4</sub>), and cationic liposomes) techniques. The physical methods deliver DNA into cells through disrupting the plasma membrane. Although these methods can be efficient in some cell lines, they often produce inconsistent transfection results and are toxic to cells. In addition, the physical methods require that the target tissues be exposed in order to achieve DNA transfer. Chemical methods using either DEAE-dextran (Vaheri and Pagano, 1965) or CaPO<sub>4</sub> (Graham and Van der Eb, 1973) have been popular methods for transfecting cells in vitro. Plasmid DNA complexes with DEAE-dextran or coprecipitates with CaPO<sub>4</sub> are absorbed onto the cell membranes and subsequently internalized into the cells. Although commonly employed, gene delivery using these methods suffers from low and inconsistent transfection efficiencies.

#### 1.1.2.2 Gene delivery employing liposomes

The development of cationic lipids as agents for transfection (Felgner et al. 1987) has proven to be the most effective non-viral gene transfer method in vitro. The first generation of lipid-based gene carrier system contains two working components: a positively charged cationic lipid and a helper lipid which is

"fusogenic". Advantages associated with this liposomal gene transfer system are: 1) plasmid DNA readily complexes with the cationic liposomes, 2) the positively charged cationic liposome-DNA complex allows for interaction with the negatively charged cell plasma membrane, 3) the fusogenic component of cationic liposomes could promote destabilization of the endosome membrane, 4) there is essentially no limitation on the size of a gene that can be packaged, and 5) potential targeting to specific tissues and cell types. This system is also being investigated as a vector for in vivo gene therapy for treatments focused on melanoma (Nabel et al. 1993) and cystic fibrosis (Fasbender et al. 1995, Yoshimura et al. 1992). Systemically administered complexes of cationic liposomes and plasmid DNA have been shown to transfect cells in vivo (Hong et al., 1997; Li et al., 1997; Liu et al., 1995; Zhu et al., 1997; Song et al., 1997; Templeton et al., 1997; Thierry et al., 1995; Zhu et al., 1993).

Unlike viral-based systems, lipid-based systems do not have the complications of mutagenicity and immunity. However, their transfection potencies are relatively lower. A successful gene transfer requires that the integrity of a transfected gene of interest is retained until it reaches the nucleus, i.e., the DNA must traverse the cell membrane, the cytoplasm and the nuclear membrane without considerable degradation occurring. One major limitation in the lipid-based system is the lack of a component that assists in mediating the release and directing the genetic materials into the nuclei. Figure 1.1 depicts the major cellular barriers that hinder the plasmid DNA from being transcribed during liposome-mediated gene delivery.

The present study describes the discovery that the cation calcium ( $Ca^{2+}$ ) is a potent cofactor in enhancing the transfection efficiencies of lipid-based transfection systems. Two liposomal systems were examined: the common plasmid DNA-cationic liposome complexes and the recently developed stabilized plasmid-lipid particles (SPLP). Both transfection activities as well as intracellular processing of liposomes and plasmid DNA were determined. Furthermore, we have also attempted to examine the mechanism by which  $Ca^{2+}$  facilitated the enhanced transfection.



Figure 1.1. Schematic illustration of the cellular barriers encountered during liposome-mediated transfection in vitro.

#### 1.2 Lipids

Lipids are defined as the water-insoluble molecules in cells that are soluble in organic solvents. Lipids in the form of fatty acids are stored as an energy reserve. More importantly, lipids provide the basic bilayer structure of the cell plasma membrane, which serves as a relatively impermeable barrier to the passage of most water-soluble molecules. It is the amphipathic nature of lipid molecules that permits them to form bilayers spontaneously in aqueous solution, with their hydrophobic tails buried in the interior and their hydrophilic heads exposed to water (Fig. 1.2A). Besides the plasma membrane, organelles such as the mitochondrion, nucleus, endoplasmic reticulum and golgi apparatus also employ lipids to form a membrane barrier between the cytosol and their internal content. Lipid composition can vary dramatically between the different sides of the same membrane, as well as among different cells or organelle membranes. Finally, lipid bilayers serve as matrices with which membrane proteins associate. The fluid mosaic model (Singer and Nicolson, 1972) depicts the asymmetry of the lipid bilayer, which provides the fluid-like framework for the attachment of various membrane proteins (Fig. 1.2B)

#### 1.2.1 Chemistry and physics of lipids

Fatty acids and triacylglycerols are the forms of lipids used for energy storage and energy metabolism. The three major types of lipids in cell membranes are phospholipids, cholesterol, and glycolipids. The following section describes the lipids relevant to the investigation of this thesis.

# Hydrophilic head Aqueous buffer Hydrophobic tail

# Lipid molecule

Α

Lipid bilayer



Figure 1.2. Lipids as components of membrane bilayer. (A) Amphipathic nature of lipids in bilayer configuration. (B) Fluid mosaic model depicting a membrane bilayer containing proteins, lipids, and glycosylated proteins ().

## 1.2.1.1 Phospholipids

The major lipid constituent of eukaryotic cell membranes is phospholipids, which contain a glycerol-based backbone and phosphate-containing head groups. Phospholipids are ampiphiles with both hydrophilic and hydrophobic moieties, which promote them to assemble into bilayer structures. The major phospholipids found in biological membranes include: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI), and cardiolipin (CL). The structures of the phospholipids used in this work are shown in Figure 1.3A.

The structures of both the chain and headgroup components among lipids contribute to the different functions. The acyl chains of phospholipids vary in their chain length and degree of unsaturation. Increasing unsaturation in the hydrocarbon region tend to maintain the bilayer in a fluid state, whereas more saturated membranes are less flexible since they tend to be more tightly packed. Phospholipids also differ in their headgroups in that PS, PG, PI and PA are anionic while PC and PE are zwitterionic. The headgroup sizes also affect the degree of packing in the membrane: smaller size headgroups allow for tighter packing than larger ones. Headgroup structures can also have an effect on the particular lipid phase organization that an individual lipid adopts upon hydration, which will be addressed in the following lipid polymorphism section.

## 1.2.1.2 Sphingolipids

Sphingosine-based lipids are another major species in the eukaryotic

## A. Phospholipids





**B.** Sphingolipid



Ceramide: R = COR'', R' = H(R'' = hydrocarbon)

C. Cholesterol



Figure 1.3. Structures of naturally occurring lipids used in this work.

Membranes. Sphingolipids are derived from the long-chain amino alcohol sphingosine. The hydrophobic portion, called ceramide, contains a mixture of fatty acids that are amide-linked to the amino group of sphingosine (Fig. 1.3B). The ceramide moiety can be embedded along with the hydrophobic chains of phospholipids and other membrane lipids.

#### 1.2.1.3 Cholesterol

Eukaryotic plasma membranes contain large amounts of cholesterol (Fig. 1.3C), up to one molecule for every phospholipid molecule. Cholesterol molecules orient themselves in the bilayer with their hydroxyl groups close to the polar head groups of the phospholipid molecules. As a result, the fluidity of the membrane is partly affected by the presence of cholesterol. Those regions of the hydrocarbon chains that are closest to the polar head groups are immobilized, while the rest of the chains remain flexible. In addition to affecting fluidity, cholesterol decreases the permeability of lipid bilayers to small water-soluble molecules. It is also suggested that cholesterol enhances both the flexibility and the mechanical stability of the bilayer.

#### 1.2.1.4 Cationic lipids

The only biologically existing cationic lipids are sphingosine and stearylamine. The first synthetic cationic lipids were *N*,*N*-didodecyl-*N*,*N*-dimethylammonium bromide (Kunitake and Okhata, 1977) and 1,2-dimyristoyloxy-3trimethylamminopropane (DMTAP) (Eibl and Wooley, 1979). It was not until the

synthesis of the cationic lipid dioleyloxypropyl-trimethylammonium chloride, DOTMA, by Felgner and coworkers that the application of cationic liposomes in transfection was demonstrated (Felgner et al., 1987). DOTMA contains a permanent positive charge in the quaternary amine headgroup (Fig. 1.4), which allows for electrostatic interaction with nucleic acids such as plasmid DNA and oligonucleotides, as well as interaction with cell plasma membrane. Together with a helper lipid, dioleoylphosphatidylethanolamine (DOPE), Felgner's group reported the transfection activity of the cationic lipid consists of the plasmid-DNA liposome complexes. Since then, a variety of cationic lipids have been prepared including the one used in this study (dioleyldimethylammonium chloride or DODAC) (Fig. 1.4). The structures of these cationic lipids can be varied with the amount of positive charges, types of quaternary amines, protonatable headgroups, ester linkages between the alcohol of glycerol and the carboxyl of the fatty acid, or an ester linkage between the tails and the glycerol (Felgner et al., 1994; Gao and Huang, 1991; Liu et al., 1997).

## 1.2.1.5 Poly(ethylene glycol) (PEG) lipids

PEG is a flexible hydrophilic polymer with repeating units of ethylene glycol (-[O-CH<sub>2</sub>-CH<sub>2</sub>]<sub>n</sub>-) coupled to the head group of a common phospholipid. PEG lipids are designed to allow the liposomal system to avoid the reticuloendothelial system (RES), resulting in increased circulation lifetimes (Lasic et al, 1991; Woodle and Lasic, 1992). PEG lipids create a highly solvated polymer layer at the membrane surface, which serves as steric barrier that prevents the binding of serum proteins onto vesicles. Such steric hindrance also reduces the body's molecular



*N*-[2,3-(dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA)



*N*,*N*-dioleyl-*N*,*N*-dimethylammonium chloride (DODAC)

Figure 1.4. Structures of the synthetic cationic lipids DOTMA and DODAC.

recognition processes from labeling the liposome as foreign for subsequent uptake and removal by RES cells (Allen, 1994). In addition, it has been found that PEG can sterically inhibit membrane fusion between membrane vesicles (Holland et al., 1996). The presence of PEG can also stabilize non-bilayer forming lipids, such as PE, in various bilayer lipid mixtures (Holland et al., 1996b).

#### **1.2.2** Structural behavior of lipids

Lipids adopt particular organizations or phases upon hydration. Such structural behavior of lipids can be influenced by both lipid types and the surrounding environment. Depending on the temperature, phospholipids in the bilayer can be in two different states, the gel or liquid crystalline state. In addition, individual lipids or lipid mixtures can adopt different lipid phases, commonly known as lipid polymorphism. Both gel to liquid phase transition and lipid polymorphism are discussed in the following sections. Furthermore, the relationship between lipid polymorphism and membrane fusion is also presented.

## **1.2.2.1** Gel and liquid crystalline phase transition

When a synthetic lipid bilayer made from a single type of phospholipid changes from a liquid state to a rigid crystalline (or gel) state, this change of state is called a phase transition. Most lipid species in isolation can undergo a transition from gel to the fluid state at a characteristic melting point. Such phase transitions can be monitored by a variety of techniques: including nuclear magnetic resonance (NMR), electron spin resonance (ESR), differential scanning calorimetry (DSC), and

fluorescence. At physiological temperatures most, and usually all, membrane lipids are fluid (Cullis and Hope, 1985).

#### 1.2.2.2 Lipid polymorphism

The ability of lipids to adopt different structures on hydration is commonly referred to as lipid polymorphism (Gruner et al., 1985). Extrinsic factors such as divalent cations, ionic strength, pH, and membrane protein are known to have an influence on lipid structural preferences (Cullis and de Kruijff, 1979; Killian and de Kruijff, 1986; Tilcock et al., 1988). Lipid polymorphism can be studied using X-ray diffraction, <sup>31</sup>P- and <sup>2</sup>H-NMR, and freeze-fracture techniques (Cullis et al., 1985). The three general features include bilayer, hexagonal ( $H_{\parallel}$ ), and micelles (Fig. 1.5). The characteristics of bilayers include a broad, asymmetric <sup>31</sup>P NMR spectrum with a low-field shoulder and high-field peak, and a flat, featureless fracture plane in H<sub>II</sub> phase system exhibits <sup>31</sup>P NMR spectra with reversed freeze fracture studies. asymmetry which are narrower by a factor of two. Freeze fracture techniques also give rise to a regular corrugated pattern as the fracture plane cleaves between the hexagonally packed cylinders. In general, unsaturated lipids with small headgroups ("cone" shape) such as dioleoyl-phosphatidylethanolamine (DOPE) adopt hexagonal structures in isolation, while lipids with symmetrical headgroup and chain ("cylindrical" shape) such as PC adopts the bilayer phase (Cullis et al., 1986; Rilfors et al., 1984). In biological membranes, a significant number of lipid species prefer or could adopt the H<sub>II</sub> arrangement at physiological condition or in the presence of



Figure 1.5 Polymorphic phase behavior of lipids.

extrinsic factors such as low pH and the presence of Ca<sup>2+</sup> (Tilcock, 1986) (Table 1). It is suggested that these lipids play a role in events such as membrane fusion, exocytosis and endocytosis.

## 1.2.2.3 Membrane fusion

Membrane fusion is an event that is vital to the functioning of many cells. occurring in processes such as intracellular membrane trafficking, exocytosis and endocytosis, cell division, viral infection, and intracellular membrane transport (Wilschut and Hoekstra, 1991). Membrane proteins could facilitate fusion by inducing local dehydration (Zimmerberg et al., 1991) and perturbations in the lipid bilayer through their insertion into membranes (Chernomordik et al., 1997; Kanaseki et al., 1997; Monck and Fernandez, 1994). Proteins involved in mediating intracellular membrane fusion include the ATPase *N*-ethylmaleimide-sensitive factor (NSF), soluble NSF attachment proteins (SNAPs), and vesicle and target membrane-associated SNAP receptors (designated vesicular (v)- and target (t)-SNAREs, respectively) (Chapman, 1995; Hay and Scheller, 1997; Rothman, 1994). SNAPs bind directly to the membrane receptors and mediate the binding of NSF. The ATPase NSF activates the SNARE complexes, which bring membranes into close proximity. This process excludes water from the interface between the membranes and promotes spontaneous fusion between the dehydrated bilayers (Mayer, 1999). It is hypothesized that fusion proteins act to facilitate the merger of the phospholipids of living membranes by the same pathway found in phospholipid

Table 1. Polymorphic phase preferences of common lipids in biological membrane (Tilcock, 1986). (L = lamellar;  $H_{II}$  = hexagonal; M = micellar).

Types of lipids	Phase preference at physiological conditions	Other conditions
Phospatidylcholine	· L	
Sphingomyelin	L	
Phosphatidylethanolamine	H <sub>II</sub>	
Phosphatidylserine	L	H <sub>II</sub> : divalent cations, pH≤3.5
Phosphatidylglycerol	L	H <sub>ll</sub> : at high salt and temp.
Phosphatidylinositol	L	
Cardiolipin	L	H <sub>II</sub> : divalent cations, pH≤3, high salts
Phosphatidic acid	L	H <sub>II</sub> : divalent cations, pH≤3.5, high salts
Cholesterol		Induces H <sub>II</sub> in mixed lipid systems

bilayer fusion. The following section describes the mechanisms involved in fusion proposed from studying model liposomal systems.

Although it is difficult to show fusion processes in vivo, studies on model membrane systems have provided circumstantial evidence in support of the hypothesis that fusion is dependent on the polymorphic capabilities of lipids. It is suggested that intermediates in bilayer-nonbilayer phase transitions also provide intermediate structures for membrane fusion (Siegel et al., 1989). Three models have been proposed that address the membrane fusion mechanism (Fig. 1.6).

The first model describes the mechanism involved in membrane fusion via inverted micellar intermediates (IMI), which are formed between the two closely apposed bilayers in the fusion process. Evidence includes the presence of an isotropic <sup>31</sup>P NMR signal together with structures referred to as "lipidic particles" in the fractured surfaces of fused vesicles in freeze fracture electron microscopy (EM) (Cullis and de Kruijff, 1979; Cullis et al., 1986; Verkleij, 1984). The second model depicts a "stalk" intermediate (Chernomordik et al., 1985; Markin et al., 1984) that is formed by coalescence of the outer monolayers of the apposing membranes. The intermediates are thought to expand radially to form a trans-monomer contact (TMC). The TMC continue to radially expand to achieve a single bilayer diaphragm between the two apposed vesicles, termed a "hemifusion intermediate". The resulting tension would lead to rupture of the hemifusion intermediate and membrane fusion (Chernomordik and Zimmerberg, 1995). The last model, known as the "modified stalk model", also involves formation of TMC (Siegel, 1993). The model proposes that instead of expanding radially, membrane fusion could be

Apposed bilayer



Figure 1.6. Proposed models of membrane fusion and intermediate structures.
directly mediated by local diaphragm rupture in the TMC. Evidence for this comes mainly from different intermediates observed in TRC-TEM at various temperature below and above the transition temperature ( $T_H$ ) (Siegel, 1993; Siegel et al., 1994; Siegel and Epand, 1997).

### 1.3 Liposomes

During the late 1960s, it was found that isolated phospholipids of living cells spontaneously reformed into vesicles which later came to be known as liposomes (Bangham et al., 1965). Liposomes have been employed as model membrane system in studies of lipids behavior, membrane permeability, as well as membrane protein functions and lipid-protein interactions. The capacity of liposomes to entrap molecules also render them useful as drug delivery systems for therapeutic purposes (Gregoriadis, 1993).

### **1.3.1** Classification of liposomes

In general, liposomes can be classified into three categories: multilamellar, large unilamellar, and small unilamellar vesicles (Fig. 1.7).

### **1.3.1.1** Multilamellar vesicles (MLVs)

Hydration of a lipid film spontaneously gives rise to MLVs, which are heterogeneous in size with diameters ranging from 0.5 to 10  $\mu$ m. MLVs are composed of a series of concentric bilayers separated by narrow aqueous spaces (Bangham et al., 1965). As a result, MLVs have very low trapping volumes (0.5  $\mu$ l/ $\mu$ mol) and are not useful for drug loading. Transbilayer distributions of solutes can be equilibrated by a freeze-thaw process (Mayer et al., 1985). The regular arrays of bilayers in MLVs, on the other hand, made them ideal for physical and motional analysis on bilayer organization of individual lipids within a membrane structure employing NMR (Cullis et al., 1985).



Figure 1.7. Classification of liposomes. Schematic representation and freeze fracture electron micrographs of (A) MLVs, (B) LUVs, and (C) SUVs are shown. The bar in the EM picture represents 200 nm.

### **1.3.1.2** Large unilamellar vesicles (LUVs)

LUVs are unilamellar system with diameters ranging from 50 to 200 nm. LUVs can be prepared from MLVs through the use of detergents or organic solvents by a number of methods which include reverse phase evaporation (Szoka and Papahadjopoulos, 1978), detergent dialysis (Mimms et al., 1981), and extrusion (Mayer et al., 1986; Szoka and Papahadjopoulos, 1980). As LUVs have a single bilayer and a large trapped volume (1-2  $\mu$ l/ $\mu$ mole for 100 nm size liposomes), they are most commonly employed as model membrane systems and as drug carriers.

### **1.3.1.3** Small unilamellar vesicles (SUVs)

SUVs can be prepared from MLVs by subjecting the MLVs to ultrasonic irradiation (Huang, 1969) or by passage through a French press (Barenholzt et al., 1979). The diameters of SUVs are typically around 25 to 40 nm. Their small size limits their use in model membrane studies. In addition, SUVs' trapped volume (0.2-0.5  $\mu$ l/ $\mu$ mol) is often too small to allow for studies of permeability or ion distributions between the internal and external aqueous compartments. The small curvature of SUVs also gives rise to a high degree of instability (Parente and Lentz, 1984; Schuh et al., 1982).

### 1.3.2 Preparation of LUVs

A number of methods exist for preparation of LUVs, which involve solubilizing the lipids in an organic solvent and subsequently hydrating them in aqueous buffer.

### **1.3.2.1** Extrusion techniques

Extrusion is the most convenient method for producing LUVs (Mayer et al., 1986). MLVs are repeatedly extruded under moderate pressures (less than 500 psi) through polycarbonate filters of defined pore size. LUVs in the range of 50 to 200 nm can be generated, depending on the pore size of the filter employed.

### 1.3.2.2 Detergent dialysis techniques

Detergents may also be used to form LUVs (Almog et al., 1986; Kiwada et al., 1985; Lasch et al., 1983; Szoka and Papahadjopoulos, 1980). Detergents exist as monomers dispersed in solution at low detergent concentrations. When the concentration increases to a specific level, referred to as the critical micellar concentration (cmc), "micelles" or clusters of detergent molecules are formed. Detergents present in one micelle can constantly exchange with other monomers present in the bulk solution or in other micelles (Lindman and Wennerstrom, 1980). During formation of LUVs, the lipid is first dissolved in detergent micelles and the detergent is then removed by dialysis. Detergents most suitable for dialysis are those with high cmc values (>1 mM) and small micelle size (Dencher and Heyn, 1978; Furth, 1980).

#### **1.4 Lipid-mediated DNA transfection**

· E \*

Lipid-mediated gene transfer is gaining in popularity in both in vitro and in vivo cell transfection studies (Felgner et al., 1994; Felgner et al., 1987; Liu et al., 1995; Liu et al., 1997; Templeton et al., 1997; Van der Woude et al., 1995). This thesis employs two lipid-based gene delivery systems. One system involves complexing plasmid DNA to the external bilayer of the liposomes, commonly referred to as plasmid DNA-lipid complexes (Felgner et al., 1987). In the other system, the stabilized plasmid-lipid particles (SPLP), DNA is entrapped inside the liposomal bilayer (Wheeler et al., 1999). The following section describes the characteristics of the two systems, the mechanisms involved in the transfection process, as well as cofactors that have been used in conjunction with the liposomes for achieving higher transfection efficiencies.

### **1.4.1 DNA-cationic lipid complexes**

As mentioned in the introductory section, the liposomal mixtures in the complex system usually contain a cationic lipid and a helper lipid. DNA associates with the cationic liposomes through electrostatic interactions, and the resulting plasmid DNA-lipid complexes are heterogeneous in structures and sizes. Several structural models have been proposed: including the "bead on string" complexes (Felgner and Ringold, 1989), the "lamellar" complexes (Gustafsson et al., 1995), and the "cylindrical" complexes (Sternberg et al., 1994) (Fig. 1.8). The "bead on string" model was first observed using metal shadowing EM (Gershon et al., 1993), which depicted several positively charged liposomes attaching to the DNA as a string.

## A Bead on string complexes



## B Lamellar complexes



# C Cylindrical complexes





Evidence of the "lamellar" complex came from cryo-TEM (transmission electron microscopy) (Gustafsson et al., 1995), X-ray diffraction and optical microscopy (Radler et al., 1997; Spector and Schnur, 1997). In this model, DNA is condensed by the cationic liposomes and is trapped between fused lipid bilayers arranged in a typical MLV pattern. Sternberg's group proposed the "cylindrical" model, in which a strand of DNA was coated by a monolayer of cationic lipids and/or co-lipids as evidenced using freeze fracture EM (Sternberg et al., 1994). Since the structural features of plasmid DNA-lipid complexes are complicated, there is currently no consensus as to which structure is ultimately responsible for mediating transfection.

Studies have been done to investigate the mechanism by which plasmid DNAcationic liposome complexes transfect cells. EM pictures reveal that the transfection complexes are internalized into cells mainly through endocytosis and that fusion takes place inside the endosomes (Farhood et al., 1995; Friend et al., 1996; Hui et al., 1996; Stegmann and Legendre, 1997; Wrobel and Collins, 1995; Zabner et al., 1995). The mechanism involved in the complex escaping from endosomes and plasmid DNA releasing from liposomes is still not clear. It has been suggested that DNA could be released through endosomal destabilization mediated by either the fusogenic lipid DOPE (Farhood et al., 1995; Hui et al., 1996; Litzinger and Huang, 1992) or via anion/cation lipid exchange between the cationic lipids in the liposomes and anionic lipids in the endosomal membranes (Bhattacharya and Mandal, 1998; El Ouahabi et al., 1997; Xu and Szoka, 1996).

A number of physical characteristics limit the application of plasmid DNA-lipid complexes for in vivo gene transfer. Complexes are cleared rapidly by the RES in

vivo (Huang and Li, 1997) and the transfected gene is mainly delivered to "first pass" organs such as lung, liver, and spleen (Huang and Li, 1997). As DNA is only partially protected, it is susceptible to DNase and serum degradation (Li et al., 1999). In addition, complexes are toxic to cells both in vitro (Felgner et al., 1994) and in vivo (Li and Huang, 1997). As a result, much effort has been devoted to the development of small lipid-based DNA formulations for systemic in vivo applications.

### 1.4.2 Stabilized Plasmid-Lipid Particles (SPLP)

Since DNA is a large molecule, stable and efficient entrapment of DNA inside liposomes have been found to be quite a challenging process (Baru et al., 1995; Fraley et al., 1979; Fraley et al., 1980; Lurquin, 1979; Nakanishi et al., 1985; Nicolau and Rottem, 1982). Recently, a novel system containing DNA within the lipid bilayer known as SPLP have been developed and characterized (Wheeler et al., 1999). Prepared by a detergent dialysis technique, SPLP contain high levels of the fusogenic lipid DOPE (>80%) and low levels of DODAC (<10%), and are stabilized by the presence of a PEG coating. This system has high DNA entrapment efficiency (>70%), is homogeneous and small in size (~70 nm), and offers full protection for the plasmid DNA against nuclease degradation (Wheeler et al., 1999).

Although the physical characteristics appear ideal for in vivo application, the transfection potency of SPLP is quite limited (Wheeler et al., 1999). Further studies have found that SPLP are deficient in two main areas: the PEG coating and low level of cationic lipid limits the level of association with cells and uptake into cells, and



Figure 1.9. Schematic structure of stabilized plasmid-lipid particles.

it may inhibit efficient release from the endosomes and subsequent delivery of the DNA into the nuclei (Mok et al., 1999).

### **1.4.3** Cofactors employed in lipid-based mediated transfection

Efforts to improve the transfection properties of lipid-based gene transfer systems have led to the incorporation of cofactors, which could be generally classified into the following categories (Table 2): 1) lysosomotropic agents that interfere with the endosomal-lysosomal pathway, 2) polycations that assist in condensing and protecting the plasmid DNA from nuclease digestion, and 3) DNAbinding proteins that assist in directing DNA nuclear import. Agents such as chloroquine and ammonium chloride interfere with the maturation of the endosomes to lysosomes by preventing the acidification process inside the endosomes (Haensler and Szoka, 1993). However, it has been reported that these lysosomotropic agents could be inconsistent in their enhancing effect, as they have been shown to actually inhibit transfection activity with certain types of cationic liposomes (Farhood et al., 1995). Polylysines and protamines are among the DNAcondensing agents employed as cofactors with liposomes. A 3- to 9-fold increased in transfection has been reported (Gao and Huang, 1996; Li and Huang, 1997). Polycations are cytoplasmic proteins and tend to remain in the cytosol (White et al., 1989). As a result, the complex needs to dissociate before transcription of the plasmid DNA can take place. Histones serve as both a DNA-condensing agent and nuclear directing element; a ~5-fold increase in transfection has been observed by including the histone H1 in the transfection complex (Boulikas and Martin, 1997).

Table 2. Types and actions of common cofactors employed in lipid-based mediated transfection.

Cofactors	Action of cofactors	Transfection enhancement
Chloroquine Ammonium chloride	Prevent entry into lysosomes	Vary with different liposomal formulations (Farhood et al., 1995)
Polylysine Protamines	Condense DNA	~ 3 to 9-fold (Gao and Huang, 1996)
Histones Nuclear localization peptides	Condense DNA, facilitate nuclear entry?	~ 5-fold (Boulikas and Martin, 1997)

# 1.5 Calcium (Ca<sup>2+</sup>)

Eukaryotic cells maintain very low concentrations of free Ca<sup>2+</sup> in their cytosol (~10<sup>-7</sup> M), while the extracellular Ca<sup>2+</sup> concentrations is very much higher (~10<sup>-3</sup> M). Such Ca<sup>2+</sup> gradients are maintained by Ca<sup>2+</sup>-binding proteins within the cytosol, as well as Ca<sup>2+</sup> pumps in the plasma membrane, mitochondrion, and intracellular Ca<sup>2+</sup>-sequestering compartments. A small influx of Ca<sup>2+</sup> increases significantly the concentration of free Ca<sup>2+</sup> in the cytosol. It is well known that the influx of Ca<sup>2+</sup> down its steep concentration gradient in response to extracellular signals rapidly activates Ca<sup>2+</sup>-sensitive response mechanisms in the cells.

## 1.5.1 Biological activities of Ca<sup>2+</sup>

 $Ca^{2+}$  acts as a strong intracellular messenger in a wide variety of cellular responses. There are two major  $Ca^{2+}$  signaling pathways: one used mainly by electrically active cells and the other used by almost all eukaryotic cells (Baker and Knight, 1984; Berridge, 1993; Fewtrell, 1993; Tsien and Tsien, 1990). During membrane depolarization in electro-excitable cells such as neurons, an increase in cytosolic  $Ca^{2+}$  triggers release of the neurotransmitter acetylcholine. In eucaryotic cells, the binding of extracellular signaling molecules to cell-surface receptors are coupled to the release of  $Ca^{2+}$  sequestered in intracellular compartments, which in turn activate subsequent signal transduction pathways. Specific  $Ca^{2+}$ -binding proteins in the cells such as calmodulin rely on the presence of  $Ca^{2+}$  to form a functional complex, which binds to other target proteins and triggers their cellular function. Other activities such as cell proliferation and gene expression are also

regulated by  $Ca^{2+}$  (Berridge, 1993; Hardingham et al., 1997). In addition, attention has been devoted to examining the roles of  $Ca^{2+}$  in regulating cytoplasmic-nuclear transport and its role in gene expression (Bading et al., 1997; Hardingham et al., 1998; Petersen et al., 1998; Santella and Carafoli, 1997). Of particular interest is the role of  $Ca^{2+}$  in membrane fusion processes. It has been found that the increase in  $Ca^{2+}$  concentration can be localized to the site of exocytosis (Erxleben et al., 1997; Llinas et al., 1992; Tse et al., 1997). Intracellular membrane trafficking such as ER to Golgi transport (Baker et al., 1997). Beckers and Balch, 1989), endosome fusion (Colombo et al., 1997), and vacuole fusion (Peters and Mayer, 1998) also require the presence of  $Ca^{2+}$ . Finally, several  $Ca^{2+}$  binding proteins, such as synaptotagmin and syncollin, have been shown to interact with SNAREs in a  $Ca^{2+}$  dependent manner during intracellular membrane fusion (Edwardson et al., 1997; Sudhof and Rizo, 1996). It has been suggested that  $Ca^{2+}$  functions as a mediator of protein-lipid interactions at membrane fusion interfaces (Montal, 1999).

## **1.5.2** Influence of Ca<sup>2+</sup> on lipid polymorphism

The most widely studied model system for membrane fusion is the Ca<sup>2+</sup>/PS system (Papahadjopoulos et al., 1977). Ca<sup>2+</sup> has the capacity to induce the formation of a rigid dehydrated complex with PS, causing the vesicles to aggregate, to release their internal aqueous contents, and to form large structures called "cochleate" cylinders. It has also been proposed that Ca<sup>2+</sup> acts either by neutralizing the charge repulsion between negatively charged liposomes, thereby promoting dehydration and bilayer contact through the formation of interbilayer Ca<sup>2+</sup>-PS

complexes (Portis et al., 1979), or by inducing formation of non-bilayer fusion intermediates (Tilcock et al., 1984).

On the other hand, studies of lipid polymorphism in mixed lipid systems, which have properties that more closely resemble those of biological membranes, could offer greater insight to membrane fusion processes. Phospholipids that adopt the bilayer phase in isolation can stabilize hexagonal-preferring lipids into an overall bilayer organization in mixed lipid systems. It has been found that addition of Ca<sup>2+</sup> to mixed lipid systems could trigger H<sub>II</sub> phase formation, by inducing either phase separation or phase transition (Tilcock et al., 1984; Tilcock and Cullis, 1981). The ability of Ca<sup>2+</sup> to sequester acidic lipid components into separate bilayer domains is referred to as phase separation, while phase transition involves Ca<sup>2+</sup> inducing the formation from bilayer to H<sub>II</sub>. In systems containing neutral lipids such as PE and PC and acidic lipids such as PS and PA, it has been demonstrated that Ca<sup>2+</sup> could segregate the acidic component into bilayer domains that usually exhibit gel-state characteristics (Tilcock et al., 1988). In PE/PS systems, Ca<sup>2+</sup> could segregate the PS component into the "cochleate" morphology, leaving PE to adopt the H<sub>II</sub> organization it favors in isolation (Bally et al., 1983). In unsaturated model systems containing lipid species such as DOPE and DOPG, Ca<sup>2+</sup> is unable to induce phase separation but instead triggers a bilayer to hexagonal H<sub>II</sub> phase transition in both lipids (Tilcock In unsaturated lipid systems containing cholesterol, Ca<sup>2+</sup> also et al., 1988). promotes H<sub>II</sub> formation by inducing lipid phase changes in all participating lipids (Cullis and de Kruijff, 1978; Tilcock et al., 1982; Tilcock et al., 1984). Table 3 summaries the effect of  $Ca^{2+}$  in mixed lipid systems in which a lamellar to H<sub>II</sub> phase

Table 3. Effect of  $Ca^{2+}$  on mixed lipid systems. (PE = phosphatidylethanolamine, PS = phosphatidylserine, PG = phosphatidylglycerol, PI = phosphatidylinositol, CL = cardiolipin, DOPE = dioleoylphosphatidylethanolamin, DOPS = dioleoylphosphatidylserine, Chol = cholesterol).

Types of mixed lipid system	$Ca^{2+}/PS$ (mol ratio) at bilayer $\rightarrow$ H <sub>II</sub>
PS/PE (30:70, mol/mol)	1
PG/PE (30:70, mol/mol)	. 1
PI/PE (15:85, mol/mol)	1
CL/PE (30:70, mol/mol)	0.5
DOPE/DOPS/Chol (1:1:1, mol/mol/mol)	0.5

transition is induced. Finally, the ability of  $Ca^{2+}$  to promote bilayer to  $H_{II}$  phase transition in lipids systems containing PEG lipids has also been investigated (Holland et al., 1996). In mixtures of non-bilayer forming lipids such as PS/PE liposomes, the presence of PEG could actually prevent  $Ca^{2+}$ -induced fusion (Holland et al., 1996). However, the  $Ca^{2+}$ -induced fusogenic activity is restored upon addition of acceptor liposomes (POPC) to which the PEG-lipid can exchange. Since it has been proposed that the intermediates between bilayer and  $H_{II}$  play a role in membrane fusion, and that  $Ca^{2+}$  is often required for fusion events to proceed in vivo, studying the effect of  $Ca^{2+}$  on lipid polymorphism is of significant value in understanding membrane fusion.

# 1.5.3 Roles of Ca<sup>2+</sup> in transfection

Ca<sup>2+</sup> has been used together with phosphate in the CaPO<sub>4</sub>-mediated transfection (Graham and Van der Eb, 1973). Despite its popularity, relatively little work has been done to investigate the mechanism by which CaPO<sub>4</sub> mediates transfection. It is believed that plasmid DNA coprecipitates with CaPO<sub>4</sub> and the complex is internalized into the cells via the endocytic pathway (Loyter et al., 1982). How the DNA is released from the CaPO<sub>4</sub> complex and how it enters the nuclei for transcription is not clear. It is only recently that Ca<sup>2+</sup> has been identified to be an efficient cofactor of polycation-mediated gene transfer (Haberland et al., 1999; Zaitsev et al., 1997). These polycations, which include histone H1, high mobility group (HMG) DNA-binding proteins HMG1 and HMG17, are nuclear proteins that could bind and condense DNA as well as assist in facilitating nuclear import.

### **1.6 Thesis Objectives**

The studies of this thesis focus on examining the effect of Ca<sup>2+</sup> on lipid-based transfection properties. Chapter 2 describes the influence of Ca<sup>2+</sup> on plasmid DNA-cationic liposome complexes. Studies were carried out to confirm the specific activity of Ca<sup>2+</sup> and the effect it has on the complex-mediated transfection. Chapter 3 discusses the influence of Ca<sup>2+</sup> on the encapsulated or SPLP system, in which a dramatic transfection enhancement was observed. Experiments were performed to elucidate the mechanism by which Ca<sup>2+</sup> facilitated the SPLP transfection potency. Ultimately, the goal is to develop lipid-based gene carrier systems with enhanced transfection potency.

#### CHAPTER 2

# CALCIUM ENHANCES THE TRANSFECTION POTENCY OF PLASMID DNA-CATIONIC LIPOSOME COMPLEXES

### 2.1 INTRODUCTION

Plasmid DNA-cationic liposome "complex" systems formed by incubation of plasmid with positively charged liposomes have been used widely as transfection agents in vitro and have promising potential for in vivo applications (Alton & Geddes, 1995; Liu et al., 1995; Nabel et al., 1993). Studies on the mechanism of cationic liposome-mediated gene transfer indicate that complexes enter cells mainly by endocytosis (Friend et al., 1996; Legendre & Szoka, Jr., 1992; Wrobel & Collins, 1995; Zabner et al., 1995; Zhou & Huang, 1994) and that the plasmid escapes into the cytoplasm as a result of endosomal destabilization facilitated by a fusogenic "helper" lipid such as dioleoylphosphatidylethanolamine (DOPE) (Farhood et al., 1995; Litzinger & Huang, 1992). It has been suggested that anionic lipids in the endosomal membranes also play a role in facilitating plasmid DNA release by interacting with cationic lipid in the complexes (Wattiaux et al., 1997; Xu & Szoka, Jr., 1996). The efficiency of cell transfection by plasmid DNA is greatly enhanced by the presence of the cationic liposomes, however transfection by complexes remains Particular obstacles include escape from the a relatively inefficient process. endosome following endocytosis, intracellular nuclease degradation of plasmid (Lechardeur et al., 1999), as well as entry of the plasmid DNA into the cell nucleus (Zabner et al., 1995).

Efforts to improve liposomal gene transfer systems have focused primarily on designing novel cationic lipids and developing liposome formulations containing different "helper" lipids (Felgner et al., 1994; Gao & Huang, 1991; Legendre & Szoka, Jr., 1992; Paukku et al., 1997; Templeton et al., 1997). Other approaches include the incorporation of fusogenic peptides. DNA-condensing agents or targeting ligands such as nuclear localization signals to improve DNA transfer (Dzau et al., 1996; Gao & Huang, 1996; Hagstrom et al., 1996; Ibanez et al., 1996; Li & Huang, 1997; Mizuguchi et al., 1996). Relatively little work has focused on the effect of Ca<sup>2+</sup> on the transfection potency of plasmid DNA-cationic liposome complexes. Traditionally.  $Ca^{2+}$  has been used to enhance in vitro transfection by the  $Ca^{2+}$ phosphate precipitation method (Graham & Van der Eb, 1973). The uptake of DNA by cultured cells is believed to be facilitated by the formation of a Ca<sup>2+</sup> phosphate-DNA co-precipitate, which enters the cell by endocytosis and subsequently is transferred into the nucleus after escape from the endosomal compartment (Loyter et al., 1982; Orrantia & Chang, 1990). Other studies have investigated the role of Ca<sup>2+</sup> in increasing the rate of endocytosis and macro-molecular nuclear uptake (Eliasson et al., 1996; Epstein et al., 1992; Perez-Terzic et al., 1996). Recently, a non-liposomal gene transfer system employing DNA-binding elements has shown enhanced transfection efficacy in the presence of  $Ca^{2+}$  (Bottger et al., 1998; Haberland et al., 1999; Zaitsev et al., 1997). It was suggested that Ca<sup>2+</sup> could increase the rate of endocytosis (Zaitsev et al., 1997) or facilitate endosomal release (Haberland et al., 1999).

In this work we have studied the effect of Ca<sup>2+</sup> on cationic liposome-mediated

cell transfection using a bovine hamster kidney (BHK) cell line. Experiments were conducted to demonstrate the specificity of  $Ca^{2+}$ -enhanced transfection and to examine the mechanism whereby  $Ca^{2+}$  improves transfection. The results indicate that the presence of  $Ca^{2+}$  during complex-mediated transfection facilitates improved intracellular delivery of cationic lipid and plasmid DNA, leading to enhancements in transgene expression of 3 to 20-fold in a number of different cell lines.

### 2.2 MATERIALS AND METHODS

### 2.2.1 Plasmids

The pCMV $\beta$  plasmid, which encodes the lacZ gene for the  $\beta$ -galactosidase ( $\beta$ -gal) protein and the pCMVGFP plasmid, which expresses the green fluorescence protein (GFP), were both driven by a cytomegalovirus (CMV) promoter to assess expression. All plasmids were obtained from J. Thompson at Inex Pharmaceuticals Corporation (Burnaby, BC).

### 2.2.2 Cell lines

293 (transformed primary embryonal human kidney), BHK (Bovine hamster kidney), CV-1 (African Green Monkey kidney), HS578T (human breast ductal carcinoma), and SK-OV3 (human ovary adenocarcinoma) cell lines were obtained from American Tissue Culture Collection (ATCC CCL-10, Rockville, MD). With the exception of HS578T cells which required an additional supplement of bovine insulin (10µg/ml), all cell lines were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100µg/ml of streptomycin (Pen-Strep). All cell lines were maintained as a monolayer at 37°C in a humidified atmosphere containing 5.0% CO<sub>2</sub>.

### 2.2.3 Lipids and Chemicals

*N*,*N*-dioleyl-*N*,*N*-dimethylammonium chloride (DODAC), <sup>14</sup>C-labelled DODAC, and *N*, *N*-distearyl-*N*,*N*-dimethylammonium chloride (DSDAC) were obtained from

Dr. S. Ansell at Inex Pharmaceuticals Corporation. *N*-[2,3-(dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) was purchased from GibcoBRL (Burlington, ON). 1,2-dioleoyl-3-phosphatidylcholine (DOPC) and 1,2-dioleoyl-3phosphatidylethanolamine (DOPE) were obtained from Northern lipids (Vancouver, BC). Dimethyl-dioctadecylammonium Bromide (DDAB) was purchased from Avanti Polar Lipids (Alabaster, AL). Calcium chloride (CaCl<sub>2</sub>), magnesium chloride (MgCl<sub>2</sub>), and sodium chloride (NaCl) were obtained from Fisher Scientific (Fair lawn, NJ). Ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) was purchased from Sigma (St. Louis, MO).

### 2.2.4 Preparation of LUVs

All large unilamellar vesicles (LUVs) containing DODAC/DOPE were prepared in a 1:1 molar ratio. Briefly, mixtures of lipids dispensed in chloroform were dried under a stream of nitrogen gas and the residual solvent was removed under high vacuum for 2 h. The resulting lipid films were hydrated with dH<sub>2</sub>O and LUVs were obtained by freeze-thawing and extruding (Mayer et al., 1986). The sizes of LUVs were checked with a Nicomp Model 270 submicron particle sizer using quasielastic light scattering techniques. Phosphorus assays were used to quantify lipid concentrations (Fiske & Subbarow, 1925).

### 2.2.5 In vitro transfection in the presence of Ca<sup>2+</sup>

Prior to transfection with DNA-lipid complexes, cells were plated at a density of 2 x  $10^4$  cells per well of a 96-well plate overnight. 1 M CaCl<sub>2</sub> stock solution was

prepared in dH<sub>2</sub>O and sterilized by filtering. Liposomes were first added to plasmid DNA and mixed briefly by pipetting up and down several times, after which the mixtures were immediately added to tubes containing appropriate CaCl<sub>2</sub> concentrations and incubated at room temperature for 30 min before diluting to the final volume with DMEM culture media containing 10% FBS, which were then used to transfect cells. CaCl<sub>2</sub> concentrations added to the transfection complexes were calculated with respect to the final volume of the transfection medium applied to cells. Cells were incubated with the transfection complexes for 4 h unless otherwise noted, after which culture media was replaced and cells were further incubated at 37°C, 5% CO<sub>2</sub> overnight until assaying for gene expression.

### 2.2.6 Determination of transgene expression

 $\beta$ -gal expression was assayed by using the substrate Chlorophenol red galactopyranoside (CPRG) (Boehringer Mannheim, Germany) at 1 mg/ml to induce color development, which was measured at 540 nm with a microplate EL-309 autoreader (Bio-Tek Instruments) as described previously (Mok & Cullis, 1997). Absorbance readings were calibrated according to a  $\beta$ -gal standard (Sigma). Total cellular protein was determined by using the Micro BCA Protein Assay Reagent Kit (Pierce, Illinois), which was subsequently used to normalize the  $\beta$ -gal activities of each transfection. For the fluorescence analysis of GFP expression, cells were plated at 5 x 10<sup>5</sup> cells per well of a 100 x 20mm tissue culture dish the night prior to transfection. Cells expressing GFP were directly visualized and photographed at the indicated time point (24, 48, and 72 h) using a Zeiss (Axiovert S100) fluorescence

microscope with an XF100 filter set (Ex=475 nm, Em=535 nm) from Omega Optical (Vermont, USA).

# 2.2.7 Intracellular uptake studies employing <sup>14</sup>C-radiolabeled lipids

BHK cells were plated at 7.5 x  $10^5$  cells per 25 cm<sup>2</sup> cultured flasks the dav before transfection. Trace amounts of <sup>14</sup>C-radiolabeled DODAC were incorporated into DODAC/DOPE liposomes. For each transfection, 2 μg pCMVβ was complexed to the liposomes at a 1.5 charge ratio (mole of positive charges per mole of negative charges) in the presence or absence of 5 mM Ca<sup>2+</sup>. Transfection media was removed from the cells at 1, 2, or 4 h post transfection. For the 24 h time point, transfection mixtures were allowed to incubate on cells for 4 h. after which complete media was replaced for the remaining time. Cells were detached by trypsinization and washed in isotonic buffer (250 mM sucrose, 3 mM MgCl<sub>2</sub>, 50 mM HEPES, pH 7.2). One quarter of the cells was analyzed as the whole cell fraction, while the remaining cells were subjected to nuclear fractionation according to a previously described protocol with modifications (Olnes & Kurl, 1994). Briefly, cells were treated with hypotonic buffer (10 mM HEPES pH 8, 10 mM KCl, 3 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 0.1 mM EGTA) on ice for 15 min. Cells were then lysed by the addition of 0.6% Nonidet P40 (NP-40) (BDH Laboratory Supplies, England) and mixed by gentle inversions. Nuclei were pelleted by centrifuging at 500 x g for 2 min, and pellets were washed using nuclei resuspension buffer (20 mM HEPES pH 8, 25% glycerol, 1.5 mM KCl, 0.2 mM EDTA). Both cell and nuclei pellets were lysed by the

addition of 250  $\mu$ l Sovable and digested for 2 h at 60°C. Solublized samples were subjected to scintillation counting in a Beckman LS3801 scintillation counter.

### 2.2.8 Dot blot and Southern blot analyses

Experimental protocols were the same as the <sup>14</sup>C-labeled lipid uptake experiments, except that all cells were trypsinized after incubation in the transfection mixtures for 4 h. The centrifuged pellets were washed with isotonic buffer and subsequently lysed by using the lysis buffer (10 mM Tris, pH 7.5, 0.5% SDS, 1 mM EDTA) containing Pronase E at 1 mg/ml (Sigma) overnight at 37°C. Genomic DNA and delivered plasmid DNA were isolated according to a published protocol (Sambrook et al., 1989). Extracted DNA was resuspended in TE buffer. DNA recovery was determined by measuring the absorbance at 260 nm. 2 µg of total DNA from each sample was either dot blotted onto a nylon transfer membrane (Amersham) with a set of pCMV $\beta$  standards (0 to 10 pg) or loaded into a 1% agarose gel and size fractionated at 60 V for 2 h for the Southern analysis. Both blots were hybridized overnight at 68°C to a <sup>32</sup>P-labeled plasmid DNA probe, which was prepared with BamHI cut-pCMV $\beta$  plasmid using the <sup>T7</sup>QuickPrime<sup>TM</sup> Kit (Pharmacia Biotech). Blots were washed 3 times at 68°C with 2x SSC containing 0.1% SDS, and were then exposed on a Phosphorimager screen which was subsequently scanned (Molecular Dynamics – Phosphorimager<sup>™</sup>SI).

### 2.3 RESULTS

# 2.3.1 The transfection potency of plasmid DNA-lipid complexes is increased in the presence of Ca<sup>2+</sup>

In order to examine the effect of  $Ca^{2+}$  on complex-mediated transfection. BHK cells were transfected with plasmid DNA-cationic lipid complexes prepared in the presence of increasing concentrations of calcium (0 to 100 mM). Plasmid DNA  $(pCMV\beta)$  was complexed with DODAC/DOPE liposomes at a cationic lipid to plasmid DNA charge ratio of 1.5 (mol of positive charges/mol of negative charges). These complexes were immediately mixed with the appropriate concentration of Ca<sup>2+</sup>, incubated for 30 min before diluting to the final volume with DMEM culture media, and then applied to cells. As shown in Figure 2.1, no  $\beta$ -gal activity was detected when cells were transfected with calcium and plasmid DNA alone. However, β-gal activities were clearly enhanced in cells that were transfected with complexes incubated in the presence of  $Ca^{2+}$ . In particular, up to 20-fold increases in transgene expression were detected at Ca<sup>2+</sup> concentrations between 5 and 25 mM. Transfection potencies decreased for  $Ca^{2+}$  concentrations at 50 mM or greater, where more than 20% reductions in total cellular protein levels were observed, Cellular protein levels at Ca<sup>2+</sup> indicating cytotoxicity (data not shown). concentrations below 50 mM were similar to those of cells transfected with lipid-DNA complexes prepared without calcium. In order to avoid cell damage, Ca2+ concentrations of 10 mM or less were employed in subsequent experiments.



Figure 2.1 Effect of increasing Ca<sup>2+</sup> concentrations on the transfection activities of plasmid DNA-cationic liposome complexes. pCMV $\beta$  (0.25 µg) in the presence (•) or absence (•) of DODAC/DOPE liposomes was added to Ca<sup>2+</sup> and incubated for 20 min at room temperature prior to transfecting BHK cells. DODAC/DOPE was complexed to pCMV $\beta$  at a charge ratio of 1.5 (mol/mol). Cells were exposed to the complexes for 4 h and then the transfection mixtures were replaced with complete DMEM media for a further 20 h incubation before assaying for  $\beta$ -gal expression, as outlined in Materials and Methods. The error bars represent the standard deviation of three experiments.

# 2.3.2 The proportion of cells transfected is increased in the presence of Ca<sup>2+</sup>

Increased transfection in the presence of Ca<sup>2+</sup> could be due to cells expressing a higher level of the delivered gene or a greater proportion of cells being transfected, or a combination of both. In order to examine the transfection efficiency in a population of cells, transfection was monitored employing fluorescent microscopy. BHK cells were transfected with pCMVGFP at a 0.75 cationic lipid-toplasmid DNA charge ratio in the presence or absence of 2.5 mM Ca<sup>2+</sup>. Complete DMEM medium was replaced at the end of the 4 h transfection period and cells were incubated in normal growing conditions for indicated periods of time. GFP expression was visualized at 24, 48, and 72 h post-transfection (Fig. 2.2). Since the plasmid DNA-cationic liposome complexes were prepared at a 0.75 charge ratio, only a small population of cells was expected to show expression of the GFP transgene (Fig. 2.2, Col. A). When transfected with complexes incubated in the presence of Ca<sup>2+</sup>, a much higher proportion of cells expressing GFP could be detected (Fig 2.2, Col. B). As the delivered plasmid resulted in transient expression, the number of cells expressing GFP decreased as cells continued to grow and divide over the 72-h time period. Nevertheless, a significant number of cells transfected with the Ca<sup>2+</sup>-enhanced complexes continued to express the transgene over the 72h time period. These data demonstrate that the increase in transfection efficiency is due, at least in part, to a higher proportion of cells being transfected when Ca<sup>2+</sup> is present in the transfection complex mixtures.



Figure 2.2. Fluorescence microscopy of BHK cells transfected with pCMVGFP using complexes in the presence of Ca<sup>2+</sup>. pCMVGFP (1  $\mu$ g) was complexed to liposomes at a charge ratio of 0.75 and incubated on cells for 4 h in the absence (column A) or presence (column B) of 5 mM Ca<sup>2+</sup>. Cells were grown continuously on tissue cultured dishes with supplement of fresh DMEM media and splitting as required. Photographs of cells expressing GFP were taken at 24, 48, and 72 h post-transfection using a Zeiss fluorescence microscope with an XF100 filter set.

### 2.3.3 Cation-dependent transfection enhancement is Ca<sup>2+</sup> specific

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In order to determine whether Ca<sup>2+</sup> specifically enhances transfection, the effect of CaCl<sub>2</sub>, MgCl<sub>2</sub>, and NaCl were compared. Increasing concentrations (0 to 50 mM) of CaCl<sub>2</sub>, MgCl<sub>2</sub>, and NaCl were incubated with pCMVβ-DOPE/DODAC complexes, which were subsequently used to transfect BHK cells. As shown in Figure 2.3A,  $\beta$ -gal expression was enhanced only in the presence of Ca<sup>2+</sup>. No significant increase in transgene expression was observed when transfection was performed in the presence of Mg<sup>2+</sup> and only a slight increase was detected at higher concentrations of Na<sup>+</sup>. To further confirm the Ca<sup>2+</sup> specific nature of this stimulatory effect, the ability of EGTA to inhibit Ca<sup>2+</sup>-enhanced transfection was examined. EGTA was either pre-incubated with Ca<sup>2+</sup> for 5 min prior to the addition of cationic liposomes and plasmid DNA, or it was added to the transfection complexes after the three components had been incubated for 30 min at room temperature. Figure 2.3B shows that the Ca<sup>2+</sup>-enhanced transfection was significantly reduced in the presence of EGTA, whether it was added before or after the complex formation. Taken together, these results indicate that transfection was enhanced specifically by Ca<sup>2+</sup>.

# 2.3.4 Ca<sup>2+</sup> increases the rate of transfection by complexes

The effect of  $Ca^{2+}$  on transfection as a function of the time complexes were incubated with cells was investigated. Complexes prepared in the presence or absence of  $Ca^{2+}$  were incubated with cells for 30 min to 4 h. At the appropriate time points, transfection medium was removed and replaced with complete medium.



Figure 2.3. Specificity of Ca<sup>2+</sup> for enhancing transfection. (A) pCMV $\beta$  (0.25 µg) was complexed with DODAC/DOPE (charge ratio 1.5) in the presence of increasing concentrations (0 to 50 mM) of CaCl<sub>2</sub> (•), MgCl<sub>2</sub> (•), or NaCl (•). (B) Effect of EGTA on Ca<sup>2+</sup>-enhanced transfection. An equimolar amount of EGTA was added to Ca<sup>2+</sup> (5 mM) either prior to the addition of liposomes and DNA, or after the formation of the Ca<sup>2+</sup>-enhanced DNA-lipid complexes. 1. untransfected cells; 2. DNA-lipid complexes; 3. Ca<sup>2+</sup>-enhanced DNA-lipid complexes; 4. EGTA incubated with Ca<sup>2+</sup> prior to addition of liposomes and plasmid DNA; 5. EGTA added after formation of Ca<sup>2+</sup>-enhanced DNA-lipid complexes. The error bars represent the standard deviation of three experiments.

Cells were further incubated overnight before assaying for  $\beta$ -gal expression. At the 1 h time point, only minimal transgene expression was detected in cells transfected with the control plasmid DNA-cationic lipid complexes (Fig. 2.4). Higher gene expression was observed at longer incubation periods, where maximum  $\beta$ -gal activity was achieved when the transfecting mixtures were incubated on cells for 3 to 4 h. On the other hand, exposure of cells for just 30 min to transfection mixtures prepared in the presence of Ca<sup>2+</sup> resulted in transgene expression as high as the maximum transfection level achieved after a 4 h incubation of complexes prepared in the absence of Ca<sup>2+</sup>.

# 2.3.5 Increased intracellular delivery of lipid is observed in the presence of Ca<sup>2+</sup>

The shorter incubation period required for gene expression employing plasmid DNA-cationic lipid complexes prepared in the presence of  $Ca^{2+}$  suggests increased rates of intracellular delivery of complexes. The kinetics of cationic lipid uptake were therefore examined. DODAC/DOPE liposomes labeled with a trace amount of <sup>14</sup>C-radiolabeled DODAC were used to prepare complexes in the presence or absence of  $Ca^{2+}$ . Lipid uptake was monitored over a 24-h time period. In the absence of  $Ca^{2+}$ , cellular lipid uptake increased up to the 4 h time point and remained stable for the remaining time (Fig. 2.5). Enhanced lipid uptake was observed for cells transfected with complexes prepared in the presence of  $Ca^{2+}$ . At the 1 h time point, cellular lipid uptake similar to the maximal level of complexes prepared without  $Ca^{2+}$  was achieved. This was equivalent to a more than 2-fold



Figure 2.4. Effect of incubation time on transfection employing control DNA-lipid and Ca<sup>2+</sup>-enhanced DNA-lipid complexes. Transfection mixtures prepared in the presence ( $\blacksquare$ ) or absence ( $\bullet$ ) of Ca<sup>2+</sup> were incubated with BHK cells for 0.5, 1, 2, 3, and 4 h. At the appropriate time points, complete media was replaced and cells were allowed to recover overnight and  $\beta$ -gal activity was assayed. Liposomes were complexed to 0.25 µg pCMV $\beta$  at a 1.5 charge ratio. The error bars represent the standard deviation of three experiments.



Figure 2.5. Kinetic analysis of the intracellular delivery of lipids in the presence of Ca<sup>2+</sup>. Uptake of <sup>14</sup>C-radiolabeled DODAC was examined over a 24-h time period for both whole cell (solid lines) or nuclear (dotted lines) fractions, prepared as described in Materials and Methods. Cells transfected with DNA-lipid complexes prepared in the presence ( $\blacksquare$ ) or absence ( $\bullet$ ) of 5 mM Ca<sup>2+</sup> were solubilized at the indicated time points (1, 2, 4, and 24 h) and assayed by scintillation counting to determine lipid uptake. The error bars represent the standard deviation of three experiments.

increase in intracellular lipid uptake when compared to the control complexes at the same time point. This greater than 2-fold increase was maintained as the transfection process continued through the 4-h incubation period. It is interesting to note that lipid uptake continued to increase over the 24-h time period (after removal of the complexes at 4 h), suggesting continued uptake of complexes adsorbed to the cell surface. Examination of the nuclear fraction showed that the level of nuclear associated lipid remained low even in the presence of Ca<sup>2+</sup> (Fig. 2.5).

# 2.3.6 Increased intracellular delivery of intact plasmid DNA is observed in the presence of Ca<sup>2+</sup>

Intracellular delivery of plasmid DNA was determined using the dot blot assay, and the integrity of the plasmid was examined employing the Southern blot analysis. Cells were exposed to complexes prepared in the presence or absence of calcium. The levels of plasmid DNA uptake for the different systems were compared after isolation of DNA from cells that had been transfected for 4 h, as described in Materials and Methods. The results of the dot blot and Southern blot analyses are shown in Figure 2.6. On average each cell took up ~8,000 copies of plasmid DNA when cells were transfected with complexes prepared in the absence of Ca<sup>2+</sup>. This is consistent with previous work indicating that readily transfectable cells take up ~10<sup>5</sup> plasmids when transfected by complexes (Tseng et al., 1997; Zabner et al., 1995). As shown in Figure 2.6A, when cells were transfected with the complexes prepared in the presence of Ca<sup>2+</sup> the number of plasmids taken up by each cell increased by more than 2.5-fold. A Southern analysis of the delivered DNA showed some


Figure 2.6. Analysis of plasmid DNA uptake using dot blot and intracellular integrity using Southern blot. (A) Intracellular delivery of plasmid DNA determined by the dot Plasmid DNA delivered by: 1. DODAC/DOPE with Ca<sup>2+</sup>; 2. blot assay. DODAC/DOPE and 3. untransfected cells. (B) Southern blot analysis of intracellular plasmid DNA. Lanes 1: pCMVβ standard; 2: TE buffer; 3-5: untransfected cells; Lanes 6-8: plasmid delivered by DODAC/DOPE; and 9-11: plasmid delivered by DODAC/DOPE prepared with Ca<sup>2+</sup>. 2  $\mu$ g pCMV $\beta$  complexed to liposomes at a 1.5 charge ratio. Cells were transfected for 4 h, in the presence or absence of 5 mM Ca<sup>2+</sup>. Genomic and plasmid DNA (total DNA) were purified from the cell lysates as described in Materials and Methods. 2 µg of total DNA were dotted onto a nitrocellulose membrane or loaded into a 1% agarose gel, which was run at 60 V for 2 h. Blots were hybridized to a <sup>32</sup>P-labeled probe overnight for detection of plasmid using a phosphorimager. The number of plasmid copies was quantitated according to a plasmid standard and the results normalized by dividing by the number of cells. The error bars represent the standard deviation of three experiments.

degradation of the delivered plasmid for both the control and calcium-enhanced lipid-DNA complexes, as indicated in Figure 2.6B by the smearing of the DNA bands and the appearance of additional DNA conformations as compared to the control plasmid. However, the results demonstrate that significantly more intact plasmid DNA is present in cells transfected with complexes prepared in the presence of  $Ca^{2+}$ .

# 2.3.7 The level of transfection enhancement is dependent on the time of Ca<sup>2+</sup> addition

Experiments were performed to determine if the point at which  $Ca^{2+}$  is added during preparation of the complexes affects the level of transfection enhancement. Three situations were investigated using equivalent concentrations of  $Ca^{2+}$  (5 mM) for each. In the first situation,  $Ca^{2+}$  was incubated with either the DODAC/DOPE liposomes or the pCMV $\beta$  for 15 min prior to adding the other component, after which the mixtures were allowed to incubate for another 15 min before being using for transfection. In the second case, liposomes and DNA were incubated for 30 min and  $Ca^{2+}$  was added just prior to applying the transfection mixtures to cells. In the last case, liposomes and plasmid DNA were mixed together and immediately added to  $Ca^{2+}$  as described in Materials and Methods, and the three components were incubated for 30 min before applying to cells.

As shown in Figure 2.7, the greatest enhancement occurred when  $Ca^{2+}$ , plasmid DNA, and liposomes were mixed and incubated together. Incubation of plasmid DNA with  $Ca^{2+}$  before the addition of liposomes resulted in similar



Figures 2.7. Effect of the order of  $Ca^{2+}$  addition on transfection potency. Cells were transfected with DNA-cationic lipid complexes (0.25 µg DNA) that were mixed with 5 mM  $Ca^{2+}$  at different stages. 1. liposomes and plasmid DNA mixtures added to  $Ca^{2+}$  simultaneously; 2.  $Ca^{2+}$  mixed with cationic liposomes first; 3.  $Ca^{2+}$  mixed with plasmid DNA first; 4.  $Ca^{2+}$  added just before transfection and after formation of DNA-lipid complexes. The error bars represent the standard deviation of three experiments.

transfection levels. However, a more than 50% reduction in transfection potency was observed when  $Ca^{2+}$  was incubated first with the cationic liposomes and then with plasmid DNA. Most significantly, transfection levels were considerably reduced (~5-fold) when  $Ca^{2+}$  was added to pre-formed plasmid DNA-cationic lipid complexes immediately prior to the application to cells. These data suggest that the stimulatory effects of  $Ca^{2+}$  depend to some extent on the ability of  $Ca^{2+}$  to interact directly with the plasmid DNA in the complex.

# 2.3.8 Ca<sup>2+</sup>-enhanced transfection for complexes is observed in a variety of cell lines

In order to illustrate the generality of the transfection enhancing effect of  $Ca^{2+}$ , the influence of  $Ca^{2+}$  on complex-mediated transfection of a number of different cell lines was examined. Previous work has shown that the type of cell employed can cause large variations in cationic liposome-mediated transfection efficiencies (Harrison et al., 1995). This variation was also observed for the cell lines tested here. Transfection employing pCMV $\beta$ -DODAC/DOPE complexes prepared in the presence or absence of Ca<sup>2+</sup> was performed on BHK, CV-1; 293, SKOV-3, and HS578T cells. In the absence of Ca<sup>2+</sup>, BHK cells showed the highest transfection was observed in the presence of Ca<sup>2+</sup> for all cell lines, although different cell lines demonstrated different degrees of enhancement (from 2 to 80-fold).



Figure 2.8. Effect of  $Ca^{2+}$  on transfection for different cell lines. Cells were transfected with 0.25 µg pCMV $\beta$  complexed to DODAC/DOPE in the presence (dashed bars) or absence (open bars) of  $Ca^{2+}(5 \text{ mM})$ . Cells were exposed to the complexes for 4 h and then the transfection mixtures were replaced with the appropriate culture media for a further 20 h before assaying for  $\beta$ -gal expression. The error bars represent the standard deviation of three experiments.

# 2.3.9 Ca<sup>2+</sup> enhances the transfection potency of complexes containing a variety of cationic lipids

The stimulatory effect of Ca<sup>2+</sup> on the transfection properties of plasmid DNAcationic lipid complexes containing a variety of cationic lipids were examined. Prior to application to cells, plasmid DNA was complexed to liposomes containing either 50 mol% of DODAC, DOTMA, DSDAC or DDAB and 50 mol% DOPE. Enhanced βgal expression was observed for all the cationic liposome formulations examined in the presence of Ca<sup>2+</sup>, with DNA-lipid complexes containing DOTMA showing the highest transfection efficiency, followed by DODAC, DSDAC, and DDAB (Fig. 2.9A). It was of interest to include DODAC/DOPC liposomes in order to see if Ca<sup>2+</sup> could enhance transfection for a "nonfusogenic" formulation. It has been demonstrated that only the fusogenic lipid DOPE is able to show helper activity in facilitating cationic liposome-mediated gene transfer (Farhood et al., 1995; Felgner et al., 1987; Mok & Cullis, 1997). Results showed that cells transfected with the DODAC/DOPC formulation exhibited very low levels of β-gal activity. Transfection efficiencies increased more than 5-fold when Ca<sup>2+</sup> was included, although the enhanced transfection was still significantly lower than for the DOPE-containing formulation in the absence of Ca<sup>2+</sup>.

The ability of Ca<sup>2+</sup> to enhance transfection for the DODAC/DOPC system was further investigated for increasing concentrations of Ca<sup>2+</sup> in complexes prepared at a 1.5 charge ratio. As shown in Figure 2.9B, significant enhancements in transfection were observed in the presence of 6 to 25 mM Ca<sup>2+</sup>, where increases in  $\beta$ -gal activities from 0 to 600 milliunits per mg of total cellular protein were observed. As



Figure 2.9. Effect of  $Ca^{2+}$  on transfection properties of different liposomal formulations. (A) Transfection properties of complexes containing DODAC, DOTMA, DSDAC, or DDAB prepared at a 1:1 molar ratio with DOPE, as well as a "nonfusogenic" formulation (DODAC/DOPC), in the presence (dash bars) or absence (solid bars) of 10 mM  $Ca^{2+}$ . DNA was mixed with 1.  $Ca^{2+}$  only; 2. DODAC/DOPC; 3. DODAC/DOPE; 4. DOTMA/DOPE; 5. DSDAC/DOPE; or 6. DDAB/DOPE. (B) Effect of increasing  $Ca^{2+}$  concentration on transfection properties of "nonfusogenic" DNA-lipid complexes. DNA was complexed to DODAC/DOPC and added to 0 to 100 mM  $Ca^{2+}$ . All liposome formulations were complexed to 0.25 µg pCMV $\beta$  at 1.5 charge ratio. The error bars represent the standard deviation of three experiments.

was observed for the DODAC/DOPE system, DODAC/DOPC transfection diminished when 50 mM or higher  $Ca^{2+}$  concentrations were used.

### 2.4 DISCUSSION

The studies reported here show that the presence of  $Ca^{2+}$  during formation of DNA-cationic lipid complexes can result in significant improvements in transfection properties in vitro. Three areas warrant further discussion. First, it is of interest to compare the improvement in transfection efficiency achieved in the presence of  $Ca^{2+}$  with that achieved by other protocols. A second area concerns the interactions of  $Ca^{2+}$  with the components of the complexes. Finally, we discuss possible mechanisms whereby  $Ca^{2+}$  results in enhanced transfection potency of plasmid DNA-cationic lipid complexes.

Compared to viral vectors, plasmid DNA-cationic liposome complexes are relatively inefficient gene transfer agents (Zabner et al., 1995). Transfection efficiency may be limited by the ability of complexes to enter the cell, release of plasmid from the endosomal compartment, the stability of plasmid in the cytoplasm, and entry of plasmid into the nucleus. A variety of agents have been proposed to enhance the transfection potency of complexes by removing one or more of these limitations. Lysosomotropic agents such as chloroquine have been suggested to promote escape of endocytosed materials from the endosome (Farhood et al., 1995; Felgner et al., 1994). However, although some increase in transfection has been detected (~ 4-fold), the effect of chloroquine has been found to vary for different types of complexes to the extent that it could inhibit transfection (Felgner et al., 1994). DNA-binding molecules such as polylysine have been proposed to inhibit breakdown of intracellular DNA and thus increase transfection, resulting in a 3 to 9-fold increase in transfection efficiency (Boulikas & Martin, 1997; Chiou et al., 1994;

Gao & Huang, 1996). The results presented here demonstrate that the presence of Ca<sup>2+</sup> can enhance the transfection potency of complexes by factors ranging from 3 to 20-fold depending on  $Ca^{2+}$  concentrations employed. Optimal Ca<sup>2+</sup> concentrations to stimulate DNA-cationic lipid complexes transfection ranged between 5 and 25 mM, which was a higher extracellular Ca<sup>2+</sup> concentration when compared to the level found in DMEM culture media, which normally contained ~1.8 mM Ca<sup>2+</sup>. The generality of this stimulatory effect was confirmed by transfecting a number of different cell lines as well as employing various cationic liposomal systems, which further established  $Ca^{2+}$  as a potent stimulatory agent for enhancing cationic liposome-mediated cell transfection. In this regard a recent report has shown that Ca<sup>2+</sup> ions can enhance nuclear protein-mediated transfection but not cationic liposome-mediated transfection (Haberland et al., 1999). This discrepancy likely arises from preparation of the complexes in the absence of Ca<sup>2+</sup>, with subsequent addition of Ca<sup>2+</sup> immediately prior to application to cells. The results presented here show that the transfection potency is significantly reduced under such situations.

It is likely, but not yet proven, that the ability of Ca<sup>2+</sup> to enhance the transfection properties of complexes involves a direct association with the plasmid DNA in the complex. Two lines of evidence support this conclusion. First, the observation that efficient transfection in the presence of calcium is observed at lower cationic lipid-to-plasmid DNA charge ratios than in the absence of calcium suggests that calcium may substitute to some extent for cationic lipid in the complex. Second, enhanced transfection potencies were observed if complexes were incubated with

 $Ca^{2+}$  for 30 min immediately after complex formation, but transfection levels were much reduced if  $Ca^{2+}$  was added immediately before application to cells. It has been shown that plasmid in plasmid DNA-cationic lipid complexes is at least partially protected from the external aqueous environment (Wheeler et al., 1999), which would preclude rapid interaction with external  $Ca^{2+}$ .

The mechanism whereby  $Ca^{2+}$  enhances the transfection potency of complexes appears to be directly related to an ability to increase uptake of complexes into cells, resulting in increased levels of intact intracellular plasmid DNA. The mechanism whereby  $Ca^{2+}$  could stimulate uptake is not yet clear, but likely arises from increased intracellular levels of  $Ca^{2+}$ . It has been shown that increased intracellular  $Ca^{2+}$  levels could lead to increases in the rates of endocytosis (Eliasson et al., 1996; Epstein et al., 1992) and thus could promote the uptake of complexes. The dependence of transfection enhancement on the time and order of  $Ca^{2+}$  addition suggests that complex-associated  $Ca^{2+}$ , as opposed to free  $Ca^{2+}$ , plays a primary role in stimulating uptake. It is possible that the complex-associated  $Ca^{2+}$  can gain access to the intracellular compartment more readily, or that the complex-associated  $Ca^{2+}$ simply represents the largest pool of  $Ca^{2+}$  in the endosome following uptake of the complex.

In summary, we have shown that  $Ca^{2+}$  can act as a potent stimulatory agent to enhance transfection properties of cationic DNA-lipid complexes. The protocol is simple and appears to be of utility in a variety of cell lines and for a variety of cationic lipids. The mechanism whereby  $Ca^{2+}$  stimulates transfection appears to involve

enhanced uptake of plasmid DNA. It is anticipated that this protocol will find general applicability as a means to enhance cationic liposome mediated transfection.

#### **CHAPTER 3**

# CALCIUM ENHANCES THE TRANSFECTION POTENCY OF STABILIZED PLASMID-LIPID PARTICLES

#### 3.1 INTRODUCTION

In order for gene therapy to be clinically useful, an effective and safe gene delivery system is required. Viral vectors are relatively efficient gene delivery vehicles, but suffer from a variety of limitations such as the potential for reversion to the wild type and immune response concerns. As a result, considerable efforts have been made to develop non-viral gene delivery systems (Hope et al., 1998; Peeters et al., 1996; Worgall et al., 1997; Yei et al., 1994). Plasmid DNA-cationic liposome complexes (lipoplexes) are currently the most commonly employed non-viral gene delivery vehicles (Chonn and Cullis, 1995; Felgner, 1997). Lipoplexes can exhibit good transfection properties in vitro but have certain limitations in vivo. For example, lipoplexes are large, highly charged systems that are rapidly cleared from the circulation following systemic administration and can also elicit toxic side effects (Harrison et al., 1995; Hofland et al., 1997; Huang and Li, 1997; Templeton et al., 1997).

Recent work from this laboratory has shown that plasmid DNA can be encapsulated in small (~ 70 nm diameter) "stabilized plasmid-lipid particles" (SPLP) that consist of a single plasmid encapsulated within a bilayer lipid vesicle (Wheeler et al., 1999). These SPLP contain the "fusogenic" lipid dioleoylphosphatidylethanolamine (DOPE), low levels of cationic lipid and are

stabilized in aqueous media by the presence of a polyethylene glycol (PEG) coating. SPLP have systemic application as they exhibit extended circulation lifetimes following intravenous (i.v.) injection, accumulate preferentially at distal tumour sites due to the enhanced vascular permeability in such regions and can mediate transgene expression at these tumour sites (Tam et al., 2000). The levels of transgene expression in vivo are greater than can be achieved with naked DNA or complexes but are lower than may be required for therapeutic benefit (Zhang et al., 1999; Mok et al., 1999). Efforts have therefore been focused on developing SPLP that have more potent transfection properties.

In previous work we have shown that Ca<sup>2+</sup> can enhance the transfection potencies of plasmid DNA-cationic lipid complexes by 20-fold or more (Lam and Cullis, 2000). This enhanced potency was ascribed to increased cell uptake of the complexes when Ca<sup>2+</sup> is present. In the present work we examined whether Ca<sup>2+</sup> could also enhance the transfection activity of SPLP. It is shown that Ca<sup>2+</sup> can result in up to 600-fold enhancements in SPLP transfection potency. Surprisingly, it was found that these enhanced transfection activities did not result from enhanced cell uptake, but appear to be related to an ability of Ca<sup>2+</sup> to destabilize the endosomal membrane following endocytosis. Finally, the effect of Ca<sup>2+</sup> on the transfection potency of SPLP formulations containing either a cationic PEG lipid (CPL) or higher DODAC content was examined.

### 3.2 MATERIALS AND METHODS

### 3.2.1 Materials

*N*,*N*-dioleyl-*N*,*N*-dimethylammonium chloride (DODAC) was obtained from Dr. S. Ansell 1-O-(2-(a-methoxyethyleneglycol)succinoyl)-2-Nand arachidoylsphingosine (PEG-CerC<sub>20</sub>) was synthesized by Dr. Z. Wang at Inex Pharmaceuticals Corporation (Burnaby, 1.2-dioleoyl-sn-alycero-3-BC). phosphoethanolamine (DOPE), and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were obtained from Northern Lipids (Vancouver, BC). 1,2-dioleoyl-snglycero-3-[phospho-L-serine] (DOPS) and 1,2-dioleoyl-sn-glycero-3phosphoethanolamine-N-(Lissamine Rhodamine B Sulfonyl) (Rh-DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol, octylglucopyranoside (OGP), HEPES, MgCl<sub>2</sub>, and NaCl were obtained from Sigma Chemical Co. (St. Louis, MO). DEAE Sepharose CL-6B anionic-exchange column materials were obtained from Sigma Chemical Co. (St. Louis, MO). The luciferase assay kit was purchased from Promega Corp. (Madison, WI). Picogreen dsDNA detection reagent was obtained from Molecular Probes (Eugene, OR). Plasmid DNA (pCMVLuc) coding for the luciferase reporter gene under the control of the human CMV immediate early promoter-enhancer element was obtained from Inex Pharmaceuticals Corporation (Burnaby, BC). Bovine hamster kidney (BHK) cells were obtained from the American Tissue Culture Collection (ATCC CCL-10, Rockville, MD) and cultured in Dulbecco modified Eagle medium (DMEM) supplement with 10 % fetal bovine serum (FBS), 100 U/ml of penicillin and 100

 $\mu$ g/ml of streptomycin. BHK cells were maintained as a monolayer at 37 °C in a humidified atmosphere containing 5.0 % CO<sub>2</sub>.

### 3.2.2 Preparation of SPLP

SPLP were prepared as described by Wheeler et al. (Wheeler et al., 1999) with some modifications. Briefly, a total of 10 µmoles of DODAC, DOPE, PEG-CerC<sub>20</sub> (7:83:10; mol/mol/mol) were dissolved in chloroform and dried under a stream of nitrogen gas. Residual solvent was removed under high vacuum for 2 h. The resulting lipid film was hydrated in 1 ml of HBS buffer (20 mM HEPES and 150 mM NaCl, pH 7.5) containing 0.2 M OGP with continuous vortexing. Plasmid DNA (400  $\mu$ g/ml) was added to the hydrated lipids and the mixtures were dialysed against HBS buffer for 36 to 48 h with 2 buffer changes. Non-encapsulated plasmid was removed by DEAE anion exchange chromatography and empty lipid vesicles were removed by employing a sucrose density gradient as previously described (Mok et al., 1999). For the high DODAC content formulation (DODAC/DOPE/PEG-CerC<sub>20</sub>, 14:76:10, mol/mol/mol), SPLP were initially prepared in HBS buffer containing 30 mM sodium citrate as described previously (Zhang et al., 1999). SPLP were characterized with respect to plasmid entrapment using a previously described Picogreen assay (Zhang et al., 1999) and sized using quasielastic light scattering.

# 3.2.3 Transfection in the presence of Ca<sup>2+</sup>

Prior to transfection, BHK cells were plated at a density of 1 x  $10^4$  cells per well in a 96-well plate overnight. 200 mM CaCl<sub>2</sub> stock solution was prepared in

dH<sub>2</sub>O and sterilized by filtering. 0.5  $\mu$ g plasmid DNA encapsulated in SPLP was used per well of transfection. SPLP were first added to appropriate concentrations of Ca<sup>2+</sup> as required by the experiment, after which culture media was added to the mixtures to obtain the final transfection volume of 100  $\mu$ l/well. Ca<sup>2+</sup> concentrations added to the SPLP were calculated with respect to the final volume of the transfection medium applied to cells. The final volume contained 20 % vol Ca<sup>2+</sup> and SPLP mixtures and 80 % vol culture media. Cells were incubated with the transfection complexes for the appropriate time periods before assaying for gene expression as described previously (Wheeler et al., 1999). Relative luciferase activity was normalized against total cellular protein determined by using the Micro BCA protein assay reagent kit (Pierce, Illinois).

### 3.2.4 Determination of SPLP uptake into cells

BHK cells were plated at 1 x 10<sup>5</sup> cells per well of 12-well plates the day prior to the experiment. SPLP cellular uptake determination was performed by incorporating 0.5 mol % Rh-DOPE into the lipid formulations (DODAC/DOPE/PEG-CerC<sub>20</sub>/Rh-DOPE; 7:82.5:10:0.5 mol/mol/mol) and SPLP were prepared using the detergent dialysis method as described previously. SPLP mixed with increasing concentrations of Ca<sup>2+</sup> (0 to 14 mM) were added to cells at a lipid dose of 80 nmoles in complete media (1 ml final volume). After incubation at 37 °C for 4, 8, and 24 h, cells were washed with PBS and lysed by the addition of buffer containing 0.1 % TX-100 in 250 mM phosphate buffer (pH 8.0). Rhodamine fluorescence of the lysate was measured on a Perkin Elmer Luminescence Spectrophotometer using  $\lambda_{ex}$  of 560

nm and  $\lambda_{em}$  of 590 nm with slit widths of 10 and 10 nm, respectively. Lipid uptake was determined by comparing lysate fluorescence to that of a lipid standard normalizing it to the total cellular protein.

### 3.2.5 Fluorescence microscopy studies

Fluorescence microscopy was employed to visualize the intracellular lipid distribution following uptake of large unilamellar vesicles (LUV). Cells were incubated with LUV with the same lipid composition as the SPLP formulation except that 4 mol % Rh-DOPE was incorporated to serve as the fluorescence marker (LUV composition: DODAC/DOPE/PEG-C<sub>20</sub>/Rh-DOPE, 7:79:10:4). LUV were prepared by using the extrusion method as described previously (Lam and Cullis, 2000). The transfection media was replaced with complete media prior to analysis under the fluorescence microscope. Fluorescence micrographs were taken on an Axiovert 100 Zeiss Fluorescent microscope (Carl Zeiss Jena GmbH) using a rhodamine filter from Omega Opticals (Brattleboro, VT) with the following specifications,  $\lambda_{ex}$ =560 ± 20 nm, 600 nm LP, and DC 590 nm.

### 3.2.6 Intracellular processing of plasmid DNA

BHK cells were plated at  $3 \times 10^5$  cells per well of 6-well plates the day prior to the experiment. 2.5 µg plasmid DNA encapsulated in SPLP were incubated with cells for 2, 4, and 8 h, in the absence or presence (8 mM) of Ca<sup>2+</sup>. At the appropriate time points, cells were washed with PBS and external SPLP were removed by trypsinization. Trypsinized cells were pelleted by centrifugation and

cells were resuspended and washed with isotonic buffer (250 mM sucrose, 3 mM MgCl<sub>2</sub>, 50 mM HEPES, pH 7.2). Subsequently, pelleted cells were lysed by incubating with 250 µl of lysis buffer (10 mM Tris, pH 7.5, 0.5% SDS, 1 mM EDTA) containing Pronase E at 1 mg/ml (Sigma) overnight at 37°C. DNA (genomic DNA and delivered plasmid DNA) were extracted as described previously (Sambrook et al., 1989). DNA recovery was determined by measuring the absorbance at 260 nm. 6 µg of total DNA from each sample was either dot blotted onto a nylon transfer membrane (Amersham) with a set of pCMVLuc standards (0 to 5 pg) or loaded into a 1% agarose gel and size fractionated at 60 V for 2 h for the Southern analysis. Both blots were hybridized overnight at 68°C to a <sup>32</sup>P-labeled plasmid DNA probe, which was prepared with *Pst*l cut-pCMVLuc plasmid using the <sup>T7</sup>QuickPrime<sup>TM</sup> Kit (Pharmacia Biotech). Blots were washed 3 times with 2x SSC containing 0.1% SDS, and were then exposed on a Phosphorimager screen which was subsequently scanned (Molecular Dynamics – Phosphorimager<sup>TM</sup>SI).

## 3.2.7 <sup>31</sup>P NMR spectroscopy

Solid-state <sup>31</sup>P NMR spectra were recorded with broad-band decoupling at 81.02 MHz on a Bruker MSL 200 spectrometer, using a 3.8- $\mu$ s 60° pulse and a 1.5-s repeat time. The free induction decay (FID) was accumulated over 2500-3000 scans and was Fourier transformed with 50-Hz line broadening. Phospholipid mixtures (25  $\mu$ mol of total phospholipid) were dispersed to form multilamellar vesicles (MLV) by vortex mixing in 2 ml of buffer (20 mM HEPES buffer, pH 7.4). Increasing concentrations of Ca<sup>2+</sup> were titrated into the vesicles by adding aliquots of

a 200 mM CaCl<sub>2</sub> stock solution. Ca<sup>2+</sup> equilibration was ensured by performing three cycles of freeze-thawing. The temperature was maintained at 25 °C with a Bruker variable temperature unit. A mixture of phosphoric acid/D<sub>2</sub>O was used as the reference for chemical shifts in all <sup>31</sup>P NMR spectra.

# 3.2.8 Entrapment of Ca<sup>2+</sup> inside SPLP

SPLP (DODAC/DOPE/PEG-CerC20/Rh-DOPE, 10:79.5:10:0.5, mol/mol/mol) were prepared as described in citrate buffer (150 mM sodium citrate and 150 mM citric acid, pH 4). Non-encapsulated plasmid was removed by DEAE anion exchange chromatography equilibrated in HBS buffer (pH 7.5) and empty lipid vesicles were removed by employing a sucrose density gradient as previously described (Mok et al., 1999). Ca<sup>2+</sup> loading was performed by incubation of the DNAloaded vesicles with 2.5 mM CaCl<sub>2</sub> and the ionophores A23187 (0.1µg/µmole lipids) for 30 min at room temperature. Unloaded Ca<sup>2+</sup> and ionophores were removed by dialysis in HBS buffer with 2 buffer changes. Internal Ca<sup>2+</sup> concentrations were determined in the absence and presence of TX-100 (0.2%) by employing the membrane non-permeant absorbant indicator Asenazo III (0.1 mM in 10 mM HEPES buffer, pH 7), against a standard curve generated employing CaCl<sub>2</sub>, Ca<sup>2+</sup> levels were measured by measuring absorbance at 650 nm. Internal concentrations of Ca<sup>2+</sup> were found to be ~175 mM. SPLP were characterized with respect to plasmid entrapment using a previously described Picogreen assay (Zhang et al., 1999) and sized using guasielastic light scattering.

### 3.2.9 Insertion of CPL

Prior to CPL insertion, SPLP were prepared as described in the previous section with some modification. SPLP containing a total of 10 µmoles of DODAC, DOPE, PEG-CerC<sub>20</sub>, and Rh-DOPE (7:82.5:10:0.5; mol/mol/mol/mol) were hydrated in 1 ml of HBS buffer (20 mM HEPES and 150 mM NaCl, pH 7.5) containing 0.2 M OGP with continuous vortexing. Plasmid DNA (400  $\mu$ g/ml) was added to the hydrated lipids and the mixtures were dialysed against HBS buffer for 36 to 48 h with 2 buffer changes. Non-encapsulated plasmid was removed by DEAE anion exchange chromatography. CPL insertion was performed after the DEAE column as described (Fenske et al., submitted). Briefly, CPL stocks in methanol labeled with a dansyl fluorescence marker were added to the vesicles to give the desired molar ratio (4 mol % CPL relative to vesicle lipid). CPL and SPLP were incubated for up to 3 h at 60 °C, and cooled on ice to room temperature. Both empty lipid vesicles and non-inserted CPL were removed by using sucrose density gradient centrifugation (at step gradients of 1%, 2.5%, and 10% sucrose). The insertion levels of CPL were quantitated by using the Perkin Elmer Luminescence Spectrophotometer as described previously (Fenske et al., submitted). Briefly, the initial dansyl/rhodamine  $(D/R_i)$  fluorescence ratio prior to sucrose density gradient and the final D/R  $(D/R_f)$ ratio of the isolated CPL-SPLP were measured. Rhodamine flouresence was assayed at  $\lambda_{ex}$  = 560 nm and  $\lambda_{em}$  = 590 nm, while dansyl fluorescence was assayed at  $\lambda_{ex}$  = 340 nm and  $\lambda_{em}$  = 510 nm, with slit widths of 10 and 10 nm. The %-insertion was calculated as follows:

# %-insertion = $([D/R]_{f})^{*}100/(D/R)_{i}$

CPL-SPLP were further characterized with respect to plasmid entrapment the Picogreen assay and sized using quasielastic light scattering.

#### 3.3 RESULTS

# 3.3.1 The transfection potencies of SPLP are dramatically enhanced in the presence of Ca<sup>2+</sup>

Previous work has shown that SPLP, particularly SPLP stabilized by PEG-CerC<sub>20</sub>, exhibit very low levels of transfection in vitro (Wheeler et al., 1999; Mok et al., 1999). We have also shown that the transfection potencies of plasmid DNAcationic lipid complexes can be enhanced up to 20-fold when Ca<sup>2+</sup> is present in the transfection medium (Lam and Cullis, 2000). Here we extend these studies to monitor the effects of Ca<sup>2+</sup> on the transfection potency of SPLP. SPLP prepared from DOPE/DODAC/PEG-CerC<sub>20</sub> (84:6:10; mol:mol:mol) lipid mixture and pCMVLuc employing the detergent dialysis method were purified from empty vesicles and unencapsulated plasmid as described in Materials and Methods. Appropriate amounts of CaCl<sub>2</sub> were then added to the SPLP preparation to give rise to the desired Ca<sup>2+</sup> concentrations following dilution into the media before applying to the BHK cells. The BHK cells and the SPLP were incubated together for 24 h, after which the transfected cells were assayed for luciferase expression. As shown in Figure 3.1, the presence of Ca<sup>2+</sup> resulted in dramatic enhancements in luciferase expression levels, with a ~600-fold increase in SPLP transfection potency observed at the optimal Ca<sup>2+</sup> concentrations. This Ca<sup>2+</sup>-mediated increase in transfection is significantly greater for the SPLP system than previously observed for plasmid DNAcationic lipid complexes. The optimal concentrations of Ca<sup>2+</sup> required for stimulating SPLP transfection potencies were in the range of 8 to 10 mM, somewhat lower than



Figure 3.1. Ca<sup>2+</sup> enhances the transfection potency of SPLP in a specific manner. Increasing concentrations of CaCl<sub>2</sub> ( $\bullet$ ), MgCl<sub>2</sub> ( $\blacksquare$ ), or NaCl ( $\bullet$ ) (0 to 14 mM – final concentrations) were titrated into SPLP prior to their addition to cells. 0.5 µg of pCMVLuc plasmid encapsulated in SPLP (DODAC/DOPE/PEG-CerC20; 7:83:10 mol/mol/mol) vesicles was used to transfect cells plated at 1 x 10<sup>4</sup> cells/well of 96-well plates. Cells were incubated with SPLP for 24 h, and Luc activity was measured as described in Materials and Methods. The error bars represent the standard deviation of three experiments.

that required (5-25 mM) for optimal stimulation of the transfection potencies of plasmid DNA-cationic lipid complexes. Finally, the ability of  $Ca^{2+}$  to stimulate the transfection potency of SPLP was specific. As shown in Figure 3.1, if MgCl<sub>2</sub> or NaCl was substituted for CaCl<sub>2</sub> no enhancement in transfection potency was observed.

### 3.3.2 SPLP are stable in the presence of Ca<sup>2+</sup>

SPLP with the PEG-CerC<sub>20</sub> coating are highly stable systems that exhibit extended circulation times in vivo, protect encapsulated plasmid from external nucleases and do not interact readily with cells (Monck et al., 2000; Mok et al., 1999; Wheeler et al., 1999). It is therefore important to demonstrate that the enhanced transfection properties of SPLP in the presence of Ca<sup>2+</sup> were not due to destabilization of the SPLP leading to enhanced cell uptake. The stability of the SPLP in the presence of Ca<sup>2+</sup> was examined employing quasi-elastic light scattering (QELS) to detect changes in size and the Picogreen fluorophore assay to detect DNA leakage. For the QELS experiments, CaCl<sub>2</sub> was added to the SPLP size or size distribution was observed (Fig. 3.2A). For the plasmid release experiments, SPLP were incubated at 37 °C in HBS buffer containing 10 % FBS in the presence or absence of 8 mM Ca<sup>2+</sup>. Plasmid release was assayed over 24 h employing the Picogreen assay. No plasmid release was observed (Fig. 3.2B).

# 3.3.3 Ca<sup>2+</sup> does not influence the cellular uptake of SPLP

The ability of Ca<sup>2+</sup> to enhance the transfection activity of plasmid DNA-



Figure 3.2. Stability of SPLP in the presence of  $Ca^{2+}$ . (A) Effect of  $Ca^{2+}$  on vesicle size mean diameters. Increasing concentrations of  $Ca^{2+}$  (0 to 50 mM) were titrated into SPLP, and vesicle mean diameters were determined using quasielastic light scattering. (B) Percent plasmid DNA retention in the presence of  $Ca^{2+}$ . SPLP were incubated at 37°C in HBS (1), 10% FBS (2), or 8 mM  $Ca^{2+}$  w/ 10% FBS (3). An aliquot was taken from each sample and levels of Rd fluorescence were determined in the absence and presence of TX-100 (0.2%) as described in Materials and Methods.

cationic lipid complexes has been attributed to an increase in the uptake of the complexes into cells in the presence of  $Ca^{2+}$  (Lam and Cullis, 2000). In this regard, the low transfection potencies of SPLP as compared to complexes arise, at least in part, from very low levels of cellular uptake of SPLP (Mok et al., 1999). It was therefore important to determine whether  $Ca^{2+}$  stimulated SPLP transfection potencies by increasing SPLP uptake into cells. SPLP containing 0.5 mol % Rh-DOPE were employed to determine SPLP uptake into BHK cells in the presence of up to 14 mM  $Ca^{2+}$  as described in Materials and Methods. The SPLP were incubated with cells for 4, 8, and 24 h and the levels of intracellular lipid determined. Lipid uptake at each time-point was normalized against total cell protein in order to account for cell growth. As shown in Figure 3.3, the results indicate that  $Ca^{2+}$  did not significantly increase the cellular uptake of SPLP even though the transfection potencies of the SPLP varied by several hundred-fold over the range of  $Ca^{2+}$  concentrations tested.

# 3.3.4 Fluorescence studies indicate enhanced endosomal destabilization following uptake of LUV in the presence of Ca<sup>2+</sup>

The fact that uptake of SPLP is not stimulated by addition of Ca<sup>2+</sup> suggests that the Ca<sup>2+</sup>-dependent enhancement of transfection must arise from more efficient utilization of SPLP that are accumulated. One possibility is that Ca<sup>2+</sup> somehow facilitates destabilization of endosomes following uptake of SPLP, thus enhancing intracellular delivery of plasmid. Previous work has shown that endosomal destabilization following uptake of vesicles containing fluorescently-labeled lipids can



Figure 3.3. Influence of Ca<sup>2+</sup> on the cellular uptake of SPLP. SPLP containing 0.5 mol % Rh-labeled DOPE (DODAC/DOPE/PEG-CerC20/Rh-DOPE; 7:82.5:10:0.5 mol/mol/mol) were employed to monitor cellular lipid uptake. 80 nmoles of lipid vesicles prepared in the presence of Ca<sup>2+</sup> (0 to 14 mM – final concentrations) were incubated on cells for the appropriate time periods. Levels of lipid uptake were determined by measuring Rh fluorescence at 4 h (•), 8 h (•), or 24 h (•) as described in Materials and Methods. The error bars represent the standard deviation of three experiments.

be detected by fluorescence microscopy as a diffuse intracellular fluorescence, whereas uptake into stable endosomes gives rise to a localized "punctate" appearance (Felgner et al., 1987). In order to be able to visualize the effects of Ca<sup>2+</sup>. LUV with the same lipid composition as the SPLP but with a high level of the fluorescent lipid Rh-DOPE were constructed (DODAC/DOPE/PEG-C20/Rh-DOPE, 7:79:10:4, mol/mol/mol/mol). These Rh-labeled LUV were incubated with BHK cells in the presence and absence of 10 mM Ca<sup>2+</sup> and the cell morphology was examined at 8 h by fluorescence microscopy. Similar levels of rhodamine fluorescence were detected in the absence or presence of Ca<sup>2+</sup>, in agreement with the guantitative measurements of SPLP uptake noted in the previous section. However, as shown in Figure 3.4, the appearance of the cells as detected by fluorescence microscopy was quite different in the presence or absence of Ca<sup>2+</sup>. Although some punctate structures are observed, BHK cells containing the fluorescently-labeled LUV exhibited a more diffuse pattern when  $Ca^{2+}$  was included. In the absence of  $Ca^{2+}$ . the fluorescence pattern was largely punctate, consistent with retention of the LUV in the endosomal compartments.

#### 3.3.5 Intracellular processing of delivered plasmid DNA

The preceding fluorescent microscopy results suggest that Ca<sup>2+</sup> enhances transfection by destabilizing the endosomal compartments, thus enhancing cytoplasmic delivery of the SPLP-associated plasmid. If SPLP plasmid can escape from the endosome more readily in the presence of Ca<sup>2+</sup>, it should avoid breakdown in the lysosomal pathway and more intact intracellular plasmid DNA should be



Figure 3.4. Influence of  $Ca^{2*}$  on cell morphology following uptake of LUV labelled with Rh-DOPE as detected using fluorescence microscopy. LUV (DODAC/DOPE/PEG-C<sub>20</sub>/Rh-DOPE, 7:79:10:4 mol/mol/mol) were prepared as indicated in Materials and Methods and amounts corresponding to100 nmoles lipid were incubated with cells (plated at  $1\times10^5$  cells per well of a 12-well plate) in the absence (A) or presence (B) of calcium (10 mM). At the 8 h time point, transfecting media was replaced with complete DMEM media and cells were examined using fluorescence microscopy. Fluorescence micrographs were taken as indicated in Materials and Methods. present. A dot blot assay was employed to measure intracellular delivery of plasmid DNA, and the integrity of the plasmid was examined by using the Southern blot analysis. Cells were incubated with SPLP in the absence or presence of 8 mM Ca<sup>2+</sup> for 2, 4, and 8 h. The levels of intact, intracellular plasmid DNA for the different systems were compared after isolation of DNA from the cells as described in Materials and Methods, and the results are shown in Figure 3.5. As shown in Figure 3.5A, when cells were transfected with the SPLP in the presence of Ca<sup>2+</sup> the amount of intact plasmid in the BHK cells was increased by approximately 10-fold after an 8 h incubation period. This is also reflected by a Southern analysis which showed that more intact plasmid DNA was present in cells transfected with SPLP prepared in the presence of Ca<sup>2+</sup> (Fig. 3.5B). Such enhanced levels of intact plasmid DNA were not observed when Mg<sup>2+</sup> was substituted for Ca<sup>2+</sup>, demonstrating the specificity of Ca<sup>2+</sup> (Fig. 3.5B).

# 3.3.6 Ca<sup>2+</sup> destabilizes bilayer lipid structures in a manner consistent with an ability to destabilize endosomal membranes

Recent work suggests that cationic lipids stimulate intracellular delivery of macromolecules such as plasmid DNA by combining with anionic lipids and forming ion pairs that destabilize bilayer membranes by inducing non-bilayer (H<sub>II</sub> phase) structure (Hafez et al., 2000; Hafez and Cullis, submitted). In this regard, it is well known that Ca<sup>2+</sup> can destabilize lipid bilayers containing acidic lipids such as phosphatidylserine (PS) in combination with unsaturated PE's by inducing the non-



Figure 3.5. Influence of Ca<sup>2+</sup> on the integrity of SPLP plasmid following uptake of SPLP into BHK cells. SPLP (DODAC/DOPE/PEG-C<sub>20</sub>, 7:83:10 mol/mol/mol) containing 2.5 µg plasmid DNA were used to transfect BHK cells as described in Materials and Methods. At appropriate time points (2 h, 4 h, or 8 h), DNA was extracted from the cells and intracellular plasmid DNA was detected by hybridization to a specific <sup>32</sup>P-labeled plasmid DNA probe. (a) Levels of plasmid DNA uptake determined by dot blot analysis in the presence ( $\blacksquare$ ) or absence ( $\bullet$ ) of 8 mM Ca<sup>2+</sup>. (b) Integrity of intracellular plasmid DNA using the Southern blot analysis. Lanes 1 and 11: pCMVLuc control; lanes 2, 5, 8 and 12: untransfected control; lanes 3, 6, 9 and 13: cells transfected with SPLP; lanes 4, 7, 10 and 14: cells transfected with SPLP and 8 mM Ca<sup>2+</sup>; and lane 15: cells transfected with SPLP and 8 mM Mg<sup>2+</sup>. The error bars represent the standard deviation of three experiments.

bilayer hexagonal H<sub>II</sub> phase structure (Hope and Cullis, 1979; Tilcock and Cullis, 1981; Bally et al., 1983). It has also been shown that  $Ca^{2+}$  can induce H<sub>II</sub> phase structure in related systems containing phosphatidylcholine (PC) and cholesterol. For example, addition of  $Ca^{2+}$  to mixtures of DOPC/DOPE/DOPS/Cholesterol (1:1:1:3; molar ratios) also triggers bilayer to hexagonal H<sub>II</sub> phase transitions (Tilcock et al., 1984). It may therefore be proposed that  $Ca^{2+}$  stimulates SPLP transfection by acting in concert with the cationic lipid in the SPLP to destabilize the lipid bilayer of endosomal membranes.

In order to illustrate this potential we investigated the Ca<sup>2+</sup>-dependent polymorphism of MLV composed of DOPC/DOPE/DOPS/Cholesterol (1:1:1:3; molar ratios) in the absence and presence of small amounts of DODAC emploving <sup>31</sup>P NMR. Considerable previous work has shown that MLV composed of phospholipids in the bilayer organization give rise to asymmetric <sup>31</sup>P NMR line shapes with a low field shoulder and high field peak, whereas phospholipids in the hexagonal H<sub>II</sub> phase give rise to a line shape with reversed asymmetry that is a factor of two narrower (Cullis and de Kruijff, 1979). As shown in Figure 3.6A, in the absence of DODAC,  $Ca^{2+}$  is able to stimulate a transition from the bilayer to the hexagonal H<sub>II</sub> phase as reported by <sup>31</sup>P NMR at the Ca<sup>2+</sup>-to-DOPS ratio of 0.5:1. Alternatively, in MLV containing small amounts of DODAC (DOPC/DOPE/DOPS/Cholesterol/DODAC; 1:1:1:3:0.25; molar ratios), Ca<sup>2+</sup>-to-DOPS ratios of only 0.25:1 are required to induce predominantly H<sub>II</sub> phase structure (Fig. 3.6B). The narrow central peak may arise from small lamellar structures or lipid in non-bilayer structures such the as cubic phase in which component phospholipids experience isotropic motional averaging.



Figure 3.6. <sup>31</sup>P NMR spectra of various model membrane systems in the presence of Ca<sup>2+</sup>. Ca<sup>2+</sup> was titrated into MLV composed of (A) DOPE/DOPS/DOPC/Chol, 1:1:1:3; or (B) DOPE/DOPS/DOPC/Chol/DODAC, 1:1:1:3:0.25, to achieve Ca<sup>2+</sup>/DOPS ratios ranging from 0:1 to 0.5:1 (molar ratios). Equilibration of the cations was ensured by three cycles of freeze-thawing. Spectra have been normalized to the same peak height. Experiments were carried out as described in Materials and Methods.

# 3.3.7 External Ca<sup>2+</sup> is required to enhance SPLP transfection potency

It was of interest to determine whether  $Ca^{2+}$  encapsulated within the SPLP could stimulate transgene expression. As detailed elsewhere (Wheeler et al., 1994),  $Ca^{2+}$  can be loaded into large unilamellar vesicles (LUV) in response to a pH gradient (inside acidic) when the  $Ca^{2+}$  ionophore A23187 is present. Internal  $Ca^{2+}$  concentrations as high as 250 mM can be achieved. As described in Materials and Methods, SPLP could be readily prepared at pH 4 in the presence of a citrate buffer and then the external pH could be raised to pH 7.5 following the detergent dialysis procedure. Addition of external  $CaCl_2$  and ionophore then resulted in loading of  $Ca^{2+}$  into the SPLP to achieve internal concentrations of ~ 175 mM. However, as shown in Figure 3.7, the presence of encapsulated  $Ca^{2+}$  did not result in significant enhancement of SPLP transfection potency.

# 3.3.8 Effect of Ca<sup>2+</sup> on SPLP systems containing cationic PEG lipids and higher levels of DODAC

One of the limitations with SPLP is that the system is not readily taken up by cells as a result of limited cationic lipid and presence of PEG on the vesicles (Mok et al., 1999). Recently, a new class of cationic lipid known as cationic poly(ethylene glycol) lipid conjugates (CPL) has been synthesized in our laboratory (Chen et al., 2000). CPL employed in this study contains a hydrophobic ceramide anchor, which is attached to a hydrophilic PEG spacer that is linked to a cationic headgroup made of four lysine residues. It has been shown that CPL enhance interactions between the liposomes and cell plasma membrane, thus leading to higher cellular binding and



Figure 3.7. External Ca<sup>2+</sup> was responsible for stimulating transfection. Ca<sup>2+</sup> was loaded employing A23187 in the presence of a pH gradient as described in Materials and Methods. Increasing concentrations of Ca<sup>2+</sup> (0 to 14 mM) were added to both SPLP (**■**) and Ca<sup>2+</sup>-containing SPLP (**●**) prior to DMEM dilution. 0.5 µg of pCMVLuc plasmid encapsulated in SPLP were used to transfect cells plated at 1 x 10<sup>4</sup> cells/well of 96-well plates. Luciferase activity was measured as described in Materials and Methods. The error bars represent the standard deviation of three experiments.
uptake (Chen et al., 2000). Experiments were carried out to determine the influence of Ca<sup>2+</sup> on the CPL-SPLP system. In addition, SPLP containing higher DODAC content (14 mol %) were also investigated, as enhanced transfection potencies have been observed for such systems (Zhang et al., 1999). For the CPL-SPLP preparation, plasmid DNA was loaded into liposomes using the previously described detergent dialysis method and CPL were inserted into the preformed SPLP using a well characterized insertion method (Fenske et al., submitted). CPL were inserted to obtain a final 4 mol % insertion efficiency, as this level has been shown to provide good cellular binding and uptake (Fenske et al., submitted). Ca<sup>2+</sup> at 8 mM was added to the SPLP and CPL-SPLP preparations, diluted into the media before applying to the BHK cells. Gene expression was determined by assaving for luciferase 24 h after incubation of the BHK cells together with the transfecting liposomes. As shown in Figure 3.8. Ca<sup>2+</sup> was also required for the improved SPLP systems. In particular, a  $\sim$ 2000-fold increase and a 10<sup>5</sup>-fold increase in transfection were detected for the SPLP containing either higher DODAC content or CPL, respectively.



Figure 3.8. Effect of Ca<sup>2+</sup> on SPLP systems containing cationic PEG lipids or higher amounts of DODAC. SPLP containing higher DODAC content (14 mol %) or CPL (4 mol %) were used to transfect cells in the presence (dashed bars) or absence (open bars) of 8 mM Ca<sup>2+</sup>. 0.5  $\mu$ g of pCMVLuc was used in each formulation in each transfection experiment. Cells were exposed to the vesicles for 24 h before assaying for Luc expression, as outlined in Materials and Methods. The error bars represent the standard deviation of three experiments.

### 3.4 DISCUSSION

This study demonstrates that Ca<sup>2+</sup> gives rise to a large enhancement of SPLP transfection potency in vitro. Three areas warrant further discussion. These include the possible mechanisms whereby Ca<sup>2+</sup> results in an enhanced transfection potency of SPLP, the relation between the results presented here and previous demonstrations that Ca<sup>2+</sup> can improve the transfection potency of plasmid DNA-cationic lipid complexes (Lam and Cullis, 2000) and the implications for the design of SPLP that exhibit enhanced transfection potency in vivo. I will discuss these topics in turn.

The mechanism whereby Ca<sup>2+</sup> stimulates the transfection potency of SPLP must account for several observations. First, the enhanced transfection appears to result from higher intracellular levels of intact plasmid in the presence of Ca<sup>2+</sup>; however these higher levels of plasmid do not arise from increased uptake of SPLP into cells. Second, the process is associated with a reduction in the "punctate" appearance of cells following uptake of fluorescently labeled SPLP. Finally, the effect is Ca<sup>2+</sup> specific. The first two observations are clearly consistent with enhanced endosomal destabilization of the BHK cells following endocytosis of SPLP and the question that remains is how Ca<sup>2+</sup> could promote this destabilization in a specific manner. In this regard, there is presently no consensus as to how endosomes can be destabilized to enhance release of their contents, however a number of leading observations have been made. Chief amongst these is the observation that cationic lipids can dramatically enhance the intracellular delivery of macromolecules such as plasmids and antisense oligonucleotides (Barron et al.,

1999; Bennett et al., 1992) and that this process appears to rely on an ability of cationic lipids to destabilize endosomal membranes, thus facilitating intracellular release of endosomal contents (Wattiaux et al., 1997; Xu and Szoka, 1996). Recent work has shown that cationic lipids exhibit as a general property the ability to combine with anionic lipids to form non-bilayer hexagonal H<sub>II</sub> phase structure (Hafez et al., 2000; Hafez and Cullis, submitted), leading to the proposal that the mechanism whereby cationic lipids destabilize endosomes relies on an ability to disrupt the bilayer organization of the endosomal membrane. In the same vein, if Ca<sup>2+</sup> could disrupt bilayer organization and induce H<sub>II</sub> phase structure similar enhancements in intracellular delivery would be expected.

There is considerable evidence that  $Ca^{2+}$  can induce H<sub>II</sub> phase structure in previously bilayer lipid systems containing anionic lipids and that this effect is  $Ca^{2+}$ specific in that other cations such as Mg<sup>2+</sup> either cannot induce H<sub>II</sub> structure or require higher concentrations to produce similar effects (Bally et al., 1983; Tilcock et al., 1984). As shown in this work,  $Ca^{2+}$  can induce H<sub>II</sub> phase structure in bilayers composed of DOPC:DOPE:DOPS:cholesterol and can act in synergy with low levels of the cationic lipid DODAC to trigger H<sub>II</sub> phase formation. It is difficult to directly relate the model membrane behaviour to the behaviour inside the endosome, however it should be noted that uptake of an SPLP results in delivery of only low levels of cationic lipid. Assuming the endosome has a diameter of 200 nm and the lipid composition determined elsewhere (Kobayashi et al., 1998) it is straightforward to show that uptake of a 70 nm diameter SPLP containing 7 mol% DODAC provides only 5% of the cationic lipid necessary to form ion pairs with all of the anionic lipid in

the endosomal membrane. This contrasts with the situation for lipoplexes, as uptake of a lipoplex system of ~ 90 nm diameter corresponds to uptake of sufficient cationic lipid and "helper" lipid to induce H<sub>II</sub> phase organization for the entire endosomal membrane (Hafez and Cullis, submitted). It is possible that the enhanced sensitivity of SPLP transfection potency to Ca<sup>2+</sup> as compared to lipoplexes may reflect this difference. In particular it may be suggested that the uptake of SPLP with low levels of cationic lipid results in a requirement for Ca<sup>2+</sup> in order to achieve maximum destabilization of the endosomal membrane, whereas uptake of a lipoplex results in delivery of sufficient cationic lipid and helper lipid to destabilize the endosomal membrane independent of Ca<sup>2+</sup> levels. In summary, the results reported here support the possibility that Ca<sup>2+</sup> enhances transfection of SPLP by promoting endosomal destabilization in synergy with cationic lipid. Such a proposal is also in agreement with the observation that the addition of Ca<sup>2+</sup> to lysobisphosphatidic acid (LBPA), a major endosomal anionic lipid (Kobayashi et al., 1998), results in formation of the H<sub>II</sub> phase (I. Hafez, unpublished results).

A surprising aspect of this study concerns the discrepancy between the influence of  $Ca^{2+}$  on the transfection properties of plasmid DNA-cationic lipid complexes previously reported (Lam and Cullis, 2000) and the results reported here for SPLP. In particular, the previous work demonstrated that  $Ca^{2+}$  could enhance the transfection potency of complexes by up to 20-fold and that this could be attributed to enhanced uptake of the complexes into the cells, rather than enhanced endosomal release. The surprising aspect is that the increased transfection potency of SPLP in the presence of  $Ca^{2+}$  could not be related to increased uptake of SPLP.

by the cells, whereas Ca<sup>2+</sup> caused at least a 2-fold increase in uptake of complexes as evidenced by uptake of both lipid and plasmid (Lam and Cullis, 2000). It is likely that this discrepancy is related to the much different physical properties of SPLP as compared to complexes. Complexes are large, positively charged systems containing high (equimolar) levels of cationic lipid whereas SPLP are small, stable, essentially neutral vesicles with a PEG coating that contain low levels of cationic lipid.

The final topic of discussion concerns extension of the results presented here to generate SPLP that exhibit enhanced transfection potencies in vivo. A remarkable aspect of the results presented here is that the Ca<sup>2+</sup> stimulation of SPLP transfection potency is even more pronounced for systems containing CPL, which results in enhanced uptake into cells. These results clearly indicate that SPLP should be used in conjunction with Ca<sup>2+</sup> whenever possible. As emphasized elsewhere (Wheeler et al., 1999, Zhang et al., 1999), SPLP have been designed for systemic applications where long circulation lifetimes and accumulation at disease sites such as tumour sites is required. Achievement of enhanced transfection potency by increasing the local concentration of Ca<sup>2+</sup> in vivo is not a straightforward proposition. As indicated here, encapsulation of Ca<sup>2+</sup> within the SPLP did not result in increased transfection potency, indicating that Ca<sup>2+</sup> is required outside the SPLP in order to give rise to enhanced transfection. It is possible that strategies aimed at increasing surface Ca<sup>2+</sup> concentrations by attachment of Ca<sup>2+</sup>-chelating agents to SPLP may give rise to enhanced in vivo transfection and these and related strategies are under active investigation.

In summary the results presented here demonstrate that  $Ca^{2+}$  dramatically enhances the transfection potency of SPLP in vitro. The effect is  $Ca^{2+}$ -specific and appears to reflect increased destabilization of the endosomal membrane with associated release of SPLP-associated plasmid into the cytoplasm rather than increased cellular uptake of SPLP. Finally, the ability of  $Ca^{2+}$  to destabilize the endosomal membrane would be consistent with its ability to induce non-bilayer H<sub>II</sub> phase structure in model membrane systems in synergy with cationic lipids.

#### **CHAPTER 4**

### SUMMARY AND FUTURE DIRECTIONS

# 4.1 SUMMARY

The studies performed in this work were aimed at examining the effect of  $Ca^{2+}$  on two lipid-based gene transfer systems. We first examined and characterized the effect of  $Ca^{2+}$  on the plasmid DNA-lipid complexes. Then the influence  $Ca^{2+}$  has on the SPLP system and its mechanism of stimulating transfection was investigated and compared with the larger complexes.

Chapter 2 describes the observation that inclusion of Ca<sup>2+</sup> significantly increased the transfection potency of plasmid DNA-lipid complexes and the subsequent experiments designed to characterize the Ca<sup>2+</sup> effect. Evidence of Ca<sup>2+</sup> acting as a cofactor for cationic liposome-mediated transfection comes from the observation that cells transfected with Ca<sup>2+</sup> and plasmid DNA alone did not lead to any transgene activity. The specificity of Ca<sup>2+</sup> was verified by comparisons with other cations such as Mg<sup>2+</sup> and Na<sup>+</sup>, as well as by the demonstration of the inhibitory effect of EGTA. Furthermore, since the other cations did not stimulate similar increases in transfection, the Ca<sup>2+</sup>-mediated enhancement was likely not associated with an electrostatic effect between the cations and the plasmid DNA-lipid complexes. Stimulation of transfection was dependent on the Ca<sup>2+</sup> concentration, with optimal transfection ranging from 6 to 25 mM. Cytotoxicity was observed at 50 mM Ca<sup>2+</sup> or higher, indicating that cells could only tolerate extrinsic Ca<sup>2+</sup> concentration below 50 mM in the presence of cationic liposome.

from fluorescence analysis of cells transfected with the plasmid encoding GFP, the increase in the complex transfection potency was related to a higher population of cells expressing the transgene. The rate of transfection was found to be faster, suggesting that  $Ca^{2+}$  facilitated the cellular delivery of plasmid DNA-lipid complexes. This was confirmed by results from kinetic analysis of radiolabeled-lipids and dot blot assays of plasmid DNA, in which the delivery of plasmid DNA-lipid complexes was enhanced from 2- to 4-fold. More significantly, a higher level of intact plasmid DNA was observed inside the cells when  $Ca^{2+}$  was included. Finally, it was the  $Ca^{2+}$  added simultaneously with plasmid DNA and cationic liposomes that gave rise to the highest transfection enhancement. These results suggest that it was likely the complex-associated  $Ca^{2+}$ , rather than free  $Ca^{2+}$ , that was ultimately responsible for facilitating transfection. The general application of  $Ca^{2+}$  as an efficient cofactor was confirmed by evaluating its influence in a number of different cationic liposomal formulations as well as by transfecting a number of different cell lines.

The finding that  $Ca^{2+}$  enhanced plasmid DNA-lipid complex transfection potency suggested that it could exhibit similar stimulatory effects for SPLP, a system with physical characteristics more suitable for in vivo application but which suffers from limited transfection activities. Chapter 3 presented the results of the experiments designed to examine the influence of  $Ca^{2+}$  on SPLP. Transfection data showed that a >600-fold increase in SPLP transfection efficiencies was achieved in the presence of  $Ca^{2+}$ , indicating that  $Ca^{2+}$  exhibited a more potent effect for SPLP than for the complexes. QEL showed that the vesicle diameter and size distribution remained unaltered, and picogreen DNA leakage assays by incubating SPLP at

37°C and 10% FBS showed that plasmid DNA was still encapsulated inside vesicle bilayers in the presence of Ca<sup>2+</sup>. These results clearly demonstrated that SPLP maintained their integrity, at least prior to cellular entry. Similar to the complexes, the Ca<sup>2+</sup> effect on SPLP was found to be quite specific. On the other hand, the optimal Ca<sup>2+</sup> range was lower and narrower (8 to 10 mM) with SPLP when compared to the complexes. It was also unexpected that, despite the dramatic enhancement in transfection activity, no significant increase in SPLP delivery was detected. We hypothesized that Ca<sup>2+</sup> could assist in overcoming an intracellular barrier that could potentially lead to an increase in transfection. One obvious barrier for SPLPmediated gene transfer was the escape from endosomes and release of the plasmid DNA being retained inside the vesicles. Evidence suggesting that endosomal destabilization occurred came initially from fluorescence studies of cells transfected with Rh-labeled LUV with a lipid composition similar to SPLP, which exhibited a more diffuse rather than punctate cytoplasmic distribution in the presence of Ca<sup>2+</sup>. Normally, endosomes mature into lysosomes and their internal content is subjected to degradation by lysosomal enzymes. By destabilizing endosomes, the delivered plasmid DNA could better escape the degradative pathway. Indeed, both dot blot and Southern blot analysis showed that increased levels (2- to 10-fold) of intact plasmid DNA was detected in the presence of Ca<sup>2+</sup>. Furthermore, no such increase in intact plasmid was detected when Mg<sup>2+</sup> was employed, indicating that endosomal destabilization is facilitated specifically by Ca<sup>2+</sup>. Using <sup>31</sup>P NMR and a model membrane system, we demonstrated that Ca<sup>2+</sup> had the ability to mediate lipid phase transitions from the bilayer to H<sub>II</sub> phase. In addition, the NMR results showed that

Ca<sup>2+</sup> works in synergy with the cationic lipid. Lipid reorganization from bilayer to H<sub>II</sub> has been suggested to signify membrane fusion, and recent findings indicate that cationic lipid plays an important role in promoting H<sub>II</sub> phase structure (Hafez and Cullis, submitted). The synergistic effect between Ca<sup>2+</sup> and cationic lipid is further demonstrated when even higher transfection enhancement was observed when Ca<sup>2+</sup> was employed in SPLP systems containing higher cationic lipid content. A ~2000-fold increase in transfection activity was achieved using a SPLP formulation containing higher DODAC content (14 mol %) and a 10<sup>5</sup>-fold increase in transfection with the CPL-SPLP system, which contains a novel cationic lipid coated on the vesicle surface.

# 4.2 FUTURE DIRECTIONS

The investigations described in this study introduced a new area in the development of liposomal-mediated transfection systems, at the same time new questions are brought forth. Although our observations allow us to propose some possible pathways in which  $Ca^{2+}$  facilitates enhanced transfection, the underlying mechanism of how  $Ca^{2+}$  stimulates transfection is still unclear.

It is not apparent why Ca<sup>2+</sup> is only effective within a certain concentration range and why the working range would vary between the complexes and SPLP. By comparison, the SPLP was more sensitive to the Ca<sup>2+</sup> concentration than the larger complexes, in that transfection efficiency rises dramatically at 8 to 10 mM and was almost as dramatically inhibited at higher concentrations. Unlike the complexes, in which cell toxicity was correlated with less gene expression at higher Ca<sup>2+</sup>, no toxic effect was observed with SPLP.

In addition to driving endosome destabilization, it is possible that the influx of  $Ca^{2+}$  with the liposomes could affect the  $Ca^{2+}$  gradient across the cell plasma membrane. Being a potent intracellular messenger,  $Ca^{2+}$  is known for its effect in modulating various cellular functions. As a result, cellular activities that could potentially affect gene expression might be stimulated with the influx of  $Ca^{2+}$ . It would be useful to determine the levels of  $Ca^{2+}$  influx during transfection mediated by liposomes. Examination of specific proteins that could be turned on by  $Ca^{2+}$  could also be an indicator to determine whether other cellular activity was affected by the  $Ca^{2+}$  influx.

The preliminary observations noted in Chapter 3 that Ca<sup>2+</sup> exhibits even

greater stimulation for SPLP containing CPL or more cationic lipid also need to be characterized further to determine the maximum levels of transfection that may be achievable. Also, it is of particular importance to try to extend these results to in vivo situations. It is possible that incorporation of  $Ca^{2+}$  chelators or co-delivery of  $Ca^{2+}$  in liposomes may prove useful.

The interest in liposomes as carriers of macromolecules is based on their potential to enclose and protect diverse materials of biological interest and to deliver them, functionally intact and in significant quantities, to the interior of many cell types. In order to generate the most efficient liposomes as gene carriers, both the extracellular and intracellular barriers must be overcome. The presence of PEG coating on the SPLP vesicles appears to overcome the extracellular barriers, which include opsonins, phagocytes, extracellular matrix, and degradative enzymes. The current improvement in the transfection potencies of SPLP using Ca<sup>2+</sup> appears to be associated with an intracellular effect. It would be useful to examine other endosomal destabilizing agents, especially ones that could be encapsulated with the plasmid DNA. Finally, since the addition of positive charges on the SPLP is based on non-specific targeting of engineered liposomes, it would be interesting to combine the Ca<sup>2+</sup> effect with the attachment of a specific targeting element onto the SPLP in order to improve the specificity of gene expression.

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